

ORAL PRESENTATIONS

SUNDAY, DECEMBER 16

Symposium 1: Cell Fate Decisions

1

Lgr5 Stem Cells in self-renewal and cancer.

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The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. We originally defined *Lgr5* as a Wnt target gene, transcribed in colon cancer cells. Two knock-in alleles revealed exclusive expression of *Lgr5* in cycling, columnar cells at the crypt base. Using an inducible Cre knock-in allele and the Rosa26-*LacZ* reporter strain, lineage tracing experiments were performed in adult mice. The *Lgr5*⁺ crypt base columnar cells (CBC) generated all epithelial lineages throughout life, implying that it represents the stem cell of the small intestine and colon. Similar observations were made in hair follicles and stomach epithelium.

Single sorted *Lgr5*⁺ stem cells can initiate ever-expanding crypt-villus organoids in 3D culture. Tracing experiments indicate that the *Lgr5*⁺ stem cell hierarchy is maintained in these organoids. We conclude that intestinal crypt-villus units are self-organizing structures, which can be built from a single stem cell in the absence of a non-epithelial cellular niche. The same technology has now been developed for the *Lgr5*⁺ stomach stem cells.

Intestinal cancer is initiated by Wnt pathway-activating mutations in genes such as APC. As in most cancers, the cell of origin has remained elusive. Deletion of APC in stem cells, but not in other crypt cells results in progressively growing neoplasia, identifying the stem cell as the cell-of-origin of adenomas. Moreover, a stem cell/progenitor cell hierarchy is maintained in early stem cell-derived adenomas, lending support to the "cancer stem cell"-concept.

Fate mapping of individual crypt stem cells using a multicolor Cre-reporter revealed that, as a population, *Lgr5* stem cells persist life-long, yet crypts drift toward clonality within a period of 1-6 months. *Lgr5* cell divisions occur symmetrically. The cellular dynamics are consistent with a model in which the resident stem cells double their numbers each day and stochastically adopt stem or TA fates after cell division. *Lgr5* stem cells are interspersed between terminally differentiated Paneth cells that are known to produce bactericidal products. We find that Paneth cells are CD24+ and express EGF, TGF- α , Wnt3 and the Notch ligand Dll4, all essential signals for stem-cell maintenance in culture. Co-culturing of sorted stem cells with Paneth cells dramatically improves organoid formation. This Paneth cell requirement can be substituted by a pulse of exogenous Wnt. Genetic removal of Paneth cells in vivo results in the concomitant loss of *Lgr5* stem cells. In colon crypts, CD24+ cells residing between *Lgr5* stem cells may represent the Paneth cell equivalents. We conclude that *Lgr5* stem cells compete for essential niche signals provided by a specialized daughter cell, the Paneth cell.

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Gene Regulatory Networks Governing Hematopoietic Stem Cell Development and Identity.

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Several studies have addressed questions about transcriptional regulation within particular hematopoietic cell compartments. Few, however, have attempted to capture the transcriptional

changes that occur during the dynamic transition from one compartment to another. We have profiled gene expression as multipotential progenitors underwent commitment and differentiation to two alternative lineages, focusing on the first 3 days of differentiation when the majority of decisions about cell fate are made. We have combined this with genome-wide identification of the targets of three key transcription factors before and after differentiation; GATA-2, usually associated with the stem/progenitor compartment; GATA-1 (erythroid); and PU.1 (myeloid). We used correlation analyses to associate transcription factor binding with particular modules of co-expressed genes, alongside detailed sequence analysis of bound regions. Dynamic modelling of TF relationships has predicted novel interactions that have been validated experimentally. These approaches have highlighted novel regulators of stem cell fate decisions and - informed our understanding of GATA factor switching. Overall, the data reveal greater degree of complexity in the interplay between GATA-1, 2 and PU.1 - in regulating hematopoiesis than has hitherto been described, and highlights the importance of a genome-wide approach to understanding complex regulatory systems. A significant challenge in the field is how to relate these types of population-based data to the action of transcriptional regulators within single cells where cell fate decisions ultimately are effected. As a step toward this, we have generated single cell profiles of gene expression for a limited set of transcriptional regulators in self-renewing and committed blood cells and used these data to build a stochastic computational model, which affords exploration of commitment scenarios *in silico*. The data highlight the 'noisiness' of transcription in multipotential cells, and we have computationally captured modes of regulation that may contribute to heterogeneous gene expression. We suggest that individual cells may enter lineage commitment through different routes; data on instruction of commitment through perturbation of individual regulators is in support of this view.

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Asymmetric cell division and spindle orientation in neural stem cells - from *Drosophila* to humans.

Juergen A. Knoblich¹; ¹*Institute of Molecular Biotechnology, Austrian Academy of Sciences, Vienna, Austria*

When we think of mitosis, we commonly have a process in mind where a cell gives rise to two identical daughter cells. In whole organisms, however, many cell divisions are actually asymmetric and give rise to two daughter cells of different size, shape or developmental fate. Asymmetric cell divisions are particularly important in stem cells, as they allow those cells to generate both self-renewing and differentiating daughter cells, an ability that is common to all stem cells. We therefore use stem cells in the developing brain of both fruitflies and mice as a model to understand the principle mechanisms that regulate and orient asymmetric cell divisions. More recently, we have extended our efforts to mammalian model systems, where mutations in regulators of basic cell biological processes like the orientation of the mitotic spindle are known to cause strong brain malformations resulting in severe mental retardation. As recent experiments have shown striking differences between human and mouse brain development, we have made an effort to establish experimental strategies where those regulators and their effects on brain development can be studied in a human setting.

Bruce Alberts Award for Excellence in Science Education

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International Institute for Collaborative Cell Biology and Biochemistry (IICCB). Building a network to share and inspire.

L. C. Cameron¹; ¹Laboratorio de Bioquímica de Proteínas, Universidade Federal do Estado do Rio de Janeiro, Rio de Janeiro, Brazil

In 2000 we organized the first international symposium on myosin V in my home city of Rio de Janeiro. That meeting became part of the foundation for a series of courses focused on hands-on research training and education workshops to train the next generation of international scientists, with particular emphasis on Central and South American students who have limited opportunities to interact directly with international scientists. My colleagues and I founded The International Institute for Collaborative Cell Biology and Biochemistry (IICCB). The IICCB is a network of world experts who want to share their expertise and knowledge to inspire students, who in future will be the scientific leaders in the respective fields of biochemistry, cell biology and biotechnology. Our vision is to invigorate a new generation of international scientific cooperation by exposing young scientists to diverse, multidisciplinary learning experiences via organizing various workshops, conferences and symposia. At these events, leading established scientists connect with each other and share their wealth of experience with the next generation of scientists who, in turn, act as ambassadors to their colleagues. We organized more than 25 of these workshops, international conferences and courses in Brazil, Uruguay, Mexico, and the United States (we had more than 1600 attendees). Subject areas include topics in cell biology (calcium signaling, intracellular transport, and other aspects of the cytoskeleton), biochemistry, biophysics, and systems biology. These training courses have had a great impact on the students, exposing them to North American and European science. Many have gone on to work in the laboratories of the U.S. and European faculty who have participated, and the courses have sparked multiple intercontinental collaborations. We are now planning to expand to other regions of Brazil and Americas and to motivate colleagues to promote courses in other Latin America countries, Africa and Asia. In addition, we are working to consolidate the IICCB with more dependable funding from national and international agencies. We hope that we can continuously grow a friendly network of science all over the world.

E.E. Just Lecture

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Decoding the biology of human genome polymorphisms in African Americans.

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The completion of the Human Genome Project introduced a new knowledge system for decoding biology based on the science of information structured in DNA sequence variation. Single nucleotide polymorphisms (SNPs) are a system of common variation widely distributed across the genome, where two or more forms of the DNA sequence are found at a given site, with the rarest form occurring at a frequency of one percent or greater in the population. Recently, our Biophysics Research and Development Group has applied first principles of thermodynamics and statistical physics in studying the informatics of SNPs, as dynamic sites in the genome. From our perspective of SNPs as complex dynamical systems, we derived a new biophysical metric for interrogating the information content (IC) present in SNP haploblocks. This metric facilitates translation of biochemical sequence variation into a biophysical metric

derived from Boltzmann's canonical ensemble used in information theory. Our normalization of this information metric (NIC) allows for comparison of SNP haploblocks across different sites in the genome. We found that low NIC scores in the human leukocyte antigen-disease related (HLA-DR) region, illumined molecular pathways of innate immune mechanisms functional in host adaptation to environmental stressors, such as pathogens. In our approach, we were able to relate NIC to biologically relevant functional knowledge embedded in the structure of common sequence variation. We are exploring new perspectives of human population biology interfacing genomics and theoretical physics at the frontier of life sciences.

Minisymposium 1: Cancer Cell Biology

6

In vivo imaging of dynamic interactions within the haematopoietic stem cell niche.

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Haematopoietic stem cells (HSC) reside within the bone marrow, therefore their microenvironment (or niche) has been traditionally inaccessible to direct observation. Despite numerous functional studies demonstrating that several bone marrow stroma cells regulate HSC function and number, still very little is known about the anatomical location of HSC during steady state and in response to several types of stimuli, including leukaemia development. It is an open question whether HSC firmly localise within a specific niche or whether they migrate between different niches, defined by distinct bone marrow microenvironments, where they receive different stimuli. Other open questions are whether leukaemia development affects HSC-niche interactions and whether leukaemia stem cells (LSC) and HSC compete for niche space.

Confocal/two-photon hybrid microscopy allows visualization at single cell resolution of transplanted haematopoietic stem and progenitor cells (HSPC) in the calvarium bone marrow of live mice. FACS-purified, ex-vivo labelled HSPC populations can be visualised upon their initial arrival in the niche (homing) and following the first divisions (early engraftment). We showed that long-term repopulating (LT) HSPC selectively localize proximal to osteoblasts within a few hours from transplantation, whereas their progeny are more distal and we collected further evidence that LT-HSPC and their progeny localization near osteoblasts correlates with positive transplantation outcome. Finally, we used a mouse model of acute myeloid leukaemia based on retroviral transduction of the MLL-AF9 oncogene and observed that leukaemic cells corresponding to different stages of disease progression follow distinct homing patterns. In particular, granulocyte-monocyte precursors (L-GMP), an established LSC population, share the same homing pattern of normal GMP rather than that of LT-HSPC. This indicated why MLL-AF9 LSC are insensitive to niche-derived signals that otherwise affect normal HSC function.

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Autophagy dependent secretion of interleukin-6 facilitates cancer cell invasion.

J. Debnath¹, R. Lock¹, C. Kenific¹, E. Salas¹; ¹Pathology, University of California, San Francisco, San Francisco, CA

To date, the pro-tumor functions of autophagy have been largely attributed to its ability to promote tumor cell fitness and survival. We now demonstrate a new role for autophagy regulators (ATGs) in facilitating tumor cell motility, extracellular matrix invasion, and in vivo metastatic capacity. In Ras-transformed epithelial cells, RNAi-mediated depletion of ATGs

profoundly inhibits invasive behavior in three-dimensional (3D) culture and restores multiple aspects of normal epithelial architecture, including polarized deposition of basement membrane and cell-cell junctional integrity. Furthermore, lung metastases in vivo are attenuated upon autophagy inhibition in Ras-transformed epithelial cells and in Polyoma Middle T (PyMT) breast tumor cells.

Importantly, the invasion defect in autophagy-deficient cells is completely rescued upon treatment with conditioned media from autophagy-competent counterparts, indicating that intact autophagy is required for the elaboration of secreted pro-invasive factors. In support, we identify the pro-invasive cytokine interleukin-6 (IL6) as one of the critical factors whose secretion is ATG dependent. Upon ATG knockdown, Ras-transformed cells fail to secrete IL6 into the conditioned media, although both IL6 transcription and translation remain intact. Moreover, function-blocking studies and rescue experiments using recombinant IL6 substantiate that this cytokine is both necessary and sufficient to restore invasion in autophagy-deficient cells. In addition to IL6, autophagy-defective cells exhibit reduced levels of other pro-invasive molecules, including Wnt5a and matrix metalloproteinase 2 (MMP2). Addition of Wnt5a partially rescues the invasion defect in autophagy depleted H-Ras^{V12} cells, while pharmacological MMP-2 inhibition potently suppresses invasion. Overall, these results support that autophagy mediates the coordinate production of multiple secreted factors that favor invasion in oncogenic epithelial cells. They also point to a broader role for autophagy in carcinoma progression, namely facilitating invasion during dissemination and metastasis.

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GATA3 suppresses metastasis, promotes differentiation and modulates the tumor microenvironment by regulating microRNA-29b expression.

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Despite advances in our understanding of breast cancer, patients with metastatic disease have poor prognoses. GATA3 is a transcription factor that specifies and maintains mammary luminal epithelial cell fate, and its expression is lost during breast cancer progression. Indeed, low GATA3 expression correlates with poor prognosis in human patients. Here, using human and mouse breast cancer cells, we show that GATA3 promotes differentiation, suppresses metastasis and alters the tumor microenvironment by inducing miR-29b expression. Consistent with miR-29b being downstream of GATA3, miR-29b is enriched in better prognostic, luminal breast cancers and increases luminal gene expression. Mechanistically, miR-29b inhibits metastasis by directly targeting a network of pro-metastatic regulators involved in angiogenesis, collagen remodeling and extracellular matrix proteolysis, including *VEGF*, *ANGPTL4*, *PDGF*, *LOX* and *MMP9*. In addition, miR-29b targets *TGFB*, thereby indirectly regulating epithelial plasticity. Loss of miR-29b, even in GATA3-expressing cells, increases metastasis and promotes a mesenchymal phenotype. This discovery that regulating tumor microenvironmental genes and differentiation through a GATA3-miR-29b axis inhibits cancer metastasis opens up new possibilities for therapeutic intervention in breast cancer.

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aPKC iota/lambda regulates Hh signaling during basal cell carcinoma growth.

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Basal cell carcinoma (BCC) initiation and expansion requires high levels of Hedgehog (Hh) signaling. While Smoothed (Smo) inhibitors are effective, early tumor resistance illustrates the

need for additional downstream targets for therapy. Here we identify atypical Protein Kinase C ι/λ (aPKC) as a novel BCC oncogene essential for Hh signaling. Genetic knockdown using shRNA against aPKC, or pharmacological inhibition of aPKC, inhibits proliferation, Hh signaling, and ciliogenesis of the murine BCC cell line ASZ001. Genome-wide transcriptome analysis of BCC cells reveals pharmacological inhibition of aPKC or Smo target similar pathways. aPKC is a Hh target gene that is overexpressed in mouse and human BCCs and functions downstream of Smo to bind and phosphorylate Gli1, resulting in maximal DNA binding and Hh activation. Consistent with its role in regulating Hh signaling, application of a topical aPKC inhibitor suppresses Hh signaling and tumor growth in primary murine BCC tumors. As acquired drug resistance is a growing problem, we also demonstrate Smo antagonist-resistant BCC cells and human tumors overexpress active aPKC and pharmacological inhibition of aPKC suppresses proliferation. These results demonstrate polarity signaling is critical for Hh-dependent processes and suggest aPKC may be a new therapeutic target for the treatment of naïve and resistant BCCs.

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BRAF^{V600E} and PI3'-kinase signaling pathways cooperate to regulate protein translation in human melanoma cells.

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The most common genetic alteration in metastatic melanoma is a T1799A transversion that encodes BRAF^{V600E} resulting in the constitutive activation of the BRAF→MEK→ERK MAP kinase pathway. In many cases, the conversion of BRAF^{V600E} expressing melanocytes to melanoma cells also requires activation of the PI3'-kinase signaling pathway, which can occur through the silencing of the tumor suppressor PTEN, a PI3'-lipid phosphatase. To explore the mechanisms of cooperation between the BRAF^{V600E} and PI3'-kinase signaling pathways, we applied pharmacological inhibitors of each pathway to BRAF^{V600E} expressing human melanoma-derived cell lines. Our data demonstrated that blockade of BRAF^{V600E}→MEK→ERK signaling inhibited the phosphorylation of the cap-dependent translational regulators, p70^{S6K}, ribosomal protein S6 (rpS6), and 4EBP1, whereas inhibition of PI3'-kinase only suppressed rpS6 and 4EBP1 phosphorylation. In addition, blockade of BRAF^{V600E}→MEK→ERK or PI3'-kinase also led to the inhibition of melanoma cell proliferation. Moreover, simultaneous inhibition of both BRAF^{V600E} and PI3'-kinase signaling showed that these two pathways cooperate to regulate protein synthesis through mTORC1-dependent effects on rpS6 and 4EBP1. However, pharmacological inhibition of the downstream PI3'-kinase effector, AKT, only had modest effects on cell growth and p70^{S6K}, rpS6, or 4EBP1 phosphorylation, even in melanoma cells that contain mutated or amplified AKT. Taken together, these data suggest that BRAF^{V600E} and PI3'-kinase cooperate to regulate melanoma proliferation and protein translation independent of AKT activation and combined targeting of BRAF^{V600E} and PI3'-kinase signaling may help to enhance the therapeutic efficacy in patients with mutations that activate both of these pathways.

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Widespread potential for growth factor-driven resistance to anti-cancer kinase inhibitors.

J. Settleman¹, T. Wilson¹; ¹Genentech, South San Francisco, CA

Mutationally-activated kinases, including EGFR, HER2, ALK, ABL, PDGFR, c-KIT, and BRAF, define a clinically validated class of targets for cancer drug therapy. However, the efficacy of kinase inhibitors in patients whose tumors harbor such alleles is invariably limited by innate or acquired drug resistance. As molecular mechanisms of resistance have begun to be elucidated,

a recurrent theme is the engagement of survival signals redundant to those transduced by the targeted oncogenic kinase. Cancer cells typically express multiple receptor tyrosine kinases (RTKs), and many of these can potentially mediate signals that converge on common and critical downstream cell survival effectors - most notably, PI-3 kinase (PI3K) and MAP kinase (MAPK). Consequently, a change in the production or availability of ligands for such RTKs, either through autocrine tumor cell production or paracrine contribution by tumor stroma, could confer resistance to inhibitors of an oncogenic kinase with a similar downstream signaling output. Indeed, using a large panel of kinase-“addicted” cancer cell lines, we found that the vast majority of such cells can be “rescued” from drug sensitivity by simply exposing them to one or more RTK ligands that engage redundant survival effectors. Among the findings with immediate clinical implications was the observation that hepatocyte growth factor (HGF), which is widely expressed in tumour stroma, confers resistance to the BRAF inhibitor PLX4032 in BRAF mutant melanoma cells, and to the HER2 kinase inhibitor lapatinib in HER2 amplified breast cancer cells. These observations highlight the extensive redundancy of RTK-transduced signalling in cancer cells and the potentially broad role of widely expressed RTK ligands in both innate and acquired resistance to drugs targeting oncogenic kinases.

Minisymposium 2: Cell Mechanics and Intermediate Filaments

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From isolated filaments to polymer-bundles in cells.

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The polymeric nature of intermediate filaments (IF) leads to a great variety of intracellular structures which determine mechanical properties and thereby the diverse cytoskeletal functions. In order to understand the physical basis of the phenomena that are observed in cells, we combine *in vitro* experiments involving purified protein with cell studies.

In a bottom-up approach, the mechanical rigidity of individual IFs, characterized by their persistence length, is measured while taking into account confinement effects which cellular components encounter *in vivo*. In a cell, however, the situation is more complex since the individual IFs form networks of bundles and thereby interactions within and between such supramolecular assemblies have to be taken into account. The structure of the bundles is assessed by novel X-ray nanodiffraction methods while the dynamics are captured by fluorescence life-cell imaging. All the above mentioned experiments are performed in specifically tailored microfluidic sample environments. By this approach we are able to mimic and precisely control close-to-physiological conditions

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The effect of small heat shock proteins on intermediate filament networks.

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Despite their importance and abundance, very little is known about the physics of intermediate filaments. The largest subgroup of the intermediate filament protein family comprises keratins, with 28 type I (acidic) and 26 type II (basic) keratins. Their principal assembly subunit is a heterodimeric coiled-coil complex of one acidic and one basic keratin. Due to the antiparallel

fashion in which these dimers connect to tetrameric complexes, the basic module for filament assembly, the resulting filaments are apolar and exhibit a surprising degree of dynamics and restructuring within cells. While the assembly kinetics in the early stages of filament assembly has been addressed, their growth and association into filamentous networks is still an enigmatic process. By in vitro studies with reconstituted 8/18 keratins, we demonstrate an inherent tendency to interact with each other under physiological salt conditions. The structure of the resulting network is determined by the competition of filament elongation and lateral association to bundles. Small heat shock proteins modulate the association kinetics and cause a drastic alteration of network structure and morphology. This seems to be an essential tool for cells to regulate the organization of their intermediate filament cytoskeleton.

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Vimentin dynamics and microtubule crosstalk during fibroblast migration.

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Overexpression of the intermediate filament protein vimentin is a key biomarker for metastasis. Conversely, fibroblasts lacking vimentin expression are defective in wound healing and in maintaining persistent, directional migration. These data suggest that vimentin might play an important role in regulating cell migration. However, very few studies have addressed the dynamics and function of vimentin polymers in migrating cells. One clue to vimentin's role in migration comes from its interactions with microtubules, another cytoskeletal polymer that establishes and maintains cell polarity. It has been documented that vimentin assembly depends on microtubules and microtubule dependent motor proteins. However, whether vimentin affects microtubule organization and dynamics in migrating cells is unknown. To address these questions, we investigated vimentin dynamics in randomly migrating fibroblasts expressing mEmerald-Vimentin. We found that vimentin is a highly dynamic filament on a slightly longer time scale than microtubules. Vimentin filaments are oriented parallel to the direction of migration, are recruited quickly into the leading edges of migrating cells and dynamically cycle from the exterior to the interior of the cells even in stationary cells. Therefore, contrary to previous belief, vimentin is a highly dynamic polymer that changes structure concurrent with cell movement. The fact that vimentin localizes dynamically to the same places as microtubules during migration but on a slightly longer timescale suggested that vimentin might help stabilize the direction of movement perhaps by feeding back to microtubules. In order to analyze the dynamics of vimentin and microtubules simultaneously, we tracked microtubule growth by following microtubule plus ends over time. We find that a subset of dynamic microtubules follow along vimentin tracks and another subset follow vimentin towards the leading edge and then continue past the vimentin track into the leading edge of cells. From these data, we hypothesize a template model whereby microtubules enter the leading edge first and recruit vimentin fibers, which in turn serve as a support for the more dynamic microtubules to continue growth in that direction, thus establishing a positive feedback loop leading to increased memory of cell polarity. Without vimentin, microtubules would still establish polarity but lose polarity more rapidly leading to decrease in migration in a given direction.

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Cell stiffness correlates with cell volume.

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Cells usually have a certain size, and their size has been shown to be correlated to gene expression across species. There are careful studies about standard cell sizes of different species, as well as cell size changes during stages of the cell cycle. Furthermore, people have shown that cells change their spreading area, stiffness, gene expression and even stem cell fate when grown on substrates of varying stiffness. However, whether cell size changes for the same cell type under varying micromechanical environments, and whether this is related to the observed phenomenological changes is not known. Here we show that height and volume of single adherent cells decrease when grown on a stiffer 2D substrate, while spreading area increases. When cell spreading area is confined by micropatterning on stiff substrate, we find cell volume is dependent inversely on their spreading area. We further measure cell stiffness with optical magnetic twisting cytometry. By controlling their volume in three different ways – varying substrate stiffness, cell spreading areas, and osmotic pressure in the medium – we find that cell stiffness correlates with cell volume but not substrate stiffness. Cells can be soft on a stiff substrate by changing only the spreading area; they can be stiff on a soft substrate by increasing osmotic pressure. Furthermore, we show that vimentin may be involved in cellular volume regulation.

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Lamin mutations that cause muscle defects disturb nuclear mechanics and nucleo-cytoskeletal coupling

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Objective: Mutations in the *LMNA* gene that encodes the nuclear envelope proteins lamin A and C cause a plethora of human diseases (laminopathies), including muscular dystrophies, cardiomyopathies, and familial partial lipodystrophy. It remains unclear how mutations in a single gene that is ubiquitously expressed result in such often tissue-specific diseases. Since lamins A and C are the main contributors to nuclear stiffness, we hypothesized that lamin mutations associated with muscular phenotypes could impair the structural properties of the nuclear lamin network, weakening the nuclear integrity and resulting in cells more susceptible to mechanical stress.

Methods: We measured nuclear stiffness in fibroblasts derived from patients with diverse laminopathies and in lamin A/C-deficient mouse embryonic fibroblasts (MEFs) engineered to stably express physiological levels of specific lamin A mutants. In a subset of cells, we also probed nucleo-cytoskeletal coupling with a custom-developed microneedle assay. To address the effect of diverse lamin mutations on the ultrastructural level, we observed the *in vitro* assembly of purified mutant and wild-type lamins. To assess the *in vivo* effect of lamin mutations in muscle, we measured nuclear stiffness in body wall muscle of *Drosophila melanogaster* models of muscle laminopathies.

Results: Patient fibroblasts carrying *LMNA* mutations associated with muscular dystrophies had ‘softer’ nuclei than cells from healthy controls. In contrast, fibroblasts from lipodystrophy patients had normal nuclear mechanics. Extending our studies to MEFs expressing a panel of lamin A mutations, we found that four of the 15 lamin A mutations tested caused decreased nuclear stiffness. Importantly, all four mutations were associated with laminopathies affecting muscle tissue, whereas mutations linked to lipodystrophy had no effect on the structural function of lamin A. Of note, most mutations linked to muscular disease also showed disturbed nucleo-cytoskeletal coupling. Extending these studies to muscle tissue, we found that mutant lamins alter the stiffness of nuclei in isolated *Drosophila* body wall muscle, where nucleo-cytoskeletal coupling is also perturbed.

Conclusions: *LMNA* mutations associated with muscular laminopathies can cause impaired nuclear mechanics, which is consistent with increased cellular sensitivity to mechanical stress that could contribute to the muscle-specific phenotypes associated with the laminopathies. In conclusion, our results demonstrate the importance of lamins A and C on nuclear mechanics in laminopathies, but also indicate that additional factors such as altered nucleo-cytoskeletal coupling influence the disease outcome.

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The nuclear mechanostat that scales with tissue stiffness and amplifies lineage: lamin-A,C.

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Tissue can be soft like brain, stiff like muscle, or rigid like bone. Proteomic profiling of human and mouse tissues and cells reveals that the nucleoskeletal protein lamin-A,C scales with various collagens and with tissue microelasticity, E. Among the many cell structure and nuclear components quantified here, lamin-A,C acts most clearly as a “mechanostat” in increasing as $\sim E^{0.7}$, whereas B-type lamins are nearly constant. Lamin-A,C dominates in stiff tissues and has been implicated in aging and diseases that impact muscle, bone, and fat but rarely brain or marrow, and nuclei in stiff tissue cells also prove much stiffer than nuclei from softer tissues. Mesenchymal stem cell differentiation *in vitro* further shows that lamin-A,C amplifies lineage signals from matrix, with low lamin-A,C favoring a soft tissue fate and high levels favoring stiff tissue. Regulation of lamin-A,C occurs at multiple levels, with conformational changes in isolated nuclei revealing its direct response to stress. Systematic relations thus exist between tissue stress and stiffness and the nucleus.

Minisymposium 3: Cell Migration and Motility

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Olfactory microvillous neurons arise from the neural crest in a Sox10-dependent manner.A. Saxena¹, B. N. Peng¹, M. E. Bronner¹; ¹Biology, Caltech, Pasadena, CA

The cranial ganglia and sense organs (nose, ears) arise from two cell types: neural crest cells and ectodermal placodes. Both undergo cell migrations and/or dynamic cell rearrangements to reach their final configuration. Interestingly, most cranial peripheral neurons are derived from the placodes, with glia cells coming from the neural crest. In the olfactory system, the classical view has been that the olfactory placode forms all olfactory sensory neurons. In contrast, here we show that cranial neural crest cells migrating from the midbrain/rostral forebrain-level neural tube are the primary source of microvillous sensory neurons within the olfactory epithelium.

Using photoconversion-based fate mapping and live cell tracking coupled with laser ablation in zebrafish embryos, we followed neural crest precursors as they migrated from the neural tube to surround the olfactory epithelium where they condensed to form the nasal cavity. Eventually, a subset of these cells, coexpressing Sox10 protein and a neurogenin1 reporter, ingressed into the olfactory epithelium, intercalated amongst placode-derived cells, and differentiated into microvillous sensory neurons. Loss-of-function analysis revealed a critical role for Sox10 in microvillous neurogenesis. Taken together, these findings demonstrate for the first time a cranial neural crest migratory contribution to olfactory sensory neurons and provide important insights into the assembly of the nascent olfactory system.

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Coordinate Control of Core EMT Regulatory Factor function is Essential for Migratory and Invasive Behavior.C. LaBonne¹; ¹Department of Molecular Biosciences, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Evanston, IL

A small group of core transcription factors, including Twist, Snail, Slug, and Sip1, control epithelial-mesenchymal transitions (EMTs) and motility during both embryonic development and tumor metastasis. Little is known, however, about how these factors are coordinately regulated in order to mediate the requisite behavioral and fate changes in a context dependent manner. We recently showed that a key mechanism for regulating Snail protein function during neural crest development is through modulating its stability. We have extended these studies to show that the stability of Twist and Sip1 is also regulated by the ubiquitin-proteasome system, and remarkably, that the same E3 ubiquitin ligase that regulates Snail, partner of paired (Ppa), also controls the function of these other core EMT factors. This demonstrates that a common regulatory mechanism has evolved to coordinately control the function of these structurally diverse proteins as they direct migratory and invasive behavior.

Building upon these important findings, we present evidence that members of the group of core EMT regulatory factors physically interact with one another to control each others function, and that their activities are coordinately regulated by a key extra-cellular signaling pathway. Collectively, these provocative findings suggest that the factors that regulate developmental and pathological EMTs and neural crest migration constitute a molecular machine whose activities are tuned temporally and spatially to ensure their proper, coordinated, function. We report a small molecule inhibitor that can be used to intervene in this coordinated regulation in a highly temporally specific manner to allow novel insights into the control of migratory behavior.

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Role of inflammatory regulator NLRP10-like on macrophage and neutrophil migration behaviors during microglia development in zebrafish.

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Nod-like receptor (NLR) genes have essential functions in innate immunity as intracellular pattern recognition receptors and mediators of inflammation. Here we report a characterization of a mutation, st73, that causes a loss of CNS-resident macrophages called microglia in zebrafish, but no overt general morphological phenotypes. The st73 lesion introduces a premature stop codon at the beginning of a novel member of the NLR gene family that lacks leucine rich repeats, which are present in all human NLR family members except NLRP10. Based on this shared domain structure, we tentatively named the zebrafish gene NLRP10-like. Despite the absence of microglia in larvae, we found that microglia precursor cells (primitive macrophages) are present in mutants. In the mutants, primitive macrophages are anomalously large and rounded, and they lack the ability to migrate into the brain to form microglia. Furthermore, we also uncovered striking defects in the other innate immune cells, the neutrophils, that are indicative of systemic inflammation. Contrary to the migration-deficient macrophages in the st73 mutants, we found an upsurge of highly motile neutrophils in circulation, on the yolk, as well as inside the brain, which is normally devoid of neutrophils. High levels of mRNA encoding pro-inflammatory cytokines, including interleukin-1 beta and tnf-alpha, confirms the presence of inflammation in the mutants. Taken together, our data implicate NLRP10-like as an endogenous repressor of inflammation, and also suggest that inflammation can both promote and inhibit migration of innate leukocytes depending on the cellular context.

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A GEF/GAP modulator defines locomotory and invasive protrusion polarity in migrating tumor cells.

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Locomotory and invasive protrusions are essential first steps that precede tumor cell invasion and migration. Spatial control of actin polymerization is necessary to achieve directional protrusion during cell migration. By using a RhoC biosensor we show that the localization of RhoC activity is crucial to maintain actin polymerization asymmetry in order to sculpt the amplitude and the shape of locomotory and invasive protrusions. This spatial localization of RhoC activity is necessary to place active cofilin right at the leading edge of locomotory protrusions by phosphorylating cofilin behind it. Here we show that RhoC activation at leading edge protrusions is regulated by a p190RhoGEF/p190RhoGAP module that spatially restricts actin barbed end distribution to shape lamellipodia during protrusion. The same mechanism is used to shape invadopodia into needle shaped protrusions. This mechanism reveals a dynamic plasticity in the distribution and amplitude of barbed ends, which can be modulated by fine-tuning RhoC activity by upstream GEFs and GAPs for directed cell motility. p190RhoGEF/p190RhoGAP are able to dynamically tune the location and amplitude of actin barbed ends in protrusions through direct effects on RhoC/Cofilin pathway. In addition, our data show that RhoC is an important component of the cellular steering-mechanism during chemotaxis by defining where actin polymerization takes place in response to external signals.

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Focal adhesion kinase (FAK) reactivates endocytically recycled integrin to allow the reassembly of focal adhesions.

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Integrin recycling and focal adhesion (FA) turnover are key events in cell migration. Yet, the relationship between integrin recycling and focal adhesion turnover is not well-defined. In particular, how the activation state of the integrin is controlled during endocytic recycling and reassembly into FAs is unknown. Here, we used the synchronous disassembly of FAs and accompanying integrin endocytosis triggered by microtubule regrowth (Ezratty et al., 2009; Chao & Kunz, 2009) as a model system to understand factors involved in the recycling of integrins and their reassembly into FAs upon return to the cell surface. In 3T3 fibroblasts, FA reassembly began 30 min after microtubule-induced FA disassembly and correlated with a return of endocytosed $\alpha 5 \beta 1$ integrin to the cell surface. $\alpha 5 \beta 1$ integrin was detected in both Rab5- and Rab11-positive endosomes and dominant negative Rab5 and Rab11 blocked FA reassembly. Rab11 knockdown also blocked FA reassembly and the return of surface integrin. Focal adhesion kinase (FAK) was detected in the Rab11 recycling endosomes and FAK mutants FAK-Y397F (autophosphorylation/Src binding site) and FAK-K454R (kinase dead) rescued FA disassembly but not reassembly in FAK^{-/-} cells, showing that FAK kinase activity is critical for FA reassembly but not for disassembly. Src associates with FAK-pY397 and has been implicated in FA reassembly (Yeo, et al., 2006). We found that Src was transiently associated with newly formed FAs and that both Src inhibitor (PP2) and FAK inhibitor (PF228) reversely blocked FA reassembly (without affecting disassembly). Interestingly, PP2, but not PF228 blocked the recycling of integrins to the cell surface, suggesting that Src functioned in returning integrin to the cell surface, but FAK functioned only after the return. To test whether FAK functioned in integrin activation following integrin recycling, we used Mn²⁺ to artificially activate integrins in FAK and Src inhibited cells. Mn²⁺ stimulated FA reassembly only in cells treated with PF228, showing that integrin activation bypasses the requirement for FAK during FA reassembly. Similarly, Mn²⁺ rescued cell adhesion to fibronectin in FAK inhibited but not Src inhibited cells. These results provide the first evidence that FAK and Src kinase activities contribute to FA reassembly by controlling recycling and reactivation of endocytosed integrin. The need for reactivation of recycled integrins suggests that only unengaged integrin is returned to the cell front to ensure that integrin will be available for establishing new FAs during cell migration.

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LOV-TRAP: A broadly applicable, genetically encoded system to control protein activity with light through controlled sequestration at membranes.

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The kinetics and subcellular localization of protein activity is precisely controlled to regulate protein interactions and to create specific signaling networks. To study this important aspect of cell signaling, genetically encoded methods are being developed to control protein activity with light in living cells and animals (1-4). Here we describe a new approach that promises to be broadly applicable and is simple to apply. Through an in vitro selection from a protein domain library with more than 10¹³ unique sequences using mRNA display, we developed Zdark, a

small protein based on the Z domain (5) that binds only to the dark state of the LOV2 domain from *Avena sativa* phototropin (Figure 1). The LOV2 domain undergoes a large, reversible conformational change induced by light between 400 and 500 nm. Zdark binds to the dark state of LOV2 with a K_d of ~ 100 nM, but shows no detectable binding to the lit state. In our new method (dubbed LOV-TRAP for LOV trapping and release of active protein) the LOV domain is anchored at an intracellular membrane (to date the mitochondrial and plasma membranes) and the protein of interest is fused to Zdark. In the dark, the Zdark-protein fusion is sequestered at a membrane where it cannot interact with its targets (here the mitochondria). Upon irradiation, LOV binding of the Zdark-protein conjugate breaks down, releasing the Zdark-protein fusion. Release is rapid (< 0.5 secs) and reversible. We have demonstrated this for repeated light/dark cycles and have introduced mutations to vary the kinetics of return to the dark state. To date, we have completed caging of VAV2, Rac1 and RhoA. Progress with other targets will be described, as will the use of the approach to control protein dimerization. Crystal structure and NMR data will be presented to illuminate the Zdark-LOV interaction. Applications of LOV-TRAP in studies of cell motility will be described.

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Minisymposium 4: Integrated Research and Teaching and Its Benefits to Faculty and Students

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Integrated Introductory Science Curriculum for Undergraduates at Princeton.

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For the past eight years, we have offered a series of courses, taken in the freshman and sophomore years that provide students with strong quantitative preparation for a major in any scientific discipline. The program is designed to help students retain the connections between their ultimate choice of major and the other disciplines. The curriculum is founded on the expectation that much of the most important science of the future, though based on the classical disciplines, will lie in areas that span two or more of them. The curriculum covers the core material of introductory physics, chemistry, biology (genetics and biochemistry), and computer science, all in an integrated manner in the equivalent of six semester courses taken in the first two years of college. The central role of mathematics as a universal language of science is emphasized throughout. The integrated science program is proving to be excellent preparation for a very broad range of majors (and ultimately, careers). About $\frac{1}{2}$ the students major in some area of biology, about one-third in physics or computer science, with the remainder scattered over the remaining scientific disciplines and engineering. The curriculum is especially valuable for students interested in bridging the traditional barriers between the biological and the physical sciences.

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Beyond the cookbook: a rigorous, research-based lab course for all.

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Introductory laboratory courses at large research universities often suffer from a "cookbook" methodology, insufficient focus on the scientific method, and lack of faculty involvement, all of which reinforce the perceived schism between research and teaching. We have undertaken a complete revision of the Stanford University introductory biology laboratory course, taken by 250 students per year, with the aim of providing students a rigorous, research-based experience that addresses an important question and uses modern research tools.

Based on a previous experience with a lab course of more limited size, we assessed that the target sophomore-level students are best engaged by research problems relevant to human biology, but are best able to accomplish research using a model organism. We chose to use yeast cells to assess the phenotypic defects of mutant alleles of the human p53 tumor suppressor derived from tumors. Over the 10-week quarter, students work as pairs in small lab sections. They assess the location of their mutation on the p53 structure and make testable hypothesis about the possible defect based on p53-related background information. The students assay transcriptional activation of reporter genes in yeast, test the level of their p53 mutant protein, assay DNA binding in vitro, and evaluate nuclear localization. In some experiments students can customize the assay for their mutant (e.g. incubation temperature, transcriptional reporter). In all experiments, the instructors stress the importance of controls and the power of comparing data with other students working on the same p53 mutant allele. The course culminated with a group poster session, again stressing interaction and discussion.

Although there is great variability in experimental skill levels, most students were able to generate data that, when considered in aggregate with all groups working on the same allele, led to a consistent model for the defect associated with that allele. We plan to engage course students in summer research projects to generate complete and validated data that can be submitted for publication.

Revision of a large, existing laboratory course presents particular challenges. We developed the course over a two-year pilot period, run concurrently with the existing course, with a smaller number of students. We used assessment during the pilot period to direct changes to the course. Most satisfyingly, students in the pilot course identified aspects of authentic research such as data analysis and analytical thinking as the most important aspect of the course at the end of the quarter, concepts that were largely missing from the previous course.

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The Genomics Education Partnership: An undergraduate team research experience.

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Research into how students learn science has confirmed that inquiry-driven research experiences that address important biological questions are more successful at producing scientifically literate students with good critical thinking skills than traditional content-driven laboratory classes. The Genomics Education Partnership (GEP), a consortium of faculty from over 90 colleges and universities with different characteristics and missions, uses comparative

genomics to engage students in research within the regular academic year. GEP curriculum has been adapted to many different settings, from a short module in a genetics course to the core of a laboratory course. The current GEP research project focuses on the evolution of the Muller F element (a largely heterochromatic domain) in five *Drosophila* species (*D. melanogaster*, *D. erecta*, *D. ananassae*, *D. grimshawi* and *D. mojavensis*). GEP undergraduates work to improve the draft genomic sequences and/or annotate these improved sequences. More than 4.2 million bases of draft sequence have been improved and hundreds of gene models produced using evidence-based manual annotation, leading to student poster presentations and collaborative publications in the scientific literature. The flexibility in the way GEP materials can be used means that each faculty member can adapt the materials in a manner that best suits his or her unique pedagogical goals. Anonymous surveys of GEP faculty and students show a high level of satisfaction with this active-learning approach. GEP students show similar self-reported gains in scientific problem solving and data assessment when compared to students who have participated in traditional summer research, as well as comprehension gains on a quiz about genes and genomes. The GEP pedagogical model offers an exciting opportunity for institutions with limited genomics resources and/or expertise to engage undergraduates in a research experience that will more fully prepare them for future careers in the life sciences. We invite additional faculty to join us (no previous training in bioinformatics required; see <http://gep.wustl.edu>; training workshops will be held summer 2013). Supported by HHMI grant # 52005780 to SCRE.

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The flip-side of integrating research and teaching: the research laboratory as a classroom.

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Undergraduate independent research projects provide opportunities to aid student growth towards the independence required for a professional career. In addition to producing publishable work, the goal of an independent study project is to help students transition from viewing knowledge as something that is provided to realizing that they can participate in the analysis, interpretation, and creation of new knowledge. Just like any other skill, the expertise required to become an effective scientist is learned. However, students accustomed to traditional courses can struggle when faced with less structured experiences like independent study. Even if a student is receiving course credit for an independent project, traditional coursework can seem more important because of the internal checkpoints (exams and assignments) by which a student can easily gauge success. Additionally, since the emphasis for independent research projects is often placed on the actual doing of experiments, students can be left without a proper understanding of the preparation, effort, and “skills away from the bench” required of an effective researcher. Here we describe student-centered approaches designed to aid students in transitioning from traditional coursework to independent research by overlaying a structure onto independent study. Projects are built around a formal syllabus. They also contain specific mechanisms to assess student progress, generating feedback useful in improving the experience for all involved. From the onset of the project, student involvement is key to developing an individualized plan that is tailored to the goals of both mentor and student. Written materials relevant to working as a scientist (such as searching the scientific literature, presenting one’s own research, and keeping a lab notebook) are provided to supplement oral instruction. To introduce students to the idea of external review and accountability, a portion of their final grade comes from an external reviewer. Assessments in the middle and at the end of the experience allow for the students and mentors to revise and improve the project for the next iteration. Student feedback indicates that participants have a realistic understanding of the

complexity of the scientific endeavor, beyond what is necessary to complete their specific experiments. Those that choose to enter graduate programs are well prepared--those that have chosen other careers have developed a skill set that will help them to be successful professionals.

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Rising to the challenge of 'Vision and Change in Undergraduate Biology Education'.

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In 2009 AAAS published a call for reform in undergraduate biology education based on several guiding recommendations: Integrate Core Concepts and Competencies throughout the Curriculum, Focus on Student-Centered Learning, Promote a Campuswide Commitment to Change, and Engage the Biology Community in the Implementation of Change. The College of Biological Sciences at the University of Minnesota-Twin Cities has established courses and programs to meet several aspects of the recommendations found in the Vision and Change report. We provide coursework that emphasizes student-centered learning, problem solving, team responsibilities, and core concepts and competencies both at a foundational biology and in some upper division courses, including cell biology. Other instructors are noticing the increased preparation and critical thinking skills of students who have participated in student-centered courses, and graduates of these courses report they are better prepared for upper division work, lab research and graduate school than students in traditional lecture courses. We established a series of Conversations on Teaching and Learning as one element of a commitment to creating a community of biology scholars dedicated to improving undergraduate courses and contributing to the teaching and learning literature. We also have begun a Scientific Teaching Program for advanced graduate students and postdocs in biology as a way to introduce them to principles of effective teaching that include classroom diversity, active learning and assessment, as informed by current literature. All participants practice what they have learned by working on a team to create a small instructional module in their sub-discipline. As current or former teaching assistants, they typically recognize the power of this education reform and are eager to put its principles into action as the next generation of biology educators.

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A science research network: Analysis of the undergraduate experience.

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We are studying a network of undergraduate institutions collaborating in biological research to examine the impact of network participation on undergraduate researchers. Specifically, we aim to: (1) identify and characterize the individuals (i.e., "actors") with whom undergraduates interact as they conduct their research; (2) characterize the nature and function of these interactions; and (3) determine the extent to which undergraduates use the network as a bridge to form connections with new individuals in the broader scientific community.

Actors in the network include faculty principal investigators, undergraduates, and postdoctoral researchers. Surveys and interviews are our primary data collection strategies, which are designed to determine with whom undergraduates interact and their motivations for interacting, as well as the strengths, functions, and outcomes of those interactions. For each potential dyad (i.e., pair of people in the network), we asked respondents to report on the existence of interactions, and their perceptions of the importance and influence of each interaction. For the five relationships they indicate as most important, we asked interviewees to report mode,

frequency, and content of the interactions (e.g., technical, intellectual, professional, educational, personal). We also collected network emails, network wiki posts, and other network records, which we will analyze to corroborate or refute inferences made from interview data.

We are conducting a systematic content analysis of interview data and social network analysis of survey data to address the following research questions: (1) What kinds of relationships do undergraduates form and which do they consider the most important or influential? (2) To what degree is networking beyond the lab group or institution viewed as central versus tangential to undergraduates' day-to-day research experience? (3) To what extent do undergraduates' interactions with different actors reflect theoretically predicted variables related to social capital, such as brokerage (serving as a "go between"), cultural capital (demonstrating values or norms within the research group or scientific community), mentorship (forming a relationship toward the undergraduate's professional and personal development), rapport (experiencing an affinity or connection), and reciprocity (engaging in an exchange that reinforces mutual obligation)?

Results from these analyses will serve as a foundation for developing hypotheses of how participating in distributed, collaborative science research influences undergraduate researchers. These results will also lay a foundation for exploring how research networks change, especially from an undergraduate perspective.

Minisymposium 5: Molecular Motors

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RNA on the move: Single molecule reconstitution of *ASH1* mRNA transport by a class V myosin from budding yeast.

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Myo4p, one of two class V myosins from budding yeast, can continuously transport and asymmetrically localize more than 20 different mRNAs to the bud tip via actin cables. *ASH1*, the most well-studied localizing mRNA, represses mating-type switching in the daughter cell. Here we reconstitute *ASH1* mRNA transport at the single molecule level using TIRF microscopy. We previously showed that when two single-headed Myo4p/She3p motors are recruited by the tetrameric mRNA binding protein She2p, a processive complex is formed (Krementsova et al., JCB 195:631, 2011). She3p is a tightly bound subunit of Myo4p that also binds She2p. Here we increase the complexity of the system by adding Alexa-labeled mRNA cargo to purified motors and She2p near physiological ionic strength (140 mM KCl). At this salt concentration, movement is only seen in the presence of mRNA. Cargo thus stabilizes the ternary complex, which provides a necessary checkpoint to ensure that only cargo-bound motors move processively in the cell. Native *ASH1* mRNA has four different localization elements, or "zipcodes", each of which can bind She2p. To understand why localizing transcripts have multiple zipcodes, we altered zipcode number and type. The most striking difference was an increase in the frequency of motor movement on actin with increased zipcode number, suggesting that a multi-zipcode transcript more effectively recruits a pair of motors. Increasing protein and mRNA concentrations to force zipcode occupancy further enhanced frequency and run length, as expected for motion driven by multiple motors. Metal-shadowed images of motor-mRNA complexes showed as many as eight motor heads (4 dimers) bound to a single native *ASH1* mRNA. These images suggested that the full potential for transport may not be realized on

single actin filaments. A comparison of movement of native *ASH1* mRNA on single filaments versus actin bundles showed this was true: run length and frequency were both significantly higher on actin bundles, which mimic the actin cables found *in vivo*. These studies highlight how building complexity *in vitro*, by using full-length motors coupled to their native cargo, is essential to fully understand at a mechanistic level how these motors function within the cell.

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Characterization of dynein by single-molecule investigations *in vivo*.

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Cytoplasmic dynein is a motor protein that exerts force on microtubules and in doing so, drives a myriad of intracellular activities including mitotic spindle positioning and chromosome movements in meiotic prophase. To generate these forces, dynein needs to be anchored, with the anchoring sites being typically located at the cell cortex. The key question is: By what mechanism do single dyneins accumulate at sites where they can generate large collective forces? Here, we directly observe single dyneins in fission yeast, which allowed us to identify the main steps of the dynein binding process: (i) from the cytoplasm to the microtubule, and (ii) from the microtubule to the cortical anchors Mcp5. We uncovered that dyneins on the microtubule move, surprisingly, either in a diffusive or a directed manner, with the switch from diffusion to directed movement occurring upon binding of dynein to the cortex. This was further confirmed by abolishing the cortical-binding ability of the anchor (Mcp5-PH Δ). We were thus able to force dynein on the microtubule bound to Mcp5-PH Δ to switch to directed movement, providing further evidence to the idea that dynein is 'activated' upon binding to its anchor. This dual behavior of dynein on the microtubule, together with the two steps of binding, constitute the mechanism by which dyneins find cortical anchors in order to generate large-scale movements in the cell.

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Single molecule fluorescence and optical trapping applied to molecular motors: Two can do it better than one.

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Kinesin and dynein are molecular motors that move in opposite directions on a microtubule. They often act on the same cargo, causing the cargo to frequently switch direction. Whether this back-and-forth motion results from a coordinating complex or from a tug-of-war between the two motors is currently unknown. We have applied single molecule fluorescence to determine that they are undergoing a synergistic tug-of-war. By synergistic, we mean that the combination of the two motors is able to bypass roadblocks along the microtubule. Furthermore, using an in vivo optical trap, and by comparing directional stall forces in vivo and in vitro, we found when cargo is going in the positive microtubule direction, kinesin and dynein are pulling, with the dynein walking backwards. The net stall force equals the stall force of kinesin (≈ 7 pN) minus the stall forces of the number of dyneins ($1.1 \text{ pN} \times \text{ND}$, where ND, = 0 to 6). When moving in the negative microtubule direction, the stall force is just equal to a multiple of dynein's stall force ($1.1 \text{ pN} \times \text{ND}$), implying that kinesin has fallen off the microtubule.

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Molecular adaptations in dynein for generation of large forces inside cells.

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The unit generator of force inside living cells is often a motor protein that exerts a tiny force of 1-10 picoNewtons. However, many processes in cells require much larger forces and must therefore rely on motors working in a team. How collective force is generated by such teams of motors is fundamentally important, but has evaded understanding. To investigate this, we develop a new model system for optical trapping directly inside living cells that overcomes certain caveats and technical hurdles with earlier in vivo force measurements. This allows us to measure the function of endogenous cellular motor-assemblies with a precision comparable to in vitro bead assays.

We make the paradoxical observation that weak dynein motors team up to generate very large forces whereas stronger kinesins fail as a team. We show that dynein's improved teamwork arises from this motor's unique ability to vary stepsize in response to load, i.e. to shift gears (Mallik et al, Nature 2004). Each dynein motor in a team speeds up or slows down, depending on the load it experiences. Dyneins therefore bunch closer together when hauling load, and consequently share load better to work efficiently as a team. We provide multiple independent lines of experimental evidence to support this hypothesis, and bring out precise mechanistic details of this process.

These results connect single-molecule properties of motors to their biologically relevant cellular functions, and suggest that nature has designed different motor proteins to adapt them for specific functions. More specifically, our work shows why dynein, the only known geared motor, needs a gear inside cells.

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A novel split kinesin assay to identify the motor proteins that interact with distinct vesicle populations

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An essential step for understanding the regulation of kinesin-driven vesicle transport is to identify the kinesins that move specific vesicle populations. Previous studies using biochemical strategies have shown interactions between kinesins, kinesin adaptors, and putative cargo proteins, but it has been difficult to confirm that these interactions are required for vesicle transport by direct assays in living cells. To elucidate all of the kinesins that interact with a given vesicle population we developed a novel imaging-based assay. Our approach involves expression of a “split-kinesin” consisting of separate constructs encoding a kinesin tail and a kinesin motor domain that can be assembled using chemical heterodimerization. The kinesin tail contains the cargo binding domain and can bind vesicles, but it is incapable of influencing their movement because it lacks a motor domain. The motor domain is constitutively active and moves along microtubules, but cannot move vesicles because it lacks a cargo binding domain. When these two components are linked together using a rapamycin homolog paradigm, their assembly leads to a rapid and profound change in the trafficking pattern of only the vesicles that bind the expressed kinesin tail domain. By expressing a series of all relevant kinesin tails, all of the kinesins capable of binding the vesicles of interest can be identified.

We used this technique in rat embryonic fibroblasts to identify members of the Kinesin-3 family that are present on endosomes and lysosomes. Interestingly, different members of this family are associated with different vesicle populations: KIF13A and KIF13B are present on early endosomes, while KIF1A and KIF1Bbeta are present on late endosomes and lysosomes. We also showed that in some cases two different members of the Kinesin-3 family are present on the same vesicle at the same time.

In cultured hippocampal neurons, we used this strategy to identify three members of the Kinesin-3 family that mediate the selective transport of dendritically polarized cargoes. Intriguingly, two of the three dendritic kinesins also mediate the axonal transport of other vesicle populations, confirming the hypothesis that kinesins can be steered into the axon or the dendrites depending on whether they interact with an axonal or dendritic cargo.

We believe that this method could be broadly utilized for studying motor protein-vesicle interactions in a variety of cell types.

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Kinesin-mediated microtubule sliding drives axon outgrowth in *Drosophila* neurons.

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How neurons extend axons is a key long-standing question in neuronal cell biology. Recently, our lab revealed a novel role of the microtubule motor kinesin-1 in driving microtubule-microtubule sliding and formation of long processes in tissue culture cells. Here, we use *Drosophila* embryonic neurons as a model system to study whether a similar mechanism operates in axon outgrowth. By using transgenic flies that carry a photoconvertible tubulin, EOS- α tub, we are able to track microtubule dynamics at different stages of embryonic neuron development in culture. We observe that microtubules are very actively sliding in young developing neurons. Within a few minutes, microtubules photoconverted within a small area are found to be distributed throughout the full neuron by sliding. Furthermore, these sliding microtubules push directly against the cytoplasmic membrane of the growing neurite tip and

very often become looped, suggesting that sliding microtubules generate mechanical force for axon extension during neuronal development. In mature, fully-developed neurons, microtubules become very stationary, and very little sliding can be detected. The strong correlation between microtubule sliding and axon growth rate strongly suggests that microtubule sliding can contribute to axon growth. Consistent with these findings, we demonstrate that neither actin depolymerization nor inhibition of microtubule polymerization blocks axon extension at initial stages of neuronal development. Finally, we have data to show that kinesin-1 powers microtubule sliding, which is important for axon growth in young neurons. These results reveal a novel mechanism driving initial axonal outgrowth in *Drosophila* neurons. Due to the high evolutionary conservation of kinesin-1, a similar mechanism may function in other neurons.

Minisymposium 6: Regulation/Organization of the Genome

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In vivo single-molecule biochemistry of chromosome replication and segregation in bacteria.

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By using a range of live cell imaging techniques that allow us count, track, position and assess the turnover of individual components of the replication and segregation machines in *Escherichia coli*, we have established new insight into the action of these molecular machines. Furthermore, by being able to deplete or replete rapidly components of these machines in vivo, we observe the consequences of these changes to cellular and chromosome behavior. The SMC complex MukBEF, TopoIV and FtsK act in *E. coli* chromosome segregation. MukBEF accumulates in foci containing on average 10 dimer of dimer complexes, consistent with them being ATP-bound. The foci typically position at midcell and/or the cell quarter positions, in the same region as the replication origins, with the positioning being cell cycle and replication independent. The dwell time of individual complexes in these foci is ~50 s. Because Muk- cells are defective in chromosome segregation and have their origins mispositioned, we propose that MukBEF acts in chromosome segregation by correctly positioning origins and other loci. MukBEF interacts with TopoIV in vivo to target it to the replication origins, where it may preferentially act in the unlinking of newly replicated sisters.

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Elucidating chromosomal organization and dynamics in bacterial cells using fluorescence microscopy and theoretical modeling.

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A cell packages its DNA within a cellular compartment that is orders of magnitude smaller than the DNA's unconfined radius. In this state, chromosomal DNA is accessed by regulatory proteins and transiently forms looped regulatory complexes. Thus, the fundamental physical mechanisms that dictate the organization and the dynamic motion of chromosomal DNA play an important role in a range of biological processes. Our research combines experimental imaging of chromosomal DNA and theoretical modeling to reveal the in vivo chromosomal behavior within *E. coli* and *Caulobacter crescentus*. Tracking of fluorescently labeled chromosomal loci in live *E. coli* cells reveals a robust scaling of the mean square displacement as $\tau^{0.39}$. Brownian dynamics simulations show that this anomalous behavior cannot be fully accounted for by the classic Rouse or reptation models for polymer dynamics. Instead, the observed motion arises from the characteristic relaxation of the Rouse modes of the DNA polymer within the viscoelastic environment of the cytoplasm. To demonstrate these physical effects, we exploit our general analytical solution of a polymer embedded in a viscoelastic medium, which exhibits remarkable agreement with our in vivo measurements. We then experimentally demonstrate a strong dependence of the motion on ATP hydrolysis, suggesting a critical impact of biologically driven fluctuations on in vivo dynamics. We then turn to *Caulobacter crescentus* to determine the structural organization of chromosomal DNA by assessing the distribution of distances between two loci. The distance between two fluorescently labeled DNA loci of various inter-loci contour lengths n is measured in *Caulobacter crescentus*. For DNA segments less than about 300 kb, the mean inter-loci distance $\langle r \rangle$ scale as $n^{0.22}$, and cell-to-cell distributions of the inter-loci distance r is a universal function of $r/n^{0.22}$ with broad cell-to-cell variability. For DNA segments greater than about 300 kb, the mean inter-loci distances scale as n , in agreement with previous observations. Predictions from Brownian dynamics simulations of the packing of supercoiled DNA polymers in an elongated cell-like confinement are consistent with our experimental results, and simulated inter-loci distance distributions predict that confinement leads to "freezing" of the supercoiled configuration. We conclude that the chromosome structure is supercoiled locally and elongated at large length scales and that in vivo crowding prevents the chromosome from reaching an equilibrium arrangement. We show that the structural "freezing" of the *Caulobacter* chromosome is consistent with the dynamic measurements within *E. coli* by determining the length-dependent relaxation times of chromosomal DNA.

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Single molecule dynamics governing the initiation of antigen-receptor gene assembly.

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Immunological memory is a hallmark of jawed-vertebrate evolution. It is controlled by antigen-receptors that directly bind the molecules committed to memory. Antigen-receptor gene assembly or variable, diversity and joining (VDJ) recombination is a DNA rearrangement process that generates trillions of antigen-receptors during the development of the adaptive

immune system. The repertoire of receptors are partitioned amongst two cell-types, B lymphocytes carrying antibodies or immunoglobulins and T lymphocytes equipped with T cell receptors. The macromolecular machine that carries out the assembly reactions is aptly named the V(D)J recombinase, and consists of recombination activating genes (RAG) 1 and 2. The RAG proteins bind and recombine two conserved recombination signal sequences (RSS) flanking V, D and J gene segments that have a nonamer, a spacer of 12 or 23 bps and a heptamer. However, *in vitro* studies on RAG1 and RAG2 have been extraordinarily difficult, and as a result RAG biochemistry has remained in a stasis. The current state of RAG biochemistry is bolstered by fragmented successes on RSS-encoded oligonucleotide DNA substrates, limiting a comprehensive understanding of V(D)J recombination on biologically relevant DNA length scales, and previous studies conducted on long DNA substrates are at best phenomenological. To ameliorate this issue, we developed a single molecule assay to capture the entire range of behavior exhibited by RAG1/RAG2 and accessory DNA bending high-mobility group protein (HMGB1) in real-time. We observed the V(D)J recombinase binding and bending RSS-encoded long DNA substrates with single or multiple RSSs. We knocked out binding by the V(D)J recombinase using mutations in RSSs that were previously shown to ablate recombination in B lymphocytes. We directly observed cooperative binding and bending by the V(D)J recombinase and HMGB1 in the presence of one or more RSSs and we uncovered strict concentration thresholds and RSS-spacer requirements for robust cooperativity and dynamic bending. Our novel single molecule system is poised to elucidate the dynamics that go into every trillionth reaction carried out by the V(D)J recombinase.

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Theoretical model of cooperative binding in heterochromatin formation.

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Gene regulation in eukaryotes requires the segregation of silenced genomic regions into densely packed heterochromatin, leaving the active genes in euchromatin regions more accessible. We introduce a model that connects the presence of epigenetically inherited histone marks, methylation at histone 3 lysine-9 (H3K9), to the physical compaction of chromatin fibers via the binding of heterochromatin protein 1 (HP1).

We generate optimized chromatin-fiber structures from a model based on the mechanical properties of the DNA and the steric interactions of the DNA and nucleosomes. These structures, which show qualitative agreements with 30nm fibers found from *in vitro* experiments, determine the spatial position of the H3K9 sites¹. In our binding model for HP1, these sites can be either unmethylated or tri-methylated; tri-methylated H3K9 appears to be a critical epigenetic signal for heterochromatin formation. We show how the spatial arrangement of these sites leads to heterogeneous bridging of HP1 between sites, affecting the cooperative binding of HP1 molecules. We consider binding both to single fibers as well as multiple fibers packed in arrays similar to possible *in vivo* conditions.

Our model demonstrates some of the key physical features that are necessary to explain several experimental observations, including the phase segregation of heterochromatin regions and the increased HP1 concentration in these regions². In particular, we find that strong cooperative interactions among the HP1 proteins are necessary to see the phase segregation of heterochromatin and euchromatin regions. From *in vitro* experiments on HP1 binding³, we calculated the binding energy difference between the unmethylated and tri-methylated states to be small (less than 2kT). This small difference allows the cell to use physiologically realizable

concentrations of HP1 to condense highly methylated regions at higher concentrations and decondense these regions at lower concentrations. Finally, we explore under what circumstances there is a threshold of methylation over which the fibers will compact; this threshold provides a buffer against small losses of methylation such as those that occur during cell division⁴. Many of the observations that we make about the HP1 system are guided by general thermodynamics principles and thus could play a role in other DNA organizational processes such as the binding of linker histones.

¹Koslover, Elena et al. (2010) *Biophys J*, 99.

²Muller, Katharina et al. (2009) *Biophys J*, 97.

³Canzio, Daniele et al. (2011) *Molec Cell*, 41.

⁴Xu, Mo et al. (2012) *EMBO Reports*, 13.

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An octameric CENP-A nucleosomal population is present at the human centromeres throughout the cell cycle.

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Centromeres are chromosomal loci upon which the mitotic kinetochores form to mediate faithful inheritance of genetic material to the daughter cells. A key feature of centromeres is the replacement of canonical histone H3 with the histone variant Centromere Protein A (CENP-A) in centromeric nucleosomes. Presence of CENP-A is presumed to mark the centromeric identity via an epigenetic mechanism. Recently, debate over the composition of CENP-A-containing nucleosomes has been intensified by conflicting observations proposing several models and dynamics for CENP-A nucleosomes. These models vary in terms of the identity and quantity of the components, number of CENP-A molecules, nucleosomal dimensions and DNA wrapping. Thus, we aimed to address the composition of CENP-A nucleosomes by developing a high-resolution single molecule imaging method using in-vivo assembled nucleosomes. Total internal reflection fluorescence microscopy (TIRFM) was coupled to a single nucleosome-immunoprecipitation technique to visualize CENP-A nucleosomes. We then used a photobleaching-assisted copy number counting technique (PA-CNC) to determine the number of CENP-A-YFP molecules per individual nucleosome. In order to investigate the presence of other histones in CENP-A nucleosomes, we integrated indirect single-molecule immunofluorescence to our TIRFM assay. Our data suggest that CENP-A dimers and H2B molecules are found in a major population of CENP-A nucleosomes throughout the cell cycle. Characterisation of the secondary population with a single CENP-A molecule is currently underway. All together, our data suggest the presence of octameric CENP-A nucleosomes as a constitutive population throughout the human cell cycle.

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Structure of active, dimeric human telomerase.

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In eukaryotes, telomerase counteracts telomere erosion to prevent cellular senescence. Telomerase contains a large RNA subunit TER and a protein catalytic subunit TERT that reverse transcribes the TER template sequence into telomeric DNA. Whether the telomerase

functions as monomer or dimer has been a matter of debate. I will describe recent results that show that human telomerase contains two TERT subunits which both bind a telomeric DNA substrate, and that catalytic activity requires both TERT active sites to be functional, providing unambiguous evidence that human telomerase functions as a dimer. I will also present the first three-dimensional structure of active, full-length human telomerase dimer as determined by single particle electron microscopy in negative stain. This structure reveals the spatial relationship between RNA and TERT subunits, and hence provides important insights into the architecture of the telomerase enzyme.

Minisymposium 7: Signal Transduction/Signaling Networks

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Encoding cellular information through p53 dynamics in individual cells.

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Many signaling molecules exhibit complex dynamical behaviors, yet little is known how these dynamics affect cellular responses. In this talk I will focus on the dynamics of p53, a critical tumor-suppressive protein that controls genomic integrity and cell survival. The dynamics of the p53 response are different for different stimuli. Double strand breaks caused by gamma radiation, trigger a series of p53 pulses with fixed amplitude and duration. The number of pulses increases with higher damage. In contrast, UV triggers a single p53 pulse with a dose-dependent amplitude and duration. Comparison of the networks responding to these damages allowed us to identify specific molecular feedbacks that shape these distinct dynamical responses. Using a combination of single cell experiments and computational modeling, we identified a sequence of precisely timed drug additions that switch p53 dynamics from pulsatile into sustained in response to gamma radiation, and we tested the resulting behavior in cells. Our results show that different dynamical patterns of p53 alter the selection and timing of gene expression and perturb cellular outcomes. Pulsing p53 led to the activation of target genes associated with cell cycle arrest, DNA repair, and regulation of p53 itself. Genes associated with irreversible cellular fates, such as apoptosis and senescence, showed no induction in response to p53 pulses. In contrast, when p53 pulses were altered to produce a non-pulsing, sustained p53 signal, genes associated with apoptosis and senescence were induced. Our study shows that p53 dynamics affect cell fate decisions and suggests that perturbing protein dynamics in a controlled way can be a useful tool for pushing cells into a desirable outcome.

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Engineered manipulation of signaling networks: Novel control of kinase activation and interactions dissects parallel Src pathways.

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Spatio-temporal control of signaling is orchestrated by a complex network of interactions between scaffolding and enzymatic components. Current tools typically cannot activate a specific protein and simultaneously cause it to interact with one specific downstream target,

which would be of great value in dissecting the organization of signaling networks. Here we provide such a tool, and use it to determine the role of individual Src-mediated signaling pathways in stimulation of different cell morphological changes. Protrusive activity of a cell and its adhesion to extracellular matrix is regulated by Src via multiple interactions. Focal adhesion kinase (FAK) and p130Cas can bind and activate Src, propagating signals through parallel pathways. Localization of Src in the cytoplasm or at the plasma membrane also determines the function of Src. Thus, we focused specifically on activation of Src acting through FAK or through p130Cas, or at different locations (cytoplasm versus plasma membrane). An engineered, "insertable" FKBP12 protein (iFKBP) was introduced into the catalytic domain of Src, while FKBP12-rapamycin binding domain (FRB) was attached to the specific downstream effector. This rendered the kinase inactive until rapamycin, added to the extracellular medium, induced heterodimerization with FRB. Using this technology we restricted Src activation to the complex it formed with FRB-bearing downstream targets. Simple activation of Src, without targeting specific downstream molecules, led to cell spreading, reorganization of focal adhesions, and the production of filopodia and lamellipodial protrusions. Using the new approach, dubbed RapR-TAP, we showed that activation of Src specifically in complex with FAK led to focal adhesion rearrangement and only slow lamellipodial protrusion, while activation in complex with p130Cas led to rapid spreading and filopodia formation, but no apparent effects on focal adhesions. Comparing Src activation in the cytosol versus at the plasma membrane showed that membrane localization is necessary to stabilize protrusions, whereas cytoplasmic Src drives rearrangement of focal adhesions. The novel method presented here isolates specific differences between parallel Src-induced signaling events, and demonstrates the feasibility of a broadly generalizable strategy to activate specific kinase-mediated signaling pathways in living cells.

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Single-cell signaling dynamics reveal the logic of early response gene transcription driven by NF- κ B.

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NF- κ B is a family of dimeric transcription factors that regulate many genes— associated with inflammation, cellular proliferation and survival as well as carcinogenesis. In the absence of extracellular signals, NF- κ B exists in complexes with inhibitory I κ B proteins. The I κ B/NF- κ B complexes are actively exported from the nucleus and NF- κ B is therefore predominantly localized to the cytoplasm. Inducers of the canonical NF- κ B pathway promote rapid degradation of I κ B proteins, releasing NF- κ B. The unmasked nuclear localization sequence of free NF- κ B directs it to the nucleus, where it can access the promoter regions of its target genes. For this reason, the degree of NF- κ B pathway activation is often equated with the amount of nuclear NF- κ B even though the correlation between NF- κ B localization and the NF- κ B-dependent transcriptional response has not been determined in single cells. What does an individual cell 'read' from a translocation event? Does the transcriptional response reflect a simple ON/OFF switch, or is the response proportional to a specific aspect of this dynamic translocation event? In this work we examine the relationship between tumor necrosis factor (TNF)-induced NF- κ B translocation dynamics and the associated transcriptional response. We have developed a live-cell to fixed-cell workflow where we first image TNF-induced NF- κ B (p65/RelA) translocation dynamics and then count, in the same cells, the number of mRNA transcripts for NF- κ B target genes detected by single-molecule fluorescence in situ hybridization (smFISH). Correlating NF- κ B translocation dynamics to the number of transcripts for three rapidly induced NF- κ B-driven genes, we find that the quantity of nuclear NF- κ B at any single time point provides little information about its activation state. Instead, cells interpret changes in NF- κ B localization,

measuring fold-change of nuclear NF- κ B. Each cell retains a 'memory' of its pre-ligand state and evaluates translocation events relative to this previous state. We found that the relationship between fold-change of nuclear NF- κ B and transcription in individual cells could not be predicted from existing computational models of the NF- κ B pathway. These observations have led us to a new model that provides insight into the regulatory circuitry that quantitatively controls NF- κ B-dependent early response gene transcription. Our results strongly suggest that competitive protein-DNA interactions provide a 'memory' required for fold-change detection.

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Sharpin-dependent linear-ubiquitination of FADD is required for anti-apoptosis pathway.

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Ubiquitin is one of the most sophisticated and versatile post-translational modifications. Depending on how ubiquitin molecules are linked as chains and modify the substrate, they regulate different biological functions and cellular signals. Recent findings indicate that Sharpin plays a critical role in TNF- α -induced NF- κ B signaling by linear (Met 1-linked, tandem repeated, head-to-tail) ubiquitination of NEMO (NF- κ B essential modulator). Sharpin is key subunit of an E3 ligase complex, LUBAC (Linear Ubiquitin chain Assembly Complex), which specifically generates linear ubiquitin chains. The phenotype of Sharpin deficient Cpdm (Chronic proliferative dermatitis mice) mice shows heavy inflammation in multiple organs including skin, gut and esophagus. Interestingly, we observed drastic apoptosis in keratinocytes of Cpdm mice determined by cleaved Caspase 3 expression. We have reported that TNF- α -induced apoptosis in Cpdm MEFs is controlled through Caspase 8 and FADD both of which are essential components of DISC (death-inducing signaling complex). However, it remains unclear how mechanistically Sharpin regulates apoptosis signaling pathway. We found that FADD is ubiquitinated by LUBAC in vitro. Moreover, TNF- α -induced apoptosis in Cpdm MEFs was rescued by expressing FADD-linear di-ubiquitin chain chimera, strongly suggesting that Sharpin plays an important role in anti-apoptosis signaling by linearly ubiquitinating FADD. In this meeting, novel functions of Sharpin in regulation of anti-apoptosis signaling pathways will be presented.

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Regulation of mRNA export by PI3 Kinase / AKT signal transduction.

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Regulation of the nuclear export of specific mRNAs could be an important mechanism for modulating translatable, cytoplasmic levels of messages. This could be especially important in regulating the expression of genes that have mRNAs with short half-lives in the cytoplasm. UAP56, ALY/REF, and NXF1 are mRNA export factors that sequentially bind at the TRanscription/EXport (TREX) complex located at the 5' end of a nuclear mRNA. NXF1, recruited last to the complex, may subsequently bind at nuclear pores for export of the mRNP complex to the cytoplasm. These three mRNA export proteins are also reported to associate with the Exon Junction Complex (EJC) that forms 20-24 nucleotides upstream of each exon-exon junction during splicing and then remains with the processed mRNA. We used Fluorescence Recovery after Photobleaching (FRAP) as a screen to identify signal transduction pathways that regulate the binding of mRNA export and EJC core proteins in macromolecular complexes at RNA splicing speckled domains, and at an integrated chromosomal array of actively transcribed β -globin transgenes arranged in tandem. The fraction of UAP56, ALY/REF, and NXF1 tightly bound in complexes was reduced by drug inhibition of the PI3 kinase / AKT branch of the phosphatidylinositol signal transduction pathway, as was the tightly bound fraction

of the core EJC proteins eIF4A3, MAGOH, and Y14. Inhibition of the mTOR mTORC1 pathway, which can be activated by AKT, also decreased the tight binding of MAGOH. Inhibition of the AKT pathway increased the nuclear export of bulk poly(A) RNA and of a subset of spliced mRNAs. A similar increase in export after AKT inhibition was observed for sets of mRNAs transcribed from both intron-containing and intron-less histone genes. Taken together, our results show that the PI3 kinase / AKT signal transduction pathway regulates mRNA export complex assembly, affecting the rate of nuclear export and the extent of nuclear retention for specific mRNAs.

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Systematically mapping the sequence space of a protein-protein interface.

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Signal transduction pathways rely on transient yet specific protein-protein interactions. How a limited set of amino acids can enforce cognate protein interactions while excluding undesired pairings remains poorly understood, even in cases where the contacting residues have been identified on both protein partners. To tackle this challenge, we developed a systematic mutagenesis approach to examine all possible combinations of residues that support a protein-protein interaction in a bacterial two-component signaling pathway. These pathways, typically consisting of a histidine kinase and a cognate response regulator, are an ideal model system for studying protein-protein interactions as they rely almost exclusively on molecular recognition for specificity. The kinase uses four key residues to recognize the regulator in both phosphorylation and dephosphorylation reactions, and to prevent docking with all non-cognate regulators. To better understand this specificity, we comprehensively mapped the sequence space of the interface formed by *Escherichia coli* histidine kinase PhoQ and its partner PhoP. We developed a robust high-throughput assay for assessing kinase and phosphatase activity *in vivo* using fluorescence-activated cell sorting. We then used this assay to screen a library of 20⁴ (160,000) PhoQ variants in which we completely randomized the four key specificity-determining residues. Using deep sequencing, we identified ~2,500 variants that can phosphorylate and dephosphorylate PhoP as well as the wild-type PhoQ. Strikingly, PhoQ can interact with PhoP via many sets of interfacial residues that are completely different from the wild type. This combinatorial approach to mapping sequence space also reveals interdependencies between individual amino acids. For instance, although the introduction of a bulky residue into wild-type PhoQ typically disrupts the interaction with PhoP, certain changes to neighboring residues can compensate and restore interaction. Conversely, we find examples of individual mutations that are tolerated by wild-type PhoQ, but which disrupt the signaling pathway when combined. These findings highlight the power of comprehensively mapping sequence space relative to screens that only examine substitutions at individual sites. We are currently characterizing the fitness of signal-responsive mutants relative to the wild type and assessing whether these variants have other, detrimental consequences for the cell.

Minisymposium 8: Stem Cells and Induced Pluripotency

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BMP-SMAD-ID signaling axis supports reprogramming to pluripotency.

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Reprogramming somatic cells to pluripotent stem cells represents an exciting new paradigm in biology and medicine. We developed a technology to generate induced pluripotent stem cells (iPSCs) by transducing defined factors, such as OCT4, SOX2, KLF4, and C-MYC, into somatic cells. These defined factors are transcription factors that regulate gene expression important in self-renewal and pluripotency; however, the efficiency of iPSC generation remains very low. Cytokine-induced cell signaling can affect the efficiency of iPSC generation, but the precise effects and mechanisms required for the reprogramming are still unclear. We hypothesized that the activation of BMP-SMAD signaling pathway enhances the efficiency of iPSC generation. We found that the efficiency of iPSC generation from the human dermal fibroblasts (HDFs) obtained from patients of fibrodysplasia ossificans progressiva (FOP) with the R206H mutation in ACVR1 gene, which leads to hyperactivation of BMP-SMAD pathway, was much higher than that of normal HDFs. The Inhibition of BMP-SMAD signaling by adding Dorsomorphin or LDN-193189 or by transducing SMAD6 or SMAD7 to FOP-HDFs decreased the efficiency of iPSC generation. Conversely, the activation of BMP-SMAD signaling by transducing mutant ACVR1 (R206H) or SMAD1 or by adding BMP4 recombinant protein to normal HDFs in specific period of reprogramming increased the efficiency of iPSC generation. Furthermore, inhibitor of DNA binding (ID) genes, which are direct targets of BMP-SMAD signaling, increase the efficiency of iPSC generation from normal HDFs and are required for iPSC generation. Our results indicate that the BMP-SMAD-ID axis is critical for efficiently generating iPSCs. In addition, our findings suggest that human genetic diseases such as FOP may favor more efficient iPSC generation, and that these pathways could provide methods for improving the efficiency of iPSC generation.

Direct lineage reprogramming to generate neural cell types.

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Cellular differentiation and lineage commitment are considered robust and irreversible processes during development. We hypothesized that expression of neural lineage-specific transcription factors could directly convert fibroblasts into neurons and identified a combination of only three factors that suffice to rapidly and efficiently convert mouse fibroblasts into functional neurons in vitro. These induced neuronal (iN) cells expressed multiple neuron-specific proteins, generated action potentials, and formed functional synapses.

One of the outstanding question was whether a defined non-ectodermal cell can be converted into iN cells given the heterogeneity of fibroblast cultures. We therefore tested whether (endoderm-derived) hepatocytes can be reprogrammed to iN cells. Surprisingly, using the same 3 transcription factors primary mouse hepatocytes could be converted very efficiently into iN cells with the ability to generate action potentials upon depolarization as well as to form synaptic contacts with primary neurons. A genetic lineage-tracing system was employed to prove that the cell of origin of iN cells were indeed endodermal cells. Moreover, gene expression studies on the global and single cell level confirmed not only the induction of a neuronal transcriptional program but also the efficient silencing of the hepatocyte-specific expression pattern. This transcriptional silencing was extensive but not complete on both population and single cell level. We therefore conclude that iN cells are not a hybrid cell with equal identities of the starting cell and neurons, but cells with a predominant neuronal identity with an epigenetic or transcriptional memory of the starting cell population.

Finally, we reasoned it would be desirable to generate a proliferative neural precursor cell population from fibroblasts given the limitations of expandability of iN cells. We followed a similar strategy that was successful to identify the iN cell factors and screened 11 factors to eventually identify Sox2 and FoxG1 as critical factors that are sufficient to induce neural precursor cells (iNPCs) with the potential to differentiate into neuronal and astroglial, but not oligodendroglial cell types. The addition of Brn2 conferred the ability to differentiate also into oligodendroglial cells. When Sox2 was omitted from the combination iNPCs were generated with differentiation potential into both glial cell types but the neuronal differentiation was less robust with the generation of only immature neuronal cells. However, when transplanted into the myelin-deficient shiverer mouse brain, the cells incorporated well into the white matter tracts, differentiated into oligodendrocytic cells expressing an array of lineage specific markers including Olig2, O4, and myelin basic protein and ensheathed host axons.

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Regulation of adult neurogenesis by c-myc.

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Stem cells play an important role not only during development but also in maintenance of the adult organisms. Adult neurogenesis is a process through which new neurons are born in the adult brain with potential links to learning and memory as well as neurological disorders. Neurogenesis takes place in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. In the hippocampus, newborn neurons mature to become excitatory granule cells that integrate into the existing circuitry. Adult neurogenesis is driven by relatively quiescent radial glia cells that infrequently divide and give rise to a proliferative cell population, most of which differentiate to adopt a

neuronal cell fate. While the cell types involved in adult neurogenesis have been relatively well-characterized, we are far from a full understanding of the underlying molecular mechanisms. The highly conserved transcriptional regulator c-myc is an essential for development and can also act as a proto-oncogene. Only a minority of studies on c-myc has been carried out in normal adult stem cells. In general, a clear picture of c-myc function remains to emerge, due to context-dependence, overlapping expression patterns with other myc family members, weak transcriptional activator function as well as low expression levels in adult cells. c-myc is involved in diverse processes such as cell growth, proliferation, apoptosis and differentiation: phenomena intimately linked to neural stem cell biology. We have hypothesized that c-myc may have a function in early steps of adult neurogenesis.

We first confirmed expression of c-myc in the adult dentate gyrus as well as cultured adult hippocampal progenitor cells (AHPs). c-myc bound to its known transcriptional targets and regulated their expression in AHPs, suggesting that c-myc was functional in this context. Expression of c-myc was downregulated upon differentiation and knockdown of c-myc led to enhanced differentiation, whereas overexpression of c-myc inhibited differentiation of AHPs. Retroviral overexpression of c-myc in the adult hippocampus led to an early increase in proliferation followed by an inhibition of subsequent differentiation. Interestingly, further analysis suggested that c-myc might have a direct role in early differentiation. In summary, our data points to a novel role for c-myc in regulation of not only cell proliferation but also cell fate in adult neurogenesis. We will discuss possible mechanisms of c-myc function and its potential role in adult stem cell biology.

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Regulated subnuclear gene positioning restricts neural stem cell competence in *Drosophila*.

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Progressive loss of stem/progenitor competence to specify earlier-born cell fate is a fundamental process of development whose mechanisms are little understood. Using a combination of DNA fluorescent *in situ* hybridization (FISH) and immunostaining in intact *Drosophila* embryos, we visualized changes in genome architecture *in vivo* as neuroblasts (neural stem cells) undergo rapid (~1hr) transcriptional transitions within the developing central nervous system. We tracked the subnuclear position of the gene locus encoding the transcription factor Hunchback (Hb), which specifies early-born temporal identity. We found that the *hb* gene progresses through three states: transcriptionally active, transcriptionally inactive but inducible, and finally, permanently silenced. We found that when the *hb* gene is transcriptionally active or inducible, the *hb* gene locus is positioned in the nuclear interior, and the neuroblast is competent to specify early-born identity. In contrast, when the *hb* gene is permanently silenced, we found its locus is positioned at the nuclear lamina, a highly repressive domain. Strikingly, the timing of this repositioning correlates precisely with the time neuroblast competence is lost. When the nuclear lamina is disrupted, the *hb* locus is positioned away from the nuclear envelope, and the competence window, as defined by the duration *hb* is transcriptionally inducible, is extended. Furthermore, we identified a neuroblast nuclear factor, Distal antenna (Dan) whose expression perfectly matches the neuroblast competence window. Prolonging Dan expression prevented repositioning of the *hb* locus to the nuclear lamina and extended neuroblast competence, suggesting it may play a role in regulating gene architecture in neuroblasts. We propose that gene reorganization is a mechanism by which stem cell competence to specify particular fates becomes restricted during development. These data may provide insight into mechanisms that can be harnessed to reverse loss of stem cell competence for tissue replacement therapies.

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Mechanism of asymmetric division in adult stem cells.S. Srinivasan¹, M. T. Fuller¹; ¹Developmental Biology, Stanford University, Stanford, CA

Regulation of adult stem cell divisions confers regenerative capacity and plasticity in tissues. Stem cells can divide symmetrically, a mechanism used during development or after injury to expand cell numbers, or asymmetrically to maintain normal tissue homeostasis. A key mechanism in setting up the symmetric or asymmetric outcome of stem cell divisions is the programmed orientation of the mitotic spindle. The specialized microenvironment in which adult stem cells reside, the stem cell niche, may regulate stem cell divisions, and thereby tissue plasticity and regenerative capacity, by influencing the orientation of the mitotic spindle. *Drosophila* male germline stem cells (GSCs) normally divide asymmetrically, providing a system to study mechanisms regulating orientation of stem cell divisions in context of the niche. The hub, a component of the niche in this system, orients the centrosomes in GSCs during interphase and eventually the mitotic spindle to enable the asymmetric outcome of stem cell division by providing 1) spatial cues via E-cadherin mediated adherens junctions that maintain a centrosome adjacent to the hub-GSC interface throughout the cell cycle and 2) the Unpaired ligand that activates the JAK-STAT pathway in GSCs, which is required to maintain centrosome orientation. We have discovered that the forkhead domain containing transcription factor Jumu, a target of activated STAT, regulates asymmetric division in GSCs. Our data suggests that *jumu* function is not required for maintenance of GSCs at the hub via adherens junctions but is required for correct centrosome positioning in GSCs, suggesting that Jumu functions downstream of the spatial cues provided by the adherens junctions to orient centrosomes. We also discovered that the NuMA homolog *Mushroom body defect (mud)*, a target of Jumu in *Drosophila* embryos identified by the ModENCODE project, is expressed in GSCs and is required for proper centrosome orientation in GSCs. *mud* mutant GSCs exhibit normal adherens junctions between the hub and GSCs suggesting that Mud, like Jumu, functions downstream of the adherens junctions to orient centrosomes. This study demonstrates that a transcriptional network downstream of the signal from the niche regulates the outcome of adult stem cell division.

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BAF53A enforces the epidermal progenitor state by re-targeting the SWI/SNF/BAF chromatin remodeling complex away from differentiation gene promoters.X. Bao¹, J. Tang², V. Lopez-Pajares¹, S. Tao¹, K. Qu¹, G. R. Crabtree², P. A. Khavari¹; ¹Dermatology, Stanford University, Stanford, CA, ²Howard Hughes Medical Institute, Stanford University, Stanford, CA

Epidermal progenitors suppress expression of terminal differentiation genes. Recently, epigenetic mediators of DNA and histone modification have been identified as dynamically regulating such repression, including DNMT1, JMJD3, Polycomb, and HDAC1/2. These data indicate a role for multiple classes of epigenetic regulators in this process, however, the impact of epigenetic chromatin remodelers, such as the ATPase driven multi-subunit BAF (SWI/SNF) complex, is not fully characterized. To address this, we performed loss of function studies of multiple complex subunits in epidermal tissue. Conditional BAF53A (ACTL6A) gene deletion in mice was found to abolish epidermal progenitor maintenance and to induce differentiation, leading to a failure of epidermal self-renewal. Similar findings in organotypic human epidermis were observed with BAF53A depletion via RNAi, where ectopically expressed BAF53A also suppressed differentiation, indicating that BAF53A enforces the undifferentiated cell state. Depletion of key components of multiple histone acetyltransferase and chromatin remodeling complexes with which BAF53A can physically associate identified a phenocopy only with the

BAF250A (ARID1A) BAF complex subunit. Paradoxically but consistent with prior knockout mouse studies, depletion BRG1/BRM ATPase subunits impaired differentiation gene induction, suggesting that BAF53A functions as an anti-differentiation component of the BAF complex. Consistent with this, BAF53A is down-regulated during normal differentiation. Mechanistically, chromatin immunoprecipitation (ChIP) experiments demonstrated that BAF53A impairs binding of the BRG1/BRM-containing BAF complex to differentiation gene promoters, including key regulators such as of KLF4, blocking their induction. These data indicated that BAF53A maintains the undifferentiated progenitor state by opposing BAF complex-enabled differentiation via genomic re-targeting of this chromatin remodeling complex away from differentiation gene promoters.

Keith R. Porter Lecture

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Cell Biology of Virus Entry.

A. Helenius¹; ¹Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

When viruses enter animal cells, most of them follow a stepwise program that involves attachment, activation of signaling pathways, endocytosis, penetration into the cytosol, intracellular transport, and finally uncoating of the viral genome. Since all of these steps depend on host cell functions, hundreds of cellular proteins end up assisting viruses in different stages of entry and thus inadvertently participating in the demise of the cell and in pathogenesis. However, the dependence on host cell functions makes viruses sensitive to perturbations that affect cellular processes ranging from macropinocytosis to endosome maturation and nuclear import. Therefore, viruses provide powerful tools to analyze cell function, and it may be possible to inhibit infection by blocking specific host cell functions.

Using high-end light microscopy, automated high-throughput siRNA and drug screening as well as biochemical approaches, we have analyzed the entry of viruses of different families. We find five distinct entry routes with penetration occurring through the plasma membrane, in early endosomes, in late endosomes, in macropinosomes, and in the ER. In several cases, a list of host genes involved is known from screens and detailed molecular mechanisms are emerging. In the Keith Porter lecture, I will describe the general concepts in early virus/host cell interaction, and describe in some detail the entry strategies used by influenza virus, which enters by membrane fusion in late endosomes, vaccinia virus, which triggers macropinocytosis, and SV40, a non-enveloped virus that penetrates through the ER membrane.

MONDAY, DECEMBER 17**Symposium 2: New Model Systems for Cell Biology**

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Probing mechanisms of axonal and neuronal vesicle trafficking using human induced pluripotent stem cells.

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Human pluripotent stem cells provide enormous opportunities to treat disease using cell replacement and related cell therapy approaches. But human pluripotent stem cells are also an important new tool for biomedical discovery in a human model system. Since humans are a large, long-lived organism with a complex brain, a rich evolutionary history, and substantial genetic variation across large and accessible populations, there are likely to be new biological mechanisms and phenomena that can be discovered using these cells. In my talk, I'll describe our initial forays into probing basic biological features of axonal trafficking and transport in human neurons made from human embryonic and human induced pluripotent stem cells. I will also describe new insights into two different diseases: Alzheimer's Disease and Niemann Pick Type C. For Alzheimer's Disease, I will describe how we are using quantitative methods for kinesin-1 localization to test whether duplications of the amyloid precursor protein gene that cause hereditary Alzheimer's Disease cause pathological phenotypes by altering kinesin-1 loading on axonal vesicles. I will also describe new work that reveals how Alzheimer's Disease genetic risk alleles at the SORL1 locus alter neuronal phenotypes. For Niemann Pick Type C, I will discuss recent studies that have led to the identification of a previously unrecognized intracellular cholesterol transport system.

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Choanoflagellate colony development as a simple model for animal multicellularity.

N. King¹; ¹*Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA*

The evolution of animals from their single celled ancestors represents one of the major transitions in life's history. The origin of animals was shaped by extensive genomic and gene regulatory innovations, co-option of pre-existing genes to new functions in cell adhesion and signaling, rising atmospheric and oceanic oxygen concentrations, and the subsequent influence of new selective pressures. By studying choanoflagellates, the closest living relatives of animals, my lab aims to reconstruct the biology of the last common ancestor of animals. I will discuss our recent findings regarding the ancestry of animal gene families and the potential connection between genome evolution and animal origins. In addition, I will describe our development of a colony-forming choanoflagellate, *Salpingoeca rosetta*, as a new model for investigating the origin of animal multicellularity. Through our study of *S. rosetta*, we have discovered that a developmental switch in choanoflagellates is regulated by a secreted signal from environmental bacteria. Explaining how the intersection of genetic novelty, gene co-option, and environmental interactions contributed to the transition to multicellularity has important implications both for understanding early animal evolution and for identifying the foundations of animal cell biology.

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Developing a model system to study regeneration.A. Sánchez Alvarado¹; ¹HHMI/Stowers Institute, Kansas City, MO

Regeneration remains one of the last untamed frontiers of developmental biology. It is amongst the oldest biological problems known to humankind, dating back to antiquity in many cultures and, perplexingly, still awaiting for a satisfactory mechanistic explanation. As such, its history is rich, and its study encompasses the work and attention of generations of biologists. So why has so little mechanistic progress been made in this field to date? One likely explanation is that the organisms that currently make the workforce of biomedical research, having been selected primarily for their accessibility to genetic studies, are not particularly good exponents of regenerative capacities (e.g., *Drosophila*, nematodes and mice). That animal models had to be selected for their capacity to be prodigious egg layers, or have very large litters in order to do genetics are relatively modern criteria for selecting animal models. However, the advent of molecular biology, genomics and their attendant technologies allow us an unprecedented opportunity to plumb the depths of biology thus far beyond the reach of genetics research. This morning I will tell you the story of one such organism, the planarian *Schmidtea mediterranea* and how it is helping us unravel the mystery of animal regeneration.

Education Initiative Forum

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Teaching *in concert*: a novel approach to interdisciplinary collaborative project-based instruction.A. L. Goodman¹, A. Dekhtyar¹; ¹California Polytechnic State University, San Luis Obispo, CA

We developed and piloted a novel approach to interdisciplinary instruction that enables undergraduate students in life sciences to work “in concert” with computer science students to solve biological problems. Our approach relies on well-defined **interdependent** roles for biology (BIO) and computer science (CS) students in a research project.

We recognize distinct learning objectives for each major and implement them in two separate courses taught side-by-side: Bioinformatics Applications for BIO majors and Bioinformatics Algorithms for CS majors. We rely on separate lectures for each group of students. Students work as part of an interdisciplinary team in a joint project-based computer laboratory. The following workflow was applied to five increasingly difficult assignments throughout a 10-week quarter: (1) BIO students are presented with a biological question, (2) they figure out what data is required to answer the question and write program requirements, (3) CS partners write programs, (4) BIO and CS students collaborate on testing the program, (5) BIO students use the program to answer the original question. We designed the assignments around the research project conducted by the Genomics Education Partnership (GEP, www.gep.wustl.edu): annotation of fruit fly genomes and comparative analysis of different genome regions.

We tested this approach with 24 BIO and 35 CS students. Our assessment included evaluation of student artifacts by partners from another discipline and by instructors, as well as self-assessment using voluntary confidential surveys (86.4 % response rate). Program requirements produced by BIO students improved or greatly improved in 82% of the evaluations by CS students. An increase in student confidence to collaborate with colleagues on interdisciplinary work was reported by 74.5% of the respondents. Our data indicate that students appreciate and mutually benefit from interdisciplinary interactions.

The following factors appear critical to the success of our approach: (1) modular organization of the course with learning objectives explicitly aligned to the research objectives; (2) clearly defined student roles and sufficient time allotted to each assignment for meaningful in-class interactions; (3) well-balanced team constitution and sufficient problem-solving and independent learning skills to ensure team success. The independent learning skills were reinforced among biology students by using an “inverted” classroom approach, where students spent most lecture time discussing the material and homework assignments completed outside of class. We believe that our approach can be adapted to a wide range of interdisciplinary project/course combinations and seek collaborations with other programs to test this hypothesis.

Minisymposium 9: Autophagy, Self Renewal, and Cell Death

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IRE1 α induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death during ER stress.

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When unfolded proteins accumulate to irremediably high levels within the endoplasmic reticulum (ER), intracellular signaling pathways called the unfolded protein response (UPR) become hyperactivated to cause programmed cell death. We discovered that thioredoxin-interacting protein (TXNIP) is a critical node in this “Terminal UPR.” TXNIP becomes rapidly induced by IRE1 α , an ER bifunctional kinase/endoribonuclease (RNase). Hyperactivated IRE1 α increases TXNIP mRNA stability by reducing levels of a TXNIP destabilizing micro-RNA, miR-17. In turn, elevated TXNIP protein activates the NLRP3 inflammasome, causing Caspase-1 cleavage and interleukin 1 β (IL-1 β) secretion. Txnip gene deletion reduces pancreatic β -cell death during ER stress, and suppresses diabetes caused by proinsulin misfolding in the Akita mouse. Finally, small molecule IRE1 α RNase inhibitors suppress TXNIP production to block IL-1 β secretion. In summary, the IRE1 α -TXNIP pathway is used in the terminal UPR to promote sterile inflammation and programmed cell death, and may be targeted to develop effective treatments for cell degenerative diseases.

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The apoptosis inhibitor ARC alleviates the ER stress response to promote β -cell survival in diabetes.

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Type 2 diabetes involves both insulin resistance and “failure” of β -cells, the cells responsible for insulin secretion. An important component of β -cell failure is cell loss by apoptosis. ARC (Apoptosis Repressor with Caspase Recruitment Domain) is an inhibitor of apoptosis that is highly expressed in cardiac and skeletal myocytes and neurons. ARC possesses the unusual property of antagonizing both the extrinsic (death receptor) and intrinsic (mitochondria/endoplasmic reticulum (ER)) cell death pathways. We discovered that ARC protein is abundant in cells of the pancreatic islets, including >99.5% of mouse and 73% of human β -cells. Moreover, we found that deletion of nol3, encoding ARC, markedly exacerbates

hyperglycemia and further impairs glucose tolerance in diabetic (*ob/ob*) mice, and this is accompanied by pancreatic islet disorganization and increases in β -cell apoptosis. ER stress is a major component of type 2 diabetes and an important trigger of β -cell death. Genetic gain- and loss-of-function studies in cultured cells demonstrated that ARC inhibits β -cell apoptosis elicited by the ER stressors tunicamycin, thapsigargin, and the free fatty acid palmitate. Unexpectedly, inhibition of cell death by ARC is mediated by suppression of the ER stress response, a novel function of ARC. Further analysis showed that ARC acts distal to ER stress sensors PERK and IRE1 α to suppress induction of CHOP, a transcription factor that activates both genes involved in resolving ER stress and genes involved in cell death. In pancreatic islet tissue stimulated with palmitate, depletion of ARC augments CHOP abundance and apoptosis, which is dramatically rescued by deletion of CHOP. Taken together, these data demonstrate that ARC is a previously unrecognized inhibitor of β -cell apoptosis in type 2 diabetes, and its protective effects are mediated through a novel mechanism involving suppression of the ER stress response pathway.

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Developmentally programmed mega-autophagy executes nuclear destruction during yeast gametogenesis.

M. Eastwood¹, M. Meneghini¹; ¹Molecular Genetics, University of Toronto, Toronto, ON, Canada

While studies of the budding yeast *Saccharomyces cerevisiae* have provided fundamental insights into the mechanisms of autophagy, the roles of cell death pathways in yeast are less well understood. We have discovered that developmentally-programmed nuclear “death” (PND) is executed during the gametogenesis phase of the yeast lifecycle, a process more commonly known as sporulation. In response to carbon deprivation, sporulating cells subject only a fraction of their meiotic products to cellularization. Under such nutritionally-stressful conditions, we observe widespread PND. We have determined that PND is accomplished by collaborating autophagic and apoptotic-like pathways. Our live-cell imaging indicates that PND coincides with the post-meiotic permeabilization and lysis of the vacuole (lysosome). PND also occurs in the absence of autophagosome biogenesis, suggesting that it is driven by the coincident release of lytic vacuolar contents rather than canonical macroautophagy. Vacuolar rupture, or mega-autophagy, is a form of autophagy principally characterized during plant cell death and resembles lysosomal membrane permeabilization events which contribute to programmed cell death in animals. Quite interestingly, yeast PND invokes the cleavage of genomic DNA into nucleosomal fragments, a hallmark event of mammalian apoptosis. The production of nucleosomal DNA fragments requires the yeast orthologue of endonuclease G, a mitochondrial nuclease which mediates DNA fragmentation during apoptosis in metazoans. Our most recent work has focused on using genetic approaches to understand how the self-destructive events which underlie PND are coordinated with known aspects of sporulation. Mega-autophagy appears to be triggered after the progression through the pachytene stage of meiosis. Cells arrested prior to this point maintain intact vacuoles, while those arrested beyond pachytene execute robust vacuolar rupture, even if spore construction is incomplete. This suggests that the pachytene checkpoint is the final point of surveillance before the bulk degradation of the mother cell is initiated through mega-autophagy. Our characterization of yeast PND demonstrates further diversity in cell death programs, and illuminates a developmental function of conserved apoptotic-like pathways in a unicellular microbe. We also present an example of autophagic pathways coordinating to destroy whole nuclei in response to nutritional stress.

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Lysosomes as novel regulators of gene expression, organelle biogenesis, and autophagy induction.*J. Martina¹, R. Puertollano¹; ¹National Institutes of Health, Bethesda, MD*

One of the most fundamental issues in cell biology is how cells integrate growth-stimulating and inhibitory signals to ultimately regulate a diversity of key cellular functions, including gene expression, autophagy, organelle biogenesis, and cell growth. mTOR is a serine/threonine kinase that regulates proliferation, cell cycle, and autophagy in response to energy levels, growth factors, and nutrients. mTOR responds to numerous stresses and its dysregulation leads to cancer, metabolic disease, and diabetes. Activation of the mTORC1 complex occurs at lysosomes and the recruitment of mTORC1 to this compartment is regulated by the Rag family of small GTPases.

Here we described how mTORC1 controls the activation of the transcription factor EB (TFEB), a master regulator of the expression of autophagic and lysosomal genes. Under nutrient-rich conditions, mTORC1 phosphorylates TFEB in S211, thus promoting binding of TFEB to the cytosolic chaperone 14-3-3 and retention of TFEB in the cytosol. Upon amino acids deprivation, dissociation of the TFEB/14-3-3 complex results in delivery of TFEB to the nucleus and up-regulation of genes that leads to induction of autophagy, biogenesis of lysosomes, and increased lysosomal degradation.

We also identify the molecular mechanism that mediates recruitment of TFEB to lysosomes, a critical step in the regulation of this transcription factor by mTORC1. We found that TFEB interacts with active, but not inactive, Rags GTPases via the surface interface containing the switch regions of the Rags G domain. Depletion of endogenous Rags, inactivation of Rags by starvation, or expression of dominant negative Rags prevents recruitment of TFEB to lysosomes and abolishes mTORC1-dependent phosphorylation and inactivation of TFEB. In contrast, expression of active Rags is sufficient to induce association of TFEB with lysosomal membranes. The first 30 residues of TFEB are both, necessary and sufficient for interaction with active Rags and recruitment to lysosomes, while a stretch of arginines in the central portion of TFEB functions as a nuclear import signal. Importantly, Rag GTPases also mediate amino acid-dependent recruitment of the transcription factor MITF to lysosomes, thus suggesting that the Rag complex might have a more general role in the regulation of gene expression.

Overall, our work provides new insight for understanding the novel and exciting role of lysosomes as signaling centers that synchronize environmental cues with gene expression, energy production, and cellular homeostasis.

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Regulation of mammalian autophagy by class II and III PI 3-kinases through PI3P synthesis.

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Autophagy requires the biogenesis of autophagosomes (APs), which are large multilamellar vesicles that sequester cytoplasmic substrates and undergo a maturation process ultimately leading to their fusion with lysosomes. Previous studies have suggested that local production of phosphatidylinositol-3-phosphate (PI3P) by class III phosphatidylinositol 3-kinase (PI3K) (i.e., Vps34) is required for AP biogenesis at specialized sites of the endoplasmic reticulum called

"omegasomes". Remarkably, studies in the first mammalian Vps34 conditional knockout mice hinted that Vps34 may not be essential for autophagy in neurons. However, in more recent studies using different mouse models, Vps34 was proposed to be required for autophagy in T-cells, fibroblasts, heart and liver tissue. Given these two contradictory sets of findings, the precise role of Vps34 for the functionality of autophagy remains an outstanding question. Here we show by electron microscopy that AP formation still occurs in *Vps34*^{-/-} mouse embryonic fibroblasts (MEFs), although fewer APs and autophagolysosomes are formed upon nutrient deprivation. Corroborating these results, long-lived protein degradation assays show a ~50% reduction in (macro)autophagy efficacy and autophagy assays show reduced formation of LC3 puncta and LC3 conjugation as well as a dramatic reduction in the recruitment of the PI3P effector WIPI-1 to sites of AP nucleation in starved *Vps34*^{-/-} MEFs. These findings indicate that Vps34 is important, but not essential for AP formation, and suggest that alternate, autophagy-relevant sources of PI3P exist in mammals. Although Vps34 is believed to be the predominant source of PI3P, class II PI3Ks can also produce this lipid and have been shown to mediate other PI3P-dependent processes. We found that silencing class II isoforms in either the wild-type or *Vps34*^{-/-} MEFs reduces autophagy, suggesting a positive regulatory role for the class II in this process. In conclusion, our studies indicate that both class II and III PI3Ks contribute functionally-relevant pools of PI3P for AP biogenesis.

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Selective autophagy in the control of cellular homeostasis.

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Autophagy, the cellular process by which protein and organelles undergo degradation in lysosomes, can take place through very different mechanisms. This work focuses in a selective type of autophagy, known as chaperone-mediated autophagy (CMA), that contributes to the degradation of a specific subset of cellular proteins in lysosomes. CMA is active in most mammalian cell types, but its activity varies depending on cell type and cellular conditions. Although there is some level of basal CMA activity, maximal activation of this pathway occurs during stress or in conditions leading to increased amount of misfolded/damaged proteins. Degradation by CMA requires the presence of a targeting motif in the substrate protein, a set of cytosolic and lysosomal chaperones and a receptor protein at the lysosomal membrane, the lysosome-associated membrane protein type 2A (LAMP-2A).

CMA activity is upregulated in response to different type of stress such as nutritional stress, oxidative stress and exposure to compounds inducing conformational changes of particular cytosolic proteins. In addition, activation of CMA is also observed in response to agents inducing ER-stress, and this autophagic pathway remains active during the period that follows the stress. Failure to upregulate CMA after ER stress leads to cell death. We have characterized the mechanisms by which active CMA contributes to return ER to homeostasis and have analyzed the consequences for the biology of this organelle of the reduced CMA observed under different pathological conditions and in aging.

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Minisymposium 10: Cell Biology of Neurodegeneration

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Local apoptosis, mitochondrial turnover and neurodegeneration.

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Loss of synapses and death of neurons are hallmarks of neurodegenerative disease such as Alzheimer's disease, but also characteristic of normal postnatal brain development in mammals. Morphological synapse elimination -- together with its functional correlate, long-term depression (LTD) of synaptic strength -- are natural processes that sculpt the brain, akin to programmed cell death (also termed apoptosis). Remarkably, LTD and synapse elimination share common molecular mechanisms with apoptosis. The mitochondrial (or intrinsic) pathway of apoptosis, culminating in caspase-3 activation, is required for LTD in hippocampal neurons. This pathway is activated transiently, moderately and locally in the vicinity of synapses to weaken and eliminate synapses -- in the absence of cell death. Local activation of the mitochondrial apoptosis pathway in the dendrites of neurons by an optogenetic approach is sufficient to cause local loss of synapses and retraction of dendrite branches, without neuronal death. Beta-amyloid (A β , which accumulates in the brain of Alzheimer's patients) co-opts the same "synaptic apoptosis" pathway to impair synaptic plasticity, which could contribute to the synapse dysfunction and loss of Alzheimer's disease.

In Parkinson's disease (the second most common neurodegenerative disease), much evidence points to mitochondrial dysfunction as a major pathophysiologic mechanism. Mutations in PINK1 (a protein kinase) and Parkin (an E3 ubiquitin ligase) cause familial Parkinson's disease and impair mitochondrial turnover by autophagy (mitophagy). We have screened for genes that regulate mitophagy in order to identify potential therapeutic targets for improving mitochondrial quality control in neurons.

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Toxic α -synuclein oligomer accumulation and endoplasmic reticulum stress is mechanistically linked to α -synucleinopathy in vivo.

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In Parkinson's disease (PD) and other α -synucleinopathies, α -synuclein (α S) abnormalities are directly implicated in the disease pathogenesis. Currently, both α S fibrils and prefibrillar α S oligomers have been proposed as the pathogenic species. However, because the α S oligomers are only observed *in vitro*, the *in vivo* pathologic significance of α S oligomers and the *in vivo* mechanisms of neurotoxicity are unknown. To study mechanisms of α S toxicity, we examined the subcellular distribution of normal and abnormal α S in brain. We show that while most of α S is found in the cytosol, a fraction of α S, but not highly related β S, is found in the lumen of the endoplasmic reticulum/microsomes (ER/M) *in vivo*. With aging, α S level in ER increases and forms toxic oligomers that accumulate with the progression of α -synucleinopathy. Accumulation of ER/M α S oligomers is followed by the occurrence of aberrant ER stress/unfolded protein response where the ER chaperones are induced without the activation of phospho-eIF2 α /CHOP. Further, increased ER-associated polyubiquitin accumulation and activation of ER stress-associated caspases indicate that α -synucleinopathy directly causes ER dysfunction that contributes to neurodegeneration. Significantly, treatment with Salubrinal, an anti-ER stress

compound, significantly attenuates disease manifestations in both the transgenic mouse model and an AAV- α S rat model of α -synucleinopathy. The results show that α S oligomers and ER-stress are pathologically relevant for PD. Further, alleviating chronic ER stress represents a new therapeutic strategy for PD and related diseases.

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Rescue of dopaminergic neuron mitochondrial dysfunction and degeneration in parkin mutants.

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Parkinson disease (PD) is a prevalent age-related neurodegenerative disorder, the motor deficits of which result from the selective degeneration of dopaminergic neurons in the substantia nigra of the midbrain. The molecular underpinnings of PD are poorly understood, but studies of mitochondrial toxins, and the characterization of genes involved in rare heritable forms of PD implicate mitochondrial dysfunction as a primary cellular defect. However, whether genetic defects associated with heritable forms of PD influence mitochondrial function in dopaminergic neurons remains unknown. To address this question we have developed a flow cytometry-based method to measure mitochondrial membrane potential and mitochondrial abundance from individual neural subsets isolated from the adult *Drosophila* brain. Our findings indicate that down regulation of the PINK1/Parkin pathway causes depolarized mitochondria to accumulate in dopaminergic neurons, and that genetic manipulation of mitochondrial dynamics factors can rescue this phenotype. Complementary experiments utilizing isolated cholinergic neurons show that dopaminergic neurons accumulate depolarized mitochondria to a greater extent than cholinergic neurons, suggesting that dopaminergic neurons are subject to a more toxic intracellular environment or lack alternative mitochondrial quality control mechanisms. Finally, we find that the degeneration of dopaminergic neurons, observed in the brains of adult parkin null mutant animals, is rescued by genetic manipulation of the mitochondrial fission, fusion and autophagy machinery. Ongoing experiments to test hypotheses related to mitochondrial dysfunction in PD, including the prediction that genes involved in heritable forms of PD maintain cellular function by promoting the elimination of dysfunctional mitochondria, will be discussed.

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Dose-dependent and mutant-enhanced neurotoxicity in mice expressing wild type or ALS-linked mutants of FUS/TLS.

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Mutations in two ubiquitously expressed nucleic-acid binding proteins, TDP-43 (TAR-DNA binding protein-43 KDa) and FUS/TLS (fused in sarcoma/translocated in liposarcoma), cause amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Abnormal aggregates of these two proteins are present in inherited and sporadic ALS, FTLD, and other neurodegenerative diseases, independent of mutations in either gene. We show here that broad expression within the nervous system of wild type or either of two ALS-linked mutants of human FUS/TLS produces progressive motor phenotypes, including abnormal gait, glial activation,

degeneration of motor and sensory axons, and loss of spinal cord motor neurons, accompanied by denervation-induced muscle fibrillations, all of which are characteristics of ALS. Expression of wild type or ALS-linked mutants of human FUS/TLS downregulates endogenous mouse FUS/TLS expression at both mRNA and protein levels, illustrating an auto-regulation mechanism for FUS/TLS. Furthermore, generation of homozygote or double heterozygotes expressing human wild type FUS/TLS cause rapidly progressive neurological phenotypes and dose-dependent lethality accompanied by widespread neurodegeneration. Taken together, our results demonstrate that (1) mice expressing ALS-linked mutation in FUS/TLS develop earlier progressive motor deficits and early mortality than mice expressing comparable level of wild type human FUS/TLS, and (2) a surprisingly small increase in increased expression of wild type FUS/TLS sharply accelerate neurodegeneration, providing a basis for FUS/TLS involvement in pathogenesis in the absence of mutation.

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Amyloid- β signals through tau to drive ectopic neuronal cell cycle re-entry in Alzheimer's disease.

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Cognitive decline in Alzheimer's disease (AD) is caused by synaptic dysfunction, the ultimate cause of which is neuron death. Most normal adult neurons are permanently post-mitotic, but AD neurons frequently re-enter the cell cycle, fail to complete mitosis, and die up to a year later. This ectopic pathway for neuronal cell cycle re-entry (CCR) apparently accounts for the death of a substantial fraction of the neurons that are lost during typical cases of AD and thus represents a crucial, but poorly understood aspect of AD pathogenesis. We now report the fundamental features of this pathway. Exposure of primary wild type (WT) neurons to amyloid- β (A β) oligomers caused CCR and activation of 3 protein kinases, fyn, PKA and CaMKII, which respectively phosphorylated tau on Y18, S409 and S416. In tau knockout (KO) neurons A β oligomers failed to induce CCR, but did activate all 3 kinases. CCR was restored in tau KO neurons by expressing WT tau, but not tau with non-phosphorylatable Y18F, S409A or S416A mutations. Tau phosphorylation at Y18, S409 and S416 catalyzed respectively by fyn, PKA and CaMKII must therefore occur to induce CCR. In contrast, 2 other prominent tau kinases, GSK3 β and MARK/Par-1 were not activated by A β oligomers under identical conditions. To determine if neuronal CCR *in vivo* requires tau, we examined hAPPJ20 mice, which overexpress mutant human amyloid precursor protein (APP) and accumulate A β deposits, on an endogenous WT tau or tau KO background. Nearly 60% of cortical neurons in 6-month-old hAPPJ20/WT tau mice expressed the G1 marker, cyclin D1, which was undetectable in hAPPJ20/tau KO mice, or in WT or tau KO parental controls. These results establish a novel, 3 stage biochemical pathway that explains why A β toxicity requires tau, and thus represent a major advance towards understanding seminal pathogenic events in AD. An especially noteworthy feature of this destructive pathway is that it is established by A β and tau independently of their respective incorporation into plaques and tangles, the histopathological hallmarks of AD brain.

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The highly conserved CAP-Gly domain of dynactin interacts with EB3 to enhance the initiation of retrograde axonal transport of multiple cargos.

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Mutations in the CAP-Gly domain of the p150^{Glued} subunit of dynactin cause two distinct neurodegenerative diseases: an inherited form of Parkinson's disease, Perry syndrome, and

distal hereditary motor neuropathy 7B (HMN7B). Dynactin is a required cofactor for the minus-end directed microtubule motor cytoplasmic dynein. We recently reported a novel function for the CAP-Gly domain of dynactin in neurons, demonstrating that this domain is crucial for the efficient flux of lysosomes from the distal axon. Here, we show that multiple cargo types including mitochondria, early endosomes, signaling endosomes and APP vesicles all require the CAP-Gly domain of dynactin for efficient retrograde transport from the distal axon but not for sustained organelle transport along the axon. Thus, while these cargos undergo distinct forms of motility in the axon, they all require the CAP-Gly domain for efficient transport initiation. To further investigate the mechanisms involved in the initiation of dynein-mediated retrograde transport, we examined the role of dynamic microtubules. We observed an increase in the local concentration of EB3-positive microtubule plus-ends in the distal axon. This region of enhanced dynamicity of the microtubule cytoskeleton corresponds to the region of the distal axon where dynactin is enriched. The CAP-Gly domain directly interacts with EB3 and this interaction is inhibited by the neurodegenerative mutations. We find that knockdown of EBs in primary neurons disrupts transport initiation specifically from the distal axon. Together these data suggest that both dynamic microtubules and EBs are important in dynactin-mediated transport initiation. Consistent with our model, the neurodegenerative mutations in dynactin cause a failure to localize p150^{Glued} to the distal axon and an inhibition of retrograde flux. The dynein-binding protein, Lis1, is a distinct disease-associated protein that has also been proposed to function as a transport initiation factor for dynein. However, in contrast to the CAP-Gly domain of dynactin and EBs, Lis1 is required for both transport initiation from the distal axon as well as sustained bidirectional transport along the axon. Further, Lis1 is not enriched in the distal axon. These data suggest that Lis1 and dynactin act through distinct mechanisms to initiate dynein-mediated organelle transport in neurons.

Minisymposium 11: Cell Division

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Generating a dynamic kinetochore-microtubule interface.

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To ensure equal chromosome segregation during mitosis, the macromolecular kinetochore must remain attached to depolymerizing microtubules, which drive poleward chromosome movement. Depolymerizing microtubules undergo a dramatic structural change resulting in the formation of curved protofilaments. How kinetochores remain attached to depolymerizing microtubules and harness the force produced during this process has yet to be defined in vertebrates. We have analyzed key players in this process including the conserved Ndc80 complex and the metazoan Ska1 complex. We demonstrate that the conserved kinetochore-localized Ska1 complex can track with depolymerizing microtubule ends. In contrast, the Ndc80 complex, lacks tracking activity on its own. Importantly, we demonstrate that the Ska1 complex can impart its tracking capability to the Ndc80 complex. This work defines an integrated kinetochore-microtubule interface formed by the Ska1 and Ndc80 complexes that can remain attached to depolymerizing microtubules.

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Differences in Ran regulation and the microtubule-associated protein TPX2 contribute to interspecies spindle scaling in *Xenopus* egg extracts.

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A bipolar, microtubule (MT)-based spindle performs the crucial and universal function of segregating chromosomes during eukaryotic cell division, but spindle size, assembly mechanism and architecture vary depending on the organism and cell type. We previously established an egg extract system that recapitulates size differences between meiotic spindles of the frog *Xenopus laevis* and its smaller relative *Xenopus tropicalis*, and identified differential phosphorylation of the MT severing factor katanin as a spindle length scaling mechanism. However, katanin activity does not fully account for meiotic spindle differences between the two species. Interestingly, we have found that the small *X. tropicalis* spindles are resistant to inhibition of RCC1, the chromatin-bound nucleotide exchange factor that generates a gradient of RanGTP, whereas the large *X. laevis* spindles are disrupted with concomitant mislocalization of downstream factors including the spindle MT-associated protein TPX2. Interestingly, TPX2 is present at three-fold higher levels in *X. tropicalis* extract and is missing a 7 amino acid insertion found in *X. laevis* TPX2 that introduces two consensus Polo kinase phosphorylation sites. When added to *X. laevis* extract in equal amounts, both *X. tropicalis* TPX2 and an *X. laevis* TPX2 mutant lacking the 7 amino acid insertion induce dramatic MT nucleation compared to wild-type *X. laevis* TPX2. We are testing the hypothesis that phosphorylation inhibits TPX2 activity in *X. laevis* extract, causing a decrease in MT nucleation that affects spindle size. Consistent with this model, addition of excess TPX2 to *X. laevis* extract reduced spindle length. Higher levels and activity of TPX2 may decrease average MT length in the *X. tropicalis* spindle, and could also act to decrease kinesin-5 dependent MT sliding. Both of these mechanisms would limit the distance that a spindle MT slides poleward in its lifetime, thereby decreasing steady state spindle length. Thus, differences in meiotic spindle size in the two *Xenopus* species are accompanied by changes in MT nucleation and stability that reflect differences in spindle architecture and assembly pathway.

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Molecular mechanisms governing extrinsic forces in mitotic spindle organization.

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The presence of supernumerary centrosomes in cancer cells often creates the potential for catastrophic multipolar divisions. However, many cancer cells successfully divide because of mechanisms that suppress multipolar mitoses by clustering their extra centrosomes. Our functional genomic study has previously identified key pathways contributing to centrosome organization in cancer cells. In addition to spindle intrinsic forces, we found that cell adhesion patterns can determine the fate of mitosis whether they divide into two (bipolar) or more (multipolar). Yet, the mechanism by which actin and adhesion-dependent forces to control the fidelity of mitosis remain unclear at the molecular level. One model proposes that sites of strong cell matrix adhesion are imprinted as an actin rich structure called retraction fibers during mitosis, and the retraction fibers serve as cortical cues to concentrate force generators and regulators to pull centrosomes. By imaging cancer cells plated on fibronectin micropatterns, here we demonstrate that Myo10, an unconventional myosin identified from our genome-wide screen, is an essential component linking retraction fiber-mediated forces to astral microtubules (MTs) during spindle organization. Myo10 specifically localizes along and to the tips of the retraction fibers. When cells with extra centrosomes were plated on Y-shaped fibronectin

micropatterns, control cells divide tripolar toward Y-adhesion axis; however, cells depleted of Myo10 undergo bipolar division independent of retraction fiber positions, suggesting that Myo10 is a force coupler from retraction fibers to centrosomes. We have hypothesized that intrinsic properties of MTs adjacent to retraction fibers are altered to favor stable interaction between astral MTs and cell cortex. To uncover heterogeneity of MT dynamics in relation to retraction fibers, we have performed live cell imaging of GFP-EB3 with high spatio-temporal resolution in cells plated on fibronectin micropatterns where retraction fiber positions are precisely manipulated. We have developed a system that quantifies and visualizes MT dynamics in different subregions of cells by an automated computer program capable of tracking GFP-EB3 comets. This approach uncovered heterogeneity of MT dynamics around retraction fiber regions. Interestingly, our analyses have revealed that Myo10 is in part responsible for promoting the long-lived and long distance travelling MTs. Taken together, these results suggest that Myo10 is a key adhesion-dependent regulator that confers heterogeneity of mitotic cortex by coupling cell geometry/adhesion to mitotic fidelity and genome stability.

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Mechanisms of chromosome segregation on acentrosomal oocyte spindles in *C. elegans*.

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It was recently reported that kinetochores are not essential for chromosome separation in *C. elegans* oocytes, suggesting that acentrosomal spindles in these cells employ a novel segregation mechanism¹. We previously found that lateral microtubule bundles surround homolog pairs (bivalents) during prometaphase, and proposed that congression is facilitated by movement of chromosomes along these bundles². Therefore, we set out to investigate whether lateral microtubule associations could mediate segregation as well.

Using high- and super-resolution imaging we found that lateral microtubule-chromosome associations are present in oocyte spindles throughout anaphase. In early anaphase, the poles broaden so that microtubule bundles are arranged in a parallel array. Chromosomes move towards spindle poles (marked with KLP-18 and ASPM-1 antibodies), and in late anaphase were found outside most of the pole staining, near the microtubule ends. At this stage microtubule bundles in the central spindle converge while splitting around the chromosomes near the poles, maintaining their lateral associations. Therefore, chromosomes potentially move poleward along laterally-associated microtubules arranged in a parallel configuration; once segregation is complete the bundles converge and the spindle disassembles.

Multiple proteins, including members of the Chromosome Passenger Complex (CPC), form a ring around the center of each bivalent during congression². While CPC components redistribute to microtubules in anaphase, some midbivalent proteins persist as rings between separating chromosomes that elongate into linear structures and then disappear¹. Intriguingly, we found changes in the behavior of these structures in oocytes with segregation defects, suggesting the presence of a mechanism that monitors the success of chromosome separation. When lagging chromosomes were present, elongation of the ring structures into linear elements was delayed; they often remained as rings through late anaphase and in these instances microtubule bundles did not converge, instead they maintained a parallel arrangement. Moreover, while the CPC kinase Aurora B redistributed to the spindle in wild type anaphase, when lagging chromosomes were present it remained associated with the ring structures. These results reveal that ring disassembly is a regulated event that may respond to the status of chromosome separation, and raises the possibility that the rings function to space parallel microtubules so that chromosomes can move along them. We therefore propose that there is a checkpoint acting to keep the spindle in an “early anaphase” configuration until chromosomes have properly

separated, to increase the fidelity of kinetochore-independent chromosome segregation on acentrosomal spindles.

1) Dumont, et.al. NCB 12, 894-901

2) Wignall & Villeneuve. NCB 11, 839-844

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Central spindle formation through the regulation of microtubule depolymerization during cytokinesis.

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During anaphase, microtubule (MT) bundles interdigitate in an antiparallel manner to form multiple "stem bodies" in the central region of the central spindle. The stem bodies align at the cell's equatorial plane, provide scaffolds for signaling molecules that are required for progression of cytokinesis, and thus form the cell division plane. As ingression of the cleavage furrow proceeds, the stem bodies gather into a cluster at the intercellular bridge to provide a scaffold for the abscission machinery that completes cytokinesis. However, how the stem bodies align at the very center of the dividing cell and enable proper control of cytokinesis is poorly understood. We performed the live imaging of HeLa cells expressing the RACGAP1-GFP marker, and found that the position of each stem body constantly fluctuated both parallel and perpendicularly to the division axis, but was always confined to the equatorial region throughout anaphase; this uncovered the dynamic nature of the division plane. Taxol treatment led to stem body misalignment, indicating that dynamic MTs are required for proper positioning of the stem body. Cells depleted of the MT-depolymerizing kinesin Kif2A also had misaligned stem bodies, along with abnormally elongated central spindle MT bundles. The results of the functional living-cell assays showed that Kif2A controls the length of the central spindle MT bundles by localizing to the ends of the central spindle and depolymerizing the MTs in that region. Inhibition of the chromosomal passenger complex (CPC) resulted in excess accumulation of Kif2A in the inner region of the central spindle, and consequently, the central spindle became abnormally short and unstable, and it oscillated parallel to the division axis. These results suggest a model where the CPC prevents Kif2A access to the central region of the central spindle near the stem bodies, and thereby limits Kif2A-dependent MT depolymerization to the distal ends of the central spindle. We propose that this CPC- and Kif2A-dependent fine-tuning of MT depolymerization contributes to the stability and symmetry of the central spindle, which are essential for proper positioning of the stem bodies.

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A gain of function screen for miRNAs regulating mitosis.

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A growing body of evidence indicates that miRNAs play an important role in the control of cell cycle progression, particularly at the G1/S transition. The function of miRNAs in regulating the expression of genes encoding for mitotic factors, however, remains vastly unexplored.

To address the role of miRNAs in mitosis, we performed a genome-wide gain of function screen using a library of miRNA-mimicking oligomers. Based on a live-cell imaging assay, we identified several miRNAs whose overexpression perturbed mitotic progression. Detailed phenotypic follow-up analysis revealed that overexpression of some candidate miRNAs affected mitotic spindle architecture, leading to mono- or multipolar spindles. These phenotypes were associated with delayed mitotic progression and a high incidence of polyploidization.

To investigate the molecular basis of the observed phenotypes, we investigated transcriptome changes upon overexpression of the miRNA candidates. Amongst the genes whose expression was altered by candidate miRNA overexpression, we identified several genes with ontologies related to the observed phenotypes, bearing predicted target sites in their 3'UTRs. Several of these down-regulated genes were validated by expression reporter constructs as direct targets of the candidate miRNAs. Moreover, siRNA-mediated depletion of these direct targets elicited similar phenotypes compared to over-expression of the corresponding regulatory miRNA. Our study thus identifies miRNAs that regulate mitosis genes, and suggests that misregulation of these regulatory miRNAs can lead to aneuploidy by mitosis failure, which may have implications for cancerogenesis.

Minisymposium 12: Cell-Cell and Cell-Matrix Interactions

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How cells exploit extracellular matrix fibrils as mechano-chemical signal converters.

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Since the physical and biochemical properties of extracellular matrix provide critical differentiation cues to cells, from stem cells to angiogenesis to cancer, it is of major importance to gain mechanistic insights how mechanical stretching of extracellular matrix molecules can alter various cell functions. Cells utilize mechanical forces to actively assemble and stretch the ECM fibers that surround them, and finally to remodel their microenvironments. To learn at the molecular level how mechanical forces are sensed and are translated into biochemical signals that can regulate the underpinning mechanotransduction events, many new assays and techniques have to be developed to probe how the stretching of proteins alters their structure-function relationships. With a focus on fibronectin, FRET-probes and stretch assays will be discussed that allow to probing the mechanical strain of fibronectin in cell culture and in microtissues, and how the stretching of proteins might alter their binding sites, as well as their biochemical display. Also bacteria take advantage of the fact that cells pull on extracellular matrix fibers and can thereby destroy bacterial binding motifs. For example, the multivalent binding motif on fibronectin that is recognized by the adhesins that *Staphylococcus aureus*, can be destroyed by stretching the fibers. We then analysed the design of altogether 32 bacterial adhesins that all target the same N-terminal fibronectin type 1 domains and found that they differ significantly in their ability to detect the mechanical strain of fibronectin fibers. The mechanical strain of extracellular matrix has never been considered before in the description of adhesion and host invasion by bacteria before. Finally, we analysed how stem cells respond to (or perhaps even utilize) the stretching of extracellular matrix fibers to regulate differentiation processes. Deciphering how proteins can serve as mechano-chemical signalling switches is thus not only essential to learn how cells probe and respond to their environments, but it has also far reaching implications in tissue engineering, systems biology and medicine.

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Dynamics and molecular organization of focal adhesions described at the nanoscale.C. C. DuFort¹, M. J. Paszek¹, M. Rubashkin¹, M. Davidson², K. Thorn³, V. M. Weaver⁴;¹Department of Surgery, University of California, San Francisco, San Francisco, CA, ²National High Magnetic Field Laboratory, Florida State University, Tallahassee, FL, ³Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA,⁴Departments of Surgery & Anatomy & Bioengineering and Therapeutic Sciences, University of California, San Francisco, San Francisco, CA

Focal adhesions are the conduits through which cells receive and interpret mechanical and physical cues from the extracellular matrix and serve a critical role in the transmission of force, wound healing, motility, and morphogenesis. While their role as intricate molecular machines is well appreciated, little is known about their overall organization at the nano-scale. It has been previously hypothesized that focal adhesion complexes are highly spatially conserved structures. However, our results suggest that there are specific nano-scale changes to focal adhesion architecture that appear to be directly correlated with regions of varying cell-generated contractility and motility phenotypes. To further our understanding of focal adhesion structure and function, the three-dimensional organization and spatial orientation of proteins comprising focal adhesion complexes with precisions of ~5 nm along the optical axis and diffraction-limited laterally was revealed with a scanning-angle variant of fluorescence interference contrast microscopy. Utilizing this technique, a library of focal adhesion protein locations including paxillin, focal adhesion kinase, vinculin, N and C-terminal labeled talin, and zyxin was developed. Each protein was found to be stratified to distinct layers and localizations within individual focal adhesion complexes over a vertical range of ~60 nm. To observe how these proteins were evolving over time, dynamic studies in 3D of adhesions present in cells with varying motility phenotypes was performed through the development and utilization of adhesion tracking algorithms. We focused our studies on the proteins paxillin and vinculin, which have both been implicated in force-dependent mechanotransduction and focal adhesion maturation. Our results show that paxillin and vinculin are localized into spatially distinct regions relative to one another during processes such as adhesion maturation, where we find that paxillin moves downward relative to vinculin, which stayed in approximately the same vertical position. We also observe that there is a dependence upon nano-scale adhesion architecture on mechanical force, in which paxillin undergoes a marked increase in height of approximately 8 nm after 5 minutes of inhibition of cell contractility with the Rho kinase inhibitor Y-27632. Additionally, we describe the effects of Rac1 and RhoA activation on focal adhesion maturation and organization dynamically at the molecular level, with implications for understanding the complex protein-protein interactions occurring during processes such as maturation, motility, and force-transmission during mechanotransduction.

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New single-chain Rac1 biosensor shows GTPase function in invadopod dynamics.Y. Moshfegh¹, J. Bravo-Cordero¹, J. Condeelis¹, L. Hodgson¹; ¹Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Members of the Rho family of small GTPases are heavily involved in cell motility and migration, and are thought to participate in the regulation of cancer metastasis. To study the function of Rac1 in highly invasive tumor cell migration, we developed a novel, genetically-encoded Rac1 FRET biosensor based on an intra-molecular design. The new biosensor is a substantial

improvement over the previous-generation Rac1 biosensor because of its single-chain arrangement, ensuring an equimolar distribution of FRET donor and acceptor, and producing a more accurate readout. Additionally, our design maintains the correct C-terminal lipid modification of full-length Rac1, enabling proper interaction with upstream regulators (GEFs, GAPs, and GDIs).

We used this new biosensor to explore the molecular basis for the involvement of Rac1 in the turnover dynamics of invadopodia, which are F-actin-rich protrusions with proteolytic activity that are exclusive to invasive tumor cells and are thought to be crucial for cell invasion and metastatic phenotypes. The biosensor has shown that there is an exclusion of Rac1 activity from the core of the invadopodial structures of MTLn3 rat carcinoma cells. This exclusion dissipates and Rac1 activity is elevated when stable invadopods disappear, suggesting that a lack of Rac1 activity is necessary for invadopod maintenance, and activation of the GTPase could possibly be involved in disassembly. PAK1, one of the major downstream effectors of Rac1, phosphorylates cortactin, which is a key protein in these structures, and upon this kinase activity, PAK1 releases cortactin. Taken together, we have developed a model for the mechanism of invadopod disassembly, where Rac1 activation in the core of an invadopodium will induce kinase activity of PAK1, which will phosphorylate cortactin, causing a destabilization of the invadopod's principal structural molecule and therefore leading to dissolution. This mechanism may be critical for the proper turnover of the invasive structures during migration and invasion of tumor cells in vivo, where a balance of invasive invadopodial matrix-degradation activity and the protrusive force of leading edge projections must be carefully coordinated to achieve a maximally invasive phenotype.

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The tumor suppressor adenomatous polyposis coli controls the direction a cell extrudes from an epithelium.

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Cell extrusion is the process of active removal of either live or apoptotic cells from epithelium. In this process, cells are squeezed out through an actomyosin based mechanism. Cells can extrude apically into the lumen or basally into the underlying tissue, depending on whether actin and myosin contract at the cell base or apex, respectively. We previously found that microtubules in cells surrounding a dying cell target p115 RhoGEF to the actin cortex to control where contraction occurs. However, what controls microtubule targeting to the cortex and whether the dying cell also controls the extrusion direction were unclear. Here we find that the tumor suppressor adenomatous polyposis coli (APC) controls microtubule targeting to the cell base to drive apical extrusion. Whereas wild-type cells preferentially extrude apically, cells lacking APC or expressing an oncogenic APC mutation prefer to extrude basally. Surprisingly, although APC controls microtubule reorientation and attachment to the actin cortex in cells surrounding the dying cell, it does so by controlling actin and microtubules within the dying cell. APC disruptions that are common in colon and breast cancer may promote basal extrusion of tumor cells, which could enable their exit and subsequent migration.

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Localized tensional forces on PECAM-1 elicit a global mechanotransduction response via the integrin-RhoA pathway.

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Mechanical forces regulate cell behavior and function during development, differentiation, and tissue morphogenesis. In the vascular system, forces produced by blood flow are critical determinants of not only morphogenesis and function, but also pathological conditions such as atherosclerosis. Endothelial cells (ECs) have numerous mechanotransducers, including platelet endothelial cell adhesion molecule-1 (PECAM-1) at cell-cell junctions and integrins at cell-matrix adhesions. Recent work has focused on integrin-mediated force transduction and force-dependent assembly of adhesion complexes. However, the processes by which forces are transduced into biochemical signals and subsequently translated into cellular responses downstream of PECAM-1 are poorly understood. Here, we use magnetic tweezers to examine mechanochemical signaling in response to direct force application on PECAM-1. We demonstrate that localized tensional forces on PECAM-1 result in, surprisingly, global signaling responses. Specifically, force-dependent activation of phosphatidylinositol 3-kinase (PI3K) downstream of PECAM-1 promotes cell-wide activation of integrins and the small GTPase RhoA. Activation of these signaling molecules is required for force-induced changes in cytoskeletal architecture, including growth of focal adhesions and adaptive cytoskeletal stiffening. Taken together, our work provides insight into the spatiotemporal regulation of mechanochemical signaling downstream of PECAM-1. Furthermore, we provide the first evidence of a global signaling event in response to a localized mechanical stress. Importantly, these data provide a possible mechanism for the differential stiffness of vessels exposed to distinct hemodynamic force patterns *in vivo*.

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Inhibition of PI3K/mTOR leads to adaptive resistance in matrix-attached cancer cells.

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Previously we found that phosphatidylinositol 3-kinase (PI3K) activation is critical for anchorage-independent survival of cancer cells, due to its ability to prevent apoptosis and rescue metabolic impairments of cells lacking natural matrix attachment. Because alterations in the PI3K pathway are common in breast, endometrial, and ovarian cancers and are thought to serve as major drivers of proliferation and survival in these cancers, we investigated the effects of drugs that inhibit PI3K/mTOR on growth and survival of tumor cell spheroids cultured in reconstituted basement membranes. Interestingly, there was a dichotomous response to PI3K/mTOR inhibitors in the tumor spheroids; matrix-attached cells were resistant to PI3K/mTOR inhibitors, whereas cells in the middle of the spheroids, lacking matrix-attachment, underwent cell death. The resistance of matrix attached cells is associated with up-regulation of a cellular program that resembles evolutionarily well-conserved responses to nutrient/growth factor deprivation and leads to up-regulation of signaling pathways required for cancer cell survival, such as IGF1R, EGFR and Bcl-2. Inhibition of components of this program abrogates resistance to PI3K/mTOR inhibition in immortalized and primary human breast and ovarian cancer cells as well as ovarian cancer xenografts (Cancer Cell (2012), 21:227-39).

We have now further characterized the components of this pathway leading to the resistance of matrix-attached cancer cells, and more specifically studied the role of the integrins and cell adhesion molecules required for the survival of the cancer cells under PI3K/mTOR inhibition. Several integrins are upregulated upon PI3K/mTOR inhibition both at transcriptional and translational level. Inhibition of beta1 integrin is not sufficient to abrogate the resistance, but rather inhibition of several integrins and/or downstream signaling components is needed to inhibit the adaptive resistance in matrix-attached cancer cells. We are currently further investigating how matrix attachment provides a permissive context for upregulation of the adaptive response to PI3K/mTOR inhibition.

Minisymposium 13: Intracellular Sorting and Trafficking

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TMEM115 as a Golgi stack protein regulating retrograde transport.

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The Golgi apparatus is a central sorting station for proteins destined for various post-Golgi compartments as well as proteins recycling between the early secretory pathways. In order to identify new Golgi proteins, we have search for transmembrane proteins at Human Protein Atlas at <http://www.proteinatlas.org/> and carefully analysed the localization of 103 proteins. Several Golgi-localized proteins were identified and two (TMEM115 & TMEM87A) of them have conserved counterparts in the yeast and fly. We have analysed in detail TMEM115, which was earlier reported as PL6 with its lost expression in renal clear cell carcinomas (J. Pathol. (2008) 214, 46-57). Double-labeling of TMEM115 with various Golgi markers suggests that it is primarily localized to the Golgi stack and its C-terminal hydrophilic region was revealed to be exposed to the cytoplasm. Functionally, brefeldin A-induced recycling of Golgi proteins back to the endoplasmic reticulum was inhibited when TMEM115 level was reduced by knockdown or increased upon over-expression, suggesting proper levels of TMEM115 is important for the recycling pathway. Co-immunoprecipitation experiment showed that TMEM115 interacts with all the subunits of COG complex, whose absence or knockdown also inhibited retrograde recycling. Knockdown of TMEM115 in HeLa cells also reduced cell proliferation. These results suggest that TMEM115 is a Golgi stack protein regulating retrograde transport and is involved in cell proliferation.

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Multiple factors function together to regulate ER structure and dynamics.

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The endoplasmic reticulum (ER) has an elaborate and dynamic structure that is conserved throughout eukaryotes. We are interested in how ER shape is regulated to generate a functional organelle. We have studied three major mechanisms that influence the 3-D structure of the ER. ER shape is determined by membrane shaping proteins, dynamics on the cytoskeleton, and interactions with other organelles. These forces work together to distribute this organelle throughout the cytoplasm and generate the complexity of ER functional domains. In turn, we have recently demonstrated that ER shape and dynamics also influence the distribution and dynamics of other organelles. I will discuss the dynamic interplay between the various mechanisms that regulate the elaborate structure of the ER and the organelles that it contacts.

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A molecular network for the transport of the TI-VAMP/VAMP7 vesicles from cell center to periphery.

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The compartmental organization of eukaryotic cells is maintained dynamically by vesicular trafficking. SNARE proteins play a crucial role in intracellular membrane fusion and need to be targeted to their proper donor or acceptor membrane. The molecular mechanisms that allow for the secretory vesicles carrying the v-SNARE TI-VAMP/VAMP7 to leave the cell center, load onto microtubules, and reach the periphery to mediate exocytosis are largely unknown. Here, we show that the TI-VAMP/VAMP7 partner Varp, a Rab21 guanine nucleotide exchange factor, interacts with GolginA4 and the kinesin 1 Kif5A. Activated Rab21-GTP in turn binds to MACF1, an actin and microtubule regulator, which is itself a partner of GolginA4. These components are required for directed movement of TI-VAMP/VAMP7 vesicles from the cell center to the cell periphery. The molecular mechanisms uncovered here suggest an integrated view of the transport of vesicles carrying a specific v-SNARE toward the cell surface.

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The ARL3 Arf-like GTPase targets myristoylated and farnesylated proteins to the membrane of primary cilia via its UNC119 and PDE6D effector proteins.

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The membrane of the primary cilium is a highly specialized compartment that organizes proteins to achieve spatially ordered signaling. Disrupting ciliary organization leads to diseases called ciliopathies, with phenotypes ranging from retinal degeneration and cystic kidneys to neural tube defects. How proteins are selectively transported to and organized in the primary cilium remains unclear. Using a proteomic approach, we identified

ARL3 effector proteins including the UNC119 and PDE6D, which we find are carrier proteins for a host of ciliary myristoylated proteins and farnesylated proteins. Previously, we found UNC119 uses a phenylalanine-rich β -sandwich to bind myristoylated ciliopathy protein nephrocystin-3 (NPHP3) and a group of other myristoylated proteins [Genes & Development 25: 2347 (2011)]. The ARL3 GTPase cycle facilitated transport of these proteins through the ciliary transition zone and insertion into the ciliary membrane.

We now show that PDE6D, a prenyl-binding protein acts as a specific facilitator for prenylated proteins to cross the transition zone. Using tandem affinity purification, we identified the ciliary farnesylated protein INPP5E involved in ciliopathies (Joubert/MORM syndromes) and other proteins as a cargo of PDE6D. PDE6D binds to INPP5E in a farnesyl-dependent manner, via a leucine-rich β -sandwich structure similar to UNC119. Mutations in the ARL3 binding domain of PDE6D fail to bind ARL3, but are still localized to the ciliary base. Specific mutations in many of the components of the ARL3 trafficking mechanism cause strong ciliopathies, showing both retinal degeneration and cystic kidney disease (nephronophthisis). A first mechanism by which ARL3 crosses the transition zone will be presented. We will also detail how the trafficking of INPP53, a 5'-inositol phosphatase can create specific ciliary membrane subdomains with distinct phosphoinositide isoforms and associated phosphoinositide binding proteins. Our studies of ARL3 effectors detail a general mechanism by which prenylated and myristoylated

proteins can be inserted into the ciliary membrane to organize specialized signaling regions and how this mechanism is compromised in many retinal-renal ciliopathies.

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Glycosphingolipid-driven membrane bending in clathrin-independent endocytosis of pathogens and signaling receptors.

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Several endocytic processes exist that do not require the activity of clathrin, and it has been a conundrum to know how the plasma membrane is bent in these cases. Our previous studies have uncovered a novel mechanism through which nanodomain construction by glycosphingolipid-binding toxins (Shiga and cholera toxins) or polyoma viruses (SV40) induces membrane curvature changes and drives the clathrin-independent formation of plasma membrane invaginations, leading to the endocytic uptake of these pathogens or pathogenic factors into cells (Nature 450, 670-675; NCB 12, 11-18). We could show that actin polymerization on Shiga toxin-induced endocytic tubule membranes alone is sufficient to induce scission in a process that requires membrane reorganization and domain formation (Cell 140, 540-553). Our data suggests that tubule membranes are poised in a metastable state such that an appropriate inducer can cause lipid segregation, thereby generating domain boundary forces that trigger line tension-driven squeezing of the tubules membranes leading to scission. We could indeed provide evidence that Shiga toxin-induced endocytic tubules are also enriched in glycosphingolipid species that are not the direct toxin receptors (Traffic 11, 1519-1529). This lipid co-sorting mechanism is dominant over curvature-mediated lipid sorting, and to be efficient, theoretical arguments suggest that the tubule membrane compositions must be close to demixing. The possibility that this concept can be generalized opens exciting perspectives on how lipid repartitioning can be exploited for membrane mechanics (Cell 142, 507-510). With Jitu Mayor, we have now analyzed how cortical actin dynamics contributes to glycosphingolipid clustering on active membranes, which facilitates toxin uptake into cells (manuscript in preparation). With Rob Parton, Katharina Gaus, and Robert Nabi, we have studied cellular proteins that use glycosphingolipids for membrane mechanics, thereby regulating the cell surface dynamics of various markers with critical roles in physiological processes such as cell migration. We found that galectin-3 functionally integrates structural carbohydrate information of cargo proteins (CD44, beta1-integrin...) with the membrane mechanical potential of glycosphingolipids to drive the glycosphingolipid-dependent formation of clathrin-independent carriers (manuscript in revision). Galectins thereby appear to function as adaptors that cluster glycosylated receptors and glycosphingolipids into nanoenvironments that then undergo membrane curvature changes according to similar mechanisms as we have described for Shiga and cholera toxins and SV40.

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CORVET and HOPS functions in *C. elegans*.

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CORVET and HOPS are tethering complexes in the endosomal pathway in yeast. They share a common core and differ only in two subunits, which are important for the interaction with Rab GTPases. We show here that both complexes also exist in metazoans, although one of the CORVET subunits is not conserved in *C. elegans* and mammalian cells. In contrast, one of the core components, the SM protein Vps33 is present in two isoforms VPS-33.1 and VPS-33.2, each of which is specific for one complex. The effect of knockdown of individual HOPS and CORVET components was studied in oocytes, in epithelial cells and in coelomocytes. These

analyses revealed that the predominant role of the CORVET complex is to promote homotypic fusion of early endosomes and the fusion of endosomal carriers with early endosomes, while the HOPS complex performs functions in endosome maturation and endolysosome formation. Moreover, different tissues appear to depend more on HOPS function than others. The specificity of CORVET and HOPS complex function is at least in part dependent on the individual VPS-33 subunit. Finally, our analysis reveals functions of individual HOPS and CORVET components that are independent of the function of the tethering complexes.

Minisymposium 14: Microtubule Organization and Dynamics

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Conformation-based mechanisms in a microtubule polymerase.

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Stu2p/XMAP215/Dis1 family proteins are evolutionarily conserved regulatory factors that use $\alpha\beta$ -tubulin-interacting TOG (tumor overexpressed gene) domains to catalyze fast microtubule growth. Catalysis requires that these polymerases discriminate between unpolymerized and polymerized forms of $\alpha\beta$ -tubulin, but how they do so has remained unclear. To address this question, we determined the structure of the TOG1 domain from Stu2p bound to yeast $\alpha\beta$ -tubulin. TOG1 preferentially binds a “curved” conformation of $\alpha\beta$ -tubulin that cannot be incorporated into microtubules, contacting α - and β -tubulin surfaces distinct from those involved in microtubule assembly. TOG1 binding to $\alpha\beta$ -tubulin apparently excludes equivalent binding of a second TOG domain. Conformation-selective interactions with $\alpha\beta$ explain how TOG-containing polymerases discriminate between unpolymerized and polymerized forms of $\alpha\beta$ -tubulin, and how they selectively recognize the growing end of the microtubule.

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Knockdown of microtubule polymerase XMAP215 can increase microtubule polymerization in *Xenopus laevis* neuronal growth cones.

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Proper neural connections, essential to nervous system function, depend upon precise navigation by the neuronal growth cone. A fundamental problem in growth cone cell biology is how guidance pathways are integrated to coordinate cytoskeletal dynamics, thus driving accurate steering. To address this question, we focus on the plus-ends of microtubules (MTs), which explore the growth cone periphery and play a key role in growth cone steering. MT plus-end dynamics are regulated by a conserved family of proteins called ‘plus-end-tracking proteins’ (+TIPs). Yet, it is still unclear how +TIPs interact with each other and with plus-ends to control MT behavior, especially in the developing nervous system. Here, we address this question by investigating the function of the traditional MT polymerase, XMAP215, during neurite outgrowth. When XMAP215 function is reduced, neurite outgrowth is severely compromised. By using automated, quantitative imaging analysis following acquisition of high-resolution live-imaging data of tagged +TIPs within cultured *Xenopus laevis* growth cones, we were surprised to discover that partial knock-down of XMAP215 leads to a 30% increase in MT plus-end velocity. This is unexpected given that XMAP215 is known to have MT polymerase activity. There are at least two possible mechanisms by which this increase in MT plus-end velocity may occur in

growth cones, either by affecting the polymerization/depolymerization rate directly, or by changing MT translocation rates through the regulation of MT interaction with other cellular structures. We have tested between these two models using quantitative fluorescence speckle microscopy, and we find that MT translocation rates are indistinguishable between controls and XMAP215 knockdown. Thus, reducing the function of the traditional MT polymerase XMAP215 leads to increased rates of MT polymerization in cultured *Xenopus* growth cones. We are currently investigating the mechanism by which this occurs, as well as how these changes in growth cone MT polymerization correlate with the reduced axon outgrowth phenotype that we also observe.

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Microtubule-associated proteins control microtubule nucleation from templates.

M. Wieczorek¹, S. Bechstedt¹, G. Brouhard¹; ¹Biology, McGill University, Montreal, QC, Canada

Cells nucleate new microtubules continuously, but the kinetics of nucleation is arguably the least understood aspect of microtubule dynamics. While microtubules can form spontaneously in vitro, γ -tubulin ring complexes (γ -TURCs) serve as template structures for microtubule nucleation in cells. Many microtubule-associated proteins (MAPs) have also been implicated in microtubule nucleation, but their direct role in the templating process is unclear. To determine the role of MAPs in templated nucleation, we have developed a quantitative assay that allows us to study the kinetics of microtubule nucleation from centrosomes (γ -TURCs), axonemes, and single microtubule templates using purified components. We found that templated nucleation is highly cooperative and that this cooperativity is independent of the type of template used. Performing the assay in the presence of different MAPs indicates that both catastrophe frequencies and growth rates influence the ability of a template to nucleate microtubules. Certain MAPs also changed the strength of cooperativity in the nucleation process. We directly demonstrate that MAPs can both enhance and inhibit templated nucleation by distinct mechanisms. These findings provide a means by which cells can control the numbers and locations of their microtubules throughout development and the cell cycle.

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The kinesin-8 Kip3 drives a distinct dynamic to non-dynamic midzone transition required to terminate spindle elongation.

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Kinesin-8 is a microtubule (MT) motor with conserved roles in chromosome alignment and spindle function. Its importance in facilitating spindle disassembly at the end of mitosis was recently demonstrated in budding yeast. Combining molecular genetics with live imaging and FRAP analysis our data reveal a novel role for the budding yeast Kinesin-8, Kip3, in regulating spindle length during anaphase. In order to examine the role of Kip3 prior to spindle disassembly we challenged the cells with an anaphase arrest. Control spindles stopped elongating as they obtained a length approximately equal to the cell diameter. However, in the absence of Kip3 spindles exceeded the cell length by at least 2-fold with elongation rates similar to typical anaphase, suggesting that the same activities that drive anaphase elongation continue to drive hyper-elongation. Surprisingly, spindles in other mutant cells, such as *ip1-321*, in which spindles slightly over-elongate maintained their bent morphology without progressing into hyper-elongation during anaphase arrest. These results eliminate the idea that Kip3 suppresses spindle length in conjunction with cortical compression forces, and implicate a role for Kip3 at the spindle midzone. FRAP of the midzone showed that MTs were highly dynamic during

spindle elongation. In control spindles, MT dynamics were dramatically suppressed once spindles obtained proper length. However, in the absence of Kip3, MTs remained highly dynamic. Furthermore, in *kip3* deletion cells with straight, buckled, or hyper-elongated spindles we found that midzone lengths were unaltered compared to control cells, demonstrating that excess MT polymerization in the absence of Kip3 at the midzone is tightly coupled to antiparallel MT sliding and spindle hyper-elongation. To decouple these processes we deleted the antiparallel sliding motor Kip1, Kinesin-5, which reduces rates of anaphase spindle elongation. In *kip1, kip3* deletion strains the midzone length was significantly longer compared to those in *kip3* deletion strains. Taken together, our results support a model in which Kip3 acts as a switch to drive a distinct dynamic to non-dynamic midzone transition in order to prevent spindle hyper-elongation and maintain structural integrity of the spindle. RR was supported by T32 HL094282 & the American Heart Association postdoctoral fellowship.

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Microtubule function is diversified by tubulin carboxy-terminal tails.

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Microtubules provide tracks for diverse transport and force generation processes in cells. Although a number of microtubule-binding proteins and motors have been identified, how the microtubule track contributes to these mechanisms is poorly understood. The carboxy-terminal tail regions (CTTs) of α - and β -tubulin subunits are likely to play a critical role. CTTs facilitate electrostatic interactions at the microtubule surface, are major sites of tubulin posttranslational modification, and exhibit high sequence variation amongst tubulin isoforms in humans. CTTs may, therefore, represent important hubs for regulating microtubule function by modulating interactions at the surface. Here we investigate the roles of CTTs by examining a series of mutants that alter CTT structure in budding yeast. We find that CTTs are not essential for life, but loss of α - or β -tubulin CTTs causes discrete defects in microtubule function. Neither is necessary for microtubule stability; however, the β -tubulin CTT specifically promotes microtubule dynamics. To identify how CTTs contribute to function, we use synthetic genetic array analysis to map the genetic interaction networks of CTT mutants. Our results indicate that mutants in α - and β -tubulin have divergent consequences, including effects on EB1 and dynein. We investigate these phenotypes using live-cell analysis of protein and network dynamics. In addition, our screen identified many unexpected interactions that suggest novel roles for tubulin in cells.

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TUBB3 mutations cause axon misguidance and cytoskeletal changes in congenital fibrosis of the extraocular muscles 3 (CFEOM3).

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Congenital Fibrosis of the Extraocular Muscles (CFEOM) is a group of eye movement disorders that can result from genetic errors in cranial axon growth and guidance. We previously identified the CFEOM3 gene as TUBB3, which encodes the neuron-specific β 2-tubulin isotype III. TUBB3 mutations cause CFEOM as well as additional nervous system disorders associated with aberrant axon guidance and maintenance, which may result from changes in microtubule dynamics and/or abnormal MAP binding. Several disease-associated TUBB3 substitutions reside at putative kinesin motor interaction sites on β 2-tubulin, such as E410K, which causes severe CFEOM3, anterior commissure/corpus callosum hypoplasia with associated social and

intellectual disabilities, Kallmann syndrome (anosmia and hypogonadotropic hypogonadism), facial weakness, and progressive axonal sensorimotor polyneuropathy. Although clinical abnormalities suggest multiple axon guidance defects caused by mutations in this cytoskeletal gene, molecular mechanisms remain unclear. Previously, we showed altered microtubule dynamics in several CFEOM mutations. We have now developed a mouse model harboring the human TUBB3E410K mutation and found axon misguidance in multiple regions of the Tubb3E410K embryonic nervous system. Moreover, through biochemical and mass spectrometry studies, we have found alterations in motor protein-microtubule interactions. Therefore, altered motor-microtubule interactions, which may affect microtubule dynamics and/or axonal transport, may explain the etiology of CFEOM3, as well as elucidate molecular pathways essential for axon guidance and circuit formation in the mammalian nervous system.

Minisymposium 15: Physical and Computational Tools for Cell Biology

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Analysis of single-cell transcriptomes reveals gene expression states that drive key transitions in cellular subpopulations.

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Biological phenomena in multi-cellular populations are fundamentally driven by the collective properties of individual cells that make up the population. Despite this, our understanding of molecular networks from either cell cultures or tissues are based on measurements made from the entire population with the implicit assumption that all cells behave similarly or that averaged signals from the population accurately describe the underlying molecular architecture of all cells in that group. A growing body of data collected from individual cells has started to challenge these basic assumptions and increasingly suggests that properties driving lineage choice, developmental fate and disease states emerge from the transcriptional and signaling architectures of distinct sub-populations of cells. We have developed a system for the routine preparation of hundreds of full-length mRNA-sequencing libraries in parallel, enabling detailed descriptions of transcription in cell populations at the level of the individual cell. Characterization of transcriptional heterogeneity within nominally homogeneous K562 cell populations in tissue culture reveals a wide spectrum of variation in gene expression and coupling of modules within signaling networks. In addition, we are investigating the changes that drive key transitions during cell differentiation and tumor formation.

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Quantitative dissection of gene regulation through DNA loop formation.

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The study of genetic circuits has reached a state of maturity that permits direct and stringent comparison between theories of transcriptional regulation and the response of living cells. We have developed and tested such models of gene expression based on equilibrium statistical mechanics. The models require only a few input parameters: binding energies of proteins, copy numbers of molecular components, and the mechanical properties of DNA. These parameters are measured in simple control experiments and used to predict gene expression levels for genetic circuits involving different combinations of these parameters. Such first principles

biological models require no fitting parameters and make quantitative and precise predictions about gene regulation over a wide range of parameter space.

Equations derived from these models define the input-output function of each genetic circuit, and predict how each system component modulates gene expression. We independently tuned each parameter of the model and experimentally tested whether the gene circuit responded as predicted. Model predictions were tested by creating these genetic circuits in *E. coli*, and using fluorescent reporters to quantify the level of gene expression for each circuit. We tuned transcription factor copy number over two orders of magnitude and varied the operator binding energies resulting in several orders of magnitude change in dissociation constants.

Next we implemented the model to examine the role of DNA mechanical properties in the context of transcription factor-mediated loop formation. The tetrameric transcription factor Lac repressor is able to increase the level of gene repression by binding to two operators and looping the intervening DNA. We use this system to ask whether the sequence-dependent flexibility of the DNA in the loop influences gene expression. By incorporating DNA sequences with known sequence-dependent flexibilities into the looping region, we found that the intrinsic flexibility of the looping DNA does not influence repression. DNA sequences shown to be flexible *in vitro*, including in our own tethered particle motion assays, do not lead to increased repression. We resolve the lack of *in vivo* sequence dependence by theoretically and experimentally exploring the role of the DNA-bending protein HU in loop formation. We found that after knocking out HU, sequence-dependent loop formation is restored *in vivo*, and use the models to suggest how the presence of DNA-bending proteins buffers away the influence of sequence-dependent mechanics on gene regulation.

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Heterogeneity in cell-matrix adhesion as an indicator of metastatic state.

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Cancer cells have great genetic diversity, which may be reflected in the variation in metastatic potential. As it has been difficult to determine a comprehensive set of genetic markers to define a metastasizing cell population, we propose to examine a common behavior, i.e. cell-matrix adhesion, that may be differentially regulated in metastasizing versus non-metastasizing cancer cells. To assess cell-matrix adhesion strength in a heterogeneous population of breast cancer cells, we employed a spinning disc device where cells adhering to matrix-coated substrates were exposed to radially-dependent shear. Within a cell population, those at the center or edge experience low or high matrix detachment forces, respectively. Exposure to acute shear for a highly metastatic cancer cell line, i.e. MDA-MB-231, resulted in a broadly distributed population of cells unlike the sigmoidal, homogeneous shear response observed from non-metastatic, non-malignant, or somatic cell lines, MCF7, MCF10A, NIH 3T3 fibroblasts, respectively. This suggests that adhesion strength differences may scale with cell state with the most metastatic cells coming from high shear regions. Magnesium and calcium concentration differences have been reported between stroma and tumors, which are also involved in regulating integrin activation. While there was an up to 2-fold increase in adhesion strength for MCF7 or MCF10A cells in the presence of 0.5 mM magnesium, MDA-MB-231 cells exhibited a 10-fold increase in adhesion strength and also developed a sigmoidal, homogeneous shear response when exposed to 0.5 mM magnesium and/or calcium. The average attachment strength as well as the restoration of attachment-homogeneity gradually increases with cation concentration with the most drastic increase occurring between 10 μ M to 0.5mM, which falls within the physiological range for both stroma and tumor. This observation indicates that variances in attachment

strength in the absence of cations may be dependent on cell state. Indeed, when selecting only strongly attaching cells (in the absence of exogenous cations), we found that those cells not only maintain their (strong) attachment phenotype over time (up to 48 hours tested so far), but also differ in cell behavior – the mitosis rate is nearly doubled compared to the bulk of cells. These findings suggest a potential mechanism to differentially regulate metastasizing cell adhesion, and also support using population-based adhesion assays to assess adhesion heterogeneity and select those cells that may have the highest metastatic potential.

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Sensitive imaging of cellular processes using two-photon polarization microscopy (2PPM).

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Membrane proteins are difficult to study, due to their requirement of a lipid membrane for function. We have taken advantage of the cell membrane requirement for exceptionally sensitive functional imaging of membrane proteins, using two-photon polarization microscopy (2PPM). 2PPM can be used for imaging of G-protein activation, changes in intracellular calcium concentration, and other cellular processes, in living cells and organisms, with sensitivity comparable to, or even exceeding that of current FRET probes. Crucially, in contrast to FRET, polarization fluorescence microscopy only requires presence of a single fluorescent protein. Therefore, many existing constructs can be used as optical probes of molecular processes involving membrane proteins. Furthermore, polarization microscopy offers a clear path towards development of new genetically encoded optical probes of membrane protein function, including a usable genetically encoded optical sensor of cell membrane voltage. Our results indicate that in many biological applications, FRET is likely to be complemented or even replaced by 2PPM.

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Opening windows into the cell: Focused ion beam micromachining of eukaryotic cells for cryoelectron tomography.

E. Villa¹, M. Schaffer¹, F. J. Bäuerlein¹, A. Rigort¹, J. Plitzko¹, W. Baumeister¹; ¹Max Planck Institute of Biochemistry, Martinsried, Germany

Cryo-electron tomography (cryo-ET) provides unprecedented insights into the 3-D macromolecular organization of cells in their native state. However, the thickness of most cells makes them inaccessible to cryo-ET. I will show how focused ion beam (FIB) milling can be used to prepare 200-500 nm lamellae from intact cells, opening large windows into the cell's interior, exposing their landscapes at molecular resolution. Advanced computational analysis makes the identification of macromolecular complexes possible, yielding visual proteomes of cells. I will discuss the use of cryo-ET and FIB milling to study the structural dynamics of the nuclear pore complex, one of the largest macromolecular machines in the cell. It is composed of hundreds of proteins, selectively controlling all traffic between the nucleus and the cytoplasm. The architecture of the NPC is central to understanding nuclear transport. However, due to its sheer size, its local environment and its dynamic nature, determining its structure at molecular resolution remains a challenge for conventional techniques. Combining FIB milling, cryo-ET, and image processing enables the study of the NPC in its native environment, free of the distortions caused by purification. This approach has not only revealed the NPC architecture at unparalleled resolution, but also captured different conformational states in action. I will further illustrate the use of cryo-FIB/ET to study diverse cellular environments at molecular detail,

including actin networks, the architecture of cell division, and the distribution of macromolecular complexes within organelles such as mitochondria.

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Voltage imaging in vivo with a new generation of rhodopsin-based indicators.

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Reliable, optical detection of single action potentials in an intact brain is one of the longest-standing challenges in neuroscience. We recently showed that a number of microbial rhodopsins exhibit intrinsic fluorescence that is sensitive to transmembrane potential. One class of indicator, derived from Archaerhodopsin-3 (Arch), responds to voltage transients with a speed and sensitivity that enable near-perfect identification of single APs in cultured neurons [Nat Methods. (2011). 9:90-5]. We extended the use of these indicators to an in vivo context through the application of advanced imaging techniques to the larval zebrafish. Using planar-illumination, spinning-disk confocal, and epifluorescence imaging modalities, we recorded electrical activity in a variety of structures, including the brain and heart, in a noninvasive manner. Transgenic lines expressing Arch variants in defined cells enable comprehensive measurements to be made from specific target populations. In parallel, we also extended the capabilities of our indicators by improving their multiphoton excitability and overall brightness. Microbial rhodopsin-based voltage indicators now enable optical interrogation of complex neural circuits, and electrophysiology in systems for which electrode-based techniques are challenging.

TUESDAY, DECEMBER 18**Symposium 3: Prokaryotic Communities**

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Manipulating quorum sensing to control bacterial pathogenicity.*B. Bassler¹; ¹Molecular Biology, HHMI and Princeton, Princeton, NJ*

Cell-cell communication in bacteria involves the production, release, and subsequent detection of chemical signaling molecules called autoinducers. This process, called quorum sensing, allows bacteria to regulate gene expression on a population-wide scale. Processes controlled by quorum sensing are usually ones that are unproductive when undertaken by an individual bacterium but become effective when undertaken by the group. For example, quorum sensing controls bioluminescence, secretion of virulence factors, biofilm formation, sporulation, and the exchange of DNA. Thus, quorum sensing allows bacteria to function as multi-cellular organisms. Bacteria make, detect, and integrate information from multiple autoinducers, some of which are used exclusively for intra-species communication while others enable inter-species communication. New studies show that interfering with quorum sensing can be used to control bacterial virulence in globally important pathogens. These findings suggest an alternative to traditional antibiotics.

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Immune defense of the intestinal epithelial surface.*L. Hooper^{1,2}; ¹Department of Immunology, The University of Texas Southwestern Medical Center, Dallas, TX, ²Howard Hughes Medical Institute, Dallas, TX*

The mammalian intestine is home to ~100 trillion bacteria that perform important metabolic functions for their hosts. The proximity of vast numbers of bacteria to host intestinal tissues raises the question of how symbiotic host-bacterial relationships are maintained without eliciting potentially harmful immune responses. We discovered that resident intestinal bacteria trigger epithelial cell expression of RegIIIgamma, a member of the C-type lectin family of carbohydrate binding proteins. RegIIIgamma directly kills Gram-positive bacteria and thus represents a novel class of antibacterial proteins. We have elucidated the mechanistic basis for RegIIIgamma bactericidal activity by showing that it kills bacteria by forming an oligomeric pore in the bacterial inner membrane. Using cryoelectron microscopy, we determined the structure of the hexameric pore assembly at subnanometer resolution. *In vivo* studies have revealed that RegIIIgamma is essential for maintaining a ~50 micron zone that physically separates the microbiota from the small intestinal epithelial surface. Loss of host-bacterial segregation in *RegIIIgamma*^{-/-} mice is coupled to increased bacterial colonization of the intestinal epithelial surface and enhanced activation of intestinal adaptive immune responses by the microbiota. Together, our findings reveal that RegIIIgamma is a fundamental immune mechanism that promotes host-bacterial mutualism by regulating the spatial relationships between microbiota and host.

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Exploring the cell biology of 2-methyl hopanoids, an ancient class of microbial lipids.

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Efforts to understand the roles of lipids in various aspects of microbial cell biology have gained momentum in recent years in recognition that the lipid composition of bacterial membranes, much like their eukaryotic counterparts, is exquisitely ordered at small spatial scales and may impact protein localization and activity. Hopanoids--steroid analogs in bacteria--are a fascinating class of lipids whose biological functions are poorly understood. Today, they are made by over 200 bacterial species, including some pathogens. Yet hopanoids are ancient: their molecular fossils can confidently be extracted from sedimentary rocks that are billions of years old. One such hopanoid fossil, 2-methyl hopane, has been used as a biomarker to date the rise of oxygenic photosynthesis because cyanobacteria were thought to be the dominant 2-methylhopanoid (2-MeBHP) producers in nature. However, over the past few years, our laboratory has challenged this interpretation by demonstrating that other bacteria can produce 2-MeBHPs in significant quantities under strictly anaerobic conditions. Using two photosynthetic bacteria (*Rhodospseudomonas palustris*, an anoxygenic phototroph and *Nostoc punctiforme*, an oxygenic phototroph) as model hopanoid-producing organisms, we elucidated the hopanoid biosynthetic and transportation machinery, investigated the regulation of 2-MeBHP production in particular, explored the phenotypes of hopanoid-deficient mutants, determined hopanoid subcellular localization and probed the ecological distribution of 2-MeBHP producers in various environments. We are currently using biochemical and biophysical methods to gain a more mechanistic understanding of hopanoid functions. Our data show that 2-MeBHPs are not linked to the process of oxygenic photosynthesis; rather, they suggest that 2-MeBHPs may facilitate certain types of symbiotic relationships between bacteria and plants/algae. Exploring the cell biology of hopanoids in modern bacteria not only provides an opportunity to gain insight into ancient microbial communities, but affords the chance to explore the processing and function of steroid analogs in living systems where they are not essential.

Minisymposium 17: Cell Biology of Regeneration

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Positive feedback in the β -catenin destruction complex promotes reliable cell fate decisions of intestinal cells.

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Homeostasis is the maintenance of a constant condition by feedback control. Tissues that self-renew and regenerate after exposure to environmental perturbation require layers of control to maintain homeostasis. The epithelial lining of the intestine, a prime example of a homeostatic system, completely renews itself every ~5 days. Amazingly, in response to continuous mechanical, chemical and pathogen-derived wounding, intestinal stem cells produce multiple cell types at just the right ratio throughout the entire life of the organism. Wnt signal transduction, through β -catenin-regulated transcription, controls intestinal tissue homeostasis and is dysregulated in many cancers. Typically, β -catenin levels are kept low in non-renewing

tissues by the β -catenin destruction complex, consisting of the scaffold protein Axin, Glycogen Synthase Kinase 3 (GSK3), Casein Kinase 1 (CK1), and Adenomatous Polyposis Coli (APC). Mechanisms underlying activation and inactivation of this complex remain poorly understood. Using high-content imaging of intestinal cells, we evaluated the response of single cells to Wnt signaling. We found cells respond in an “all-or-none” binary fashion without intermediates. To identify feedback control responsible for binary behavior, we performed biochemical studies in *Xenopus* egg extracts and uncovered positive feedback between Axin and GSK3. Mathematical modeling of this feedback loop predicts bistability in the activity of the β -catenin destruction complex as a whole. We are validating our model by pharmacologically and genetically perturbing the positive feedback between Axin and GSK3. Our findings elucidate molecular design features that convert a graded morphogen, such as Wnt, into discrete binary cell fate decisions and provide a mechanism for cellular memory of Wnt stimulation when the stem cell microenvironment is disrupted. This behavior has important consequences in the intestinal stem cell niche, where stem cell number must be tightly maintained to prevent loss or overgrowth of the epithelium.

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Role of Mob1 in regeneration of a single cell.

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Understanding the processes behind the regeneration of cells and tissues is an important cell biological question. Cells need to identify and react to specific damages in and around them, and in order to understand these processes at a basic level we need a model that exhibits a similar regeneration of specialized structures in the context of a single cell. Perhaps the most classical system for studying regeneration in single cells, pioneered by Thomas Hunt Morgan, F.R. Lillie, Vance Tartar and others, is *Stentor coeruleus*, a large ciliate, ~1mm long, with a highly patterned cell cortex and the ability to regenerate and reorganize after surgical or chemical manipulations. Its large size and complex organization rival those of metazoan organisms and the ease of surgical manipulations gives *Stentor* significant advantages over other ciliate models. Using the traditional surgical techniques unique to *Stentor* as well as modern RNA interference (RNAi) methods, visualization techniques, and genomic sequencing I will revive *Stentor* as a model for studying the regeneration of cell polarity and organization. We have partially sequenced *Stentor's* macronuclear genome and shown that RNAi machinery is functional in *Stentor*. Using an RNAi approach we have begun a systematic investigation of the molecular basis of single-cell regeneration. Mob1 is a highly conserved kinase regulator and is known to interact with NDR/LATS kinases. It plays a role in a variety of functions including apoptosis, mitosis, morphogenesis, and cell proliferation. Recent work in *Tetrahymena thermophila* suggests that Mob1 also performs important roles in ciliates. Results for RNAi of Alpha-Tubulin and Mob1 in *Stentor* result in dramatic changes in cell polarity and organization of the cortex, showing that we can analyze single-celled regeneration in *Stentor* at a molecular level. Knocking down Alpha-Tubulin, a key structural component in the cortex, results in cortical defects and problems with cell regeneration. This is very different from the Mob1 knockdown, which results in the drastic elongation of cells, cortical aberrations, and an apparent loss of proper cell proportions. Using RNAi in conjunction with the unique microsurgical methods available in *Stentor*, it should be possible to restore this classical system to its previous status as a central model for understanding the basic principles of cell polarity, biological pattern formation, and cellular regeneration.

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Self-renewal and fate specification of skin stem cells.T. Chen¹, E. Fuchs¹; ¹Rockefeller University, New York, NY

Adult stem cells (SCs) sustain tissue maintenance and regeneration throughout the lifetime of an animal. They often reside in specific signaling niches that orchestrate the stem cell's balancing act between quiescence and cell cycle re-entry based upon demand for tissue regeneration. How SCs maintain their capacity to replenish themselves following tissue regeneration is still poorly understood. Here, we use RNA interference (RNAi)-based loss-of-function screening as a powerful approach to uncover transcriptional regulators governing SC self-renewal and regenerative potential. Hair follicle (HF) SCs provide an ideal paradigm. They've been purified and characterized from their native niche in vivo, and in contrast to their rapidly dividing progeny, they can be maintained and passaged long-term in vitro. Focusing on nuclear proteins/transcription factors enriched in SCs versus progenies, we screened ~2,000 shRNAs for their impact on long-term but not short-term self-renewal in vitro. To address the physiological relevance of our findings, we selected one candidate, Tbx1, surfacing in the screen. Expressed in many tissues, this transcription factor has not been studied in the context of SC biology. By conditionally ablating Tbx1 in vivo, we show that tissue regeneration during homeostasis occurs normally but is dramatically delayed. Devising an in vivo assay for SC replenishment, we then show that when challenged with repetitive bouts of regeneration, the Tbx1-deficient SC niche becomes progressively depleted. Addressing mechanism, we discover that Tbx1 acts as an intrinsic rheostat of BMP signalling, the gatekeeper governing the transition between SC quiescence and proliferation in HFs. Our results validate the RNAi screen and underscore its power in unearthing new players governing SC self-renewal and tissue-regenerative potential.

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Growth coordination during tissue regeneration requires nitric oxide signaling.J. Jaszczak¹, A. Dao¹, A. Halme¹; ¹Department of Cell Biology, University of Virginia School of Medicine, Charlottesville, VA

The regeneration of tissues is often tightly coupled to developmental progression. Our group has been using *Drosophila melanogaster* as a model system to understand how developmental signals impact the regenerative capacity of tissues, and in turn how regenerating tissues alter developmental signals. Using this system, we have previously described a developmental checkpoint that is activated by localized tissue damage and delays developmental progression to allow for regenerative repair of the damaged tissue. We and others have observed that activation of the developmental checkpoint performs another important function: to coordinate the growth of the regenerating tissue with the growth of other undamaged tissues. Recently, we have identified an important role for nitric oxide signaling in growth coordination during regeneration through mutation and mis-expression of the sole *Drosophila* nitric oxide synthase (*Nos*) gene. We have found that *Nos* activity is both necessary and sufficient for growth restriction in undamaged tissues during regenerative repair, and likely exerts its effects on growth through inhibiting signaling through the steroid hormone ecdysone. In support of this, we have shown that ecdysone signaling is necessary for the growth of undamaged imaginal discs, the up-regulation of *Nos* activity can block ecdysone signaling, and that *Nos*-dependent growth inhibition during delay can be overcome by the ectopic addition of ecdysone. Whereas recent published experiments have demonstrated that nitric oxide can promote ecdysone signaling during development, our observations suggest that there may be a separate pathway by which nitric oxide can inhibit ecdysone signaling in response to tissue damage, and that this pathway is critical for maintaining proper tissue proportion following damage. We are currently

examining how nitric oxide limits ecdysone signaling, the role of ecdysone in regulating tissue growth, and how regenerating tissues remain unaffected by systemic growth inhibition.

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The vasculature guides the collective migration of Schwann cells during peripheral nerve regeneration.

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The peripheral nervous system (PNS) has remarkable regenerative capacities following injury including the ability to restore a fully cut nerve. This process requires axons to find their way across a bridge of new tissue, often several millimetres in length, which forms to reconnect the severed ends of the nerve. We have previously shown that Schwann cells migrate collectively to guide the regrowing axons across the 'bridge', in a process directed by fibroblasts at the nerve stumps (Parrinello et al., Cell 2010). In this study, we have addressed the molecular and cellular mechanisms that control the direction of Schwann cell migration to ensure the successful delivery of axons to the distal stump. We have found that polarised blood vessels form inside the bridge rapidly following injury and prior to Schwann cell migration. We find they form in response to an angiogenic signal from macrophages - which are the major cellular component of the bridge. Importantly, we show that the migrating cords of Schwann cells use the blood vessels *in vivo* as "tracks" to find their way across the bridge. Moreover, we can recapitulate this behaviour *in vitro* using 3D blood vessel models. Significantly, when we disrupt the correct polarisation of the blood vessels *in vivo*, the Schwann cell cords lose their directionality leading to defective nerve repair. This study provides further novel insights into the mechanisms important for promoting nerve repair following injury and further highlights the complexity of the multicellular response required for successful regeneration of an adult tissue.

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Identification of factors critical for planarian nervous system regeneration.

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Planarians possess an extraordinary capacity for regeneration. While superficially simple, the planarian brain exhibits regional specialization and is composed of a wide variety of neural cell types. Importantly, as one of only a handful of animals with the ability to regenerate their central nervous systems *de novo*, these freshwater flatworms provide a unique opportunity to study regeneration of complex nervous tissues *in vivo*. Despite some progress in understanding regeneration of the planarian brain, several important, mechanistic questions remain: What extrinsic and intrinsic factors direct planarian stem cells to neural (or specialized neural) fates? How are newly regenerated neurons reconnected with existing neural or target tissues? How are neural connections reestablished faithfully to restore function after regeneration? To discover factors key to these processes, we used high throughput sequencing and identified hundreds of transcripts significantly upregulated or downregulated at key time points during head regeneration in the planarian *Schmidtea mediterranea*. The expression patterns of these genes suggest a variety of potential functions; while several genes are expressed in the nervous system (either ubiquitously or in a limited manner), others are expressed in stem cells, stem cell progeny, or other tissue types (intestine, etc.). We are currently evaluating the function of many of these genes in brain regeneration using RNA interference. Already, we have identified two conserved transcription factors with uncharacterized roles in planarian regeneration. One LIM domain protein, expressed medially in the nervous system, is required for connection of the two

lobes of the planarian brain during regeneration. Additionally, knockdown of a basic helix-loop-helix transcription factor results in fusion of the two photoreceptors at the midline. The mechanistic insight revealed by this study will illuminate the coordination of cellular events required for planarian nervous system regeneration, contributing to the broader goal of recapitulating the complex type of nervous system regeneration required for functional restoration in humans.

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Transition in the division mode of cortical neural stem cells.

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In the developing mammalian neocortex, elongated neuroepithelial cells function as neural stem cells. These cells initially undergo symmetric divisions to proliferate and then later divide asymmetrically to continuously generate a self-renewing daughter (radial glia or apical progenitors) and a differentiating daughter cell (neurons and intermediate progenitors). In the neurogenic mode, self-renewing daughters of radial glial divisions retain their epithelial structures, including both apical and basal processes, therefore maintaining the pseudo-stratified brain organization. However, our recent live-imaging studies have revealed that, on occasion, these radial glia undergo oblique cleavages that split the cell into an apical and basal part. The apical daughters inherit only the apical membrane and differentiate. In contrast, the basal daughters, which inherit only the basal process, migrate out from the ventricular zone and become a different type of self-renewing progenitors that continue asymmetric neurogenic divisions. These progenitors closely resemble a major type of progenitors seen in the outer subventricular zone (OSVZ) of gyrencephalic animals, termed the basal or outer radial glia (Fietz et al., 2010; Hansen et al., 2010), indicating that the basal (outer) radial glia are also present in rodents, but as a minor cell population. Thus, it appears that this type of progenitor is commonly found during mammalian cerebral development, and the self-renewing progenitors in the developing mammalian cortex undergo two transitions in the division mode; first, from symmetric to asymmetric divisions of radial glia, and then later on, from radial glia to basal radial glia. The second transition can shift radial glia to a new niche (OSVZ), which might have a role in the brain size expansion during mammalian evolution. Mutant mice with defects in cleavage plane orientations generate a number of the basal radial glia, and using these mice will allow us to analyze the properties and genetic defects of basal radial glia.

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Asymmetric cell division in stem cells during CNS development.

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The mammalian brain develops from a layer of progenitor cells located on the apical side of a neuroepithelium. Initially, the progenitor pool expands by symmetric divisions. Later, progenitors divide asymmetrically and generate only one self-renewing daughter cell. The other daughter

cell either migrates towards the cortical plate to differentiate into a neuron (direct neurogenesis) or becomes an intermediate progenitor generating two neurons after one additional symmetric division (indirect neurogenesis). In symmetric progenitor cell divisions the mitotic spindle is always parallel to the neuroepithelial surface, but asymmetric divisions can also have vertical or oblique spindles. Whether this reorientation of the mitotic spindle is actually responsible for the establishment of asymmetry during those divisions is highly controversial and unclear.

We have addressed this question by generating conditional mutants and overexpression lines for the mouse *Inscuteable* (*mlnsc*) gene, the homolog of a key regulator of spindle orientation in *Drosophila*. We find that mutating *mlnsc* almost completely abolishes oblique and vertical mitotic spindles while *mlnsc* overexpression has the opposite effect. Surprisingly, our data indicate that spindle reorientation is not essential for the asymmetric outcome of the progenitor divisions but strongly influences the balance between direct and indirect neurogenesis. Thus, lineage decisions in the vertebrate brain are influenced by the orientation of progenitor divisions. As modified lineages and increased indirect neurogenesis are highly correlated with the expanded cortical size in primates, our data might be relevant for human brain evolution as well. By genome-wide RNAi screening in *Drosophila* neural stem cells, we have identified a large set of genes essential for lineage specification. We have started to analyze their mammalian homologs and will present results on their functional conservation.

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Neural stem and progenitor cells and the evolution of the cerebral cortex.

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Our group studies the molecular and cellular mechanisms of neurogenesis in the developing neocortex in the context of mammalian brain evolution, specifically the various types of cortical stem and progenitor cells and their modes of division. In terms of their cell biology, two principal classes of cortical stem/progenitor cells can be distinguished. One class comprises stem/progenitor cells exhibiting bipolar morphology and apical-basal cell polarity that divide at the ventricular, i.e. apical, surface of the ventricular zone. These are the neuroepithelial cells and apical radial glial cells, which are collectively referred to as apical progenitors (APs). The other class comprises stem/progenitor cells dividing in a more basal, abventricular location, notably the subventricular zone, which are collectively referred to as basal progenitors (BPs). These fall into two subclasses (i) radial glia-related progenitors exhibiting monopolar morphology and basal, but not apical, cell polarity, referred to as basal radial glial cells (bRGCs); and (ii) progenitors exhibiting nonpolar morphology and lacking overt apical-basal cell polarity, which comprise transit-amplifying progenitors (TAPs) that undergo multiple rounds of cell division and intermediate progenitor cells (IPCs) that undergo only one round.

Our group has been studying the following issues related to these progenitor cells in the embryonic mouse, ferret, marmoset, macaque and human neocortex:

- (1) the various lineages from APs to BPs and neurons and their impact on neuron number;
- (2) the machinery underlying BP delamination, focusing on the primary cilium and centrosome;
- (3) the relationship between cell polarity and cleavage plane orientation in the context of symmetric versus asymmetric progenitor cell divisions;
- (4) the role of the microcephaly gene *Aspm*;
- (5) the cholesterol-binding apical membrane protein prominin-1/CD133;
- (6) prominin-1-bearing extracellular membrane particles released into the ventricular fluid from the midbody and primary cilium of APs, and their role in differentiation;

(7) the basal process of APs and bRGs and its role in extracellular matrix-dependent, integrin-mediated progenitor self-renewal;
 (8) the role of cell cycle length in stem and progenitor cell proliferation versus differentiation;
 (9) the comparative analysis of the transcriptomes of the various progenitor types in embryonic mouse and fetal human neocortex, and the cross-species transfer of transcriptomes by microinjection of single progenitors in organotypic slice culture.
 Recent insights relevant for understanding the evolution of the neocortex will be presented.

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Shootin1 acts in concert with Kif20b to promote polarization of migrating neurons.

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Shootin1 has been ascribed a role in regulating polarization of primary hippocampal neurons. We set investigating the role of Shootin1 in polarization of migrating neurons based on the proposed analogy with the process of polarization in primary hippocampal neurons. Shootin1 is highly expressed in the developing cortex, is detected in the microtubule associated proteins fraction, and its knockdown affects neuronal migration and polarization in vivo. Furthermore, a member of the kinesin superfamily, Kif20b, was identified as a novel Shootin1 interacting protein, and we mapped the binding domain to a fifty-seven amino acid sequence. The direct interaction and binding affinities between the minimal binding domain of Kif20b and Shootin1 were analyzed using surface plasmon resonance based technology. Furthermore, these proteins interacted in vivo, and in primary hippocampal neurons Kif20b knockdown reduced the mobilization of Shootin1 to the developing axon as evident by immunostaining and fluorescence recovery after photobleaching (FRAP) analysis, thus suggesting that Shootin1 is a novel Kif20b cargo. In the developing brain, either knockdown of Kif20b or expression of the minimal binding domain inhibited neuronal migration. Functional interaction between Shootin1 and Kif20b is suggested from migration assays in vivo. Collectively, our data demonstrate the importance of Shootin1 and its interaction with Kif20b to the dynamic process of neuronal polarization and migration.

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A role for cilia in dendrite extension.

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The cilium is a cellular organelle involved in motility and sensation. Non-motile primary cilia are found in many cells of the human body, including the nervous system. Previously considered to be mostly vestigial, there has been a resurgence of interest in primary cilia due to their link to a range of developmental and adult disorders in humans known as ciliopathies. Most ciliopathy-associated proteins are located at the base of the cilium in a complex structure called the ciliary transition zone.

In the nematode worm *C. elegans*, cilia are found exclusively at the tip of the dendrites of sensory neurons. Loss of cilia results in impaired perception of the external environment, but does not affect viability or fertility of the animal, making *C. elegans* an excellent model to study cilia and ciliopathy-associated proteins. In this study, we set out to characterize the *C. elegans* ortholog of CEP-290 (mutated in Meckel-Gruber and Bardet-Biedl syndromes, as well as Nephronophthisis) and its interactions with other previously described transition zone proteins associated with those disorders. Our work supports the existence of two functional modules at the transition zone, one comprising the nephrocystins NPHP-1 and NPHP-4, the other CEP-290, MKSR-2 and other Meckel syndrome proteins.

Co-deletion of any two components of these two modules results in short ciliated dendrites. In these mutants, dendrites fail to extend due to a lack of attachment of the dendritic tip during neuronal migration. Dendrites display correct numbers of properly formed cilia, but mispositioning results in impaired ciliary function. This adhesive function is restricted to the transition zone, since mutations in other ciliary structures do not affect dendrite extension.

In conclusion, our work describes a novel and unsuspected function for cilia in cellular architecture, maintaining dendritic tip attachment during neuronal migration.

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Molecular and cellular mechanisms of dendrite remodeling in *Drosophila* sensory neurons.

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Developmental dendrite pruning is a general mechanism that is required for maturation of neural circuits, yet how neurons can specifically eliminate unwanted branches remains elusive. During *Drosophila* metamorphosis, the larval-specific dendrites of class IV sensory neurons are once pruned and replaced by adult-specific processes, providing an ideal model to genetically dissect molecular mechanisms that control dendrite pruning. Using in vivo calcium imaging, we found localized calcium transients in dendritic branches during metamorphosis. Once the calcium transients happened in dendritic branches, the branches were pruned 3-4hr after continuous transients. The localized transients are mediated by calcium influx through voltage-gated calcium channels (VGCCs), and blockage of the VGCCs activities impaired dendrite pruning, suggesting that the localized calcium transients are required for elimination of the unwanted dendritic branches. Further genetic and biochemical studies indicated that the PKA signaling pathway likely functions downstream of the calcium transients in dendrites. Based on these observations, we propose a novel model in which the localized calcium transients act as a temporal and spatial cue to trigger dendrite pruning in class IV sensory neurons during metamorphosis. I will discuss how the calcium-dependent dendrite pruning in *Drosophila* neurons might relate to the axon/dendrite pruning observed in the mammalian nervous system during the critical period.

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Minisymposium 19: Cell Polarity

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Tracking shallow chemical gradients by actin-driven wandering of the polarization site.

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Many cells are remarkably proficient at tracking very shallow chemical gradients, despite considerable noise from stochastic receptor-ligand interactions. Motile cells appear to undergo a

biased random walk: spatial noise in receptor activity may determine the instantaneous direction, but because noise is spatially unbiased it is filtered out by time-averaging, resulting in net movement up-gradient. How non-motile cells might filter out noise is unknown. Using yeast chemotropic mating as a model, we demonstrate that a polarized patch of polarity regulators “wanders” along the cortex during gradient tracking. Computational and experimental findings suggest that actin-directed membrane traffic contributes to wandering by diluting local polarity factors. The pheromone gradient appears to bias wandering via interactions between receptor-activated G β and polarity regulators. Artificially blocking patch wandering impairs gradient tracking. We propose that the polarity patch undergoes an intracellular biased random walk that enables noise filtering by time-averaging, allowing non-motile cells to track shallow gradients.

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An optogenetic analysis of the minimal requirements for bud-site selection.

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An autocatalytic complex of four proteins (Cdc42, Cdc24, Bem1, PAK) is associated with the selection and maintenance of a single site of polarity in budding yeast, and positive feedback in this complex is required to amplify stochastic fluctuations in Cdc42 activity during symmetry-breaking polarization. However, the relevance of this feedback is not clear for situations where cells polarize towards an established spatial cue, and there has been no direct demonstration that artificially localized cues can be recognized and amplified solely through this mechanism. In order to experimentally manipulate signaling processes such as polarity establishment, we have created tunable, light-controlled interacting protein tags (TULIPs) based on genetic fusion with a photosensory LOV2 domain from plants and an engineered PDZ domain binding partner. These domains can be fused to cellular proteins of interest or localization motifs, thereby making their interaction light-regulated. We have shown that the tags function in yeast and mammalian cells and are readily adaptable to diverse signaling pathways. Here, we use TULIPs tags to confer light sensitivity to the activation of Cdc42 in yeast, thereby eliciting light-directed specification of the site of budding. We show that the multifunctional Cdc42 guanine nucleotide exchange factor (GEF), Cdc24, is sufficient to provide a spatial cue upon localized recruitment to the cortex, and that this artificial cue can override the endogenous cue. Furthermore, the polarity-specifying ability of Cdc24 is lost when either its GEF activity or its ability to interact with the scaffold Bem1 is ablated, even when a wild-type copy of Cdc24 is present. These results suggest that several different functionalities are required to trigger positive feedback and successfully specify the site of budding. Future experiments will explore the timing, cell-cycle dependence, and molecular requirements of light-directed polarity specification.

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Cell polarity: Mechanochemical patterning.

S. W. Grill¹; ¹MPI-CBG and MPI-PKS, Dresden, Germany

Recent advances in our understanding of the coupling of mechanical and biochemical processes for the purpose of cellular polarization will be presented. I will focus on a particular example, polarization of the *C. elegans* zygote, a classic example for mechanochemical patterning. In this system actively generated flow of the actomyosin cell cortex triggers polarization in terms of the intracellular distribution of markers of polarity. I will present biophysical principles of actively generated cortical flow, and I will discuss the two-way feedback between mechanical and biochemical networks. This work provides insight into general mechanisms of biological patterning that go beyond spatial patterning in the framework of reaction-diffusion.

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Human bone-marrow mesenchymal stem cells regulate biased DNA segregation in response to cell adhesion asymmetry.

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Biased DNA segregation is a mitotic figure in which the chromosomes carrying the original DNA strand and those carrying the copy do not segregate equally between the two daughter cells. It has been documented in various species ranging from bacteria to mammals. In mouse muscles, biased DNA segregation has been shown to occur in adult stem cells and to regulate asymmetric cell fate (Rocheteau et al., Cell, 2012). However the absence of DNA bias in asymmetric divisions of mouse hematopoietic stem cells (Kiel et al., Nature, 2007) but its occurrence in human tumour-derived cells (Pine et al., PNAS, 2010) feed the debate on the relationship between stemness, asymmetric division and biased DNA segregation. The parameters influencing this bias have not been identified yet. No clear conclusions could be firmly drawn owing to the low frequency of biased DNA segregation in all these conditions and to the difficulty to clearly identify daughter cells in situ. Here we used pulse-chased EdU DNA labelling and video-microscopy to monitor cell division and quantify DNA segregation bias in human bone marrow-derived mesenchymal stem cells. The low rate of division in these cells was overcome by following a thousand of cells in parallel for each donor sample. Thereby we clearly showed that biased DNA segregation does occur in human stem cells. To investigate the role of cell adhesion to its micro-environment in the regulation of this event, cells were plated on micro-patterned extra-cellular matrices. We found that the rate of 10% of cells displaying biased DNA segregation observed in unconstrained cells or cells plated on symmetric micro-patterns increases to 50% on asymmetric micropatterns. To the best of our knowledge, it is by far the highest rate ever reported for this event which is no longer anecdotic. Importantly, this sensitivity of asymmetric extra-cellular cues was specific to stem cells, reproducible for all tested donor, and was not observed in human skin-derived fibroblasts used as a control of differentiated cells. These data demonstrate that stem cells have a unique capacity to feel and respond to the asymmetry of their adhesion pattern by increasing massively their capacity to segregate the chromosomes carrying the original strand in one of the daughter cells only.

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Bazooka forms a platform that integrates polarity and cell cycle progression in *Drosophila* male germline stem cells.

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Many stem cells are known to divide asymmetrically to balance self-renewal and differentiation. In *Drosophila* male germline stem cells (GSCs), asymmetric division is achieved by spindle orientation, which is predetermined during interphase by stereotypical positioning of centrosomes. The correct centrosome orientation is further monitored by a checkpoint mechanism (the centrosome orientation checkpoint (COC) to ensure asymmetric division. Here we show that bazooka (Baz), a homolog of mammalian Par-3, is an integral component of the COC. In GSCs, Baz forms small foci at the hub-GSC interface, and orchestrates the subcellular localization of the centrosome and the spectrosome, a germline-specific ER-like organelle implicated in the COC. Par-1, a known component of the COC that localizes to the spectrosome, functions through the phosphorylation of Baz. We further show that the two opposing microtubule motors, dynein and kinesin, functions in the COC through their ability to

position the spectrosome and the centrosome, respectively. We propose that the Baz patch forms a platform to integrate the centrosome orientation status and cell cycle progression .

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Mechanisms of SPB specification during spindle orientation in yeast.

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In many asymmetrically dividing cells, the centrosomes are segregated in a non-random manner between the two daughter cells, with the centrosome containing the oldest centriole generally segregating to the renewing cell, and the younger one to the differentiating daughter. This phenomenon is conserved in budding yeast, where the mother cell and the bud inherit the two centrosome-equivalents, called the spindle pole bodies (SPBs), in a non-random manner. Indeed, budding yeast cells divide asymmetrically, producing a smaller and rejuvenated daughter cell at the surface of the bigger and slowly aging mother cell. Forming the poles of the mitotic spindle, the SPBs are produced through semi-conservative duplication, leading to the generation of a new SPB at the side of the old one. Remarkably, the new SPB segregates to the mother cell, and the old one to the bud, in more than 95% of mitosis. How the cell distinguishes the old from the new SPB is unknown. However, previous work has established that the microtubule and actin associated protein Kar9 is recruited virtually exclusively to the microtubules emanating from the old SPB and drives its orientation towards the bud. We are investigating the mechanisms specifying the fates of the SPBs. These studies revealed that the SPB components Nud1 and Cnm67 are absolutely essential for this function. In this process, the Nud1 protein functioned upstream of the mitotic exit network (MEN), a signaling cascade related to the Hippo pathway in metazoans and which mediated the establishment of Kar9 asymmetry towards the old SPB. MEN fulfills this function in conjunction with Cdk1 in metaphase, and at least in part through direct phosphorylation of Kar9 on S197, 332 and 429. Additional mutants interfering with proper specifications of the SPBs comprise kinases of the morphogenetic checkpoint, which control the timing of the metaphase-to-anaphase transition, as well as acetylation and deacetylation enzymes. Together, our data suggest a model in which SPB specification is linked to the SPB duplication cycle at bud emergence.

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Aging, neurodegeneration and trinucleotide disease: Insight from *Drosophila*.

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Human neurodegenerative diseases, like Huntington's disease and the spinocerebellar ataxias, are late-onset progressive neurodegenerative disorders for which few cures or treatments are available. To develop new approaches, we have been using the simple model organism *Drosophila* to provide insight that we then extend to the human disease. We use the human disease gene to recreate the disease toxicity in the fly, and then take advantage of powerful molecular genetic approaches in the fly to define pathways and mechanisms.

These studies have revealed multiple pathways involved in neurodegeneration, including toxicities due to RNA pathways. These processes include toxic activities of the mRNA encoding the disease proteins for repeat expansion diseases, RNA binding proteins and their altered activities, and modulation by miRNAs, including novel aspects of miRNA regulation such as 3'end trimming by the exonuclease Nibbler. A key miRNA we identified that links age-associated processes with long-term brain integrity is miR-34. Taken together, these findings emphasize

and highlight the diversity of ways in which altered RNA pathways and metabolism impacts brain integrity, and interplays with gene function in brain degenerative disease.

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Ubiquitin-dependent aggregation of misfolded proteins in the absence of HSP70 chaperones.

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The heat shock protein-70 chaperone family is a key component of the cellular quality control system, mediating protein folding, refolding and preventing aggregation. The primary role of Hsp70 chaperones in ubiquitin-dependent degradation of misfolded proteins has been perceived to be the delivery of misfolded proteins to E3 ubiquitin-ligases for ubiquitylation.

In order to assess the precise role of Hsp70 chaperones in yeast protein quality control and degradation, we focused our study on substrates of the yeast E3 ligase, Doa10, which undergo ubiquitylation and subsequent degradation. We have previously identified a Doa10-recognized degradation signal (degron) at the C-terminal tail of the functionally conserved kinetochore protein, Ndc10. Degradation of protein substrates containing this degron requires the Hsp70 chaperones Ssa1 and Ssa2. Deletion of both SSA1 and SSA2 caused stabilization of an ER-localized substrate carrying an Ndc10-degron, but only slightly reduced its ubiquitylation levels. This implies that the function of Ssa1/2 is required downstream to ubiquitylation. Expression of the Ndc10 degron fused to GFP in DOA10-knockout cells led to its accumulation in the nucleus, in a soluble form. In contrast, deletion of SSA1/2 led to aggregation of the GFP-labeled degron, while the deletion of DOA10 together with the deletion of SSA1/2 significantly decreased aggregate formation. These results indicate that the aggregation of substrates containing the Ndc10 degron is strongly dependent on their ubiquitylation. Furthermore, the aggregates in SSA1/2-deficient cells were selectively retained in yeast mother cells undergoing budding, consistent with known asymmetric inheritance of deleterious proteins. As such, these cells could also provide a physiological paradigm for studying age-dependent accumulation of ubiquitylated protein aggregates.

We propose that the Hsp70 chaperones assist the degradation of misfolded proteins by two means: supporting substrate recognition by the ubiquitin-ligation apparatus and maintaining ubiquitylated proteins in a soluble form. This ensures safe delivery to the proteasome and protects cells from the possibly deleterious effects of misfolded protein aggregation.

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Systematic genetic interaction mapping of protein folding and stress response pathways in the human ER.

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Systematic mapping of genetic interactions is a powerful approach to gaining a comprehensive mechanistic understanding of complex biological processes, and has successfully been applied to the yeast endoplasmic reticulum (ER) and other areas of biology in microorganisms. However, systematic genetic interaction mapping has thus far not been applied to mammalian cells. A genetic interaction map of the human ER would likely yield important insights into mammalian-specific ER functions.

We have now developed an RNA interference-based platform that allows us to conduct highly quantitative genetic screens and genetic interaction mapping in mammalian cells. To probe human ER function, we used the bacterial subtilase cytotoxin (SubAB), which kills cells by specifically cleaving BiP/GRP78, the Hsp70 chaperone of the ER, and thereby inducing the

unfolded protein response (UPR) and apoptosis. We have identified a large number of genes that modulate the response of human cells to SubAB: Host factors required for SubAB trafficking to the ER, many of which we also identified for other ER-trafficking toxins; factors contributing to protein load in the ER; a variety of chaperones and other protein folding factors; signaling components of the UPR; and previously uncharacterized factors. Surprisingly, factors implicated in other types of cellular stress responses also modulated sensitivity to subAB, hinting at possible cross-talk between different homeostatic and stress response pathways. We are now systematically mapping genetic interactions between these factors to understand how they interact in pathways, and in order to pinpoint putative functions for previously uncharacterized proteins.

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H₂S and fasting protect against hypoxia-induced disruption of proteostasis in *C. elegans*.

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Cells and organisms must keep the proteome in a well-folded, functional state even in stressful conditions. Maintaining proteostasis requires the coordination of pathways that control protein translation, folding, and turnover. We are interested in the mechanisms by which animals integrate cellular stress responses with proteostasis networks. We have found specific hypoxic O₂ concentrations that disrupt proteostasis in *C. elegans*, as measured by aggregation and toxicity of polyglutamine-containing proteins. We demonstrate that H₂S, an endogenous gaseous signaling molecule, can protect against and reverse the effects of hypoxia on proteostasis. H₂S exposure activates the conserved hypoxia-inducible transcription factor, *hif-1*. However, we find that *hif-1* is necessary but not sufficient to protect proteostasis in hypoxia, suggesting that other factors are involved in the effects of H₂S to improve proteostasis. In a screen to identify factors that coordinate the response to hypoxia with proteostasis network we identified the AMP-associated kinase (AMPK) subunit *aak-2*. The *aak-2* mutant animals do not show increased protein aggregation in hypoxia. In contrast, age-associated polyglutamine protein aggregation occurs normally in the *aak-2* mutant animals. In parallel studies we found that fasting protects animals against hypoxia-induced protein aggregation. AMPK has been shown to mediate some effects of increased lifespan by dietary restriction. Thus, we hypothesized that AMPK integrates the effects of food deprivation and hypoxia on proteostasis. Consistent with this model, *aak-2* mutant animals show increased protein aggregation in hypoxia after fasting. We propose that AMPK signaling regulates proteostasis to control allocation of resources in stressful hypoxic conditions. We are also testing the hypothesis that beneficial effects of H₂S are mediated by AMPK.

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The chaperome as a therapeutic space in aging and neurodegenerative diseases.

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Chaperone networks act as regulators of proteostasis, safeguarding the proper folding of the proteome and preventing aggregation. While numerous chaperones and co-chaperones have been implicated in aging and disease using candidate gene approaches, our understanding of

chaperone connectivity and functional cooperativity at a systems level is incomplete. We utilized a series of comparative genomics and bioinformatics methodologies to identify the repertoires of 219 *C. elegans* and 332 human chaperone genes belonging to fifteen distinct protein families and specific subcellular compartments, collectively referred to as “chaperomes”. To establish the organizational properties of the chaperome, we combined physical protein-protein interactions (PPIs) and gene expression data from public databases and generated integrated *C. elegans* and human chaperome networks. We hypothesized that the chaperome network would reveal specificity when challenged to respond to the dynamics of aging or aggregation prone client proteins implicated in human neurodegenerative diseases such as Alzheimer’s, Huntington’s and Parkinson’s disease. Examination of expression patterns of chaperome genes during aging revealed distinct network communities of activation and repression that included representatives from all chaperone families. Strikingly, the subsets of chaperones that are activated or repressed during aging showed similar accelerated changes in brain tissue from AD, HD and PD patients. Following chaperome-wide perturbation studies in *C. elegans*, we validated and refined a functional chaperome subnetwork, which includes 16 specific members from the Hsp90, Hsp70, Hsp60 (chaperonin), Hsp40, and TPR families, and new candidate genes that have not been linked directly to proteostasis. Our studies define a core chaperome as a conserved modifier of proteostasis, in aging and disease, revealing potential targets for therapeutic intervention.

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Humoral control of proteostasis.

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As an organism ages, its proteins face an increasing severity in the challenges they receive from extrinsic and intrinsic environmental perturbations. Chaperones become dysregulated, while the degradation machineries stop working properly. The protein accumulates damage and starts to misfold. At this point, the cell needs to mount a response to restore its homeostasis; however, the stress response machinery that it typically relies upon when faced with such challenges has lost its capacity to function.

This breakdown, however, does not lead to complete disorder. As the organism ages, it exhibits a degree of correlated, recognizable, and predictable changes to its physiology over time. These changes can occur synchronously across multiple tissues and organs. The phenotypic changes of aging occur in a type of concert, rather than in isolation, suggesting the residual participation of the endocrine system in the onset of age-related phenotypes. The demise of the cell thus most often occurs within the context of the simultaneous demise of the whole organism.

Our lab focuses on the questions of why an aging organism begins to lose control over the integrity of its proteome, and how this loss is communicated across its various tissues. We have taken the unique approach of breaking down a cell into its small and canonically-autonomous parts – its suborganelles and subcompartments – such that we can take a larger step back to ask how those smaller portions can communicate both with each other and with the organism as a whole. Our approaches have required us to diversify the contexts in which we ask questions: we work on model systems ranging from stem cells and nematodes to mice. We have developed and applied techniques that allow us to manipulate signaling pathways or proteins within a single tissue, cell, or an organelle within a single cell so that we can observe how that small perturbation might reverberate and effect the physiology of the whole of the organism.

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A subset of microRNAs and messenger RNAs in the nucleolus.

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During studies of myogenic differentiation with rat L6 myoblasts we found that a small set of microRNAs localizes in the nucleoli of these cells. Since these microRNAs are also present in the cytoplasm we considered their nucleolar localization to reflect a transit. This led us to ask whether they might be combining with messenger RNAs that also traffic through the nucleolus. Microarray analysis of RNA from highly purified nucleoli revealed the presence of a subset of the messenger RNAs expressed in these cells. Prevalent among these nucleolus-localized mRNAs are ones that encode connexin-like proteins involved in the cell fusion events of myogenesis taking place two days later. Target sites for two of the nucleolus-localized microRNAs were found in the 3' UTR of one of the nucleolus-localized mRNAs suggesting a docking of microRNAs on nucleolus-trafficking mRNAs prior to export in a translationally suppressed state, this suppression to be relieved later in myogenic differentiation. This is a strikingly different concept relative to the standard model of microRNA action in the cytoplasm and yet is not at variance with any prior results in the microRNA field.

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Crosslinking reveals unexpected roles for the yeast SR protein Npl3 in ncRNA metabolism.

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From the onset of transcription, nascent RNA transcripts are coated with a complement of RNA binding proteins (RBPs). This array of proteins mediates a complex series of mRNA processing events, leading to the production of a mature functional RNA. Many processing events are highly inter-dependent and, in consequence, disruption of individual RBPs can result in numerous deleterious effects. In higher eukaryotes, this is further complicated by functional redundancies between paralogous genes. The determination of direct molecular functions for these RBPs thus remains a significant challenge.

The SR proteins are a large family of RBPs in metazoans with diverse functions in pre-mRNA processing. The budding yeast genome encodes a single SR protein, Npl3, to which a multitude of roles in pre-mRNA processing have been attributed, including transcription elongation, splicing and 3' end processing. To gain insights into the direct functions and targets of this protein, we employed an *in vivo* crosslinking and sequencing approach to elucidate which transcripts it associates with and where in the RNA it binds. We found Npl3 to bind diverse RNA species, including mRNAs and non-protein coding RNAs (ncRNAs), many of which were oligo-adenylated at non-canonical sites. Non-templated adenosine stretches are hallmarks of RNA molecules destined for turnover, suggesting a role for Npl3 in targeting RNAs for degradation. To determine whether specific RNA species are enriched in the absence of Npl3, we used strand-specific tiling arrays to look for expression changes when *NPL3* is deleted. We found that

CUTs, a class of unstable ncRNAs, were enriched in the mutant strain, and the transcription units of many snoRNAs were greatly extended. The increased abundance and/or length of these different ncRNAs had dramatic effects on the expression of surrounding genes. These data suggest that although deletion of *NPL3* results in many altered mRNA processing phenotypes, these may largely be downstream of a direct function in the transcription termination and/or turnover of ncRNAs. We are currently assessing whether Npl3 functions directly in the termination of ncRNAs, or in targeting aberrant RNAs for turnover, and how it interacts with other components of these pathways.

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Cyanobacterial DEAD-box RNA helicase: Autoregulation, RNA maturation and sRNA metabolism.

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DEAD-box RNA helicases are ubiquitous enzymes found in all domains of life that rearrange RNA secondary structure. They function in every aspect of RNA metabolism, frequently in response to abiotic stress. In the cyanobacterium *Synechocystis* sp. PCC 6803, expression of the cyanobacterial RNA helicase gene, *crhR*, is regulated in response to temperature fluctuation. Physiologically, CrhR inactivation dramatically affected the photosynthetic capacity and morphology of the mutant cells in response to temperature downshift. Analysis of the expression profile of the *crhR* dicistronic operon in wild type and Δ *crhR* cells indicated that CrhR autoregulates its own expression through a combination of transcript processing and temperature-dependent mRNA and protein stabilization mediated by dramatic temperature- and mutant-induced alteration of half-life. *crhR* inactivation resulted in unregulated accumulation of both transcript and protein while multiple transcripts detected in the mutant suggested defects in RNA processing and/or degradation. 5' RACE-RCA and immunoprecipitation analysis are ongoing to establish processing sites and potential RNases (RNase II/R, E, J) involved in transcript turnover and maturation. Furthermore, tiling arrays revealed that CrhR controls expression of a subset of small RNAs in *Synechocystis*. A model integrating the ability of CrhR in unwinding and annealing RNA with a role in gene regulation during environmental acclimation involving autoregulatory pathways and small regulatory RNAs will be presented.

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The role of core RNA splicing factors in spindle assembly.

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The mitotic spindle is a complex macromolecular structure responsible for the faithful distribution of genetic material to daughter cells. Our lab has shown that the spindle is not a purely protein-based machine, since an RNA component is essential for its integrity. Furthermore, RNA splicing factors have been implicated by a number of genome-wide screens as important for cell cycle progression and mitosis. Lastly, a spindle assembly factor, TPX2, has been observed to co-purify with active spliceosomes. In order to investigate whether RNA splicing factors play a direct role in mitosis we are utilizing transcriptionally silent *Xenopus laevis* and *Xenopus tropicalis* egg extracts to reconstitute spindle assembly in vitro and isolate mitotically functioning RNAs. Interestingly, we have found that a small molecule inhibitor of spliceosome assembly, isoginkgetin, as well as antibodies with known splicing inhibitory

potential, caused defects in spindle integrity in a concentration-dependent manner. We observed similar phenotypes when we immunodepleted small nuclear ribonucleoproteins (snRNPs) with antibodies against an epitope shared by four spliceosomal snRNPs. One expectation of splicing involvement in mitosis is that introns are present in mitotic RNAs. We performed next generation sequencing on non-ribosomal RNAs from *X. tropicalis* egg extract as well as on RNAs co-immunoprecipitated with spliceosomal snRNPs. Strikingly, even though transcription had ceased in mature oocytes long before the eggs were harvested, we observed that approximately 2-4% of introns were retained. These introns represent a number of gene ontology terms with a strong enrichment of genes involved in regulation of transcription and protein phosphorylation; cell cycle, mitotic and microtubule-related terms are also represented. Of all retained introns 79% contained premature stop codons that must therefore be removed to allow for translation. Our data support the direct involvement of RNA splicing factors in mitosis and suggest the tantalizing possibility that RNA processing plays a regulatory role in cell division.

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Assembling liquid droplets of cytoplasmic RNA and protein.

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Ribonucleoprotein (RNP) granules are non membrane bound macromolecular assemblies that form from the dynamic interactions of RNA and protein. They play a central role in cell growth, embryonic development, and many other fundamental biological processes. Among dozens of examples include germ granules and processing bodies in the cytoplasm, and Cajal bodies and nucleoli in the nucleus. We show that these structures behave as liquid droplets, which assemble from soluble cytoplasmic/nucleoplasmic RNA and protein by a type of phase transition, whose set-point is biologically controlled in space and time. The size and shape of these structures is controlled by their liquid like material properties, and the mechanical and geometric constraints of their local environment. These findings shed light on both the self-assembly of RNP granules, and their function as fluid-phase micro-reactors that facilitate RNA metabolism.

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Understanding co-transcriptional pre-mRNA splicing and the dynamic rearrangements at the heart of the spliceosome.

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Eukaryotic gene expression is tightly controlled to maintain proper cellular function. Eukaryotic genes are transcribed by RNA polymerase II to generate fully-processed (capped, polyadenylated, and spliced), mature messenger RNA molecules. Although the cellular machineries that carry out these reactions (RNA synthesis and RNA processing) have typically been studied as biochemically distinct reactions, they are, in fact, temporally and spatially organized to coordinately orchestrate the proper production of a fully-processed mRNA. One of the outstanding challenges has been to identify specific factors that coordinate pre-mRNA splicing with transcription, particularly within the context of a chromatin template. We have previously shown that Gcn5, which encodes the HAT activity of the co-activator SAGA complex, mediates co-transcriptional recruitment of the U2 snRNP to pre-mRNA. As further evidence of a role for SAGA in mediating ATP-dependent rearrangement of the U2 snRNP, we have identified interactions between the DEXH/D box protein Prp5, which hydrolyzes ATP to remodel the U2 snRNP and facilitate branchpoint recognition, and components of SAGA. A mutation in PRP5

that decreases ATP-binding is suppressed when components of the SAGA complex are mutated, thereby for the first time linking SAGA with a catalytic activity that drives spliceosomal rearrangements. These results have led us to further analyze how histone acetylation contributes to U2 snRNP recruitment. We find that chromatin remodeling complexes that associate with acetylated histones physically and functionally interact with the U2 snRNP and contribute to spliceosome assembly. These data support a model whereby co-transcriptional spliceosome assembly is intimately coordinated with histone acetylation and chromatin remodeling.

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SUMOylation regulates mammalian septin functions.

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Septins are cytoskeletal proteins that assemble into non-polar filaments and higher-order structures such as rings and hourglasses. By acting as scaffolds for protein recruitment and as diffusion barriers for subcellular compartmentalization, septins play key roles in diverse cellular functions such as cytokinesis and ciliogenesis. In addition, septins play a crucial role in bacterial infection, by entrapping intracytoplasmic bacteria into cage-like structures and impairing their cell-cell spreading capacity. Yeast septins were among the first proteins reported to be modified by SUMOylation, a ubiquitin-like post-translational modification. However, whether mammalian septins could be modified by SUMO, and what roles this modification may have in septin functions remain unknown. We found that different members of the human septin family can be SUMOylated. We show that non SUMOylatable mutants of septins are impaired in their ability to form cages around intracytosolic bacteria. We also demonstrate that SUMOylation of septins is critical for mammalian cytokinesis. Together, our results reveal that SUMOylation is a key modification regulating mammalian septin functions.

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Dendritic network remodeling in *Listeria monocytogenes* protrusions.

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Several intracellular pathogens, including *Listeria monocytogenes*, display actin-based motility in the cytosol of infected cells and spread from cell to cell through membrane protrusion formation at the cell cortex. Seminal studies have revealed how these intracellular pathogens exploit the actin assembly machinery and display cytosolic motility. In contrast, the mechanisms supporting membrane protrusion formation at the cell cortex, and therefore pathogen dissemination, are still poorly understood. To bridge this gap in knowledge, we have developed large-scale RNAi treatment procedures, high-throughput fluorescence microscopy and computer-assisted image analyses to screen for cellular factors supporting *L. monocytogenes* dissemination in human epithelial cells. In addition to expected components of the actin assembly machinery, such as the ARP2/3 complex and capping proteins, we identified Actin Interacting Protein 1 (AIP1), a component of the Cofilin-dependent actin disassembly machinery. Cytosolic motility was indistinguishable from wild-type in AIP1-depleted cells.

However, AIP1 depletion resulted in a striking accumulation of membrane protrusions at the cell cortex. Using time-lapse video microscopy, we uncovered that, unlike the situation observed upon formation of actin tails in the cytosol, the ARP2/3-containing network formed at the bacterial pole was quickly remodeled into an ARP2/3-devoid network in protrusions. We used photo-activation of GFP-tagged proteins to demonstrate that this AIP1-dependent process in fact recycles actin molecules (and ARP2/3) from the actin network formed in protrusions. This recycling process is essential to fuel continuous ARP2/3-dependent actin nucleation at the bacterial pole, as protrusions elongate. To identify potential AIP1 partners, we screened for cellular factors whose depletion enhanced the cell-to-cell spreading defects observed in AIP1-depleted cells. As previously described in lamellipodia, we identified Cofilin as a central component of the AIP1-dependent remodeling machinery in protrusions. In addition, we uncovered a previously unappreciated role for the actin-depolymerizing factor homology (ADF-H) domain proteins GMF and Twinfilin 2. Thus, *L. monocytogenes* spread from cell to cell relies on the coordinated activity of machineries supporting assembly and disassembly of dendritic network in protrusions, which provides a powerful framework to investigate the mechanisms supporting actin network remodeling in cellular structures such as lamellipodia.

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Influence of host cell behavior on the invasion and cell-to-cell spread of *Listeria monocytogenes* in endothelial sheets.

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The facultative intracellular bacterial pathogen *Listeria monocytogenes* harnesses the actin machinery of its host cell to move within individual cells and from one cell to another, without exposure to the extracellular space. Mounting evidence suggests that such actin-based motility and cell-to-cell spread contribute to systemic spread of *L. monocytogenes*, whereby the bacterium spreads from the initial site of infection in the mammalian gut epithelium to subvert cellular barriers and infect distal sites, such as the placenta or the central nervous system. However, the molecular details of cell-to-cell spread remain largely uncharacterized. We are studying the ability of *L. monocytogenes* to subvert the vascular endothelium, a single layer of cells that lines blood vessels and represents a significant barrier to systemic bacterial spread. In tissue culture experiments, we have demonstrated that *L. monocytogenes* in infected macrophages can robustly infect monolayers of uninfected human umbilical vein endothelial cells (HUVEC) via direct heterotypic cell-to-cell spread, and that this spread depends on both bacterial motility and macrophage-endothelial adhesion. Additionally, we have confirmed that *L. monocytogenes* can directly invade a monolayer of HUVEC; in these latter experiments, a substantial fraction of infected cells in a sheet are infected by cell-to-cell spread from the much smaller number of initially invaded cells. Using time-lapse microscopy of infected cells, we determined that infected HUVEC exhibit surprisingly normal bulk division rate and individual cell motility, suggesting that these and other normal host processes may contribute to bacterial spread. To identify specific host factors that modulate bacterial invasion and spread in HUVEC, we conducted a quantitative, image-based siRNA screen. We have identified a number of host genes that appear to specifically regulate bacterial invasion, cell-to-cell spread or both. We are currently using time-lapse fluorescence microscopy and flow cytometry to determine whether these factors directly affect bacterial infection or instead alter infection indirectly, e.g. by altering host cell motility, cytoskeletal dynamics, or cell-cell interactions. We are particularly interested in the role of host proteins associated with adherens junctions, which have been well-characterized in *L. monocytogenes* invasion of epithelial cells but have not been clearly implicated in endothelial cell invasion or in cell-to-cell spread.

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Unique requirement for the exocyst complex in the repair of large plasma membrane wounds and host cell invasion by *Trypanosoma cruzi*.

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Host cell entry by *T. cruzi* shares mechanistic elements with plasma membrane injury and repair. Both processes require Ca²⁺-triggered exocytosis of lysosomes^{1,2}, delivery of the lysosomal enzyme acid sphingomyelinase to the outer leaflet of the plasma membrane^{3,4}, and a rapid form of endocytosis that mediates internalization of small membrane lesions such as those caused by pore forming toxins^{4,5}. *T. cruzi* trypomastigotes transiently wound host cells and enhance endocytosis, taking advantage of the ubiquitous plasma membrane repair pathway to gain access to the intracellular milieu and form a parasitophorous vacuole enriched in ceramide³. However, trypomastigotes are large (10-15 µm long) parasites that preferentially invade cells at specific peripheral sites, and it is unclear how generation of small sphingomyelinase/ceramide-dependent endosomes facilitates their internalization. Because *T. cruzi* invasion involves highly polarized mobilization of intracellular membranes^{1,3}, we investigated a possible role of the exocyst tethering complex. *T. cruzi* invasion was significantly decreased in HeLa cells deficient in Exo70 or Sec8, and both exocyst proteins were recruited to the *T. cruzi* parasitophorous vacuole during early steps of invasion. Strikingly, depletion of Exo70 or Sec8 also inhibited Ca²⁺-dependent repair of large mechanical wounds, while not affecting the cell's ability to reseal after injury with small SLO pores. Thus, the exocyst complex appears to be uniquely required for the repair of large mechanical wounds and for *T. cruzi* internalization, two processes that require substantial trafficking and fusion of intracellular vesicles at specific plasma membrane sites.

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The role of Bst-2/Tetherin in HIV transmission from primary human macrophages.

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Bst-2/Tetherin is a cellular protein that restricts HIV release and is antagonized by the viral protein U (Vpu). The restriction factor is thought to physically link budded virions to the host cell surface, preventing their release and cell-free transmission. Although Tetherin has been extensively studied in T cells and cell lines, little is known about its localization and functions in macrophages, and its role in the direct cell-cell transmission of HIV remains controversial.

Here we show that upon interferon treatment of primary monocyte-derived macrophages (MDM), Tetherin is highly up-regulated on both the mRNA and protein levels. Immunostaining shows that in IFN-treated MDM, Tetherin localizes to the cell surface as well as to an intracellular compartment positive for a marker of the trans-Golgi network. MDM infected with Vpu-deficient HIV show increased overall and cell surface levels of Tetherin, higher levels of cell-associated virus and decreased HIV release into the culture supernatant, suggesting that Tetherin restricts the release and cell-free transmission of HIV in these cells.

Direct cell-cell transmission of HIV is thought to be more efficient than cell-free propagation. One of the key structures mediating cell-cell transmission is the so-called virological synapse,

which is characterised by the accumulation of viral proteins and cellular receptors at the interface of the infected and the target cell. To investigate the role of Tetherin in cell-cell transmission of HIV from macrophages to T cells, we co-cultured infected MDM with autologous CD4⁺ T cells. We find that T cells associate with MDM within minutes, while the formation of virological synapses takes at least half an hour. qPCR experiments show that in our system cell-cell transmission of HIV from MDM to T cells is about ten fold more efficient than cell-free infection of the T cells. Using Western Blot analysis to detect HIV protein in the T cells early (24 h) after the co-culture with infected MDM, we find that T cell infection is greatly diminished in the absence of the Tetherin antagonist Vpu. Depleting MDM of Tetherin by RNAi rescues the infection of the T cells.

In conclusion we find that Tetherin efficiently inhibits the release of cell-free HIV from primary macrophages and also inhibits cell-cell transmission of HIV from macrophages to autologous CD4⁺ T cells. These results support the notion of high evolutionary pressure on HIV and related viruses to antagonize Tetherin.

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A novel role for cell polarity proteins in innate immunity to *Pseudomonas aeruginosa* infection.

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The mucosal epithelium consists of polarized epithelial cells with distinct basolateral (BL) and apical (AP) membranes that serve as a barrier to the outside world. The ability of pathogens to modulate such barrier function through manipulation of cell polarity is an emerging theme in microbial pathogenesis. *Pseudomonas aeruginosa* is a gram negative pathogen that is an important cause of nosocomial infections. In the setting of epithelial cell injury and immunocompromise, *P. aeruginosa* can unleash its potent arsenal of virulence factors to cause devastating disease. We have previously reported the novel observation that aggregates of *P. aeruginosa* can bind to the AP surface of polarized cells, often near cell-cell junctions, where, within the first 30 to 60 minutes of infection, they transform a small patch of AP membrane into one with BL characteristics. This spatial and temporal inversion of cell polarity involves the production of a host membrane protrusion that is enriched for phosphatidylinositol 3,4,5-trisphosphate (PIP3), phosphoinositol-3-kinase (PI3K), actin, and BL proteins but that excludes AP proteins. Importantly, TJs are not disrupted during the initial stages of protrusion formation, suggesting that protrusions result from localized rearrangement rather than overt loss of cell polarity. The mechanism that induces these profound polarity changes and the role that membrane protrusions with inverted polarity play in infection remains largely unexplored. In this study, we examined the molecular pathways of protrusion formation and their biological consequences. We demonstrate that protrusion formation is dependent on recruitment of a Par3/PI3K/Rac module at the site of aggregate binding, leading to de novo formation of a primordial AJ and localized activation of NFκB. We identify two bacterial virulence factors critical for these events, including the flagella and the Type-III secretion system translocation apparatus. These data reveal an unanticipated function for spatial changes in polarity in activation of core innate immune pathways in response to challenge with *P. aeruginosa* and may represent a novel mechanism by which the host senses and distinguishes between pathogens and commensals.

Minisymposium 23: Organelle Structure and Vesicle Formation

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Extended-Synaptotagmins are endoplasmic reticulum (ER) proteins that function as tethers at ER-plasma membrane contact sites.

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The endoplasmic reticulum (ER) forms a complex network of cisternae and tubules that extends throughout the cell and participates in multiple contacts with virtually all other membranes, including the plasma membrane (PM). ER-PM contact sites in cells of higher eukaryotes have been primarily studied in the context of Ca²⁺ homeostasis. However, there is growing evidence that ER-PM contacts play more general roles in the functional cross-talks between these two membranes. The molecular mechanisms shared by all eukaryotic cells that mediate such contacts remain to a large extent unknown. We have identified a family of multiple C2 domain containing proteins, the Extended-Synaptotagmins (E-Syt1, E-Syt2 and E-Syt3) as mediators of ER-PM contacts. The E-Syts are closely related to the yeast tricalbins (See Toulmay and Prinz, *J. Cell Science* 125: 49-58, 2011 and Abstract by Manford et al.). Ultrastructural analysis by electron microscopy revealed that E-Syts are ER membrane proteins, that epitope-tagged E-Syt2 and E-Syt3 are localized at ER-PM contact sites via interactions mediated by their C-terminal C2 domain, and that the overexpression of these two E-Syts induces a striking increase of ER-PM contact sites with a massive formation of cortical ER. E-Syt1 localizes to cortical ER only in a complex with E-Syt2 and/or E-Syt3. E-Syts-dependent cortical ER marks sites where STIM1 is recruited upon ER Ca²⁺ depletion. Our results, along with the results reported in the companion Abstract by Saheki, Giordano et al., demonstrate that E-Syts are molecular tethers that bridge the ER to the PM and provide new insights into the conserved molecular machinery controlling ER-PM cross-talks by direct contacts.

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Visualizing the dynamic architecture of the endocytic machinery by high resolution tracking of fluorescent proteins in yeast cells.

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Budding of clathrin-coated endocytic vesicles is driven by a highly dynamic and complex machinery consisting of over 50 different proteins. Although individual proteins have been studied extensively, their function as parts of the endocytic machinery is not well understood. Especially, it is not known how most of the protein components are organized *in situ* during vesicle budding.

We have developed a live-cell particle tracking approach that measures average positions of fluorescently tagged endocytic proteins in relation to each other during the vesicle budding process with high spatial and temporal resolution. To reconstruct the dynamic organization of the endocytic machinery we combine this tracking data with estimates of numbers of molecules, and with available structural and correlative light and electron microscopy data.

We first investigated coat-associated proteins Sla2 and Sla1. The clathrin coat is structurally well studied but the organization of other coat-associated proteins is poorly understood. Our

results suggested that the elongated rod-shaped Sla2 protein is located at the central part of the endocytic coat. By using N- and C-terminal GFP tagging we were able to resolve that Sla2 is oriented perpendicular to the plasma membrane so that its lipid binding domain is at the membrane and the actin binding domain projects away into the cytoplasm. Sla1, in contrast to Sla2, is likely located in the peripheral part of the coat. Next we tracked actin and several actin binding proteins to study the polymerization dynamics of the endocytic actin network. Two opposite models have been proposed about the direction of actin polymerization in relation to the forming vesicle. Our tracking of actin cytoskeletal proteins combined with FRAP experiments supported a model where actin filaments polymerize at the base of the invagination, not at the vesicle surface. Finally, we analyzed the dynamics of a BAR domain protein Rvs161/167 that binds to the membrane at the neck of the invagination to regulate vesicle scission. Our results suggested that Rvs161/167 starts assembling just below the coated tip of the invagination and then extends toward the base as the invagination elongates, eventually covering most of the tubular part of the invagination. The Rvs161/167 assembly ends with a catastrophic disassembly that coincides with vesicle scission. In summary, our results reveal several new insights into the mechanisms of endocytosis and demonstrate that live-cell imaging can provide detailed information about the dynamic molecular architecture of the endocytic machinery.

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The centrosome regulates the Rab11-dependent recycling endosome pathway at appendages of the mother centriole.

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The recycling endosome localizes to a pericentrosomal area through microtubule-dependent transport. The observation that the mother centriole appendage protein, centriolin, interacts with Sec15, an effector of the recycling endosome component, Rab11-GTPase, suggests an interaction between centrosomes and endosomes. For the first time we show that the recycling endosome associates with the centrosome, more precisely, with mother (older) centriole appendages. The mother centriole appendage proteins, centriolin and cenexin/ODF2, regulate the association of the endosome components Rab11, the Rab11 GTP-activating protein Evi5, and the exocyst at the mother centriole. We have developed an in vitro method for reconstituting endosome protein complexes onto isolated centrosomes. This in vitro method for reconstituting proteins has a number of applications from understanding the hierarchy of protein association at the centrosome to understanding microtubule nucleation. Our experiments illustrate that purified GTP-Rab11 but not GDP-Rab11 binds to mother centriole appendages in the absence of membranes. Furthermore, centriolin depletion displaces the centrosomal Rab11 GAP, Evi5, and increases mother-centriole-associated Rab11; depletion of Evi5 has the same effect on Rab11 localization. This suggests that centriolin localizes Evi5 to centriolar appendages to turn off centrosomal Rab11 activity. Depletion of centriolin or cenexin disrupts recycling endosome organization and function suggesting that mother centriole proteins can regulate Rab11 localization and activity at the mother centriole through Evi5 and the exocyst.

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Cavins and caveolar coat complexes.

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Caveolae are flask-shaped invaginations of the plasma membrane. They are thought to pinch off from the membrane to form endocytic vesicles, and there is good evidence that they play key

roles in endothelial permeability and transcytosis. The remarkably uniform and characteristic morphology of caveolae suggests that they are generated by specific protein-protein and protein-membrane interactions, but the number, identity and structural properties of protein complexes involved in generating caveolae are not well defined. The cavin proteins are components of caveolae, and are expressed at varied amounts in different tissues. The cavins have been shown to interact with each other, and potentially with caveolin proteins, to form caveolae.

We have used several approaches to analyse the ultrastructural, biochemical and functional properties of cavin proteins within the caveolar coat. Our new data begin to delineate the role of stable protein complexes in shaping caveolar membranes. The data highlight the paradigmatic differences between caveolar morphogenesis and the dynamic, reversible association between coat proteins and membranes observed at clathrin-coated pits and other vesicle transport steps.

In complementary experiments, we have used knockout mice to test the hypothesis that expression of different complements of cavin proteins leads to differences in the biogenesis and biochemical properties of endothelial caveolae between tissues. We analysed caveolae in endothelium from caveolin 1 and cavin 1 null mice, and from cavin 2 and cavin 3 null mice generated as part of this study. Deletion of cavin 2 causes a profound loss of endothelial caveolae in lung and adipose tissue, but has no effect on the abundance of caveolae in other tissues. Cavin 3 is not required for making caveolae. The tissue-specific effects caused by deletion of cavin 2 on caveolar abundance are accompanied by changes in endothelial morphology. The requirement for cavin 2 in caveolar biogenesis specifically in some tissues is associated with an altered size of the hetero-oligomeric complexes into which cavin proteins assemble. These data reveal unsuspected heterogeneity between endothelial caveolae, and suggest a mechanism for caveolae to carry out varied molecular functions.

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Scyl1 functions as a scaffold for multiple components of the COPI machinery.

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COPI-coated vesicles mediate retrograde trafficking from the Golgi apparatus and the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) to the ER in the early secretory pathway. We recently demonstrated that Scyl1-like 1 (Scyl1), a member of the Scyl1-like family of catalytically inactive protein kinases binds COPI coat proteins and that loss of Scyl1 disrupts COPI trafficking. We now demonstrate that Scyl1 interacts with class II Arfs, most notably Arf4, but does not bind class I Arfs. Moreover, Scyl1 co-localizes with Arf4 at the ERGIC. Scyl1 interacts with membranes through a C-terminal helical domain that is distinct from its non-overlapping binding sites for COPI and Arf4, suggesting that Scyl1 couples Arf4 to COPI on ERGIC membranes. Additionally, gel filtration chromatography reveals that Scyl1 undergoes trimerization mediated by HEAT repeats found in the central region of the protein. HEAT-repeat assemblies generate binding sites for proteins and remarkably, through the HEAT repeats Scyl1 also binds to GBF1, a guanine-nucleotide exchange factor for class II Arfs. Thus, Scyl1 appears to be a scaffold for multiple components of COPI vesicle formation and consistently, over expression of Scyl1, which will disrupt the scaffold function leads to tubulation of the ERGIC. Our data reveal Scyl1 as the first known protein to interact specifically with class II Arfs and place Scyl1 as a key organizer of the COPI protein machinery.

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A novel, conserved complex regulates the membrane association of AP-1 clathrin adaptors.

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Clathrin adaptor complexes recognize cargo proteins and recruit them into clathrin-coated vesicles. Whereas the plasma membrane-associated AP-2 adaptor complex has many known accessory factors, few regulators of the Golgi/endosome-localized AP-1 complex have been identified to date. We have used a fluorescence-based yeast genome-wide screen to systematically identify new AP-1 regulators that restore transport of the chitin synthase Chs3 to the surface of cells lacking the exomer component Chs6. Top-scoring mutants were subjected to genetic interaction mapping to find functionally-associated clusters. This analysis identified a cluster of proteins linked to AP-1 that contained known AP-1 regulators and binding partners, as well as several Golgi-localized proteins whose functions are not well understood. We discovered that two of these proteins form a complex. One subunit of this novel complex, Laa2 (Large Adaptin Associated 2), contains a canonical AP-1 binding motif that links it to the gamma-adaptin subunit of AP-1. Loss of Laa2, or mutation of its AP-1 binding site, reduces the fluorescence lifetime of AP-1 suggesting the Laa2 complex binds AP-1 to regulate its membrane association. Laa2 is related to mammalian Fez1, a Golgi-localized protein that facilitates the transport of secretory vesicles in axons. Fez1 associates with the small coiled-coil protein SCOCO, and we found Laa2 copurifies with the yeast SCOCO homolog, Slo1. Moreover, *slo1* mutants have a phenotype similar to that of *laa2* mutants, strongly suggesting Laa2/Slo1 are the yeast counterparts of the mammalian Fez1/SCOCO complex.

E.B. Wilson Medal Presentation and Address

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Hsp90 chaperone sculpting evolutionary change: a quantitative genetic and proteomic view.

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Heat shock proteins (Hsps) promote the correct folding and maturation of many other proteins in the cell. Hsp90 is an abundant and highly specialized molecular chaperone that works on a particularly interesting group of client proteins: metastable signal transducers that are key regulators of a broad spectrum of biological processes. The folding of Hsp90 clients is particularly sensitive to changes in the external and internal environment of the cell. Therefore, Hsp90 is uniquely positioned to couple environmental contingencies to the evolution of new traits. Our work has defined two mechanisms by which Hsp90 influences the acquisition of new phenotypes. First, Hsp90 can potentiate the effects of genetic variation, allowing new mutations to produce immediate phenotypes. Second, by robustly maintaining signaling pathways, Hsp90 can buffer the effects of mutations, allowing the storage of cryptic genetic variation and its subsequent release by stress. Genetic reassortment and recombination can enrich these variants in subsequent generations making them manifest even in the absence of stress. We discovered these powerful evolutionary mechanisms in fruit flies, mustard plants, and fungi, but expect them to operate in all eukaryotes.

Recently we have taken advantage of two different model systems to further address the function of Hsp90 at the organismal and molecular levels. In the budding yeast *Saccharomyces cerevisiae*, we have mapped hundreds of Hsp90-dependent traits in ecologically diverse strains. In human cells, we have systematically and quantitatively surveyed most human kinases, transcription factors and ubiquitin ligases for interaction with Hsp90 and its co-chaperone Cdc37. This analysis clarified the mechanism of Hsp90 client recognition as a two-step process: its co-chaperones provide specificity at the protein-fold level, whereas thermodynamic parameters determine client binding within a protein family. Our results in these distinct model systems provide a unified and quantitative framework for understanding the role of Hsp90 in the evolution of genomes and in shaping health and disease.

WEDNESDAY, DECEMBER 19**Minisymposium 25: Actin Organization and Dynamics**

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Identification of cation binding sites on actin that control polymerization, bending stiffness and severing by vertebrate cofilin.

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The regulation of actin assembly and modulation of filament mechanical properties are critical for actin function. It is well established that physiological salt concentrations promote actin assembly and alter the overall bending mechanics of assembled filaments and networks. However, the molecular origins of these salt-dependent effects, particularly if they involve non-specific ionic strength effects or specific ion binding interactions, are unknown. Here, we demonstrate that specific cation binding at two discrete sites situated between adjacent subunits along the long-pitch helix drive actin polymerization and determine the filament bending rigidity. We classify the two sites as “polymerization” and “stiffness” sites based on the effects that mutations at the sites have on salt-dependent filament assembly and bending mechanics, respectively. Cofilin binding is coupled to dissociation of filament-associated cations, stiffness site reorganization and enhanced actin filament bending and twisting dynamics, consistent with displacement of stiffness site cations underlying the effects of cofilin on actin filament mechanics. The work presented reveals the molecular mechanism of salt-dependent actin assembly and cofilin-mediated changes in actin filament bending mechanics and severing.

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The formin INF2 severs actin filaments through a fundamentally different mechanism from cofilin: relating biochemical function to cellular activity.

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Formins are a class of proteins that accelerate actin nucleation, then influence filament elongation rate by remaining at the barbed end. INF2 is a biochemically unique mammalian formin in that it accelerates both actin polymerization and depolymerization. Importantly, mutations in INF2 lead to the kidney disease focal and segmental glomerulosclerosis (FSGS) and the neurological disorder Charcot-Marie Tooth Disease (CMTD). I am elucidating the molecular mechanism of INF2's unique depolymerization activity, using TIRF microscopy and other biochemical assays of actin dynamics. Prior work in our lab has shown that depolymerization requires both the FH2 and WH2/DAD sequences, and occurs in two steps: a) a severing step, which requires phosphate release from the actin subunits; and b) a depolymerization step, which requires the WH2/DAD. My work reveals the following mechanistic features of INF2-mediated severing/depolymerization. First, rapid severing occurs throughout the length of the filament and not progressively from the barbed end. This suggests that INF2 binds filament sides prior to severing and accelerates phosphate release of filaments since it is capable of severing filament segments less than 50s after monomer addition. Second, INF2 can bind filament sides that are both phosphate-bound and phosphate-free, but with different stoichiometries. Additionally, INF2 can alter filament flexibility in the phosphate-free state. These results suggest that phosphate release causes a conformational change to

filament-bound INF2, allowing severing. Lastly, INF2 bound at the barbed end causes catastrophic disassembly of short filaments. Based on these results, I postulate that both filament side binding and barbed end binding by the FH2 domain changes filament architecture, which promotes severing and changes monomer dynamics at filament ends. I postulate further that the WH2/DAD enhances severing by insertional binding between actin subunits in the filament. These results contrast in several aspects with the severing mechanism employed by cofilin. This mechanistic investigation of INF2 severing/depolymerization activity gives insight on recent cellular results from our lab, which reveal a role for INF2-mediated actin dynamics in fission of both Golgi and mitochondria.

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Srv2/CAP forms six-bladed throwing stars that directly catalyze actin filament severing and disassembly.

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Actin filament severing is a critical step in the dynamic turnover of cellular actin networks. Cofilin is sufficient to sever filaments, but it has long been thought that additional factors may be required to enhance severing and account for very high rates of filament turnover observed in vivo. Srv2/CAP (cyclase-associated protein) is a conserved and widely expressed regulator of the actin cytoskeleton that until now has been proposed to function exclusively in binding and recycling actin monomers. Further, these functions have been ascribed to domains found in its C-terminal half (C-Srv2). Here, we have unveiled a new and unanticipated role for Srv2/CAP in directly catalyzing cofilin-mediated severing of actin filaments. This function is mediated by N-Srv2, and is physically and genetically separable from C-Srv2 function in actin monomer recycling. Using dual-color TIRF microscopy, we observed that N-Srv2 promotes filament disassembly by increasing the frequency of cofilin-mediated severing without affecting cofilin binding to filaments. To address the structural basis of these effects, we used electron microscopy and single particle analysis, which revealed that N-Srv2 forms novel hexameric structures resembling ninja throwing stars. An N-terminal truncation that disrupts Srv2 oligomerization severely impaired enhanced severing activity, and led to striking defects in cellular actin organization and cell polarity. In addition, genetic analysis revealed that the activities of N-Srv2 but not C-Srv2 are essential in vivo in combination with those of another cofilin co-factor, Aip1. Together, our results define a new cellular role for Srv2/CAP in directly stimulating filament severing and disassembly, and demonstrate that the activity of cofilin alone, while essential, is not sufficient for cell viability, and that additional severing co-factors are critical.

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The mechanobiochemistry of dendritic actin network assembly.

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Branched actin networks generate protrusive forces required for cell motility and movement of sub-cellular structures. Despite advances in our biochemical knowledge of dendritic actin network assembly, very little is known about how dynamic networks of close-to-physiological architecture respond to mechanical stimuli at the molecular level. We combined micropatterning with atomic force microscopy (AFM) and multi-color TIRF imaging to visualize the active, force-generating region of in vitro reconstituted actin networks in a biochemically and mechanically

defined environment. Our measurements demonstrate that Arp2/3-generated actin networks are highly sensitive to load, particularly at low forces. The network growth velocity decreases exponentially with counteracting forces, while the actin density in the force-generating region of the network increases strongly with elevated loads. This shows that net actin polymerization decreases less drastically than the growth velocity. In addition, the levels of network-associated capping protein increase in proportion to the amount of actin polymer in the network, indicating that average filament length is determined solely by the kinetics of capping protein association and is insensitive to applied force. Our AFM-TIRF measurements provide new insight into the role of capping, branching, and filament elongation in force-dependent changes in dendritic actin network assembly.

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Enabled negatively regulates diaphanous-driven actin dynamics.

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Cell protrusions are an essential component of dynamic cell movement during development and disease. Actin regulatory proteins control the architecture and dynamics of cell protrusions, forming branched actin networks for lamellipodia or unbranched actin bundles for filopodia. While many actin regulators have been identified and characterized, it remains unclear why cells need multiple actin regulators with similar functions and how this network of proteins works together to create the diverse cell protrusions seen in normal development. Enabled (Ena) and Diaphanous (Dia) provide a superb model, as both can act at the barbed end of actin filaments to promote polymerization of unbranched filaments. We previously found that Ena and Dia form a protein complex, and discovered they act in complex, non-additive ways to shape the balance of lamellipodia and filopodia in migrating epidermal cells. We hypothesized that Ena and Dia directly bind each other and this binding regulates their activity. Through yeast two-hybrid and GST pull-downs, we found that Ena's EVH1 domain binds to Dia's proline-rich FH1 domain. To test how this Dia:Ena binding interaction affects cell protrusions, we are using three parallel model systems: in vitro actin assembly assays, *Drosophila* D16 cells, and *Drosophila* embryos. To compare protrusive behavior, we developed a software program to measure cell protrusion dynamics, including filopodia number, length, and stability, and lamellipodial protrusion. Our data reveal that while both Dia and Ena drive ectopic filopodia, these have distinct properties and dynamics. Consistent with our earlier in vivo experiments, together they act in non-additive ways. The EVH1 domain is sufficient to reduce the number of ectopic filopodia driven by activated Dia, suggesting that Dia:Ena binding is a negative regulatory mechanism. To test this hypothesis, we performed pyrene-labeled actin assembly assays and found that EVH1 reduces actin assembly by Dia's FH1FH2 domains. We are now using SNAP-tagged versions of activated Dia and Ena in TIRF assays to visualize their interactions with actin filaments and each other to understand their mechanism of controlling actin dynamics. We also are testing this hypothesis by examining the functional interactions of Ena EVH1 mutants and Dia FH1 mutants that block Dia:Ena interactions, to address whether this negative regulatory mechanism requires direct binding. Together, these assays will provide insight into how similar actin regulators interact to tune cell behavior.

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Anillin regulates junctional integrity and RhoA activation at cell-cell junctions in the intact epithelium.

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My lab is focused on studying the molecular mechanisms by which Rho family small GTPases regulate cytokinesis in the context of the intact epithelium. Understanding this process is important because successful cytokinesis is essential for proper development, and cytokinesis failure can contribute to tumor formation. Anillin is a contractile ring component that is required for cytokinesis in several model organisms. Anillin can link multiple components of the contractile ring including F-actin, Myosin-2, and Septins with the plasma membrane, and it scaffolds a number of other proteins including the small GTPase RhoA and the RhoA regulator MgcRacGAP. While Anillin is important for stable furrow positioning in isolated cultured cells, little is known about Anillin's function during cytokinesis in multicellular organisms *in vivo* or whether it plays functional roles outside of cytokinesis. Here, we used *Xenopus laevis* embryos as a model system to examine the role of Anillin in regulating cytokinesis in the intact epithelium. We find that a population of Anillin is localized at cell-cell junctions throughout the cell cycle and is particularly enriched at cell-cell junctions of dividing cells. Anillin functionally regulates cell-cell junctions; both tight junctions and adherens junctions are disrupted in Anillin knock down embryos. Additionally, we observe increased intercellular spaces between cells in Anillin knock down embryos. When control embryos are mounted in fluorescent dextran, the dextran cannot penetrate into intercellular spaces. However, in Anillin knock down embryos, dextran can penetrate, indicating that the epithelial barrier function is compromised. Because Anillin is reported to interact with both RhoA and MgcRacGAP, we tested the effect of knocking down Anillin on RhoA activity at cell-cell junctions. Surprisingly, Anillin knock down results in increased spontaneous flares of RhoA activity at cell-cell junctions. These RhoA activity flares are prominent at cell-cell junctions in both dividing cells and non-dividing regions of the epithelium. These results reveal a novel role for Anillin in regulating cell-cell junctions likely via RhoA. We propose that Anillin is required to properly distribute cortical tension and RhoA activity in order to maintain cell-cell junctions. This function is particularly important during the process of cytokinesis in an epithelial context when the dividing cell must maintain and remodel cell-cell junctions with neighboring cells as well as manage the contractile forces and shape changes associated with cytokinesis.

Minisymposium 26: Cell Growth and Cell Cycle Control

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Feed-forward regulation ensures stability and rapid reversibility of a cellular state.

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Cellular transitions are important for all life. Such transitions, including cell fate decisions, often employ positive feedback regulation to establish and stabilize the new cellular state. However, positive feedback is unlikely to underlie stable cell cycle arrest in yeast exposed to mating pheromone because the signaling pathway is linear, rather than bistable, over a broad range of extracellular pheromone concentration. We show that the stability of the pheromone arrested state results from coherent feed-forward regulation of the cell cycle inhibitor Far1, which is activated both by phosphorylation and transcription. Fast regulation by phosphorylation allows

rapid cell cycle arrest and reentry, whereas slow Far1 synthesis reinforces arrest. Importantly, feed-forward regulation achieves a stable arrest without altering signaling pathway output throughout the reversible transition. Since feed-forward regulation achieves the ostensibly conflicting aims of arrest stability and rapid reversibility without loss of signaling information, we expect its frequent implementation at reversible cellular transitions.

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Evolution and conservation of G1/S regulation in fungi and beyond.

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Cyclins are central regulators of cell cycle progression and form a large super-family, with multiple members in most eukaryotes. Recent work shows that the G1–S regulatory network (cyclins and transcription factors) and its associated dynamic properties in budding yeast and mammals are conserved, despite lack of sequence homology between many regulators. In particular, the origin of fungal G1 and B-type cyclins has remained enigmatic. To address this question, I have constructed a phylogeny of the cyclin superfamily in Fungi and beyond, anchored on *Saccharomyces cerevisiae* G1/S-type cyclins (CLN) and B-type cyclins (CLB). When compared to cyclins across the eukaryotic kingdom, the fungal CLB/CLN cyclins cluster closest to B-type cyclins found in plants and animals. In the fungal lineage leading to *S. cerevisiae*, the molecular phylogeny of G1/S-type cyclins (CLN) suggests they are derivatives of an ancient B-type cyclin (CLB) duplication that occurred in an early fungal ancestor. Evidence suggests that a fungal ancestor downsized and simplified its repertoire of cyclins. The zygomycetes contain only a single B-type cyclin (CLB) and no G1 cyclins (CLN), which may represent persistence of an ancestral state. The de novo evolution of fungal G1/S cyclins (CLN) from B-type cyclins (CLB) stands in stark contrast to the strong conservation of G1-S regulatory network between budding yeast and mammals. I also examine the phylogeny of fungal G1/S transcription factors (SWI6/SWI4/MBP1) and animal G1/S transcription factors (E2F/DP). Similar to cyclin phylogeny, early Fungi are characterized by the emergence of novel SWI6/SWI4/MBP1 transcription factors and the disappearance of the original E2F/DP transcription factors. These results suggest rapid evolution of the fungal cell cycle through which novel B-type cyclins and transcription factors are able to acquire and maintain the function and dynamics of G1-S regulatory network.

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Insertion of spindle pole bodies into the nuclear envelope.

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The defining feature of eukaryotic cells is the double lipid bilayer of the nuclear envelope (NE) that serves as a physical barrier separating the genome from the cytosol. Nuclear pore complexes (NPCs) are embedded in the NE to facilitate transport of proteins and other macromolecules into and out of the nucleus. In *Saccharomyces cerevisiae* where the NE does not completely breakdown during mitosis, the microtubule-organizing center, known as the spindle pole body (SPB), must also be inserted into the NE to facilitate organization of the mitotic spindle. Although most aspects of this process are unknown or unproven at a molecular level, it is thought that remodeling of NE lipids and stabilization of the resulting highly conserved pore membrane structure are involved in the formation of insertion sites for both the NPC and SPB.

Using a combination of genetics, live cell imaging and biochemistry we have analyzed the process of SPB insertion in order to better understand the functional role of membrane proteins in NE remodeling, such as the pore membrane proteins Pom34 and Pom152 that are required for NPC assembly, the shared NPC and SPB component Ndc1 and the yeast ortholog of the conserved SUN family of inner nuclear membrane proteins Mps3. Our results suggest that the NPC and SPB compete for a shared insertion factor that we propose to be Ndc1. Levels of Ndc1 at the NPC and SPB are regulated by its interactions with Pom152-Pom34 and with Mps3, respectively. A competition model between the NPC and SPB for a shared insertion factor could be a mechanism to restrict SPB duplication to G1 phase of the cell cycle and may explain why mutation of many SPB components results in diploidization.

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Molecular mechanism for proteasomal recognition of ubiquitinated substrates described by CryoEM.

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The proteasome is the major ATP-dependent protease in eukaryotic cells, degrading unwanted cellular proteins through recognition of covalently attached polyubiquitin chains. Highly discriminate recognition of ubiquitin chains by this multicatalytic complex is crucial to cellular function. Recent studies by cryo-electron microscopy (cryoEM) offer insight into the molecular organization of the regulatory particle, which is involved in substrate recognition, unfolding, and translocation, but the structural context by which the proteasome recognizes polyubiquitin chains remains poorly understood. Using cryoEM to visualize proteasome mutants lacking one or more of the ubiquitin receptors, we now have a detailed model for polyubiquitin reception by the proteasome. Upon receptor binding of a polyubiquitin chain, the proteasome regulatory particle undergoes a conformational reorganization in which portions of the lid serve as hinged attachment points to the core particle, while the remainder of the lid repositions the ATPases for substrate delivery to the proteolytic chamber. This offers an explanation for the proteasome's dependence on the lid for efficient substrate degradation.

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Cell cycle coupled structural oscillation of centromeric nucleosomes and kinetochores in yeast.

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The centromere is a specialized chromosomal structure that regulates chromosome segregation. Centromeres are marked by a histone H3 variant. In budding yeast, the histone H3 variant Cse4 is present in a single centromeric nucleosome. Experimental evidence supports several different models for the structure of centromeric nucleosomes. To investigate Cse4 copy number in live yeast we developed a new method coupling fluorescence correlation spectroscopy and calibrated imaging. We find that centromeric nucleosomes have one copy of Cse4 during most of the cell cycle, whereas two copies are detected at anaphase. The proposal of an anaphase coupled structural change is supported by Cse4-Cse4 interactions, incorporation of Cse4, and the absence of Scm3 in anaphase. Nucleosome reconstitution and

ChIP suggests both Cse4 structures contain H2A/H2B. The increase in Cse4 intensity and deposition at anaphase is also observed in *Candida albicans*. Using the same calibrated imaging approach for kinetochore proteins, we find that some components are dynamic in anaphase. Our experimental evidence supports a cell cycle coupled oscillation of centromeric nucleosome and kinetochore structure in yeast.

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Geometric control of cell division in fission yeast: one kinase – one substrate – two effects.

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Spatial and temporal controls of cell division are essential to coordinate cell growth with division. In rod-shaped fission yeast cells, the DYRK-family Pom1 kinase couples cell size to cell cycle progression by forming plasma membrane-associated gradients emanating from the cell tips, which negatively regulate the medially localized SAD-family kinase Cdr2. Cdr2 promotes mitotic entry by inhibiting Wee1 and specifies the division plane at the cell middle by interaction with the anillin-like protein Mid1. While Cdr2 is a substrate for Pom1, the molecular mechanism underlying the negative regulation of Cdr2 by Pom1 remains elusive. In particular, *pom1* deletion affects both the localization and the output of Cdr2, but the relationship between the two effects is unclear. In agreement with this, by quantitative analysis of Pom1 and Cdr2 cortical distributions, we observe that, as cells grow, Cdr2 levels increase at the cell middle, within a widening window of basal-level Pom1.

Using an analog-sensitive *pom1-as* allele, we find that Pom1 effects on Cdr2 activity and localization are separable. Similarly, we identified a separation-of-function *pom1* allele, *pom1-305*, which produces short cells but shows only minor effect on Cdr2 localization. Conversely, an active *pom1* allele that lacks few autophosphorylation sites and spreads along cell sides, *pom1-2A*, produces long cells again with minor effects on Cdr2 localization. This suggests a differential threshold of Pom1-dependent phosphorylations on Cdr2 that separates the regulation of kinase activity from localization. We identified Pom1 phosphorylation sites on Cdr2 by SILAC and generated multiple phosphosite mutants spanning the entire Cdr2. Our experiments suggest that Pom1 regulates Cdr2 localization to the medial cortical region and its activity through phosphorylation of distinct domains. We show that Cdr2 membrane anchoring depends on a KA-1 domain, involved in phospholipid binding, and a polybasic motif, which is directly phosphorylated by Pom1. Ongoing work suggests other phosphosite mutants may specifically affect Cdr2 activity. These studies illustrate how one kinase may have two distinct effects on the same substrate to control timing and positioning of cell division.

Minisymposium 27: Development and Morphogenesis

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Cell and tissue mechanics in zebrafish gastrulation.

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Tissue morphogenesis during embryonic development is brought about by mechanical forces which are generated by the specific biophysical and motility properties of its constituent cells. It

has also been suggested that embryonic tissues behave like immiscible liquids with a given surface tension and that differences in surface tension between tissues determine their spatial configuration during embryogenesis. To understand how single cell biophysical and motility properties regulate tissue surface tension and how tissue surface tension controls tissue organization in development, we are studying the specific function of germ layer progenitor cell adhesion, cell cortex tension and motility in determining germ layer organization during zebrafish gastrulation. We found that the combinatorial activity of progenitor cell adhesion, cortex tension and motility determines germ layer tissue surface tension and that differences in germ layer tissue surface tension influence germ layer organization during gastrulation. We will discuss these findings in the light of different hypotheses explaining how single cell biophysical properties determine tissue morphogenesis in development.

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Dynamic interactions between PAR proteins modulate the cycling of actomyosin networks during *Drosophila* apical constriction.

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Apical constriction is a major mechanism underlying morphogenesis. Previously thought to be mediated by contractile actomyosin belts anchored to cell junctions, recent studies including ours have shown that many cell types apically constrict via cyclical pulses of dynamic apical actomyosin networks. What governs the cycling of these networks *in vivo* remains unclear. Using *Drosophila* amnioserosa cells during dorsal closure as a model to study apical constriction, we previously found that amnioserosa contractility is based on cyclic assembly and disassembly of apical actomyosin networks. We also found that the PAR complex, composed of the apical polarity regulators Bazooka (Baz, *Drosophila* PAR-3), PAR-6, and atypical protein kinase C (aPKC) regulate distinct phases of the actomyosin cycles: Baz enhances network duration whereas PAR-6/aPKC promote the lull time between pulses. We hypothesized that Baz may enhance actomyosin network durations by inhibiting PAR-6/aPKC and that dynamic interactions between PAR proteins may be coupled to the cycling of the actomyosin networks. A later change in the actomyosin networks allowed us to test this hypothesized mechanism. During later dorsal closure, the actomyosin networks have decreased cycling and increased persistence; a change that occurs concomitantly with a shift in PAR proteins away from cell junctions and towards the apical surface. This shift appears to involve a rebalancing of mechanisms influencing junctional and apical surface PAR localization, with the latter being more closely linked to the apical actomyosin networks. To test whether increasing apical surface Baz attenuates aPKC-mediated inhibition of the actomyosin networks, we overexpressed mutant forms of Baz which either abolish or mimic its aPKC phosphorylation site (S980A and S980E, respectively). BazS980A is known to have increased affinity for aPKC whereas BazS980E has decreased affinity for aPKC. Overexpressed BazS980A precociously localized to the apical surface, stabilized and colocalized with apical surface aPKC, and promoted precocious apical constriction. In contrast, BazS980E had no such effects despite higher expression levels. We propose that the normal apical shift of the PAR proteins leads to increased Baz-aPKC interaction and to increased competitive inhibition of aPKC kinase activity towards its other substrates. This increased Baz-aPKC interaction may relieve aPKC-mediated inhibition of the actomyosin cytoskeleton leading to reduced cycling, more persistent networks, and greater apical constriction.

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Cellular mechanisms and evolution of morphogenesis in *Volvox* and related algae.I. Nishii¹; ¹*Temasek Life Sciences Laboratory, Singapore, Singapore*

Most metazoa first build a hollow sphere through successive cell cleavages and then this spherical multicellular sheet repeatedly folds - invaginate and protrude - to form unique shapes of organs and individual. How did such supercellular morphogenetic processes arise during the evolution of multicellular organisms from their unicellular ancestors? *Volvox* and their close relatives serve as a good model for investigating such issue. This group ranges from unicellular *Chlamydomonas*, through several related species increasing cell numbers, to *Volvox* with thousands of cells. The key morphogenetic process in *Volvox* development is called inversion, in which the spherical embryo turns inside out. The pre-inversion embryo is a cellular monolayer in which neighboring cells are linked to one another at the mid-level of their cell bodies by cytoplasmic bridges. The single place where such linkages are missing is across the opening where the inversion movement will start. In onset, the cells become flask shaped by extending long, thin stalks from their outermost ends, showing analogy with a characteristic cell shape seen in metazoan morphogenesis such as gastrulation. Next, cells near the opening migrate relative to their cytoplasmic bridges until they are linked to their neighbors only at the outermost tips of their stalks, which causes the cell sheet to turn outward. Transposon-tagged inversionless mutants were isolated and five different loci were characterized. Intriguingly, these genes were involved in cell shape changes and cell migration in inversion described above but also related to ECM. *invD* and *invE* genes, which encoded a novel phospho-protein and a MAP kinase, respectively, were required for cell elongation. Phosphorylated InvE proteins were abundant before inversion but they were rapidly dephosphorylated after inversion. In addition, phosphorylation of InvD was dependent on InvE. Next, *invA* encoded a kinesin motor protein that was localized near the cytoplasmic bridges (Nishii et al. 2003) and required for the cell migration. Furthermore, *invB* and *invC* encoded a sugar nucleotide transporter and a glycosyl transferase, respectively, both have roles in processing of glycoproteins involved in the size control of ECM surrounding embryo (Ueki & Nishii 2008, 2009). Finally, regarding evolution, all five homologous genes in *C. reinhardtii* were found. Intriguingly, *invD* and its ortholog were quite divergent in sequence compared to other pairs, suggesting that the evolution of InvD from an ancestral protein might have been an important role for elongated flask-shaped cell formation. We also discuss tracing such cellular basis in inversion and localization of InvA in related species from evolutionary aspects.

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Titration of histones sets the DNA threshold for activating transcription at the mid-blastula transition in *Xenopus*.A. Amodeo¹, A. Straight², J. Skotheim¹; ¹*Biology, Stanford University, Stanford, CA,*
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In many species, the early post-fertilization divisions occur rapidly and synchronously without growth phases, cell cycle checkpoints, or transcription. These early embryos are almost entirely transcriptionally inactive and rely on maternally supplied RNAs. At the Mid-blastula Transition (MBT), embryos initiate large-scale transcription from the zygotic genome (also known as the Maternal to Zygotic Transition or MZT), add growth phases, and gain checkpoints. Previous work suggested that the MBT is initiated by the increased DNA-to-cytoplasmic ratio resulting from repeated rounds of DNA replication and cell division without cell growth. This led to the hypothesis that the progressive titration of an inhibitory factor present in the embryo allows the initiation of zygotic transcription. However, the molecular identity of the titratable factor remained unknown. To determine the inhibitory molecule, and thus prove the titratable factor hypothesis,

we have developed a cell-free system in which to study the MBT. We found that *Xenopus* egg extracts initiate transcription at threshold DNA concentrations similar to those present at the MBT in intact embryos. Using this cell free system we have biochemically isolated histones H3 and/or H4 as the proteins responsible for inhibiting transcription in response to the DNA-to-cytoplasmic ratio. We have demonstrated that exogenous, bacterially produced, H3/H4 tetramers can increase the DNA threshold for transcriptional onset in a dose dependant manner. The identification of an abundant, high affinity DNA binding molecule that can inhibit transcription in a dose dependant manner strongly supports the titration model for MBT initiation. In addition, these findings suggest a molecular mechanism in which titrating out histones progressively opens chromatin structure to allow transcription.

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Asymmetric inheritance of primary ciliary membrane in dividing neural progenitors.

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The primary cilium is a microtubule-based protrusion of the cell surface that is involved in mediating extracellular signals, such as Sonic Hedgehog (Shh). The primary cilium is nucleated by the centrosome containing the eldest centriole (mother centriole). Prior to mitosis, the primary cilium is assumed to be disassembled in order for the centrosomes to be free to serve as the poles of the mitotic spindle.

Recent findings show that asymmetric inheritance of the centrosome containing the mother centriole is related to cell fate decisions during mammalian neurogenesis. However, it is unknown whether centrosome-associated structures such as the primary cilium are involved in this asymmetrical regulation of cell fate. Therefore, we set out to investigate the fate of the primary cilium upon cell division of developing neural progenitors and the possible involvement of the primary cilium in subsequent cell fate decisions in the developing mouse neocortex.

We find that in mitotic neural progenitors and cultured cell lines, a membrane structure containing the ciliary small GTPase Arl13b is associated with the centrosome that contains the mother centriole. By surface biotinylation of the apical membrane in explanted mouse telencephalic hemispheres, we show that this structure contains ciliary membrane and is derived from the primary cilium that was present at the cell surface prior to mitosis. Using live imaging, we find that upon completion of mitosis, this centrosome-associated ciliary membrane is asymmetrically inherited by one of the daughter cells. Furthermore, we show that inheritance of the ciliary membrane causes earlier reestablishment of the primary cilium on the cell surface after division. Interestingly, the association of the ciliary membrane with one centrosome in mitotic neural progenitors decreases as neurogenesis proceeds.

We hypothesize that the observed asymmetry in primary cilium reassembly after mitosis might differentially expose daughter cells to extracellular signals, such as Shh. Therefore, we speculate that inheritance of the ciliary membrane affects cell fate decisions during mammalian neurogenesis.

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Contact-mediated long distance signaling by *Drosophila* cytonemes.

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How cells communicate with each other at long distances is key to understanding how cells cooperate to form organized tissues during development and why cells in various disease states lose or escape normal controls. Although much progress has been made identifying signaling molecules that mediate these communications – proteins such as Hedgehog, Wingless,

Decapentaplegic (Dpp, a BMP homolog), Fibroblast Growth Factor and Epidermal Growth Factor – the mechanism by which these proteins move with target specificity and in regulated amount through and across tissues remains unproven. Several proposed models postulate that some form of diffusion moves these signaling proteins through extracellular spaces. My work has investigated a different “direct delivery” mechanism whereby specialized filopodia (cytonemes) transfer signaling proteins between cells at sites of direct contact (Roy and Kornberg, *Sci Signal.* 2011; 4:pt8). Cytonemes are types of filopodia first identified in the *Drosophila* wing imaginal disc that were proposed to be involved in long distance signaling during development. My work shows that same group of cells emanate different types of cytonemes that can be distinguished by their specific response to different signaling ligands depending on the presence or absence of different protein receptors in them (Roy et al. *Science.* 2011; 332:354-358). I then show, using the GRASP GFP reconstitution method, that cytonemes from epithelial cells make direct contact with distal target cells, and also show that contacting cytonemes exchange, receive and transport ligands from source cells to recipient cells in receptor dependent manner. Finally, I show that genetic conditions that reduce cytonemes also reduce signal transduction. These findings establish that even non-neuronal cells can make direct long distance contacts for target specific and regulated signal exchange and strongly support the model of cytoneme-based movement of signaling proteins as a novel and essential mechanism for cell-cell communication.

Minisymposium 28: Membranes Organization and Lipid Dynamics

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Internalization of near infrared labeled transferrin into breast cancer cells using FRET tomography imaging.

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Transferrin (Tfn) is an iron-binding protein used to transport iron via cell surface Tfn receptors (TFR) into the cytoplasm. The dimeric nature of TFR allows the use of Förster Resonance Energy Transfer (FRET) based detection methods to identify receptor-bound, fluorophore labeled Tfn donor and acceptor pairs from unbound Tfn. We report the use of near infrared (NIR)-FRET tomographic imaging to detect internalization of Tfn based on the reduction of donor fluorophore lifetime using a multispectral time resolved fluorescence molecular tomography (FMT) imaging system. Our main goal is to distinguish breast cancer cells (T47D) from normal cells (HMEC) via quantification of cellular Tfn uptake within live mice, due to the wide use of Tfn as a carrier for anti-cancer drug delivery systems. T47D and other cancer cells show higher TFR expression and more rapidly internalize Tfn compared to HMECs in which Tfn is co-localized to early endosome 1 (EEA1) positive markers, thus validating specific cellular internalization of Tfn. In support, we demonstrate increased % FRET efficiency (E%) at higher intensity thresholds of acceptor molecules in breast cancer cells compared to controls using confocal FRET analysis. To establish *in vivo* conditions, we tested temporal lifetime measurements of several donor NIR fluorophore candidates using our time-resolved imaging platform with data fitted into a bi-exponential decay function to estimate non-FRETing donor lifetimes. NIR FRET donor-acceptor AF700-Tfn and AF750-Tfn pair was selected as the optimal FRET pair under our imaging conditions due to the longer donor lifetime and higher quantum yield of AF700. Alexafluor NIR fluorophores AF700 and AF750 conjugated to Tfn molecules (AF700-Tfn/AF750-Tfn) were used for uptake studies into T47D and HMECs. Cells were

imaged using a time-resolved wide-field illumination strategy, with whole-body functional and fluorescence tomographic data sets at high-spatial and high-spectral densities (690nm-1020nm spectral range), and acquired using a 300ps time gate and a 40ps time delay to obtain time point spread function (TPSF) over a 4.6ns time window (116 gates). Co-internalization of AF700-Tfn and AF750-Tfn into T47D cells show a positive increase in fractional amplitude of FRETing donor Tfn molecules as A:D ratios increased from 1:4 to 4:1, as expected using fluorescence lifetime based FRET tomographic imaging. In support, mean photon counts show FRET-induced quenching at 4:1 compared to the 1:4 A:D ratio. We demonstrate a detection of Tfn internalization based on FRET events in cancer cells at a threshold of approximately 1×10^4 cells, a number well below current clinical detection capabilities. We thus show proof-of-principle detection of NIR FRET in breast cancer cells in which these signals could be a powerful and non-invasive tool to identify the presence of tumors *in vivo* and to optimize targeted delivery systems based on Tfn-TFR mediated uptake into cancer cells.

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Direct imaging of sterol-enriched micro-domains that segregate vacuolar membrane proteins.

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The self-organization of lipids in membranes has been proposed to drive membrane domain formation. The most well studied lipid domains are sterol-sphingolipid enriched micro-domains (rafts) that are liquid ordered and coexist with liquid disordered portions of membranes. Rafts have been suggested to mediate the segregation of proteins and to be critical for processes such as endocytosis, viral budding, protein stability, immune receptor trafficking, and signaling. Large, stable liquid ordered rafts have been seen in model membranes. However, *in vivo* rafts have proven difficult to observe in live cells without invasive methods and seem to be much smaller and shorter-lived than those in artificial membranes, leading many to doubt whether rafts exist or play significant roles in cells. Here we show that large, stable sterol-enriched domains, which are strikingly similar to those observed in model membranes, form in the yeast vacuole membrane in response to nutrient deprivation or other stresses such as weak acid stress. All vacuolar membrane proteins tested segregate to one of two domains when cells starve. These two domains coalesce into several remarkable patterns on the vacuole surface, including a quasi-symmetrical pattern. Sterols are enriched in one of the two domains, are required for domain formation, and the sterol-enriched domain is liquid ordered. In addition, we demonstrate that liquid-ordered phases are present on vacuoles even when domains are not visible by microscopy, suggesting that the domains are present all the time but coalesce into large domains only in starvation or other stress conditions. We found that mutants lacking genes involved in lipid metabolism, vesicular trafficking, and regulating cellular entrance into quiescence lacked vacuolar lipid domains. Remarkably, these mutants were not able to recover from quiescence when treated with the TORC1-inhibitor rapamycin, suggesting that vacuolar lipid domains are required to exit the quiescence. This is the first demonstration that sterol-enriched raft-like liquid ordered domains similar to those observed in artificial membranes form in living cells and play an important role in the cellular response to stress.

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TLR4 signaling regulates a novel form of endocytosis.

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We have characterized a novel form of endocytosis in which cells create an extracellular, acidic, hydrolytic compartment (a lysosomal synapse). The lysosomal synapse allows rapid catabolism of large species or moieties tightly linked to the extracellular matrix that cannot be internalized even by phagocytosis. These species are typically involved in disease states that are coupled to an inflammatory response. Consistent with this, we find that Toll-like receptor 4 signaling is necessary for lysosomal synapse formation. Thus far, we have examined this mechanism of uptake in macrophages and dendritic cells. These cells use the lysosomal synapse to degrade aggregated lipoproteins thereby transforming them into foam cells, a key pathological feature of atherosclerotic plaques.

We find that compartment formation is dependent on local F-actin polymerization and that targeted lysosomal exocytosis provides the hydrolases necessary for extracellular catabolism. Acidification of the lysosomal synapse allows hydrolase activity. Data indicate that after partial catabolism outside the cell, remnants can be internalized and digested in lysosomes. Thus, the formation of a lysosomal synapse requires extensive rearrangement of the actin cytoskeleton, formation of a nearly sealed extracellular compartment, and targeted secretion of lysosomal contents. All of these activities require carefully coordinated signal transduction processes. Recent work has focused on the receptors and signal transduction pathways involved in the formation and function of the lysosomal synapse. Data show that one arm of the signaling is associated with Toll-like receptor 4 via the downstream adapter protein myeloid differentiation primary response protein 88. Downstream signaling also involves phosphoinositide 3-kinase, protein kinase B (Akt), Bruton's tyrosine kinase, phospholipase C, protein kinase C and calcium mobilization as important events leading to lysosomal synapse formation and function.

Akin to phagocytosis, uptake via the lysosomal synapse occurs in specialized cells. Efforts are currently underway to examine the relevance of this pathway to several other diseases, such as Alzheimer's, diabetes and cancer. Identification of the specific molecular pathways involved in this process will not only contribute to the basic understanding of endocytic mechanisms but may also provide molecular targets for the ultimate development of therapies related to a number of diseases.

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Micropatterning of plasma membranes of differentiated vertebrate cells.

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Vertebrate membranes are patterned into discrete domains necessary for critical physiological functions in multi-cellular organisms. This patterning not only involves proper delivery of proteins and lipids to different compartments (i.e. epithelial apical/basolateral membranes), but also lateral segregation of proteins within the membrane. Recently, it has been shown that yeast membrane proteins are distributed in non-homogenous patterns throughout the membrane, and that this patterning is necessary for proper protein function [1]. Here we show that the intracellular adaptor molecule ankyrin G (AnkG) is localized to discrete microdomains within mammalian cell membranes both in vitro and in vivo, including epithelial lateral membranes, axon initial segments, and cardiac T-tubules. Previous studies have shown that AnkG binds to

band 3 in erythrocytes with a low stoichiometry [2, 3], suggesting a close apposition of ankyrin to its binding partners. Surprisingly, using high resolution fluorescence microscopy, we find that AnkG and its partners are in distinct domains with only modest colocalization. These domains are independent of fixation as they can be seen in living cells. In addition, cysteine 70 of AnkG, which is palmitoylated, is necessary for the association of AnkG with these microdomains suggesting that lipid modifications may play a role in microdomain patterning. Finally, using fluorescence recovery after photobleaching, we show that AnkG binding partners may be restricted within their respective domains through tethering to the spectrin-based membrane skeleton. This is based on an increased E-cadherin mobile fraction, but no change in recovery rate, within the epithelial lateral membrane upon expression of an inducible dominant-negative consisting of the AnkG binding site from beta 2 spectrin.

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TRIM9 coordinates membrane trafficking and cytoskeletal dynamics during neuronal development.

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During the establishment of neuronal circuitry, axons and their collateral branches are guided by attractive and repulsive cues to postsynaptic targets. While many guidance receptors have been identified, how they trigger the cytoskeletal dynamics and vesicle fusion with the plasma membrane required for guidance and branching is unclear. Here we identify TRIM9, an E3 ubiquitin ligase with restricted expression in the nervous system, as a critical component at the interface of netrin signaling to the cytoskeleton and membrane trafficking in the mammalian nervous system. The TRIM9 orthologue in *c. elegans* and *drosophila* has previously been implicated in netrin-dependent axon guidance. We will present evidence that vertebrate TRIM9 functions in two critical stages of neuronal development: axonal branching, which increases the number of synaptic partners, and axonal guidance, which underlies innervation of proper targets. Vertebrate TRIM9 interacts with the netrin receptor DCC, actin regulatory Ena/VASP and MRL proteins, the exocytic t-SNARE, SNAP25, and the nonconventional myosin motor that targets DCC to the membrane, Myo 10. Inhibition of TRIM9 ligase activity in cortical neurons alters filopodia formation and their invasion by microtubules. We also observe that genetic loss of TRIM9 results in increased VAMP2-mediated exocytosis and exuberant collateral axon branching *in vitro* in the absence of netrin. Interestingly, without TRIM9, cortical neurons lose the ability to respond to netrin. Culturing cortical explants from wildtype and TRIM9^{-/-} brains indicates TRIM9 function is critical for asymmetric neurite outgrowth toward a netrin source. This suggests TRIM9 is involved in netrin-mediated axonal guidance, which we are currently investigating. Ongoing characterization of these mice has identified neuroanatomical defects caused by genetic loss of TRIM9, suggesting the functions of TRIM9 found *in vitro* are critical *in vivo* as well. The position of TRIM9 at the interface of netrin signaling to both the cytoskeleton

and membrane trafficking provides an exquisite regulation of these subcellular machines that power neuronal morphology changes in response to netrin.

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Dynamic regulation of endoplasmic reticulum-plasma membrane junctions monitored by a genetically-encoded fluorescent marker.

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Many cellular functions, including secretion, cell migration and differentiation, depend on store-operated calcium entry (SOCE). A decrease of endoplasmic reticulum (ER) calcium and the subsequent activation of an ER calcium sensor, STIM1, initiate SOCE. Reduced ER calcium induces STIM1 to oligomerize and translocate to the ER-plasma membrane (PM) junctions where the two membranes are closely apposed to each other within 20nm. The calcium channel Orai1 in the PM is then activated, which enables SOCE. Although ER-PM junctions have been observed by electron microscopy (EM) in many cell types, little is known about their formation and regulation. To help understand the dynamic regulation of ER-PM junctions, we first generated a genetically-encoded fluorescent marker of ER-PM junctions based on the translocation mechanism of STIM1. We have confirmed that this marker selectively localized to ER-PM junctions using confocal microscopy and EM, and that it did not perturb STIM1 and Orai1 translocation to the junctions nor disrupt SOCE. This marker has enabled us to visualize and track ER-PM junctions using live-cell total internal reflection fluorescence (TIRF) microscopy. We found that approximately two hundred stable ER-PM junctions exist in the adhesion surface of a single HeLa cell at resting state. During SOCE, a significant up-regulation of ER-PM junctions was observed. We further employed a cytosolic calcium chelator and a cytosolic caged-calcium reagent to demonstrate that the up-regulation of ER-PM junctions required an increase of cytosolic calcium but not a decrease of ER calcium. In summary, we have generated a marker enabling us to track the dynamic change of single ER-PM junctions in live cells and discovered cytosolic calcium-dependent regulation of ER-PM junctions.

Minisymposium 29: Nuclear Structure and Function

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The nuclear pore protein Nup98 plays a conserved role in transcriptional memory.

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The interaction of nuclear pore proteins (Nups) with genes can function to regulate transcription. In yeast, targeting of genes to the nuclear pore complex (NPC) promotes stronger transcription. For some inducible genes, interaction with the NPC is maintained for several generations after repression and serves to prime them for future reactivation, a phenomenon called transcriptional memory. We have found that a mechanistically similar phenomenon occurs in human cells. A subset of interferon γ -inducible genes are much more strongly induced in cells that have previously been exposed to IFN γ . As in yeast, this type of transcriptional memory involves a physical interaction with Nups, binding of a poised form of RNA polymerase II to the promoter and changes in chromatin structure. However, unlike yeast, the interaction of these genes with Nups occurs in the nucleoplasm. Finally, in cells transiently knocked down for Nup98, memory is lost; RNA polymerase II does not remain associated with IFN γ -inducible promoters and the rate of reactivation is dramatically reduced. These results suggest that Nup98 (Nup100 in yeast) plays a conserved role in promoting epigenetic transcriptional memory.

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In vivo* three-dimensional characterization of mRNA export through the nuclear pore complex.W. Yang*¹; ¹*Temple University, Philadelphia, PA*

Protein-receptor-facilitated nuclear export of messenger RNAs (mRNAs) through the nuclear pore complexes (NPCs) is a key step for the flow of genetic information in eukaryotic cells. However, the transport kinetics, the three-dimensional (3D) pathway and the export selectivity mechanism of mRNA-protein complexes through the NPCs remain poorly understood, although they are of fundamental interest. Here we employed a super-resolution microscopy imaging approach to three-dimensionally characterize mRNAs exporting through individual NPCs in living human cells. With a spatiotemporal resolution of 2 ms and 8 nm, the fundamental features of mRNA export through the NPCs, escaped from previous measurements of electron microscopy and single-molecule fluorescence microscopy, have been obtained: 1) mRNA complexes were decelerated and then selected at the narrowest waist of the NPC as they entered the NPC from the nucleus. Approximately one third of these incoming complexes successfully arrived at the cytoplasm, and the others aborted their export and returned back to the nucleus; 2) either the successful or the abortive mRNA export events took approximately 12 ms to interact with the NPCs; 3) In a 3D view, mRNA complexes primarily paved their passageways through the periphery around a rarely occupied central axial conduit on the nucleoplasmic side and in the central channel of the NPC, then dissociated on the cytoplasmic side of the NPC, and finally passively diffused into the cytoplasm.

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In vivo* visualization of chromosome synapsis in *C. elegans*.O. Rog*¹, *A. F. Dernburg*¹; ¹*University of California, Berkeley; and Howard Hughes Medical Institute, Department of Molecular and Cell Biology and California Institute for Quantitative Biosciences (QB3), Berkeley, CA*

Meiosis is the special cell division process that enables the production of haploid gametes. During meiotic prophase, chromosomes form linkages with their homologous partners to enable reductional segregation during the first meiotic division. Essential to this process is the pairwise alignment of homologous chromosomes along their entire lengths. In most eukaryotes this alignment is reinforced through synapsis, the polymerization of a structurally conserved structure called the synaptonemal complex (SC), which links homologous chromosomes. The SC promotes genetic exchanges (crossovers) between homologs, and likely regulates their number and location. Synapsis is a dynamic process, the details of which are difficult to infer from images of fixed cells or tissues. Our goal has been to better understand this process through analysis in living nematodes, using fluorescently tagged SC components and high-resolution time-lapse microscopy. Our observations have revealed that synapsis is rapid and highly processive. Individual chromosomes achieve full synapsis in 20-30 minutes, at a rate of ~170nm per minute. We find that initiation of synapsis is a relatively infrequent event that is rate-limiting for completion of synapsis, consistent with evidence that initiation is subject to strict regulation. Initiation occurs asynchronously on each chromosome, resulting in staggered synapsis of different chromosomes. In *C. elegans*, synapsis is accompanied by rapid chromosome motions driven by dynein, which is coupled through the nuclear envelope to one end of each chromosome. Under conditions where this motion is severely abrogated, synapsis remains processive but is ~5-fold slower. This suggests that chromosome motion promotes

homolog alignment, enabling synapsis to proceed more rapidly. Moreover, it reveals a novel function for the rapid chromosome motions that have been observed in diverse organisms during meiotic prophase.

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Assembly, dynamics and function of the synaptonemal complex (SC) in meiotic prophase nuclei.

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The SC is a highly ordered proteinaceous structure that assembles at the interface between paired and aligned homologous chromosomes during meiotic prophase. The SC has long been recognized as a hallmark feature of the meiotic program and has at least two conserved functions, in stabilizing tight associations along the lengths of homologs and in promoting maturation of recombination intermediates into crossovers. Recent work has shown that the SC also plays a role in inhibiting crossover formation during *C. elegans* meiosis, thereby limiting crossover number. Further, SC structures undergo patterned disassembly that creates an organization crucial for directing the pattern of chromosome segregation during the meiotic divisions. In addition, several lines of evidence suggest that SC assembly is part of a feedback mechanism that regulates prophase progression to assure a successful meiotic outcome.

While substantial progress has been made in identifying SC components and defining its biological roles, the SC has remained enigmatic in many ways. Although the SC normally assembles only between aligned homologous chromosomes, the SC structure itself is indifferent to homology. Moreover, SC assembly appears to be cooperative and processive, indicating a requirement for tight coordination of homolog pairing and synapsis. Further, the zipper-like appearance of the SC in EM images belies a growing realization that the SC structure is likely much more dynamic than previously appreciated. We will discuss mechanisms that regulate assembly of the SC to ensure that it only occurs in a productive manner, i.e., linking the axes of correctly paired and aligned homologous chromosomes. We will show that reducing the pool of specific SC subunits results in attenuated crossover interference, reflected in an increase in the number of cytologically-differentiated crossover sites, implying a requirement for appropriate stoichiometry to confer normal SC function. Further, we will present evidence from FRAP analysis that the "fully assembled" SC structure is indeed dynamic, undermining the view of the SC as a static scaffold.

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Corralling the microtubule tip: Nanoscale kinetochore architecture suggests an integrative model for its bidirectional motility.

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The kinetochore is a macromolecular machine that drives chromosome movement and segregation during cell division. The molecular mechanism underlying this unique, bidirectional motor depends as much on its nanoscale protein architecture as the structures and biochemical properties of individual proteins. Yet, the *in vivo* kinetochore architecture, and more specifically, the nanoscale distribution of individual microtubule (MT) binding protein molecules around and along the microtubule tip remains unknown. To define this architecture, we have developed a systematic FRET quantification technique in live cells. Our analysis reveals two microtubule-binding domains within the budding yeast (*Saccharomyces cerevisiae*) kinetochore. The first unit in this bipartite arrangement consists of Ndc80 and Dam1. The MT-binding domains of

these complexes work in close spatial proximity (< 10 nm), suggesting a functional integration of these molecules. We propose that this force generating unit is mainly responsible for MT depolymerization coupled movement. The MT-associated, plus-end tracking protein Stu2p (XMAP215) makes up the second unit. The C-terminus of this large protein situated ~ 35 nm behind Ndc80-Dam1. This multi-function protein likely participates in polymerization-coupled motility. We propose that it contributes either tip-tracking ability or destabilizes the growing tip. Thus, our reconstructed kinetochore architecture, when considered in the context of the unique structural and MT-binding properties of each individual protein, suggests an integrative model explaining bidirectional kinetochore movement that is tightly coupled with MT polymerization and depolymerization. This scheme of corralling the dynamic MT tip is likely conserved in all eukaryotes, even if organisms freely substitute proteins with analogous activities (with the exception of the Ndc80 complex) to fine-tune chromosome motility.

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The structure and function of a chromatin spring in mitosis.

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The physical properties of a long-chain polymer such as DNA and its organization inside the cell contribute in fundamental ways to replication, repair, transcription and chromosome segregation. DNA and the unique chromatin organization at the centromere function as a mechanical spring in mitosis. We apply principles of polymer physics to understand how chromatin architecture (such as DNA wrapping around nucleosomes, looping and catenation) contributes to faithful chromosome segregation. Cohesin and condensin together with pericentric chromatin constitute the spring that functions as a restoring force counterbalancing the microtubule extensional force in metaphase of mitosis. The spring exhibits asymmetric stretching, inconsistent with a simple linear spring model. We present experimental and modeling evidence for a threshold extension in the spring to switch between cohesin-condensin rich versus depleted spring constants, that accurately recapitulates in vivo chromatin and spindle dynamics.

Minisymposium 30: Prokaryotic Cell Biology

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De Novo synthesis of the cell wall in *E. coli*: Reversion of L-Forms.

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Bacterial morphology is crucial to a wide variety of cell functions, including motility, adhesion, pathogenesis. The shape of nearly all bacterial cells is determined by a rigid cell wall made of peptidoglycan. A rod-shaped cell such as *E. coli* thus faces the challenge of coordinating the nanoscale proteins responsible for peptidoglycan synthesis to construct a micron-scale sacculus. What are the principles that allow cell wall synthesis proteins to establish order over a range of length scales spanning nearly three orders of magnitude? We approached this question by examining the re-growth of the sacculus in cell wall-deficient 'L-forms' of *E. coli*, in which cell wall synthesis has been transiently disrupted by beta-lactam antibiotics. An L-form undergoing reversion begins in a spherical shape without an intact cell wall. When cell wall synthesis inhibiting antibiotics are removed, the cell generates new rod-shaped protrusions, which eventually undergo septation and adopt the normal rod morphology of *E. coli*. The

reversion of L-forms thus provides an opportunity to study morphogenesis in bacteria lacking an intact cell wall, and to study the activity of cell wall synthesis enzymes independently of their interaction with the cell wall. We used automated image analysis to quantify the morphological trajectories of reverting L-forms as they transition from rod to sphere, and to track the activity of cell wall synthesis enzymes. In particular, we examined the localization of bacterial cytoskeletal elements MreB and FtsZ, both of which are involved in cell wall growth and morphogenesis, and correlated their localization patterns with changes in cell shape. We also probed the effect of different mutants of MreB on the dynamics of the reversion process. We found that MreB determines the size of early rod-shaped protrusions in reverting L-forms, demonstrating that MreB regulates cell shape even in the absence of an intact cell wall.

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MinCD cell division proteins form alternating co-polymeric filaments.

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Most bacteria form a dynamic ring-like structure at the division site, containing the tubulin-like protein FtsZ, which constricts to produce two daughter cells. The Min proteins play an important role in the precise positioning of Z ring at the division site by oscillating between the poles in most organisms.

In *E. coli*, the three proteins MinC, MinD and MinE, encode the Min system. MinC and MinD are widely conserved in bacteria and they act together to inhibit Z ring assembly. MinD binds to the membrane in dimeric form in the presence of ATP through an amphipathic helix and also binds to MinC. MinC directly interacts with FtsZ and destabilises Z ring assembly. MinE stimulates the ATP hydrolysis of MinD and hence dissociates the MinCD complex from the membrane. So far it has been thought that a reaction/diffusion pattern-forming reaction based on MinD and MinE alone causes the oscillation of all three proteins.

Using light scattering and electron microscopy (EM) we show *in vitro* that MinC and MinD form nucleotide-dependent, alternating co-polymeric filaments, even in the absence of membrane. To better understand the interaction between MinC and MinD in the filament, we have solved the crystal structure of the MinC-MinD complex at 2.7 Å resolution. A hybrid model of the MinCD filaments, built using the MinCD and MinD dimer crystal structures correlates well with the appearance of MinCD filaments as observed in EM. Our structural analysis also identifies residues important for the MinCD interaction and helps to explain how MinE competes with MinC for the overlapping binding site on MinD.

We propose that the MinCD filament belongs to a new class of cytomotive, nucleotide-regulated filaments, adding to actin and tubulin-like systems present in many bacteria.

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How to segregate DNA without dynamic instability: Bundling of the bacterial actin-like filament AlfA is regulated by an accessory factor, AlfB.

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AlfA is a filament-forming actin-like protein in *Bacillus subtilis* that functions to actively partition the large, low copy number plasmid by which it is encoded. Previous *in vivo* and *in vitro* observations of filament dynamics have revealed a set of kinetic and structural properties (namely constitutive bundling and lack of dynamic instability) that are inconsistent with previously established models for actin-like plasmid segregating proteins such as ParM. To

understand the mechanism of AlfA-driven plasmid segregation, we imaged AlfA and its downstream DNA-binding protein, AlfB, interacting with plasmids *in vivo* and *in vitro*. Our live cell microscopy reveals that plasmids can move along existing AlfA structures or track the ends of growing ones, consistent with the idea that the AlfA polymer seen *in vivo* is actually a bundle of multiple filaments. Furthermore, these polymers can form between plasmids to push them apart, prompting us to ask how plasmids alter filament dynamics to generate this specific assembly. To address this question, we purified AlfB and found that it dramatically alters the kinetics and structure of AlfA. AlfB binds to AlfA monomers and polymers, not only increasing the critical concentration of assembly, but also preventing the otherwise very robust bundling of AlfA. The 100bp centromeric DNA region to which AlfB binds, however, rescues bundling and promotes polymerization. These observations lead us to a model of AlfA-driven plasmid segregation wherein bundles of AlfA form specifically in association with AlfB-DNA complexes. We propose that the intrinsic bundling property of the polymer, normally inhibited by a high concentration of free AlfB in the cytoplasm, functions as a capture mechanism to specifically join DNA-bound filaments to one another. Polymerization in opposite directions, driven by antiparallel bundling, would cause plasmids to be segregated from one another, ensuring their maintenance through cell division.

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Spatial regulation of protein distribution in bacterial cells.

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The formation of specialized cellular architectures often requires the establishment and maintenance of subcellular domains with a distinct lipid and protein composition. We report the identification of a novel mechanism that determines the distribution of proteins within cells of the stalked model bacterium *Caulobacter crescentus*. It depends on the establishment of a macromolecular protein complex forming at the junction between the cell body and the stalk appendage. Mutants with defects in the assembly or integrity of this structure show aberrant protein distribution patterns, which in turn lead to a significant decrease in cellular fitness. Collectively, our results demonstrate that eukaryotes and prokaryotes use similar molecular principles for the regulation of subcellular protein localization in order to ensure the proper function of cells.

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Time-resolved nanometer scale AFM imaging of antimicrobial peptide activity on live *Escherichia coli* cells.

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Understanding drug-membrane interactions is crucial to drug research and development. Bacterial membranes have a much more complex structure than mammalian cell membranes. As such, knowledge of bacterial membrane composition and organization, as well as characterization of the molecular-level responses to drug interactions, is critical to the development and assessment of effective drug formulations. Cellular drug responses involve

highly dynamic processes. However, the ability to image live cells with nanometer resolution on timescales relevant to dynamic cellular events has proven challenging. The ability of atomic force microscopy (AFM) to obtain three-dimensional topography images of biological molecules with nanometer resolution and under near-physiological conditions remains unmatched by other imaging techniques. However, with traditional AFM systems, the typically longer image acquisition times required to obtain a single high-resolution image (~minutes) has limited the ability to investigate dynamic biological processes. While recent years have shown significant progress in the development of high-speed atomic force microscopy (HS-AFM), the nature of the instrumentation that has been developed has several drawbacks in specimen size, requiring small scan sizes and flat sample surfaces. As such, the majority of biologically-related HS-AFM studies have concentrated on imaging single biomolecules with little focus on using HS-AFM to examine cellular processes. With the rapidly growing antibiotics crisis, antimicrobial peptides (AmP) are increasingly being investigated as therapeutic alternatives. Key to their success is an understanding of the mechanisms by which AmPs interact with the cell membrane and facilitate cellular death. Using HS-AFM, we have obtained the first high-resolution time sequence images of the native structure of a bacterial outer membrane, obtained directly on the surface of live *Escherichia coli* cells. The increased time resolution of HS-AFM allowed us to observe dynamic changes in the nanoscale structure of the outer membrane in direct response to the AmP CM15, at timescales relevant to the mechanism of AmP-induced cell death. The results of these HS-AFM studies have provided the first opportunity to resolve the dynamics of AmP-mediated cell death in a native cell membrane environment in real-time and with nanoscale resolution.

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Type 6 secretion dynamics within and between bacterial cells.

M. Basler¹, B. Ho¹, J. Mekalanos¹; ¹Harvard Medical School, Boston, MA

The bacterial Type VI secretion system (T6SS) corresponds to an organelle that is functionally analogous to a contractile bacteriophage tail and can power secretion of proteins between cells. The dynamics of this organelle can be readily visualized by time-lapse fluorescent microscopy using ClpV fusion proteins. This methodology has documented cell-cell interactions referred to as "T6SS dueling" that correspond to the location of protein translocation events between cells that signal assembly of new T6SS organelles. In order to characterize the contact-dependent signal that triggers T6SS dueling behavior, we have explored the ability of different bacterial species to prey upon T6SS-positive cells and T6SS-negative cells. Our results show that in the case of *Pseudomonas aeruginosa*, T6SS-dependent killing of *Vibrio cholerae* is greatly stimulated by T6SS activity occurring in those prey species. These results suggest that T6SS dueling activity occurs between heterologous species and is used to direct killing activity toward T6SS+ prey cells at the exact point of that prey cell's initial T6SS attack on the predator. In contrast, *V. cholerae* T6SS activity is repeatedly randomly distributed around the predator cell envelope. While *V. cholerae* can readily kill T6SS-negative *E. coli*, *P. aeruginosa* fail to kill this prey species efficiently suggesting that aiming of the "T6SS thrust" is essential for killing by this prey species. Imaging and quantification of prey viability further suggests that both T6SS effectors and the T6SS apparatus itself may be mediators of prey cell killing.

Symposium 4: Chromatin Dynamics

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Repressing and tethering chromosomes via molecular machines.

B. J. Meyer¹; ¹HHMI and U.C. Berkeley, Berkeley, CA

Genome stability requires that chromosomes be properly replicated, tethered, recombined, segregated, and expressed. These processes are controlled, in part, by related protein complexes, the condensins and cohesins. The talk will focus on mechanisms by which these complexes control chromosome-wide gene expression and chromosome tethering and segregation. Gene expression in multi-cellular organisms is controlled by regulatory mechanisms that function over dramatically different distances: across large chromosomal territories or along individual genes. X-chromosome dosage compensation is exemplary for dissecting gene regulation over vast distances and the role of chromosome structure in that regulation. Dosage compensation ensures that males (XO or XY) and females (XX) express equal levels of X-chromosome gene products, despite the difference in X-chromosome dose. In the worm *C. elegans*, the dosage compensation complex (DCC) is targeted to both X chromosomes of hermaphrodites to repress transcript levels across the entire X by half. The DCC resembles condensin, a protein complex required for the compaction, resolution, and segregation of mitotic and meiotic chromosomes. The DCC shares all but one subunit with two other condensin complexes in the worm, and DCC subunits also participate in other aspects of chromosome dynamics, including chromosome segregation and the regulation of crossovers between homologous chromosomes during meiosis. Emphasis will be given to the mechanisms by which the DCC recognizes and binds X chromosomes in somatic cells and the mode of DCC action, including its effects on chromosome structure and transcription. Protein complexes structurally related to condensin (the cohesin complexes) mediate the reduction of genome copy number during meiosis that is required for the production of haploid sperm and eggs. Yeast studies showed that the meiotic pattern of chromosome segregation requires that the "kleisin" subunit of mitotic cohesin be replaced by a meiosis-specific paralog. Unlike budding yeast, *C. elegans* has three meiosis-specific kleisin subunits and three distinct meiotic cohesin complexes. Work presented will show that kleisin identity dictates the biological role of the cohesin complex during meiosis, how cohesin is loaded onto chromosomes, how cohesin is triggered to become cohesive, and when, where, and how cohesin is removed from chromosomes to achieve the two successive rounds of chromosome segregation essential for the reduction in genome copy number. An important generality from our studies is that reshuffling of interchangeable molecular parts can create independent molecular machines with similar architectures but very different functions.

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How do cells establish and maintain sister chromatid cohesion?

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Sister chromatid cohesion is mediated by a multi-subunit complex called cohesin whose Smc1 and Smc3 form rod shaped proteins with ABC-like ATPase domains at one end and "hinge" dimerization domains at the other, through which they form V-shaped heterodimers that are converted to tripartite rings through association of Smc3 and Smc1's ATPase domains with the N- and C-terminal domains of cohesin's kleisin subunit respectively. Though the structures of Smc1/3 and Smc1/kleisin interfaces have been known for several years and are conserved among all Smc/kleisin complexes, that of the Smc3/kleisin interface has proved more elusive. Through a collaboration with Stephan Gruber (Max Planck, MPI), we show that N-terminal

kleisin domains bind to Smc3 ATPase heads in manner similar to that of equivalent bacterial proteins and through a mechanism that is completely different to that of the Smc1/kleisin interface. These structural insights have enabled us to cross-link chemically all three interfaces using pairs of cysteine insertions and thereby test whether cohesin rings entrap sister DNAs. Because these experiments have now confirmed the ring hypothesis, we need to understand how DNAs enter cohesin rings, what occurs to them during DNA replication, and how cohesin is prevented from releasing either DNA until separase cleaves rings open at the onset of anaphase. I will describe experiments suggesting that DNAs enter rings through Smc1/3 hinge interfaces but can subsequently exit through their Smc3/kleisin interfaces. DNA exit through opening the Smc3/kleisin interface is responsible for cohesin's turnover on chromosomes in both yeast and *Drosophila* and is blocked by acetylation of Smc3 ATPase heads by Eco1. I will also discuss the key role of cohesin's Pds5 subunit which binds to sequences near Smc3's N-terminal Smc3 ATPase binding domain and together with Wapl, which binds to Pds5, and Scc3, which binds to a central domain within kleisin, catalyzes transient dissociation of Kleisin's N-terminal domain from Smc3 ATPase heads. This releasing activity is responsible for dissociation of cohesin from chromosome arms during prophase in animal cells. Remarkably, by facilitating acetylation of Smc3's ATPase heads by Eco1 during S phase and by blocking access of the Hos1 de-acetylase during G2, Pds5 also functions to block the releasing activity that would otherwise destroy cohesion after it has been generated at replication forks.

POSTER PRESENTATIONS

SUNDAY, DECEMBER 16

Unconventional Myosins

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Single molecule analysis of Myosin-1a membrane binding dynamics in cells.

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At the enterocyte apical surface, densely packed microvilli form a barrier called the brush border. The monomeric motor protein Myosin-1a (Myo1a) forms lateral cross bridges in microvilli by linking the core actin bundles to the plasma membrane. As the most abundant motor in the brush border, Myo1a plays a key role in physiological functions at the membrane/cytoskeletal interface, including maintaining microvillar structure and membrane tension, and regulating microvillar vesicle shedding. Myo1a performs these functions while membrane bound, yet it is unknown how this motor transduces force onto a fluid lipid bilayer. Previous studies from our lab have shown that Myo1a tail homology 1 (TH1) domain binds electrostatically to liposomes containing phosphatidylserine (PS), and that Myo1a membrane binding potential is distributed throughout TH1 rather than localized to a single motif. Structural prediction studies using I-TASSER further suggest the membrane binding regions may fold into independent motifs. Therefore, distribution of lipid binding potential may result in a multi-site interaction between TH1 and the lipid bilayer. Multiple points of contact not only increase affinity, but might also slow or immobilize the diffusion of a membrane bound protein. In the case of Myo1a, this could be beneficial for imparting force onto the membrane. To test this prediction in cells, we employed single molecule total internal reflection fluorescence microscopy in combination with single particle tracking and mean squared displacement analysis. We quantitatively compared the lateral mobility of Myo1a, TH1 and Lactadherin-C2, a protein that binds to PS and diffuses with velocities similar to the lipid alone. Preliminary results indicate that Lactadherin-C2 diffuses the fastest, followed by TH1 and then Myo1a. In addition to diffusing more slowly, many Myo1a molecules also exhibit near immobility. These results suggest that the motor/actin interaction may make an unexpected contribution to controlling the dynamics of Myo1a at the membrane/actin interface. Future experiments will focus on confirming these results and will examine how TH1 structure and membrane composition affect Myo1a mobility while bound to membrane.

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Myosin1B shapes Organelles and Plasma Membrane and pulls Membrane Tubules out of Giant Unilamellar Liposomes.

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Microtubules and microtubule-associated motors (kinesins and dyneins) have a well-established role in controlling membrane shape of organelles. In contrast, the multiple roles played by the actin-associated motors (myosins) in the dynamics of intracellular membranes only begin to emerge. We have recently demonstrated that the motor activity of a myosin 1 (myosin 1b,

Myo1b) is required for the formation of tubular carriers at the Trans-Golgi Network (TGN) [1]. More recently using molecular biology, live cell imaging and biochemistry we observed that Myo1b motor activity controls filopodia formation at the plasma membrane upon EphB2 stimulation through the interaction with EphB2 receptor. Thus Myo1b may control membrane shape both in organelles and at plasma membrane. Myo1b is a vertebrate myosin-I isoform with a single-headed molecular motor. Its motor activity has been shown to display large force sensitivity [2], compatible with a control of the dynamic changes of membrane morphology by Myo1b. To further elucidate the mechanism by which Myo1b controls the membrane tubule formation, we develop an in vitro model membrane system composed of purified Myo1b bound to giant unilamellar vesicles (GUVs) containing phosphatidylinositol -4,5- biphosphate (PIP2), and actin-fascin bundles immobilized on a glass substrate. We demonstrate for the first time that Myo1b alone is able to pull membrane tubules out from a GUV along actin bundles, even though they are single-headed and non-processive. We successfully observe the dynamics of tubule elongation and estimate Myo1b density along membrane tubules. Additionally, Cryo-EM observations provide the first direct evidence at molecular resolution of the direct binding of Myo1b to PIP2 containing lipid bilayers. Based on our experimental observations and the known biophysical properties of the motor [2], we propose a physical model explaining myosin-induced membrane tubulation.

[1] C. G. Almeida, A. Yamada, D. Tenza, D. Louvard, G. Raposo & E. Coudrier (2011). Myosin 1b promotes the formation of post-Golgi carriers by regulating actin assembly and membrane remodelling at the trans-Golgi network. *Nat. Cell Biol.* 13, 779

[2] Laakso JM, Lewis JH, Shuman H, & Ostap EM (2008) Myosin I Can Act As a Molecular Force Sensor. *Science* 321(5885),133.

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Myosin 1b is required for amino acid transport in renal proximal tubule cells.

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In the kidneys amino acids in the blood are filtered in glomeruli and reabsorbed by amino acid transporters in the microvilli-rich brush border membrane of proximal tubule (PT) epithelial cells. Recent studies demonstrated that renal amino acid transport is critically dependent on the membrane protein collectrin, which is homologous to the membrane anchor region of angiotensin-converting enzyme 2. In mice collectrin knock out decreases expression of multiple amino acid transporters including the SLC proteins B⁰AT1, SIT1, XT2 (B⁰AT3) and XT3, a family of sodium-dependent neutral amino acid transporters, and causes severe aminoaciduria. Myo1b is an actin- and membrane-associated molecular motor protein found in membrane protrusions such as lamellipodia, filopodia and ruffles and associated with intracellular organelles. In this study, we show that myo1b is localized in brush border membrane of PTs in murine kidney. Myo1b colocalizes with collectrin and B⁰AT1 in the apical microvilli of cultured PT cells. Localization of myo1b at the apical membrane requires both motor activity and phosphoinositide binding. Knock down of myo1b decreases collectrin and B⁰AT1 expression and reduces leucine transport mediated by collectrin-dependent B⁰AT1 in PT cells, while phosphate transport does not change. These results suggest that myo1b is critical for the association of collectrin and/or B⁰AT1 to the brush border membrane in PT cells.

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Characterization of myosin 1e function in maintaining cell junction integrity in the kidney.

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Focal segmental glomerulosclerosis (FSGS) is a renal disorder that results in massive proteinuria and often progresses to end-stage kidney disease. Kidney visceral epithelial cells, also known as podocytes, are critical both to normal kidney function and the development of FSGS. Podocyte actin cytoskeleton and their highly specialized cell-cell junctions (also called slit diaphragm complexes) play key roles in controlling glomerular filtration.

Myosin 1e (myo1e) is an actin-based molecular motor that is expressed in renal glomeruli. Previous studies have identified two mutations in the MYO1E gene that are associated with childhood familial FSGS in humans (Mele et al., NEJM, 2011, and Sanna-Cherchi et al., Kidney Int., 2011). Our lab has also shown that disruption of the Myo1e gene in mice promotes podocyte injury and results in loss of the integrity of the glomerular filtration barrier (Chase et al., AJP Renal Physiol., 2012). Here we have used biochemical and microscopic approaches to characterize myo1e localization and protein-protein interactions in glomerular podocytes. Myo1e was consistently enriched in the slit diaphragm fraction during subcellular fractionation of renal glomeruli. Protein pulldown assays indicated that myo1e interacted with ZO-1, a tight junction protein and a component of the slit diaphragm, in vitro. Myo1e and ZO-1 were present in the areas of cell-cell contact in cultured kidney podocytes. Live cell imaging studies showed that myo1e was recruited to newly formed cell-cell junctions, where it colocalized with the actin filament bundles supporting nascent contacts. These findings suggest that myo1e represents a component of the slit diaphragm complex and may contribute to regulating junctional integrity in kidney podocytes.

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Functional role of specific regions of the tail domain of Myosin IIIA in actin bundle based motility.

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Myosin IIIA (MYO3A) is proposed to function as a transporter in parallel actin-bundle based structures such as the stereocilia of inner ear hair cells and calycal processes of photoreceptors. Interestingly, the tail region of MYO3A binds Espin 1, allowing for the transport of Espin 1 to the tips of actin protrusions. Since Espin 1 contains WH2 activity which is capable of enhancing filament elongation, the ability of MYO3A to transport and concentrate Espin 1 at the tips causes elongation of the actin protrusions. In the current study we focused on the regions of the tail domain of MYO3A that are important for its ability to move to the tips and generate actin protrusions in the absence of Espin 1. The domain structure of the MYO3A tail can be separated into 6 exons (30-35). Exon 35 contains the tail actin binding site which has been found to be critical for localization of MYO3A to the actin bundle protrusion tips. To determine if any other exon(s) (30-34) are necessary for allowing MYO3A to move to the tips of actin protrusions, we generated exon deletion constructs of GFP-tagged MYO3A and transfected them into COS7 cells. Removal of either exon 30 or 34 reduced but did not abolish MYO3A localization to filopodial tips. Interestingly, exon 30 contains a proposed calmodulin-binding IQ motif of unknown function. There are two other IQ motifs in MYO3A known to bind calmodulin and likely serve as the traditional lever arm in MYO3A. Therefore the IQ motif in

exon 30 and other regions of the tail may play a role in MYO3A based motility. Studies are ongoing examining density and length of filopodia to assess the role of specific MYO3A tail regions in generating and elongating actin protrusions.

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Protein Kinase A triggers Myosin Va-dependent melanosome transport.

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Pigment-containing melanosomes are reversibly distributed throughout the cytoplasm of melanophores in lower vertebrates. During dispersion, melanosomes are transported via kinesin-2 towards the cell periphery, where Myosin Va takes over to finalize the delivery across the actin-meshwork. During aggregation, cytoplasmic dynein moves melanosomes back towards the cell center, a process during which Myosin Va function is dispensable. However, the molecular mechanisms of regulated employment of Myosin Va during melanosome transport are still unknown. To identify factors that directly regulate and control Myosin Va function, we reconstituted melanosome transport on actin filaments *in vitro*. Consistent with the *in vivo* observations, melanosomes purified from the aggregated cell state are largely dormant, while melanosomes from the dispersed cell state exhibit directed movement on actin filaments. We rescued this 'inactivity' of Myosin Va observed in aggregated melanophores either by adding cell extract from dispersed cells or exogenous Protein Kinase A (PKA) to the isolation procedure. A PKA-specific inhibitor prevented this rescue. Conversely, the addition of cell extract from aggregated cells significantly reduced the activity of melanosomes isolated from the dispersed state. These results point towards a regulation mechanism via activation and inactivation of Myosin Va with PKA acting as the master switch. Consistent with this notion, the protein content of the melanosome-associated beta-isoform of PKA (catalytic subunit) hardly differed between the aggregated and dispersed state. Thus, PKA is ideally suited to reversibly regulate the Myosin Va activity during dispersion and aggregation.

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Class V Myosins - The Promiscuous Actin Motors and their Rab Partners.

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Class V myosins are highly processive actin motors. Mammals have three isoforms that have been implicated in the transport of a diverse range of cargoes including mRNA, viruses, organelles, and cell surface receptors. How they are recruited to specific cargo is unclear, however it is emerging that Rab GTPases are likely to be involved. To date each myosin V isoform has been found to interact with Rab8, Rab10, and Rab11. However, to our knowledge, no class V myosin has been tested in a systematic screen of the entire Rab GTPase family. To this end we used a high throughput yeast two-hybrid "living chip" assay to test myosin Va for interactions with all human Rab GTPases. In addition to the previously identified partners, we observed interactions with several other Rabs that function in overlapping endocytic and secretory transport pathways. These interactions have been confirmed biochemically and we demonstrate that myosin Va has three distinct Rab binding domains. Using site-directed mutagenesis we generated mutants that abolish individual Rab interactions and we show that, depending on the splice-isoform, Rab10 or Rab11 recruits calcium-activated myosin Va to membranes. A pool of myosin Va associates with each Rab-positive compartment. We provide evidence that in non-specialised cells myosin Va is unlikely to play a role in the trafficking of bulk cargoes, but rather acts as a dynamic tether. Our results indicate that myosin Va is a major

regulator of the topology of membrane compartments belonging to the endocytic recycling and post-Golgi secretory systems.

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A mitotic checkpoint protein regulates the dissociation of myosin V from its cargo.

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Myosin V motors, present in virtually all eukaryotic organisms, are critical for cellular differentiation, organization and function. Myosin V based transport initiates with the regulated attachment of myosin V to its cargo via cargo specific adaptors. After transport, the regulated detachment from myosin V deposits cargoes at their destinations. Insight into the regulated detachment of cargoes was revealed in studies of *Xenopus laevis* melanophores where in coordination with the cell cycle, phosphorylation of myosin V triggers the detachment of melanosomes¹.

In *Saccharomyces cerevisiae*, in coordination with the cell cycle, the myosin V motor, Myo2, transports the vacuole. Transport terminates after the vacuole reaches the bud. Vac17, the vacuole specific adaptor for Myo2, contains a PEST sequence that is required for the degradation of Vac17 and the termination of vacuole transport². We sought to determine the mechanisms that regulate the detachment of the vacuole from Myo2.

In random mutagenesis and alanine screens, we identified specific Vac17 residues critical for the detachment of the vacuole from Myo2. Notably, all residues identified were within the PEST sequence. Using phospho-specific antibodies, we show that one of these residues, Thr240, is phosphorylated *in vivo*. In an EMS mutagenesis screen we identified the spindle position checkpoint/E3 ubiquitin ligase Dma1 as a novel regulator of the detachment of the vacuole from Myo2. Interestingly, Dma1 binds directly to Vac17-phospho-Thr240 but not to *vac17-T240A*. Moreover, Dma1 colocalizes with Vac17 at the bud tip, the site where vacuole transport terminates. We generated an enzymatically inactive mutant, *dma1-I329R*, and found that Dma1 dependent ubiquitylation is required for the detachment of the vacuole from Myo2 and for the subsequent degradation of Vac17 via the proteasome.

Regulated Vac17 phosphorylation likely ensures that Dma1 recruitment and thus the termination of vacuole transport does not occur until the vacuole reaches its destination. As a checkpoint protein, Dma1 regulates mitotic exit and cytokinesis; thus, it is tempting to speculate that Dma1 terminates vacuole movement in coordination with other events that occur late in the cell cycle.

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2. Tang, F., et al., Regulated degradation of a class V myosin receptor directs movement of the yeast vacuole. *Nature*, 2003. 422(6927): p. 87-92.

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Autophagy-receptors link myosin VI to autophagosomes to mediate Tom1-dependent autophagosome maturation and fusion with the lysosome.

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Autophagy is both a crucial regulator of cell homeostasis during times of starvation and a specialised pathway for the targeted degradation of protein aggregates, damaged organelles, and pathogens. During selective autophagy, ubiquitinated cargo is recognized by autophagy receptors. These receptors contain both ubiquitin recognition motifs and interaction domains for the autophagy-related protein, LC3, thus linking autophagic membranes to ubiquitinated cargo in order to form autophagosomes. Following biogenesis, maturation leaves autophagosomes competent for fusion with the lysosome, thus facilitating subsequent cargo degradation and recycling. We have found that the unique minus-end directed motor protein, myosin VI, is required for autophagosome maturation and fusion with the lysosome. The disparate cellular functions of myosin VI are mediated by interactions with distinct binding partners that recruit myosin VI to different intracellular compartments. The ESCRT-0 protein Tom1 is a novel myosin VI adaptor protein in mammalian cells that is involved in targeting of myosin VI to early endosomes. The loss of either myosin VI or Tom1 reduces delivery of endocytic cargo to autophagosomes, thereby preventing autophagosome maturation and autophagosome-lysosome fusion. Indeed, without myosin VI, ubiquitinated protein aggregates – such as mutant huntingtin protein containing a pathological polyQ expansion – are not properly degraded and instead accumulate. Myosin VI targets to autophagosomes via the RRL motif in its cargo-binding tail domain, which is known to mediate direct interaction with the autophagy receptors T6BP, NDP52, and optineurin. Therefore, we postulate that myosin VI and Tom1 are required for endosome delivery to autophagosomes via docking to optineurin, NDP52 or T6BP. Thus our data provides insights into the mechanisms and proteins required for autophagosome maturation and their fusion with the lysosome. These studies contribute to a further understanding of the unique functions of the minus-end directed myosin VI.

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Myosin 7a Binding Protein.

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Drosophila myosin 7a (Dm7a) is an unconventional myosin that is essential for the maintenance of bristle structure in Johnston's organ (the auditory center in *Drosophila*) as well as the morphology of the fly's thorax bristles. Dm7a has previously been shown to auto-regulate its ATPase activity via a MyTH7 domain, present at the C-terminus of the myosin tail. A yeast-2-hybrid screen, using the MyTH7 as bait, yielded positive colonies for a protein we call M7BP (myosin 7a binding protein). The N-terminus of M7BP (residues 1~160) is a potential Rab-binding domain, and the C-terminus (residues 550~938), termed the myosin-binding domain (MBD), interacts with Dm7a via the MyTH7 at the C-terminus. M7BP activated the ATPase of Dm7a at low actin concentration, and the approximate affinity between the two proteins was 0.8 μ M. While purified M7BP is monomeric (based on cross-linking, analytical-centrifugation, and EM studies), when mCherry-M7BP is transfected into S2 cells along with GFP-Dm7a, it appears to facilitate oligomerization of the myosin inferred from the co-localized migration of the two fluorescent proteins to the tips of filopodia. Filopodia were observed in approximately half of the

cells co-transfected with mCherry-M7BP and GFP-Dm7a, which is similar to the effect seen with transfections using artificially zippered GFP-HMM constructs of Dm7a. Conversely, single transfections of either GFP-Dm7a or mCherry-M7BP alone do not cause filopodial formation, and transfections using truncated GFP-Dm7a (i.e. missing the C-terminal MyTH7) or truncated mCherry-M7BP (i.e. only the MBD) yields a small population (>20%) of cells with filopodia. In the latter cases we believe the myosin is activated, but still unable to oligomerize. IP experiments from fly lysates show M7BP binds at least four different Rabs (4, 5, 8, 11), and therefore in vivo oligomerization of Dm7a likely involves endogenous Rabs in S2 cells that induce a tripartite protein formation. To investigate the role of Dm7a in the fly, we over-expressed GFP-Dm7a in the *Drosophila* larval hemocytes using the UAS/GAL4 system and show that it induces the formation of filopodia in the hemocytes. Dm7a is localized to the tips of the filopodia. The hemocytes of Dm7a mutant flies show defective phagocytosis of *E. Coli*. Additionally, we conducted a lethality assay by challenging the larvae with bacteria and show that 21% of wild-type larvae eclosed into adult flies and less than 10% of Dm7a mutant larvae eclosed. This suggests that the failure of Dm7a hemocytes to phagocytose invading pathogens compromised the defense mechanism of the flies.

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Characterization of *Drosophila* myosin 7a and M7BP in the eyes.

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Mutations in crinkled (*ck*), the *Drosophila* ortholog of myosin 7a (Dm7a) caused deafness in flies due to a disorganization of Johnston's organ, which is its auditory system and abnormal bristle structures on the thorax. Both of these structures are formed by bundled arrays of actin. We have identified a binding partner of Dm7a using the MyTH7 as a bait in a yeast two hybrid system and it is named Myosin 7a Binding Protein (M7BP). M7BP protein has a potential Rab-binding domain in the N-terminus and immunoprecipitation experiments using M7BP antibody pulled down some *Drosophila* Rab proteins (Rab 4, 5, 8 and 11). We show that Dm7a, M7BP and Rab11 are expressed in the larval eye disc in the lens-secreting cone cells. Electron microscopy sections of the adult eyes of Dm7a mutants showed missing pigment granules around the primary pigment cells and at the base of the rhabdomeres of the photoreceptor cells. Scanning electron microscopy displayed abnormal bristles in the adult eyes. We are presently characterizing a P-element insertion line of M7BP. These flies also showed anomalies in the bristles in the adult eyes. We are performing excision mutagenesis to isolate more M7BP mutants.

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Loss of Myosin VIIA alters epidermal actin protrusions in developing *Drosophila* embryos.

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Myosin VIIA (MyoVIIA) is an actin based motor protein conserved in mammals and flies, and mutations in both species cause hearing loss due to defects in stereocilia or the Johnston's organ, respectively. In *Drosophila melanogaster* severe mutations in MyoVIIA are lethal or "semi-lethal" with only a very small percentage of flies surviving to adulthood. Those that survive show defects related to actin bundling in hair and bristle morphology, are deaf, and do not survive for long. In this study, we examined the function of crinkled (*ck*), the fly homolog of MyoVIIA, in embryogenesis. All embryos lacking maternal and zygotic *ck/MyoVIIA* display defects in denticle formation, the hair like growths on the ventral epidermal cells. The mutant denticle rows are disorganized with denticles displaying a variety of phenotypes including loss of

polarity, a smaller than normal size, and a branched morphology, apparently due to a failure of actin filament bundling. We used *ck/MyoVIIA* C-terminal tail truncation mutants to investigate which of its domain(s) contribute to denticle formation (Singh 2012 Duke University Ph.D Diss.) and the data suggest that the Myth4FERM domains are important for proper denticle morphology. In addition, we investigated genetic interactions of MyoVIIA with the family of ZPD proteins, another family of proteins known to affect denticle morphology. This study suggests *ck/MyoVIIA* is necessary for actin appendage formation in developing embryos, but the detailed molecular mechanisms which produce this biological effect are not yet clear (Supported by GM33830 and DC007673 to DPK and GM093592 to JLS).

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Mechanistic study of myosin X for filopodial formation.

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Myosin X, as an unconventional myosin, is best known for its filopodial tip localization and filopodia induction activity. Myosin X is involved in a series of physiology process, including cell migration, cell adhesion, phagocytosis and even essential for developmental procedures with neuronal axon guidance and blood vessel formation. A lot of studies were done but it is still vague that how myosin X promote filopodia formation through several potential mechanisms, including regulating motor activity, inducing dimerization, transporting filopodial-formation related proteins or activated by extracellular signaling. Here through structural and biochemical study, at the cellular level, we provide crucial evidence to reveal the detailed mechanism about how different domains of myosin X contribute to its filopodia tip localization and induction activity. We show that coiled coil region of myosin X folds into a novel antiparallel structure and essential for its walking along filopodia. And myosin X split PH1-PH2 tandem at the tail region function as a specific and acute cellular PI(3,4,5)P3 sensor. The C-terminal MyTH4-FERM tandem can form complex with the DCC P3 domain and is required for filopodia induction. Here we proposed a model that, after activation by the guidance cues, myosin X is recruited to membrane and restricted to the filopodia tip by its different tail regions interacting distinctly with membrane receptor and specific lipid components.

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Evolution of MyTH/FERM Myosins.

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Myosins are a highly diverse family of actin-based motors present in almost every eukaryote. A subgroup of these myosins is characterized by the presence of one or two adjacent MyTH (myosin tail homology 4)/FERM (band 4.1, ezrin, radixin, moesin) domains in their C-terminal tail regions. A M7-like MF myosin is also present in Amoebozoa but it was initially designated as an orphan and then subsequently as M22 (Kollmar & Odronitz, 2007; CyMoBase.org). The Metazoan class 7, 10 and 15 MF myosins have roles in regulating the growth of parallel actin bundles such as filopodia (M10) and stereocilia (M7, M15), as does the *Dictyostelium* M7-like MF myosin that is essential for filopod extension. These common functions of amoeboid and metazoan MF myosins as well as the availability of additional amoeboid MF myosin sequences prompted a comprehensive phylogenetic analysis of the MF myosins to reinvestigate the classification of amoeboid MF myosins and gain better insight into the evolutionary relationship between known MF myosins.

A complete collection of MF myosin sequences from a set of diverse organisms was obtained from CyMoBase.org (Odrionitz & Kollmar, 2007), aligned using MUSCLE and/or PROMALS 3D. The motor and C-terminal MF domain sequences were extracted, re-aligned and then separate phylogenetic trees generated for both domains using a variety of programs including ClustalX, FastTree and MrBayes. The initial analysis suggests that the last common ancestor of Amoebozoa and Metazoa had an M7-like MF myosin with a tail domain containing two MF domains separated by an SH3 domain. The family of M7-related proteins then underwent a dramatic expansion in the lineage leading to animals, with early gene duplications generating an M22/M10 branch as well as an M15/12/35 branch. The existence of an M22-like protein in some basal fungi, e.g. *Gonapodya*, suggests that the earliest of the gene duplications resulted in the M22 subfamily. This analysis reveals that the MF-containing myosins could be considered collectively as an M7 supergroup, perhaps analogous to M1 class that contains several divergent subgroups.

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Characterization of full-length mammalian myosin-18A.

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Class 18 myosins share a close phylogenetic relationship with the well-characterized class 2 myosins. They have been demonstrated to play roles in cytoskeletal maintenance, control of Golgi morphology and stromal cell differentiation. We purified recombinant full-length, HMM, and S1-like mammalian myosin-18A using baculoviral/Sf9 expression. Expressed myosin-18A heavy chain was shown to bind co-expressed Essential light chain (ELC) and Regulatory light chain (RLC), similar to the behavior of conventional class 2 myosins. EM and analytical ultracentrifugation of full-length myosin-18A reveal a two-headed structure at high salt, reminiscent of myosin-2, and a four-headed antiparallel structure at physiological salt concentrations. Despite the presence of a long coiled-coil and in contrast to the class 2 myosins, myosin-18A did not form filaments, even at very low ionic strength or when the RLC was phosphorylated. Binding of myosin-18A to actin was shown to be weak in both the absence and presence of ATP. Myosin-18A bound ATP and ADP weakly and did not show any activation of its very low basal ATPase rate by actin even when the RLC was phosphorylated. Homology modelling of its structure is consistent with this myosin lacking conserved amino acids known to participate in the catalysis of ATP hydrolysis. Myosin-18A was unable to translocate actin filaments in actin gliding assays and no powerstroke was observed in single molecule optical trapping experiments. An interaction with nonmuscle myosin-2 was demonstrated in vitro suggesting a possible means by which a myosin with little or no inherent motor activity may play a role in modulating cytoskeletal morphology.

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Emergent behavior in nano-patterned groups of myosin motors.

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Unconventional myosin function in a cellular process emerges from the interaction of multiple myosins on an organelle scaffold with several spatially patterned actin filaments. Currently, the role of myosins in a cellular process is inferred from the effects of disruption of myosin function using chemical or genetic approaches. In contrast, single molecule studies over the last decade have focused almost exclusively on understanding single actin-myosin interactions. To bridge this gap between single molecule and cellular function, modular well-defined assays are necessary, wherein the number, type and spatial distribution of actin-myosin interactions can be engineered. We have developed an assay platform that combines the polarized actin network derived from a fish epidermal keratocyte with DNA nano-structures engineered with precise number and spatial configuration of myosins. Here, we report on the emergent behavior of nano-structures patterned with myosin V and VI. Nano-structures with myosin V move towards the cell periphery whereas those with myosin VI moves towards the cell center. Run length increases with motor number to spans the width of the keratocytes ($n \geq 4$). Movement speed for nano-structures with multiple myosins of the same type does not depend on motor number. Surprisingly, while most myosin VI trajectories (~ 90%) are linear, a substantial fraction (~ 35%) of myosin V trajectories are highly skewed with fraction of skewed trajectories increasing with motor number. Therefore, myosin VI appears well suited to bring about linear-directed endocytic transport, whereas myosin V is tuned for dispersion of exocytic vesicles. For nano-structures with equal number of myosin V and VI, both directions of movement are equally favored (53% myosin V, 47% myosin VI). The run length of the nano-structures increases with motor number, regardless of motor type. Our results differ from findings for single filaments (79% myosin V), emphasizing the importance of actin architecture in myosin behavior. For nano-structures with both myosin V and VI, majority (> 95%) of trajectories are unidirectional, unlike bi-directional movement driven by a combination of kinesin and dynein. In an assembly with asymmetric motor composition, the net flux of trajectories is highly sensitive to the relative number of myosin V and myosin VI. This sensitivity represents a potential avenue for regulating net flux of vesicle movement *in vivo*. To understand the molecular basis for emergent behavior, we performed sensitivity analyses in stochastic simulations of nano-structure movement. Current efforts are focused on manipulating emergent behavior in our assays, based on key parameters identified from simulations, in order to extend our insights into myosin function *in vivo*.

Kinesins

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A spatial sensor of kinesin activity reveals conformational changes in KIF17 induced by EB1.

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Kinesin family motors (KIFs) are versatile microtubule stimulated ATPases that perform functions contributing to establishment and maintenance of cell architecture, cell motility and cell division. To act appropriately during these processes, kinesin activity must be regulated in space and time. Little is known about how regulation is achieved but members of kinesin 1, 2, 3

and 7 families are known to adopt compact and extended conformations that reflect autoinhibited and active states, respectively. We designed intramolecular conformation sensors encoding kinesins tagged with fluorescent proteins at their N- and C-termini. We used Förster resonance energy transfer and fluorescence lifetime imaging microscopy to directly monitor biosensors in compact and extended conformations in single cells with minimal experimental manipulation. Lifetime data was mapped on a simple graphical polar plot (the “phasor” plot) allowing us to resolve the positions and relative populations of active and inactive motors in fixed or live cells. In addition to enhanced spatial resolution garnered by this method, FRET-FLIM is also advantageous in that large data sets can be analyzed rapidly, accurately and reproducibly. We validate this approach in MDCK epithelial cells expressing biosensors of KIF17, a homodimeric kinesin-2 family member involved in MT stabilization in epithelial cells. Expression of the KIF17 biosensors resulted in MT stabilization, demonstrating they function as expected. At steady state ~30% of KIF17 is in an extended, active conformation in cells. The majority of active KIF17 localizes near the cell cortex and resides on nocodazole-resistant, stable MTs. Co-expression of EB1, which interacts with the KIF17 motor domain, induces a MT-dependent increase of KIF17 in the extended, active conformation in cells. Unexpectedly, we found EB1 was not necessary for MT stabilization by KIF17. Together, our data suggest a model in which EB1 helps maintain KIF17 in an active conformation at MT plus-ends, possibly to direct local MT stabilization.

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A role for kinesin-1 in phagosome transport in the RPE.

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Retinal pigment epithelial (RPE) cells are professional phagocytes. Each day they phagocytose the tips of the photoreceptor outer segments, as part of the turnover of these phototransductive disk membranes. Phagosomes are then transported towards the basal region of the RPE cells, where they are degraded, following fusion with lysosomes. We have been studying the roles of molecular motors in delivering RPE phagosomes to lysosomes. Here, we report our findings on the role of conventional kinesin, kinesin-1, and its coordination with the unconventional myosin, myosin-7a. First, we observed that myosin-7a and kinesin-1 associated with phagosomes, with preferences towards less mature and more mature phagosomes, respectively. These associations were observed in fixed cells and by live-cell tracking of phagosomes in cells expressing fluorescently-tagged motors. Second, using RPE cells from mutant mice, we found that normal phagosome motility and digestion is impaired in cells lacking kinesin-1 function. Our results support a relay model whereby kinesin-1 takes delivery of phagosomes from myosin-7a, in the apical RPE, and then transports them in a basal direction towards the primary lysosomes.

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Motility by the chromokinesin NOD contributes to production of the polar ejection force.

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Polar ejection forces (PEFs), which push chromosomes away from spindle poles, are primarily generated by kinesin-10 family chromokinesins. Vertebrate kinesin-10 motors produce PEFs by walking chromosomes towards the spindle equator via plus-end directed motility. Although PEFs are also present in *D. melanogaster*, it is uncertain how the *Drosophila* kinesin-10 motor NOD produces force. NOD is classified as a non-motile kinesin and is believed to generate

PEFs by end-tracking on polymerizing microtubules. Here we demonstrate that two types of chromosome-microtubule interactions contribute to PEF production by NOD. Elevated PEFs stretched NOD-coated mitotic chromatin through both lateral and end-on interactions with microtubules. NOD mutants engineered to have either plus-end directed motility or tip-tracking activity generated PEFs albeit with less efficiency than the wild-type protein, suggesting that NOD could possess both activities. Remarkably, NOD exhibited motility in microtubule gliding assays. Our findings support the hypothesis that NOD end-tracks and demonstrate, for the first time, that NOD is a motile kinesin.

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Molecular Motor Kid Plays a Role in Focal Adhesion Localization and Protein Synthesis.

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During mitosis in eukaryotes, the microtubule-based spindle assembles with the aid of the kinesin superfamily proteins (also known as KIFs). A typical KIF contains a motor domain, which binds to the microtubule, and a stalk and tail domain, which uses energy from ATP hydrolysis to drive conformational changes that generate the motile force used for cargo transport.

The two motor proteins we focus on in our lab are Eg5/KIF11/Kinesin-5 and Kid/KIF22/Kinesin-10. They both interact with microtubules and play critical roles in spindle assembly and chromosome alignment during mitosis. Eg5 is a plus-end-directed motor which helps slide anti-parallel microtubules apart. Kid is a plus-end-directed chromokinesin (chromosome arm-localization kinesin) which binds to both the chromosome and the microtubule. It contributes to the polar ejection force that pushes chromosomes away from the spindle pole. Previous studies focused on the roles of Eg5 and Kid in mitosis. However, our lab recently identified an unexpected role Eg5 plays during interphase. Loss of Eg5 led to a decrease in new protein synthesis and an increase in free ribosomes, suggesting that Eg5 may have a function facilitating protein synthesis.

On the other hand, our preliminary data for Kid suggested a potential role for Kid as an inhibitor for protein synthesis. Like Eg5, Kid was also shown to associate with ribosomes, but loss of Kid led to a general increase in protein translation. More specifically, Kid might be involved in regulating localized translation at sites of focal adhesions. First, Kid colocalized with focal adhesions by immunofluorescence. Secondly, knockdown of Kid resulted in a change in the number and distribution of focal adhesions. After knockdown of Kid in HeLa or NIH-3T3 cells, the number of focal adhesions increased and the sites of focal adhesions became more scattered throughout the cytoplasm. Finally, such changes in the distribution of focal adhesions appear to have an effect on cell adhesion as cells became less adherent. Altogether, these results suggest that Kid might play a novel role in translational regulation at focal adhesions.

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Eg5 functions as a brake during anaphase B pole-pole separation in mammalian cells.

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Our previous work showed that mitotic spindles in LLC-Pk1 epithelial cells frequently move along the long axis of the cell resulting in displacement of the metaphase spindle away from the geometrical center of the cell. Differential pole movement in anaphase B corrects spindle mispositioning, resulting in approximately equal sized daughter cells. The minus end-directed motor, dynein and its activator, dynactin, are localized to the cell cortex during anaphase and

are required for pole-pole separation during anaphase B. Here, we examine the role of the plus-end directed kinesin-5, Eg5, in spindle pole separation. Eg5, which is required for centrosome separation during spindle formation, is localized to the spindle throughout mitosis, but its role in spindle pole separation in mammalian cells remains unclear. To address this, we first verified that inter-polar microtubules overlap in the spindle midzone, using cells expressing EB1-GFP to track dynamic plus-ends. We next characterized the localization of Eg5 during anaphase in cells expressing localization and affinity purification (LAP)-tagged Eg5 from a bacterial artificial chromosome under the control of the endogenous promoter. LAP-Eg5 localized to midzone microtubules throughout anaphase, and was lost from the midzone upon treatment with the Eg5 inhibitor, S-Trityl-L-cysteine (STLC). To determine the contribution of Eg5 to anaphase B, we tracked pole movements in cells expressing GFP- γ -tubulin using multidimensional motion analysis. We show that spindle pole separation in anaphase B is characterized by an initial rapid separation followed by a secondary slow phase of separation. Addition of STLC prior to anaphase onset resulted in a faster initial pole-pole separation (from 0.03 $\mu\text{m}/\text{min}$ to 0.06 $\mu\text{m}/\text{min}$). This is consistent with Eg5 having a role as a “brake” during spindle pole separation during anaphase in mammalian cells.

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Heterotrimeric kinesin 2-dependent transport localizes odor receptors to the ciliary outer segment in adult *Drosophila*.

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Sensilla on the third antennal segment of *Drosophila* are innervated with three different types of odor sensing neurons (OSNs), expressing three morphologically distinct cilia. Each of them displays a specific set of olfactory receptors (ORs). How these ORs reach the ciliary outer segment is unclear. We have earlier shown that the *Drosophila* olfactory cilia are formed during the pupal stages and this process requires the heterotrimeric kinesin-2, comprising the motor subunits, KLP64D and KLP68D, and a non-motor accessory protein, DmKAP. Here, we present the results of an extensive genetic and developmental analysis of OR localization to the ciliary outer segments in pupae and adults. We found that the ciliary entry of a recombinant odor coreceptor, OR83::GFP, is developmentally restricted. In addition, increasing amounts of the OR83b::GFP and OR47b::GFP accumulates in the cilia with aging. A set of viable Klp64D mutants, which are found to affect the kinesin-2 entry into the cilia, selectively in adult stages, reduce the OR::GFP localizations in the adult cilia. The olfactory cilia, however, grow to the standard sizes and both the membrane and tubulin contents of the cilia remain unaltered in these mutants. Together, these observations indicate that a separate olfactory receptor transport by the heterotrimeric kinesin-2 maintains the sensory function in the adult, and provide a paradigm for future exploration of the mechanism of sensory maturation and plasticity.

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StARD9 is a Novel Transmembrane Kinesin Implicated in the Dynamics of Lysosomal Membranes Containing NPC1 and Cholesterol.

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Niemann Pick-Type C (NPC) is a rare neurodegenerative disease induced by mutations in either the NPC1 or NPC2 genes. 95% of NPC patients suffer from mutations in NPC1, a multi-pass transmembrane protein shown recently to bind cholesterol (Infante et al., 2010). While defects in cholesterol transport are recognized in NPC disease, the contributions of NPC1

cholesterol binding is not understood completely. Live-cell imaging of membranes containing NPC1 reveals that, in contrast to wild-type NPC1, mutant NPC1 membranes fail to project dynamic tubular NPC1 membranes. This suggests that membrane tubulation is a critical aspect of cholesterol transport and proteins responsible for tubulation are targets for therapeutic intervention. Mass spectrometry analysis of wild-type NPC1 and mutant NPC1 membranes isolated from the NPC24 human patient cell line was used to identify protein composition differences. The novel protein StARD9 was present in wild type membranes but absent in NPC1 mutant membranes. Sequence analysis of StARD9 reveals that the 4700 amino acid protein contains a N-terminal kinesin-like motor domain, a C-terminal START lipid binding domain, and transmembrane segments. These features suggest STARD9 is a membrane protein with cytoplasmic microtubule motor activity. Upon shRNA depletion of StARD9 activity, a five-fold decrease in membrane tubulation from NPC1 lysosomal membranes relative to untransfected cells was observed. Additionally, accumulations of cholesterol that mimic NPC disease are observed when StARD9 is depleted. These preliminary studies suggest that STARD9 is an essential membrane-associated motor protein required for tubulation of lysosomal membranes, allowing for stimulation of cholesterol efflux. To analyze full and partial length StARD9 constructs, we completed cDNA cloning of the full 4700 amino acid protein. The N-terminal kinesin motor domain, but not a p-loop motor domain mutant, labeled microtubules consistent with other kinesins. Intermediate constructs encoding the motor domain and initial transmembrane segments displayed punctate microtubule labeling, which became more robust as the C-terminus was further extended. The full length construct displayed labeling of both membranous organelles and microtubules. Membranes containing full length StARD9 and stained with LysoTracker confirmed an accumulation in lysosomes. These findings suggest StARD9 is a lysosomal transmembrane protein containing an N-terminal kinesin motor domain. We propose that StARD9 is a membrane protein responsible for driving dynamics of membranes containing NPC1.

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Modulation of ERRA Nuclear Localization and Function by KIF17.

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Kinesins are microtubule stimulated ATPases that transport a variety of cellular cargoes. Among the kinesins, KIF17 belongs to the kinesin 2 family and is a plus-end directed motor that participates in MT stabilization and polarization of epithelial cells. In a yeast-2-hybrid screen for additional factors that might contribute to its role in MT stabilization, we identified estrogen related receptor alpha (ERRA) as a novel KIF17 interacting protein. ERRA is a ligand-independent, orphan nuclear receptor that modulates cellular responses to estrogen by competing with estrogen receptor for binding to regulatory cofactors and estrogen response elements (ERRE) on target DNA. The testis-specific KIF17 isoform was shown previously to play a role in nucleo-cytoplasmic shuttling of ACT (activator of CREM mediated transcription). As such, we hypothesized that in epithelial cells, KIF17 could modulate ERRA activity by controlling its sub-cellular distribution and interaction with promoter elements on target genes. Therefore, after confirming KIF17 and ERRA interact in cells, we tested if KIF17 plays a role in regulating the localization and function of ERRA in breast epithelial cells. To do this, we injected cells with cDNAs encoding GFP-ERRA and monitored the localization of newly synthesized ERRA by time-lapse microscopy. In control cells, GFP-ERRA accumulated in nuclei by 4h after cDNA injection. Interestingly, in cells co-expressing the KIF17-tail domain, translocation of ERRA from the cytoplasm to the nucleus was attenuated 40%. We then tested whether

inhibition of ERRA nuclear localization by the KIF17 mutant also affected ERRA activity in cells. In luciferase reporter assays, expression of mutant KIF17 reduced binding of ERRA to ERRE suggesting inhibition of ERRA nuclear transport by KIF17 can alter transcriptional activity of this orphan receptor in breast epithelial cells. Collectively, these data show that KIF17 interacts with ERRA and can modulate ERRA localization and activity in epithelial cells. These results also suggest that targeted inhibition of ERRA localization could serve as a novel strategy for breast cancer treatment.

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Molecular motor KIF5A is essential for GABA_A receptor transport to the neuronal surface and is involved in inhibitory neural transmission.

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KIF5 (also known as kinesin-1) family members, consisting of KIF5A, 5B and 5C, are microtubule-dependent molecular motors important for neuronal function. Among the KIF5s, KIF5A is neuron-specific and highly expressed in the central nervous system. However, specific roles of KIF5A remain unknown. Here, we established conditional KIF5A-knockout mice, in which KIF5A protein expression was postnatally suppressed in neurons. Epileptic phenotypes were observed with electroencephalogram (EEG) abnormalities in knockout mice due to impaired GABA_A receptor (GABA_AR)-mediated synaptic transmission. We further identified reduced cell surface expression of GABA_AR in knockout neurons. Importantly, we identified that KIF5A specifically interacts with GABA_AR-associated protein (GABARAP) that is known to be involved in GABA_AR trafficking. It was shown that KIF5A regulated neuronal surface expression of GABA_ARs via interaction with GABARAP. These results provide a new insight into the molecular mechanisms of KIF5A, which regulate inhibitory neural transmission.

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Kinetochore kinesin CENP-E is a processive tracker of dynamic microtubule tips.

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Member of kinesin-7 family CENP-E is a kinetochore-associated plus-end-directed motor, which is important for faithful chromosome segregation in mitosis. CENP-E has been implicated in chromosome transport to the spindle midzone, where the microtubule (MT) plus ends are located. Here we report that once reaching the dynamic MT ends in vitro, CENP-E converts from a lateral transporter into a MT tip-tracker, stably associating with the tips of both assembling and disassembling MTs. We show that the binding between kinetochores and dynamic MT ends is destabilized in live cells when CENP-E function is perturbed via an inhibition or RNAi depletion, implying that the tip-associated CENP-E plays an important mitotic role. To determine the molecular mechanism of CENP-E tip-tracking, we characterized two purified recombinant fragments: the motor & neck domain and the C-terminal tail, harboring a MT binding site. The motor & neck domain walked on the MT wall in essentially the same manner as the full length CENP-E, while the C-terminal tail exhibited rapid diffusion along the

MT. Neither of these mutant proteins showed tip-tracking, however, this activity could be recapitulated by artificially joining them with Qdots. We analyzed these results with a coarse grained molecular model of CENP-E motility. This model successfully describes the observed tip-tracking of CENP-E, which occurs via the repeated cycles of the plus-end-directed walking, tail-mediated diffusion of the MT wall-tethered motor heads and their speedy re-association with the MT wall in the vicinity of the MT tip. This novel “tethered motor” mechanism of tip-tracking does not rely on the specific properties of the assembling or disassembling MT tips, explaining why this kinesin can tip-track bi-directionally. Together, these results establish the requirement for CENP-E in stably linking the kinetochores to dynamic MT tips, and provide a detailed molecular explanation about how CENP-E can achieve this function.

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illuminating the intraflagellar transport machinery in the chemosensory cilia of *Caenorhabditis elegans*.

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In the chemosensory cilia of *Caenorhabditis elegans* two kinesin-2-family motors, heterotrimeric kinesin-II and homodimeric OSM-3-kinesin, act together in a process called intraflagellar transport (IFT). Along the middle segment these two kinesins cooperate, however OSM-3-kinesin alone maintains the distal segment. How the interplay between these motors in IFT is regulated is unknown. Using ultrasensitive, quantitative fluorescence microscopy we are able to track both motors at endogenous expression levels deep inside the living organism. We find that initially the two motors form an IFT-train together, but while moving along the middle segment kinesin-II gradually undocks, allowing the OSM-3-kinesin train to reach terminal velocity already at the middle segment. Our results provide new insight into IFT and motor-driven processes in general.

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Polarized motor activity moves myonuclei in vivo.

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Proper positioning of the nucleus is essential for cellular and tissue functions ranging from the proper segregation of DNA, to the polarization of the cytoskeleton, to the contraction of muscle. Microtubule motors provide the force to move nuclei in many systems, yet how Dynein and Kinesin are coordinated to move nuclei is poorly understood. To address these questions we examined nuclear movement in the muscles of live, developing *Drosophila* embryos. These experiments revealed that translocating nuclei are polarized with Kinesin acting at the leading edge of the nucleus and Dynein acting at the trailing edge of the nucleus. These conclusions are supported by multiple lines of evidence that suggest at least two functions for polarized Kinesin and Dynein activity. First, the movement of nuclei is unidirectional toward the muscle pole with fewer than 2% of nuclei changing directions. However, when nuclei do change direction, the leading edge of the nucleus becomes reoriented in the new direction of movement. Moreover, translocating nuclei undergo dynamic shape changes alternating between elongated and spherical shapes. These shape changes are the result of the leading edge of the nucleus being moved forward before the trailing edge of the nucleus is moved. In Kinesin and Dynein mutant embryos, nuclear movement is slow compared to controls and the changes in nuclear shape are absent. The nuclei in Kinesin mutants retain a spherical shape

with an aspect ratio of approximately one, whereas the nuclei in Dynein mutants are in a persistently elongated shape with an aspect ratio of approximately three. That the nuclei cannot elongate in Kinesin mutants suggests that Kinesin is required to extend the leading edge of the nucleus. Similarly, that the nuclei are persistently elongated in Dynein mutants, indicates that Dynein is required to contract or release the trailing edge of the nucleus to complete a step in translocation. Together these data indicate that the activities of the microtubule motors Kinesin and Dynein are polarized on the nucleus and that the benefits of this polarization are two-fold: Polarized activity provides a means to enhance the unidirectional aspect of movement, and more importantly, enables the nuclei to dynamically change shape, thus maximizing movement efficiency in the dense environment of the embryo within the time constraints of development.

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Nuclear positioning during skeletal muscle fibers formation implicates microtubule network, molecular motors and MAPs and is essential for muscle function.

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The basic unit of skeletal muscle in all metazoans is the multinucleate myofiber, within which individual nuclei are regularly positioned. The molecular machinery responsible for myonuclear positioning is not known. Improperly positioned nuclei are a hallmark of numerous muscle diseases, including centronuclear myopathies, but it is unclear whether correct nuclear positioning is necessary for muscle function.

We performed an in vitro time-lapse video microscopy on myotubes to measure nuclei velocity and localization in early myotubes. Using a cytoskeleton targeting drugs approach and a siRNA screen on molecular motors, we identified two distinct microtubules dependent nuclei movements in myotubes. The first movement occurs just after fusion: entering nucleus of the myoblast moved towards the center of the myotube. This nuclear movement is driven by dynein/dynactin complex and regulated by Cdc42, Par6 and Par3. We also provide evidence for a role of Par6 and Par3 on myotube growth. The second movement occurs inside the myotube and is responsible for the alignment of nuclei. This nuclear movement is driven by the interaction between the kinesin Kif5b and the Microtubule Associated Protein Map7. This complex maintains an anti-parallel network of microtubules in myotubes and allows nuclei to correctly align within the myotube. We demonstrate that myonuclear positionings is physiologically important. *Drosophila* mutant larvae for the ortholog of Map7 display decreased locomotion and incorrect myonuclear positioning, and these phenotypes are rescued by muscle specific expression of Map7.

Our results show for the first time that the ability to correctly position myonuclei correlates with better muscle growth and function. Correcting nuclear positioning defects in patients with muscle diseases might benefit muscle strength and improve muscle function.

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Understanding Kinesin-Mediated Cell Wall Assembly Using Genetic and Single-Molecule Studies.

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The microtubule cytoskeleton at the cortex of plant cells guides the oriented deposition of cell wall material which determines cell shape and the overall growth and stature of plants. It is hypothesized that the Arabidopsis FRA1 kinesin, a member of the kinesin-4 family, plays a major role in this process by mediating the directional movement of cell wall-related cargo along cortical microtubules. We have characterized a T-DNA-induced knockout mutant, called

fra1(SALK), which has shorter, fatter and weaker stems as compared to wild-type controls. These phenotypes correlate with shorter pith cells and thinner walls in fiber cells. Cell wall composition analysis revealed that fra1(SALK) has 60% less glucose than wild-type controls, indicating that FRA1 mediates the synthesis and/or secretion of cellulose and/or hemicelluloses, which together make up the bulk of the plant cell wall. To study the mechanism of FRA1 function, we stably introduced a FRA1-3GFP fusion construct into our fra1(SALK) mutant. The FRA1-3GFP construct complements the fra1(SALK) mutant, indicating that the fusion protein is functional. We used highly inclined and laminated optical sheet (HILO) microscopy for single-molecule imaging of FRA1-3GFP in living Arabidopsis seedlings. FRA1-3GFP appears as discrete puncta at the cell cortex and photobleaching analysis revealed that these puncta primarily consist of FRA1 dimers. The FRA1-3GFP dimers were found to move long distances on cortical microtubules, making FRA1 well-suited for transporting cell wall-related cargo along cortical microtubule tracks. The velocity of FRA1 is about 60x faster than the velocity of the plasma-membrane-embedded cellulose synthase complexes, which synthesize and extrude cellulose microfibrils in the extracellular space. In addition, pharmacological experiments showed that FRA1 motility is unaffected by drugs that either deplete or immobilize cellulose synthase complexes at the plasma membrane. These data indicate that FRA1 is unlikely to direct the movement of plasma-membrane-embedded cellulose synthase complexes along cortical microtubules. Instead, FRA1 likely facilitates delivery of secretory vesicles containing cellulose synthase complexes and/or hemicelluloses to sites of exocytosis along cortical microtubules. We have screened for FRA1-interacting proteins using yeast 2-hybrid and identified proteins that are associated with vesicle trafficking. Together, our data provide new insights into how the FRA1 kinesin regulates plant cell wall assembly. This work is supported by NSF grant MCB-1121287.

Actin and Actin-Associated Proteins I

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Fission yeast tropomyosin promotes directed transport in cells by converting myosin-V into a processive motor.

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Most class-V myosins studied to date are dimeric motors that walk along actin filaments as single molecules. This processivity relies on a high-duty ratio in which motors spend the majority of their ATPase cycle time in the strong actin-bound ADP state. However, recent studies have shown that a sub-population of myosin-Vs are low duty ratio, non-processive motors. Here we employed a low duty ratio fission yeast myosin-V (Myo52p) to investigate the role of tropomyosin on motor function and transport. Decoration of actin filaments with fission yeast tropomyosin (Cdc8p) converts Myo52p into a processive motor. Similarly, Cdc8p-decoration activated the movement of beads coated with small numbers of Myo52p molecules (that mimic in vivo distributions). This regulation was not unique to Cdc8p because budding yeast tropomyosins and two out of three mammalian tropomyosins tested also activated Myo52p processivity. Our findings reveal a novel mechanism of regulation that facilitates sorting of myosin transport to specific actin tracks within the cell.

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Effect of mutations in the tropomyosin-binding sites on assembly of tropomodulin isoforms in skeletal myocytes..

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Tropomodulins (Tmod 1-4) cap the pointed end of the actin filament in a tropomyosin (TM)-dependent manner. Tmod/TM interactions are isoform-specific and the affinity of Tmod1 for skeletal striated α TM (stTM) is higher than that of Tmod3 and Tmod4. Earlier we showed that mutations R11K, D12N and Q144K in both TM-binding sites of Tmod1 decreased the affinity of Tmod1 to stTM making it similar to that of Tmod3 and Tmod4. The actin filament-capping ability of the Tmod1 mutants in the presence of stTM also decreased. GFP-Tmod1 WT, GFP-Tmod1 Q144K (single mutant), GFP-Tmod1 D12N/Q144K (double mutant) and GFP-Tmod1 R11K/D12N/Q144K (triple mutant) were expressed in primary chicken skeletal myocytes. CFP-Tmod3 and ChFP-Tmod4 were co-expressed with GFP-Tmod1, WT and mutants, to compare the propensity for them to assemble at thin filament pointed ends. Single, double and triple mutations in Tmod1 decreased its assembly at the pointed ends, correlating with our *in vitro* data. Surprisingly, assembly of the Tmod1 mutants was enhanced by the co-expression of Tmod3. Moreover, assembly of Tmod3 was increased in the presence of Tmod1WT in comparison to when it is expressed alone. Our data suggest that Tmod1 and Tmod3 mutually promote localization of each other at the pointed ends. Co-expression of Tmod4 with Tmod1 led to a drastic reduction of assembly for both molecules, potentially due to competition mechanism. We hypothesize that cooperation and competition in function of Tmod isoforms are influenced by their TM-binding properties. Supported by NIH grants to ASK and CCG.

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A nemaline myopathy-linked tropomyosin mutation reduces thin filament length and disrupts actomyosin crossbridge formation.

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Nemaline myopathy (NM) is the most common nonprogressive hereditary myopathy, with an incidence of 1 in 50,000 live births. NM results in muscle weakness, delayed motor development, slow fiber predominance, and appearance of actin-containing aggregates ("nemaline bodies") in muscle tissue. NM is caused by mutations in genes that encode the structural components of the sarcomeric actin (thin) filaments, which, in addition to actin, include tropomyosin, troponin, and nebulin. Previous studies have identified the mechanism for muscle weakness in NM as either thin filament length dysregulation or impaired actomyosin crossbridge activity. In this study, we examined the basis for thin filament pathogenesis in tropomyosin-based NM by testing the hypothesis that thin filament instability leads to actin depolymerization from the pointed end and resultant thin filament shortening. Using tissue from human NM patients with three different NM-causing tropomyosin mutations (*TPM2*-null, *TPM3*-R167H and *TPM2*-E181K) as well as healthy controls, we found that skeletal muscle harboring the *TPM3*-R167H mutation displays a lower thin filament length ($1.03 \pm 0.08 \mu\text{m}$) than skeletal muscle with the *TPM2*-null and *TPM2*-E181K mutations ($1.30 \pm 0.08 \mu\text{m}$ and $1.33 \pm 0.06 \mu\text{m}$, respectively) as well as healthy control muscle (ranging from $1.26 \pm 0.05 \mu\text{m}$ to $1.38 \pm 0.07 \mu\text{m}$). Thin filament shortening in *TPM3*-R167H muscle was not accompanied by changes in the distance of the N-terminus of nebulin from the Z-line, indicating that the observed shortening specifically affected the nebulin-free pointed-end extension of the thin filament. The *TPM3*-R167H mutation also

decreased cooperative thin filament activation in combination with reductions in myosin crossbridge number and active force production. This study is the first to demonstrate simultaneous thin filament length misregulation and inhibited actomyosin crossbridge formation in tropomyosin-based NM, suggesting crosstalk between these pathogenic mechanisms for NM. The results presented here have broad relevance to the development of future therapeutic approaches for tropomyosin-based NM as well as other NMs.

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The actin regulatory protein Tropomyosin 5NM1 regulates glucose uptake and GLUT4 trafficking in the mouse.

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A primary defect in Type 2 diabetes is alterations to glucose uptake in skeletal muscle and adipose tissue. Insulin-stimulated glucose uptake requires the trafficking of GLUT4-containing vesicles from intracellular stores to the cell surface. We have identified a novel population of actin filaments defined by the cytoskeletal tropomyosin (Tm) isoform Tm5NM1. Analysis of Tm5NM1 transgenic (Tg) mice suggests these filaments play a role in glucose uptake. In Tm5NM1 Tg mice, whole body glucose clearance and insulin-stimulated glucose uptake into white adipose tissue (WAT), skeletal muscle and heart was increased. This was specific to Tm5NM1, as glucose clearance was unaltered in mice expressing an alternative Tm, Tm3. Gene expression profiling (Illumina microarray), quantitative RT-PCR and Western blot analysis of WAT from the Tm5NM1 Tg mice detected an increase in genes involved in actin filament turnover and GLUT4 trafficking, including myosin motors (Myo1c, $P < 0.05$) and components of the exocyst complex (Sec8, $P < 0.005$). In keeping with Tm5NM1's reported role in stabilising actin filaments, there was a 30% increase ($P = 0.019$) in filamentous actin (detected by phalloidin staining) in Tg WAT. In 3T3L1 adipocytes, insulin-stimulation resulted in a shift in Tm5NM1 localisation to the plasma membrane, consistent with a role in GLUT4 trafficking. This effect was specific to Tm5NM1 and not other Tm isoforms. Finally, in 3T3-L1 adipocytes stably expressing Tm5NM1, there was an increase in insulin-stimulated GLUT4 movement to the plasma membrane compared to vector control cells. We propose that Tm5NM1 actin filaments promote recruitment of GLUT4 trafficking machinery that in turn results in enhanced insulin-stimulated GLUT4 translocation to the plasma membrane and glucose uptake.

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Regulation of cell proliferation mediated by the actin filament stabilizing protein, Tropomyosin 5NM1.

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The actin cytoskeleton has long been known to link signalling pathways with cellular proliferation, although the molecular mechanism(s) by which actin filaments participate in cell cycle progression are not very well understood. Tropomyosins (Tms), integral components of microfilaments, can regulate the dynamics and structural properties of actin filaments by controlling the interaction of filaments with actin binding proteins. In this study we demonstrate that a specific population of actin filaments composed of Tm5NM1 play a critical role in regulating cell proliferation. An increase in proliferation was evident in B35 cells and primary mouse embryonic fibroblast (MEFs) overexpressing Tm5NM1. In contrast, Tm5NM1 knock-down in SHEP cells or in knock-out (KO) MEFs significantly reduced proliferation. In Tm5NM1 transgenic mice, we convincingly show that the predominant phenotype seen is an increase in total fat mass due to an increase in the number of adipocytes without changes in fat cell size. Conversely, mice null for Tm5NM1 have reduced fat mass content. In order to elucidate the potential signalling pathway(s) most likely influenced by Tm5NM1, we employed the use of an antibody microarray featuring 500 pan-specific and 300 phospho-site specific antibodies to components from the major established signalling pathways. The array revealed the significant upregulation of components of the MAPK/ERK pathway including EGFR, KSR1 scaffolding protein, MEK 1 and pERK1/2 in the KO MEFs compared to WT MEFs. Whilst phosphatases, PP2A and MKP-1, known to regulate the duration of ERK signalling, were significantly downregulated. Initial western blots have indeed confirmed elevated expression of pERK1/2 seen in the KO MEFs relative to WT MEFs. More over, specific signalling pathway inhibitors demonstrate that proliferation via the PI3K pathway is Tm5NM1 independent but MAPK/ERK2 dependent. This was demonstrated using an ERK2 specific inhibitor that has no effect on the poor proliferation of the KO MEFs whilst significantly reducing that of the WT MEFs. Taken together our data strongly proposes that a specific population of actin filaments consisting of Tm5NM1 mediates cell proliferation via the MAPK/ERK2 pathway.

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Functional Analysis of Conserved Residues in Fission Yeast Tropomyosin.

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Tropomyosin (Tm) is a global regulator of actin filament function through stabilizing filaments and interacting with actin binding proteins including myosins and formins. We have taken a bioinformatics approach in a structure-function analysis of this protein in *Schizosaccharomyces pombe* (fission yeast). *S. pombe* has a single, essential Tm gene (*cdc8*) that is required for growth, formation of actin cables and the contractile ring, transport of actin patches, and conjugation. Our goal is to identify critical residues for these and other potential functions of Tm in the cell. We previously overexpressed 16 Ala/Ser mutants to screen 21 evolutionarily-

conserved surface residues for their ability to rescue growth of a *cdc8-ts* mutant (*cdc8-27*) at the restrictive temperature (35 °C). Whereas all rescued growth, three that exhibited abnormal growth or morphological characteristics were selected for further analysis: (D16A/K30A), (V114S/ E117A/H118A) and (R121A/D131A/E138A). To analyze Tm's universal function, actin binding, we expressed recombinant Tm in *E. coli* with a N-terminal AlaSer to enhance actin affinity. Whereas D16A/K30A had near-wildtype actin affinity (by cosedimentation), the binding of V114S/ E117A/H118A and R121A/D131A/E138A was too weak to measure. For cellular analysis, we created *cdc8* mutant strains using a marker gene replacement strategy in which the endogenous gene is replaced using homologous recombination; recombinants were selected using nutritional markers (Tang et al., 2011). Following selection, the recombinants were backcrossed against parental strains, and the presence of mutations was confirmed by genomic DNA sequencing. We note that the V114S/ E117A/H118A and R121A/D131A/E138A strains are genetically unstable; the backcrosses yielded colonies with phenotypes that differ from that of either parent, in addition to the expected parental phenotypes. The strains were analyzed for nuclear number (DAPI), cell and septum morphology (Calcofluor), and the actin cytoskeleton (phalloidin). All contained one or two nuclei with normal localizations, indicating that cells complete nuclear and cell division. The D16A/K30A cells contained actin cables, but the actin patches were not well-polarized, often found throughout the cell rather than being localized at the poles. Contractile rings formed but were often irregular. V114S/ E117A/H118A showed actin cables and a normal distribution of actin patches. However, the contractile ring was irregular. R121A/D131A/E138A exhibited the most severe phenotype. Actin cables were present, but the actin patches were found at the poles and throughout the cells. Often cells with two nuclei had dual diffuse contractile rings and septa that resulted in two "cell" divisions with two daughter cells and a piece of cell without a nucleus in between. We conclude that the conserved residues mutated in these strains have crucial but distinct functions in actin patch localization and contractile ring assembly that depend on interactions with other actin binding proteins, as well as actin. Supported by NIH-RO1-GM093065 and The Aresty Research Center for Undergraduates at Rutgers University.

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Multi-domain model structures of α -actinin-4 explain how external cues can regulate actin binding.

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In eukaryotic non-muscle cells, the regulation of the homodimeric actin-crosslinking protein alpha-actinin-4 (ACTN4) during cell migration involves signaling receptors with intrinsic tyrosine kinase activity, yet the underlying molecular mechanisms are poorly understood. Here we develop structural models for human ACTN4 and three of its phosphorylation states, both in the presence and absence of Ca²⁺ ions, revealing the complex network of interactions responsible for the regulation of this protein. The core structure corresponds to a complex between the C-terminal CaM-like domain of one monomer, and the N-terminal actin-binding domain of the opposite antiparallel monomer. Mutagenesis experiments validate our predictions of distinct Ca²⁺-independent phenotypes for phosphorylated Y265 and Y4/Y31 that upregulate and downregulate actin binding, respectively. A kinetic model of the predicted network that entails eight combinations of multi-domain complex structures reconciles a broad set of semi-quantitative in vitro binding data. Collectively, these studies provide insight pertinent to the molecular basis of actin crosslinking by ACTN4, while revealing a framework for understanding how post-translational modifications can result in robust signals by assembling and disassembling multi-domain complexes.

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WASH-dependent Actin Recruitment to the Maturing Phagosome.

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Phagocytosis is a receptor-mediated process whereby professional phagocytes engulf foreign particles into a membrane-bound organelle termed the phagosome. Through a coordinated series of membrane fission and fusion events the nascent phagosome matures into the highly microbicidal phagolysosome. Despite the multiple rounds of vesicular fusion, the size of the maturing phagosome remains constant, suggesting that in addition to membrane fusion membrane fission also occurs. Through these fission events, the phagocyte presumably retrieves biologically important molecules to be recycled; however, the molecular mechanisms regulating membrane fission at the phagosome remain elusive. We investigated early phagosome membrane fission by monitoring the fate of glycosylphosphatidylinositol (GPI)-anchored proteins using fluorescent membrane tracers. Our data show that membranous tubules emanate from the maturing phagosome; actin puncta, revealed by phalloidin staining, were found near the base of these tubules. We therefore speculated that actin-containing structures might be involved in driving membrane fission and cargo retrieval from the phagosome. Recent evidence has shown that the WASP and SCAR homologue (WASH) activates the actin-nucleating complex Arp2/3 on endosomes, where it participates in membrane remodeling and receptor trafficking. Expression of WASH-GFP, as well as immunolocalization of endogenous WASH showed that this protein co-localizes with the actin puncta of early phagosomes. Moreover, the sorting nexins SNX1 and 2 also co-localize with WASH-positive structures on maturing phagosomes, suggesting the involvement of retromer in WASH recruitment. Importantly, WASH-deficient primary macrophages failed to recruit actin to early phagosomes, implying that actin association with early phagosomes is WASH-dependent. These data are consistent with a model where WASH induces actin polymerization, which promotes membrane fission and efficient remodeling of the maturing phagosome. To verify this model, comparative measurements of the rate and extent of phagosome maturation in wild-type and WASH-deficient macrophages are currently underway.

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Processive acceleration of actin barbed end assembly by N-WASP.

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Motile cells direct the force of actin barbed end polymerization towards the leading edge of the membrane. WASP family nucleation promoting factors (NPFs) bind to the membrane at the leading edge and activate arp2/3 to generate a dendritic actin network. WASP family proteins also bind to actin filament barbed ends, and we recently showed that neuronal WASP (N-WASP) can remain processively attached to bundled barbed ends in an *in vitro* motility assay (Hu and Kuhn, 2012, PLoS ONE 7(2): e31385). We used total internal reflection fluorescence (TIRF) microscopy of individual actin filaments to determine whether N-WASP acts as a true processive actin barbed end assembler in the absence of arp2/3 complex. N-WASP PP-WWCA coated nanofibers were incubated with *de novo* nucleated actin filaments in chambers coated with rigor myosin. When N-WASP nanofibers captured individual barbed ends, barbed ends either continued to grow along the nanofiber at the diffusion-limited rate or, when oriented perpendicular to the nanofiber, grew at a substantially reduced rate. However, when filament densities increased to the point of substantial filament overlap, a subset of nanofiber-attached barbed ends formed prominent buckles or loops between their nanofiber tethers and myosin

attachment points. Buckling filament barbed ends showed substantial parallel association with neighboring filaments. Measurement of elongation rates showed that buckling filament barbed ends grew 3.4-fold faster than the diffusion-limited rate of unattached barbed ends in the same field. We found similar barbed end rate enhancements in N-WASP constructs with and without the native poly-proline (PP) region. We found that increasing polycationic Mg^{2+} concentrations to 10 mM did not alter barbed end acceleration rates. However, increasing Mg^{2+} from 1 to 10 mM increased the frequency of buckle formation 2.6 to 3.0 fold, consistent with a requirement of accelerated assembly on barbed end bundling. Filament bundling by divalent cation Mg^{2+} confers multimerization of barbed ends that allows their cooperative, processive binding to dense, tethered WCA-domains. Acceleration of barbed ends beyond their natural diffusion limited rate shows that, like formin and VASP, tethered N-WASP can act as a processive barbed-end assembly motor in the absence of arp2/3 complex.

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Molecular details of drebrin-cofilin cross-talk.

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The competition between drebrin and cofilin is believed to be a part of the pathway leading to synaptic dysfunction in Alzheimer's disease and epilepsy. However, up to date the cross-talk between cofilin and drebrin is poorly understood. TIRF microscopy revealed that saturating concentrations of drebrin, either full length or of its actin binding core (a.a. 1-300), inhibit but do not abolish cofilin-induced severing of actin filaments. The dependence of cofilin binding on fractional saturation of actin filaments with drebrin suggests direct competition between these two proteins. Decoration of F-actin with both cofilin and drebrin can yield a total combined binding density of 100%. This implies that these two proteins can either overcome or reverse the allosteric changes in F-actin induced by the competitor's binding. Pre-steady state kinetic experiments revealed that the binding of drebrin to actin filaments was over two orders of magnitude faster than the cooperative binding of cofilin (as evaluated by the nearest-neighbor approach; Cao W et al, 2006, JMB, 361; P:257-267). However, drebrin dissociation rate from the filaments was also faster (~10 fold) than that reported for cofilin. Despite its higher affinity for F-actin, drebrin can be displaced from the filaments by high concentrations of cofilin. This is explained by a decrease in the availability of segments of five actin protomers (unoccupied by cofilin), which constitute the drebrin binding site on F-actin. Our results contribute to a molecular understanding of the cross-talk between drebrin and cofilin.

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Regulation of axonal filopodia and collateral branches by the actin filament binding protein drebrin.

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Axon collateral branches underlie the ability of individual neurons to make connections with multiple targets. Collateral branches arise from the maturation of transient axonal filopodia, which in turn, emerge from axonal patches of actin filaments. In this study we investigated the role of the actin filament binding protein drebrin in the formation of axonal filopodia and branches by chicken sensory neurons. Live imaging of RFP-drebrin E1 and eYFP-actin in vitro revealed that drebrin is present throughout the axon shaft and is recruited to actin patches,

persisting throughout the lifespan of the actin patch. Furthermore, over-expression of drebrin increased the probability that an actin patch will give rise to a filopodium, while conversely shRNA-mediated depletion of drebrin decreased the probability that an actin patch gives rise to a filopodium. Endogenous drebrin was detected in the proximal portion of established filopodia and in the shafts of nascent branches. Over-expression of drebrin increased the number of axonal filopodia and branches *in vitro* and *in ovo*, while conversely depletion of drebrin decreased the number of axonal filopodia and collateral branches. Furthermore, expression of drebrin increased the length and branching of individual axonal filopodia, while depletion of drebrin decreased the length and branching of filopodia. *In vitro*, over-expression of drebrin increased the percentage of axonal filopodia invaded by GFP-EB3, a microtubule plus end binding protein. Furthermore, filopodia of drebrin over-expressing axons were remarkably stable, although capable of bouts of tip elongation and retraction. Collectively, the data suggest that drebrin is involved in the formation of axonal filopodia and collateral branches by regulating the emergence of filopodia from axonal actin patches and the stability of axonal filopodia. Furthermore, the data suggest that drebrin assists the entry of microtubules into axonal filopodia thereby promoting the maturation of axonal filopodia into branches.

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Cryo-electron tomography and subvolume analysis of MSP filaments derived from the amoeboid sperm of *Ascaris suum*.

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Locomotion of nematode sperm is remarkably similar to that of most other crawling cells but is powered by a system of filaments composed of major sperm protein (MSP) instead of the actin-myosin machinery typically associated with amoeboid motility. The MSP motility apparatus has been reconstituted *in vitro*, and individual MSP filaments can be generated by the addition of ATP to detergent-treated *Ascaris* sperm cytosol. Filaments formed in this way have been examined using electron cryo-tomography of both frozen-hydrated and negatively-stained samples. Together, these have allowed for the structural analysis of filaments formed in the presence of MSP accessory proteins. Filaments within complex MSP meshworks have been averaged using subvolume alignment by classification, and the resulting physiological models have been compared to tomographic reconstructions of purified MSP polymerized in ethanol as well as earlier models derived using both Fourier synthesis (King et al. 1992. JCS 101:847) and X-ray crystallography (Bullock et al. 1998. NSB 5:184). There are currently six *Ascaris* sperm proteins known to modulate MSP filament dynamics in sperm; these same proteins are absent from filaments prepared using purified MSP but may be present and bound to filaments prepared from cytosol. Model comparisons provide us with a greater understanding of how these accessory proteins effect MSP filament dynamics and influence motility. Supported by NIH Grant R37 GM29994 and by the American Heart Assoc.

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Direct binding of BAR-domain protein FAP52 to F-actin.

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BAR domain proteins have been extensively studied for their roles in membrane-binding and shaping. These domains have characteristic curved shapes, and it has been shown that they can induce this curvature in the membrane to which they bind. This has emerged as an important element in membrane remodeling, including vesiculation and tabulation. More

recently, many links have been found between membrane remodeling and the actin cytoskeleton. In a number of cases it has been assumed that BAR domain proteins interact with the actin cytoskeleton via actin-binding proteins. We can now show that Focal Adhesion Protein (FAP52), a multidomain adaptor protein of 448 amino acids containing a BAR domain and characterized as an abundant component of focal adhesions, directly binds to F-actin. FAP52 binds to F-actin in a similar manner to tropomyosin, and we have used cryo-EM to generate three-dimensional reconstructions of this complex using the BAR domain fragment of FAP52. Strikingly, the curvature of FAP52 on the actin filament is very similar to the curvature previously shown for F-BAR domains binding to membranes. These results suggest that, at least for this BAR domain protein, the interactions with the membrane and with F-actin both can be accomplished by the same protein.

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Possible link between copine A and the actin cytoskeleton.

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Copines make up a multigene family of calcium-dependent, phospholipid-binding proteins. Copine proteins consists of two C2 domains at the N terminus followed by an "A domain" similar to the von Willebrand A domain found in integrins. The C2 domain is a calcium-dependent membrane-binding motif, while the A domain is thought to be a protein-binding domain. We are studying copine protein function in the model organism, *Dictyostelium discoideum*, which has six copine genes, *cpnA*-*cpnF*. Previous research showed that *cpnA*- cells exhibited a cytokinesis defect, a developmental defect, and a defect in contractile vacuole function. To fully understand the role of CpnA in these cellular processes, we used several methods to identify proteins that interact with CpnA. We first used column chromatography and mass spectrometry to isolate proteins that bound to a CpnA-linked agarose column. One of the proteins that eluted from the CpnA-linked column and not the control column was actin. We also carried out immunoprecipitations in the absence of calcium using a polyclonal antibody to GFP with *Dictyostelium* cells expressing GFP, GFP-CpnA, or GFP-fused to the VWA domain of CpnA (GFP-Ado). Actin co-precipitated with GFP-Ado, but not GFP or GFP-CpnA. In addition, we treated cells with Triton X-100 and spun down the insoluble cytoskeletal fraction. GFP-Ado was found in the cytoskeletal pellet in the presence or absence of calcium, while GFP-CpnA was found in the cytoskeletal pellet only in the presence of calcium. GFP alone was not found in the cytoskeletal pellet. This data suggests that the VWA domain of CpnA is able to interact with the cytoskeleton in a calcium-independent manner, while full length CpnA interacts with the cytoskeleton in a calcium-dependent manner. We have also expressed a GST-tagged version of CpnA in *Dictyostelium* and have purified GST-CpnA using glutathione chromatography. We are currently carrying out experiments to test whether CpnA is able to bind directly to actin and is able to regulate actin polymerization in vitro. In addition, we are using GST-pull down assays to explore whether other candidate binding proteins are able to bind directly to CpnA in vitro.

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Coronin Effects on Actin Filaments.

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Coronin is one of the conserved actin-binding proteins and its different isoforms have been identified in many eukaryotic organisms. Coronin's mode of influencing actin dynamics in the

cell is not well understood yet. In vitro, coronin was found to regulate cofilin's severing of actin filaments in a nucleotide state dependent manner. Here, the structural effects of coronin on F-actin were examined by microscopy and in solution. Electron microscopy (EM) images show shortening of actin filaments in the presence of coronin alone. This effect varies with the nucleotide state of actin. AMP-PNP bound F-actin appears completely shredded in the presence of Crn β . Coronin's recognition of the nucleotide binding site on actin is indicated by a decrease in fluorescence emission and an increase in the accessibility of etheno-ADP to collisional quenchers in the presence of Crn β . The effect of coronin on interprotomer contacts in F-actin was explored by monitoring its impact on disulfide crosslinking reactions. We found that Crn β decreased the inter-strand disulfide cross-linking between Cys265 (H-loop) and Cys374 (C-terminal) in F-actin but increased the cross-linking rates between Cys41(DNase I binding-loop) and Cys374. Our data suggest that coronin binding has an effect on the major structural elements and the interprotomer contact regions of the actin filament.

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SPIN90, a novel actin binding protein, induces fibroblasts differentiation depending on substrate rigidity.

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Mechanical environment has been proposed to alter various cellular processes including cell motility and differentiation. However, the molecular basis for how mechanical signaling is converted into biochemical reaction for cellular events is largely unknown. In this study, we demonstrated that SPIN90 (Nck-interacting protein with SH3 domain), an F-actin binding protein, controls myofibroblast differentiation depending on substrate rigidity. Mouse embryonic fibroblast (MEF) isolated from SPIN90 null mouse protruded profound dendritic extensions in the 3D collagen matrices responding to serum, as compared with control MEFs. Interestingly, SPIN90^{-/-} MEFs tended to be able to maintain the protrusion of dendritic extensions even in 3D collagen culture at low cell densities. In addition, SPIN90^{-/-} MEFs displayed prominent development of stress fibers with enlargement of vinculin positive focal adhesions and increased the 3D collagen matrix contraction, suggesting a critical role of SPIN90 in cellular contractility. When cells were grown on collagen coated soft polyacrylamide gel on which the tension state of cell-matrix interaction is reduced, SPIN90^{-/-} MEFs showed both faster spreading than normal MEFs and focal adhesion kinase (FAK) activation, suggesting that SPIN90^{-/-} MEFs seemed to turn on the contractile machineries independent of mechanical and biochemical signaling. Finally, SPIN90^{-/-} MEFs can be highly expressed alpha-smooth muscle (alpha-SMA), a marker for myofibroblast, in the soft acrylamide gel without agonist stimulation. The newly expressed alpha-SMA is completely inhibited by Rho kinase inhibitor. In conclusion, our results suggest that SPIN90 could play important roles in regulating the cellular contractility via regulation of FAK/Rho kinase signaling when cells were in the low-tension environments, which in turn contribute to regulating myofibroblast differentiation.

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Regulation of endogenous septin scaffolds and the role of p53 and p63.

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Septins are a conserved family of genes encoding proteins involved in a variety of cellular processes including cytokinesis, polarity, actin and microtubule dynamics, membrane events and apoptosis. These GTP binding proteins are increasingly recognized as a novel part of the cytoskeleton and are now known to form hetero-oligomeric complexes and higher order structures, such as filaments and rings. The human SEPT9 gene has a complex genomic architecture with 18 different transcripts generated through shuffling of six 5' ends and three 3' ends. Two transcripts with unique 5' ends (SEPT9_v4 and SEPT9_v4*) encode the same protein. Five different amino termini isoforms are recognized (SEPT9_i1-i5) with _i4 and _i5 truncated versions of the larger isoforms. In neoplasia the relative ratio of transcripts changes and in particular there is upregulation of SEPT9_v4* mRNA, a transcript that is translated with enhanced efficiency leading to increased SEPT9_i4 protein. We have examined the effect of overexpression of the individual SEPT9 isoforms on endogenous SEPT9 and endogenous SEPT6 scaffolds. We find that SEPT9_i2, SEPT9_i4 and SEPT9_i5 over-expression in two different cell lines induces dramatic actin and cytoskeletal reorganization with the formation of filopodia around the cell periphery when compared to other isoforms. In addition, SEPT9_i4 and SEPT9_i5 expression causes disruption of both endogenous SEPT6 and SEPT9 containing filaments with granular staining visible or shorter filaments within the cytoplasm of transfected overexpressing cells. We next examined how one of these small isoforms might be regulated. Using a combination of sequence analysis and CHIP-seq we identified putative p53 and p63 binding sites upstream of the first exon of SEPT9_v4*. Using a luciferase reporter gene construct we demonstrate that at least one of these sites is regulated by wild type but not mutant p53 and by all of the TAp63 isoforms (α , β , γ) and two of the Δ Np63 isoforms (α , β). Similarly, the induction of p53 in a p53 wild type background by doxorubicin also led to the upregulation of SEPT9_v4*. This upregulation was dependent on p53 since it was lost when p53 was knocked down by siRNA. Conversely, knocking down total p63 caused upregulation of SEPT9_v4* in a background of wild-type p53 in HFK cells. Taken together this data suggests that the truncated isoforms of SEPT9 may regulate septin scaffolds and that p53 and p63 both have a role in the regulation of at least one of these isoforms.

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Self-organization in Reconstituted Bundles of Actin and Skeletal Muscle Myosin II.

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Actomyosin bundles such as muscle myofibrils, stress fibers and cytokinetic rings are used by cells to exert force, contribute to the cell's structural integrity and accomplish morphological change. While these linear structures exhibit varied architecture, two common organizational themes are a punctate distribution of myosin II and distinct patterns of actin polarity. The mechanisms that cells use to assemble and maintain these organizational features are poorly understood. To investigate the mechanisms of organization, we combined mathematical modeling with in vitro reconstitution of actomyosin bundles that contained only actin filaments and skeletal muscle myosin II thick filaments. In the presence of ADP, actin and myosin formed a loose network of stable (>1 hour) bundles ~5-50 μ m long anchored between polystyrene beads. Upon addition of ATP, the bundles contracted and became taut over 2-8 s. Over a longer

timescale of ~30 s skeletal muscle myosin II, initially distributed uniformly along the bundles, self-organized by translating along the bundle into distinct puncta reminiscent of those in the actomyosin cytoskeleton of living cells. To understand myosin II self-organization quantitatively, we developed a mathematical model in which the motions of myosin thick filaments depend on the local actin polarity. Owing to the random bundle assembly process, actin filaments have random positions and orientations, resulting in local deviations of actin polarity that follow a random walk along the bundle axis. The actin-myosin interaction propels myosins in the direction of local actin polarity, resulting in aggregation of myosin filaments at those locations where the net bundle polarity vanishes. With no fitting parameters, our model successfully reproduces the broad distribution of distances d between neighboring myosin puncta observed in our experiments. In particular, our prediction that the distribution follows a power law decay $\sim d^{3/2}$ on intermediate scales less than the filament length is in remarkably close agreement with our measurements. Thus, our results show that the local pattern of actin filament orientation is an important regulator of the larger scale organization of actomyosin structures, and that a minimal bundle of actin and myosin has the inherent capacity to self-organize into a heterogeneous banded structure without an organizing template or accessory proteins.

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Plectin contributes to the mechanical stability of keratinocytes and myoblasts.

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Plectin and its isoforms are promiscuous crosslinkers of actin filaments, microtubules and intermediate filaments (IF). Plectin is expressed in a wide variety of cell types. In epithelial cells and keratinocytes, it is also found in hemidesmosomes that link the laminin receptor $\alpha 6 \beta 4$ with the keratin IFs. Mutations in the plectin gene cause a skin blistering disorder (epidermolysis bullosa) that is also associated with a late-onset of muscular dystrophy. In both disorders, mechanical alterations of the keratinocytes and the myoblasts, respectively, are thought to be ultimately responsible for the pathological manifestation. To test this hypothesis, we measured the mechanical properties (stiffness, contractility, adhesiveness, and motility) of plectin knockout and plectin-expressing mouse keratinocytes and myoblasts. Cell stiffness was measured by pulling superparamagnetic fibronectin or laminin1-coated beads with a force of 10 nN. The magnetic force was then ramped up to 80 nN, and the cell-bead rupture force was recorded as a measure of adhesive strength. Contraction (only in myoblasts) was measured by polyacrylamide-based traction microscopy, and cell migration was determined over a time course of 5 h. In plectin-deficient myoblasts, stiffness, tractions, and adhesive strength were ~2-fold reduced, indicating that plectin is important for the mechanical stability of these cells. Plectin-deficient keratinocytes assessed under similar conditions were found not to show such effects. Confocal immunofluorescence images of laminin1-coated magnetic beads attached to keratinocytes demonstrated prominent association of the beads with the actin cytoskeleton, but not with keratin intermediate filament-linked hemidesmosomal components. This suggested that the contribution of keratin intermediate filaments to the overall mechanical stability of keratinocytes could not be assessed by this particular (bead-based) type of force measurements. Taken together, our results demonstrate that human diseases associated with plectin mutations have a cell mechanical origin, and that plectin affects the cytoskeleton in different cell types in distinct ways.

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ZASP mutations trigger disassembly of F-actin bundles in myofibrillar myopathy.

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Myofibrillar myopathies (MFM) are characterized by a degenerative process that primarily affects the actin myofibrils at the Z-disc in skeletal muscle. In this report we explore the underlying mechanisms of the myofibrillar degeneration in zaspopathy, a prototype MFM. Zaspopathy is caused by heterozygous mutations in the Z-band alternatively spliced PDZ-motif protein (ZASP). The A165V and the A147T mutations are in or near a highly conserved motif that is expressed in skeletal muscle (sZM). We have identified the sZM as a skeletal α -actin (ACTA1) binding region of ZASP. Both wild type and mutant ZASP bind to actin filaments in vitro, but only the mutant proteins cause disassembly of F-actin bundles induced by α -actinin 2. To examine the effects of the sZM mutation on skeletal α -actin in vivo, we electroporated plasmids encoding either ZASP-GFP or ZASPA165V-GFP into the tibialis anterior muscles of wild type mice (n=4). Both GFP-tagged wild type and mutant ZASP co-localized with α -actinin 2, a marker for the skeletal muscle Z-disc. By 4 weeks after the electroporation, there was streaming of GFP fluorescence from the Z-disc into the sarcoplasm accompanied by a patchy loss of the α -actinin 2 staining in the muscle fibers expressing mutant ZASP. Phalloidin staining of the same muscles showed focal accumulations of actin filaments that co-localized with ZASPA165V-GFP in the sarcoplasm. Focal accumulations of phalloidin-actin co-localized with ZASP were also seen in biopsied skeletal muscle sections from a patient with zaspopathy. Electron microscopic images of mouse muscle expressing mutant ZASP showed discrete disruption of Z-discs, loss of the normal actin filament pattern, and the presence of cellular debris, including membranous whorls reminiscent of the findings reported in the human disease. These findings were not observed in muscle fibers expressing wild type ZASP. Our observations support a gain-of-function mechanism for ZASP mutations in the pathogenesis of MFM.

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Characterization of ZASP-skeletal actin interaction and its role in a myofibrillar myopathy.

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Myofibrillar myopathies (MFM's) are a group of muscular dystrophies characterized by disruption of actin cytoskeleton in skeletal muscle. Zaspopathy, a prototype MFM, is caused by heterozygous mutations in the Z-band alternatively spliced PDZ motif protein (ZASP). The disease-causing mutations A147T and A165V are near or within an evolutionarily conserved 26 amino acid motif expressed in skeletal muscle sZM. The function of sZM and the mechanism by which mutations lead to disease are not yet known. To identify the proteins interacting with the sZM region, we performed yeast two-hybrid (Y2H) screen of human skeletal muscle cDNA library using the 132 amino acids encoded by exons 5-6-7 of ZASP (sZM_{132aa}±A165V) as bait. The most frequent prey clones (n=8 for each bait) encoded the 131 C-terminal amino acid residues of skeletal muscle α -actin 1 protein (ACTA1₂₄₇₋₃₇₇). Pair wise Y2H assays using different regions of ZASP as bait against skeletal actin validated the sZM_{132aa} as an actin-binding region of ZASP. Deletion analysis of ACTA1 in Y2H assays showed that the amino

acids 304-325 of skeletal α -actin are essential for binding to the sZM_{132aa} region in ZASP. The interaction between ZASP and skeletal actin was validated by co-immunoprecipitation (co-IP) of these two proteins from mouse muscle lysates as well as transfected Cos7 and HEK293 cell lysates. Presence of either A147T or A165V mutation did not affect ZASP interaction with skeletal actin. ZASP-actin interaction was abolished by deletion of sZM from ZASP in Y2H and co-IP assays. Direct binding of ZASP to skeletal actin filaments was examined by a co-sedimentation assay using purified proteins. After a high speed centrifugation GST remained in supernatant, whereas GST-tagged ZASP proteins sedimented with skeletal actin filaments in pellet. We are performing surface plasmon resonance assays to assess binding affinity of WT and mutant ZASP proteins to skeletal actin. Whereas both WT and mutant ZASP bound to skeletal actin, only the mutant proteins caused disassembly of α -actinin 2-crosslinked F-actin bundles in vitro and accumulation of F-actin in sarcoplasm in electroporated mouse muscle fibers (ASCB submission # 858). These results suggest that ZASP is a novel skeletal actin binding protein and that sZM is essential for this interaction. F-actin destabilizing properties of mutant ZASP point to a gain-of-function disease mechanism in MFM.

Regulation of Actin Dynamics I

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RhoA and Rac1 GTPases contribute independently to actin regulation during particle engulfment by retinal pigment epithelial cells.

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Mammalian rod photoreceptors renew their outer segments by shedding their distal, aged tips diurnally. These aged photoreceptor outer segment particles (POS) are removed by adjacent retinal pigment epithelial (RPE) cells through an $\alpha\beta 5$ integrin dependent phagocytosis mechanism that resembles the clearance phagocytosis of apoptotic cells. Particle engulfment during RPE phagocytosis requires actin-rich phagocytic cup formation at the site of bound particles. Rho GTPase family members are the primary regulators of F-actin distribution. We hypothesized that RPE cells utilize Rho GTPases to control phagocytic cup formation during particle engulfment. To determine contributions of individual Rho GTPases to particle engulfment, we investigated the phagocytic activity and F-actin distribution of immortalized or primary rat RPE cells in culture in response to POS particles isolated from pig eyes. We found that expression of a dominant-negative form of Rac1 decreased POS internalization as well as abolished formation of phagocytic cups. In contrast, inhibition of RhoA or its downstream effector Rho kinase (ROCK) increased the particle internalization rate, while activation of RhoA decreased internalization and abolished phagocytic cup formation. Treating RPE cells with the ROCK inhibitory agent Y-27632 reversed the inhibitory effect of RhoA activation on phagocytosis. However, ROCK inhibitor treatment failed to rescue phagocytic cup formation in RPE cells expressing dominant-negative Rac1. Moreover, expression of a constitutively active form of Rac1 did not restore phagocytic cup formation in cells also treated with RhoA activator. Taken together, these results indicate that activation of Rac1 and inhibition of RhoA-ROCK are independently required for phagocytic cup formation during POS engulfment by RPE cells.

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Dynamin2 orchestrates Actomyosin assembly and dynamics in non-muscle cells.

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Actin networks in non-muscle cells exist in two major architectures: a sheet-like network of branched actin filaments in lamellipodia and parallel, bundled actin filaments which, together with myosin II, comprise actomyosin of contractile stress fibers of lamellae. A current model for formation of one type of stress fiber (transverse arcs) suggests that crosslinked lamellipodial actin filaments co-assemble with myosin assemblies forming actomyosin of lamellae. Our work and that of others have implicated the large GTPase dynamin2 in influencing actomyosin of transverse arcs. To determine dynamin2's function in actomyosin assembly, we used time-lapse microscopy of live U2OS cells expressing two probes of actomyosin: GFP-myosin light chain 2 (MLC2) and mCherry-alpha-actinin. In control cells, nascent myosin assemblies first appeared within the lamellipodia then flowed rearward and coalesced with alpha-actinin-decorated actin filaments forming distinct, striated cables of actomyosin near the lamellipod-lamellum interface. In cells depleted of dynamin2, nascent myosin assemblies appeared more frequently within lamellipodia and their integration with alpha-actinin-decorated actin filaments was irregular; transverse arcs also flowed rearward more rapidly than in control cells. Since dynamin2 localized to the distal edge of lamellipodial actin networks, but not with nascent myosin assemblies, we suggest that dynamin2 orchestrated the transition of a subset of lamellipodial actin filaments into crosslinked filaments optimized for co-assembly with myosin. To test this hypothesis, we examined the spatial and temporal distribution of alpha-actinin within lamellipodia. In control cells, alpha-actinin was discontinuous, with some regions of the network enriched with alpha-actinin compared to other regions. In dynamin2-depleted cells, alpha-actinin was homogeneously distributed within the network. Thus, dynamin2 influences the spatial and temporal distribution of alpha-actinin-decorated actin filaments of lamellipodia. Dynamin2-R399A, a mutant defective in self-assembly but with basal level of GTPase activity, restored the dynamic distribution of alpha-actinin within lamellipodial F-actin and partially restored the retrograde flow of transverse arcs. Surprisingly, dynamin2-T141A, which exhibits increased GTP hydrolysis, did not restore either phenotype in dynamin2-depleted cells. Importantly, the effects of depleting dynamin2 on actomyosin did not result from perturbations in endocytosis, since internalization of either integrin β 1 or transferrin receptor were not significantly perturbed in cells depleted of 80-90% of endogenous dynamin2. Hence, we conclude that dynamin2 regulates actomyosin assembly and dynamics in non-muscle cells, most likely though its actions on lamellipodial actin filaments.

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Early cell spreading depends on actin polymerisation from integrin clusters by FHOD1.

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Matrix adhesions are critical for cell growth and differentiation, and actin network assembly at adhesion sites dynamically regulates various downstream cellular signals. The formation of adhesions depends upon a number of steps that follow integrin binding to matrix ligands and clustering that include actin polymerisation by an unknown mechanism. Actin nucleation and polymerization factors are grouped into several classes, such as the ARP2/3 complex or the formin family proteins. Here we demonstrate that formins are critical for adhesion formation. Furthermore, one of the major formins in mouse fibroblasts, FHOD1, is activated during early

cell adhesion. FHOD1 is also recruited to early integrin clusters in an Src family kinase dependent fashion, concomitantly with outward polymerisation of actin and its knockdown impairs spreading. Other major fibroblast formins on the other hand, such as mDia1 and mDia2 are activated only after the initial fast spreading phase. By showing a sequential involvement of distinct formins in cell spreading with FHOD1 as early regulator of spreading and rigidity sensing, our findings provide novel insights into the mechanisms of cell migration with implications for development and disease.

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Src Regulation of Actin Dynamics in Growth Cone Filopodia and Lamellipodia.

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Axonal growth and pathfinding is fundamental to development and regeneration of the nervous system. Src tyrosine kinase has been implicated in axonal growth and pathfinding; however, how exactly it affects the underlying cytoskeleton has remained elusive. Here, we have used quantitative fluorescent speckle microscopy to determine which specific aspects of actin and protrusion dynamics are controlled by Src activity in neuronal growth cone filopodia and lamellipodia. We manipulated and determined protein levels and activities of *Aplysia* Src2 tyrosine kinase by expressing various mutant constructs in cultured *Aplysia* neurons in a controlled manner. Our results show that the expression of constitutively active (CA) Src2 increases density and lateral movements of filopodia compared with controls, while the expression of dominant negative (DN) Src2 has opposite effects, suggesting a positive role of Src2 in filopodia formation and integration within the lamellipodial actin network. On the other hand, analysis of actin dynamics revealed that CA Src2 expression only slightly increases filopodial actin assembly rate and protrusion time, while DN Src2 expression slightly decreases actin assembly rate, suggesting a minor role of Src2 in filopodial elongation. CA Src2-expressing growth cones also spend more time in leading edge protrusion and thereby have wider lamellipodia compared with control growth cones, while DN Src2-expressing growth cones have narrower lamellipodia, suggesting that Src2 activity enhances actin assembly in growth cone lamellipodia rather than filopodia. These findings support our hypothesis that Src activity promotes actin assembly and branching in growth cone lamellipodia in an Arp2/3 complex-dependent manner, a hypothesis that will be tested in future experiments.

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The role of actin polymerization in regulating endothelial cell barrier function.

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Endothelial cells form a selectively permeable barrier in blood vessels and arteries. Endothelial cells are exposed to a variety of extracellular stimuli including shear force, pulsatile stretching, soluble factors such as growth factors and chemokines, as well as leukocyte adhesion and transmigration. Since the endothelial cytoskeleton is known to play a role in regulating vascular permeability, we sought to test the hypothesis that different stimuli may compromise barrier function via specific effects on the actin cytoskeleton of endothelial cells. In this study, we examine how the endothelial actin cytoskeleton and cell-ECM adhesions change in response to growth factors and chemokines known to induce vascular permeability as well as to membrane rupture induced by physical perturbation. We expressed a fluorescently-labeled F-actin marker with fluorescently labeled paxillin in primary human umbilical cord endothelial cells (HUVECs)

and imaged cells by total internal reflection fluorescence microscopy (TIRF). We found that endothelial cells have small (3-5 μ m) semicircular dynamic actin structures that propagate along the ventral ECM-attached surface of the cell. These adhesive F-actin waves contain paxillin and integrin, but do not degrade fluorescently labeled-gelatin. Adhesive F-actin waves are sensitive to VEGF, Thrombin and TNF α . To determine how HUVECs responded to physical disruption, we used a 440nm dye-laser to ablate small (~1-2 μ m) holes in the ventral plasma membrane and cell cortex. The wounds formed by laser ablation were consistently healed by a burst of Arp2/3-mediated actin polymerization, and myosin II contractility was not required for wound closure. We conclude that dynamic actin polymerization contributes to endothelial response to growth factors and is essential for proper closure of plasma membrane wounds.

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Regulating the Actin Cytoskeleton During Transendothelial Migration.

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Transendothelial migration (TEM), or diapedesis, is a vital physiological process by which leukocytes cross from the bloodstream into the underlying tissue. During migration to the site of an infection or tissue damage, the leukocyte must cross the endothelium. Two routes are taken by the leukocyte: either a paracellular route, which is between two adjacent endothelial cells, or a transcellular route, which is directly through one endothelial cell. Leukocytes and endothelial cells actively participate in this process by signaling to one another, and by regulation of their respective actin cytoskeletons. Much research has revealed key players within the lymphocytes during TEM, however much less is known about the mechanisms that govern regulation in the endothelial cells.

Since actin regulation via various Rho GTPases within endothelial cells is important for transendothelial migration, we hypothesized that the Arp2/3 complex regulators would play key roles during TEM. Using RNAi, we examined the role of N-WASP, WAVE2, and cortactin in TEM. Using a transwell assay we observed reduced TEM of lymphocytes through N-WASP, WAVE2 and cortactin knockdown endothelial cells. Interestingly, counting the number of transmigration events by immunofluorescence revealed a reduction in both the paracellular and transcellular routes of transmigration of lymphocytes in cortactin and N-WASP knockdown cells. However, there was no change observed for paracellular transmigration of lymphocytes in WAVE2 knockdown cells, but a decrease in the number of transcellular events. Arp2/3, WAVE2, N-WASP and cortactin are all recruited to form rings around anti-ICAM-1 coated beads, which cluster ICAM-1, form a docking structure, and initiate downstream signaling important for TEM.

In resting endothelium, WAVE2 and cortactin localize to junctions, while there is little to no localization of Arp2/3 or N-WASP at junctions. All these proteins can be strongly recruited to junctions following S1P treatment, indicating a potential role in the maintenance of the endothelial barrier. Indeed, the permeability and transendothelial resistance of the monolayers are altered following knockdown of Arp2/3 regulators, suggesting a role for each in maintaining the integrity of the monolayer. The contribution of Arp2/3 regulators to barrier integrity influences transmigration.

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Mobile Actin Densities in the Axons of Rat Sensory Neurons.

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Local and long-distance transport of cytoskeletal proteins is vital to the maintenance and growth of the axon in neurons. Though recent progress has provided insight into the movement of microtubules and neurofilaments, mechanisms underlying the movement of actin remain elusive, in large part due to rapid transition between its filament states and its diverse cellular localization and function. In this work we have undertaken the first quantitative, high-resolution characterization of actin movement in individual axons of rat sensory neurons during unimpeded growth. Cells were transfected with GFP-actin and seeded on laminin coated glass coverslips. Transient GFP-actin densities that moved bidirectionally were observed along the length of the axon. These densities were composed of filamentous actin, which our data suggest were created through bundling of actin filaments rather than through polymerization or de novo synthesis in the axon. Further, kymographic analysis was used to show quantitative and qualitative differences in the dynamics of these densities compared to other soluble proteins in the axon. Although there were long-lived densities, many arose, moved in complex bidirectional paths, and then disappeared. Despite this complexity, there was an anterograde bias in the net movement of all densities in an axon. This net displacement was consistent with rates of slow component B transport (8.5mm/day). These results suggest a novel form of actin transport. In order to test this, cells were exposed to either latrunculin or nocodazole to test the effect of altered structural integrity in the actin and microtubule cytoskeletal networks. We show that increased stability of either microtubules or actin inhibits the movement of these densities. Similarly, EHNA and BDM were used to inhibit the motor proteins dynein and myosin. Surprisingly, dynein is not responsible for the retrograde movement of actin and myosin appears to be not involved. Taken together, these results suggest a novel mechanism in which actin, unlike other proteins, is not transported along microtubule tracks. It is unclear if actin piggybacks onto short mobile microtubules or if another mechanism is at work. Regardless, this represents the first comprehensive quantitative analysis of actin movement in the axon. We have identified key players in actin transport and raise specific testable hypotheses for future study.

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***Rickettsia* motility occurs in two temporally and mechanistically distinct phases mediated by different actin nucleators.**

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Spotted fever group *Rickettsia* hijack the host actin cytoskeleton to invade, move within, and spread between host cells during their obligate intracellular life cycle. *Rickettsia parkeri* express two bacterial proteins that can activate host actin polymerization: RickA activates the host actin-nucleating Arp2/3 complex while Sca2 directly nucleates actin filaments by mimicking host formins. We sought to determine how each protein contributes to bacterial actin based-motility and actin comet tail formation. Over the timecourse of infection of cultured endothelial cells, we observed that *Rickettsia* form actin comet tails immediately after invasion (early actin tails), followed by a decrease in tail formation after 2 h post-infection, and an increase as actin tails again become numerous at 24-48 h post-infection (late actin tails). Late actin tails, as previously described, were long, composed of helical bundles of actin, and associated with polar localization of Sca2. Late tail formation was insensitive to treatment with Arp2/3 inhibitors, and

the Arp2/3 complex was not recruited to late tails. Time-lapse microscopy indicated that late actin-based movement was more rapid and followed a straighter path when compared to either early *Rickettsia* motility or *Listeria monocytogenes* actin-based motility in the same cell type. In contrast with late tails, early tails were formed only between 15 and 60 min after infection, had a distinctive short and curved appearance, and were associated with polar localization of RickA. Early tails were decorated with the Arp2/3 complex proteins Arp3 and ARPC5, and treatment with Arp2/3 inhibitors blocked early tail formation. Finally, early actin-based motility was slower and paths of movement were more curved compared to late motility. Interestingly, the parameters of early *Rickettsia* motility resemble those of *Listeria* motility, also an Arp2/3 complex-dependent process. We conclude that RickA activates the Arp2/3 complex during early actin-based motility, while Sca2 independently nucleates actin during late motility. Thus *Rickettsia* are unusual in their ability to promote two temporally and mechanistically distinct phases of actin-based motility during infection, possibly performing unique functions during the bacterial life cycle.

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Diverse strategies of actin-based motility within the *Burkholderia* genus.

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The pseudomallei group of *Burkholderia* spp. are Gram-negative facultative intracellular pathogens that use actin-based motility to facilitate their spread from one host cell to another. Previous studies identified the bacterial protein BimA as a factor that is required for actin-based motility. However, BimA sequence differences among three species from this group (*B. pseudomallei* (Bp), *B. mallei* (Bm) and *B. thailandensis* (Bt)) and previous biochemical studies of two of the three BimA proteins suggest that each ortholog mediates actin nucleation by a distinct mechanism. We sought to determine if the actin tails formed by bacteria expressing different BimA orthologs display distinct filament architectures. To enable the analysis of BimA from select agent strains (Bp and Bm), we engineered non-infectious Bt strains that lack endogenous *bimA* and instead contain *bimA* from Bp or Bm. Confocal and structured illumination microscopy of mammalian cells infected with bacteria that express BpBimA, BmBimA or BtBimA revealed actin tail morphologies unique to each ortholog. Both BpBimA- and BmBimA-expressing strains generate tails consisting of helical actin bundles similar to those observed for *Rickettsia* spp. In contrast, the BtBimA-expressing strain generated tails that were more densely packed with actin, reminiscent of *Listeria monocytogenes* actin tails. The expression of distinct BimA orthologs also resulted in different motility rates in infected mammalian cells, as well as in *Xenopus laevis* egg extract. These results support the idea that BimA from different *Burkholderia* spp. mediates actin nucleation by distinct mechanisms, and suggest that differences in polymerization mechanism and tail architecture have functional consequences for actin-based motility.

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Actin dynamics in the sea urchin egg.

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The actin cytoskeleton of the early sea urchin embryo is organized into a thin, isotropic cortex with many interspersed microvilli. And while much is known regarding the upstream and proximal regulators of actin polymerization during cell migration, it is unclear how these regulators contribute to actin organization early in development or how these regulators are themselves modulated by the cell cycle. In an effort to understand actin organization and function during the embryonic cell cycle, we employed GFP-Lifeact to follow actin filament

dynamics during the first embryonic cell division. In addition to the contractile ring, we were able to identify four distinct populations of actin filaments in sea urchin eggs, all of which were subject to changes in CDK1 activity. Upon entry into mitosis, microvilli were found to be short and dense leading up to mitotic exit, at which time they elongated at a rate of approximately 340 nm/minute. A thin submembranous population of actin filaments could also be detected that underwent a transient thinning upon anaphase onset, only to recover shortly before cytokinesis. A bright ring of nuclear actin was observed that collapsed approximately 2-3 minutes prior to nuclear envelope breakdown. Lastly, GFP-Lifeact revealed an explosive elaboration of cytoplasmic actin occurring just prior to the metaphase-anaphase transition. The polymerization of cytoplasmic actin appeared to initiate at the cell surface, moving in wave towards the cell center. However, kymograph analysis suggested that these filaments were not being transported inward, but were instead stationary, disassembling during cytokinesis. Our current efforts are focused on identifying the proximal regulators of these different actin populations, but injection of C3 transferase ablated all actin-based structures, implicating RhoA-dependent actin modulators as likely candidates.

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Pulsed accumulation of RHO-1 drives focal contractility in the early *C. elegans* embryo.

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Actomyosin contractility is a key force generator for a diverse range of developmental processes including cell polarization, cell division, cell migration and tissue morphogenesis. We are using the early *C. elegans* embryo as a model system to study a mode of contractility known as pulsed contractility, in which cycles of actomyosin assembly, contraction, and disassembly produce transient local deformations of the cell surface. Pulsed contractions occur in a variety of contexts including polarity establishment in *C. elegans*, dorsal closure in *Drosophila*, and convergent extension in *Xenopus*, but despite their widespread occurrence and apparent importance, the mechanisms that underlie their initiation and termination are poorly understood. Pulsed contractions likely represent an excitable event initiated by positive feedback, and terminated via delayed negative feedback. As a first step towards identifying key feedback mechanisms, we used dual-color near-TIRF microscopy to measure the kinetics and recruitment order of four known regulators of pulsed contractility: RHO-1 and its downstream targets ANI-1 (Anillin), F-actin and NMY-2 (non-muscle myosin II). We found that ANI-1, F-actin and NMY-2 accumulate and dissipate with nearly identical timing during pulsed contractions both in the polarizing zygote P0 and in the non-polarized AB blastomere. In contrast, a biosensor for active RHO-1 accumulates and dissipates *before* its targets in AB and *with* its targets in P0. These data suggest that pulsed contractions are driven by transient activation of RHO-1. Hypothesizing that one or more of RHO-1's downstream targets feedback to promote its further activation/accumulation, we depleted embryos of each target and measured RHO-1 dynamics. NMY-2 depletion/inactivation had no effect on RHO-1 pulses. Depletion of ANI-1 had little effect on RHO-1 or NMY-2 pulses in AB, but shortened pulse duration and increased the delay between RHO-1 and NMY-2 appearance/disappearance in P0. This suggests that neither ANI-1 nor NMY-2 are required for RHO-1 pulses, but that ANI-1 may act during polarization to more strongly couple RHO-1 and NMY-2 during individual pulses. In contrast, depolymerizing F-actin abolished RHO-1 pulses, instead producing stable co-accumulations of RHO-1, ANI-1 and NMY-2. This suggests that either F-actin assembly participates in a positive feedback loop that drives RHO-1 activation/accumulation or F-actin forms a scaffold that is required for such a feedback loop to operate. Finally, inhibiting F-actin disassembly caused an increase in RHO-1 pulse duration, suggesting that filament disassembly may be involved in pulse termination.

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Calcium influx contributes to cytoskeletal remodeling and positioning of CRAC channels at the immune synapse.C. Hartzell¹, R. S. Lewis¹; ¹Dept. of Molecular and Cellular Physiology, Stanford University, Stanford, CA

Calcium influx through Ca²⁺ release-activated Ca²⁺ (CRAC) channels is an essential signal for T lymphocyte activation and immune function. Following antigen-induced depletion of Ca²⁺ from the endoplasmic reticulum (ER), the ER Ca²⁺ sensor STIM1 accumulates at ER-plasma membrane (PM) junctions where it traps and activates Orai1, the pore-forming subunit of the CRAC channel in the PM. STIM1 and Orai1 accumulate at the immune synapse (IS) that forms between the T cell and the antigen presenting cell, raising the question of how CRAC channels become localized to the IS and whether Ca²⁺ influx through these channels influences IS formation or function. To address these questions, we stimulated Jurkat T cells on coverslips coated with anti-CD3 antibodies and monitored the organization of eGFP- or mCherry-tagged proteins at the IS using time-lapse TIRF microscopy. As T cells spread on the coverslip, actin and myosin accumulated in concentric rings around the center of the contact region, with myosin forming filamentous structures at the central boundary of the actin ring. Actin and myosin flowed continuously towards the center of the IS where they disassembled, leaving a cleared central region. ER tubules extended continuously from this central region towards the cell periphery on the tips of growing microtubules, while actin treadmilling appeared to push ER tubules back towards the center of the IS. Fluorescent puncta containing STIM1 and Orai1 accumulated in the central actin-poor region of the IS, suggesting that corraling of the ER restricts STIM1 to the center of the IS, where it can trap and activate Orai1. Interestingly, acute removal of extracellular Ca²⁺ or addition of 2-APB, a CRAC channel blocker, evoked an increase in filamentous actin at the cell footprint, inhibited actin treadmilling, and caused myosin to become disordered, indicating that Ca²⁺ influx through CRAC channels is required for cytoskeletal reorganization during IS formation. Based on these results, we hypothesize that open CRAC channels promote their own localization at the center of the IS through a feedback loop involving calcium influx, actomyosin dynamics, and ER positioning.

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GMF severs Arp2/3 complex-actin filament branches by a cofilin-like mechanism.C. A. Ydenberg¹, S. B. Padrick², M. Sweeney¹, M. Gandhi³, B. L. Goode¹; ¹Department of Biology, Brandeis University, Waltham, MA, ²Department of Biochemistry, University of Texas, Southwestern Medical Center, Dallas, TX, ³Myriant Technologies, LLC, Quincy, MA

Rapid actin assembly and disassembly are responsible for driving lamellipodial extension, propulsion of intracellular parasites, and internalization of endocytic patches. In all of these systems, actin filaments are nucleated from the sides of existing filaments by the Arp2/3 complex, a multimer consisting of two actin-related subunits and five unrelated subunits, together with a nucleation promoting factor (NPF) such as WASp. Arp2/3 complex remains bound to both the mother and daughter filaments after nucleation, creating a stable, 70°-angled, branch. ADF/cofilin plays a key role in the disassembly of all actin structures, where it contributes to disassembly by severing filaments. Previously, our group showed that GMF, a highly conserved cousin of ADF/cofilin, localizes to endocytic patches in yeast and, in vitro, binds to and inhibits nucleation by Arp2/3 complex. TIRF microscopy revealed that yeast GMF also dissociates daughter filaments from mothers, identifying GMF as the first dedicated “debranching factor”. Here, we show that these activities are conserved in murine GMF isoforms. Further, we identified functional surfaces on yeast GMF through scanning mutagenesis. From a collection of 19 mutant alleles, five compromised cell growth in a *cof1-22*

background. All 19 of the corresponding mutant proteins were purified and their activities compared. Three of the mutants defective *in vivo* also were impaired for nucleation inhibition and debranching *in vitro*. The surface defined by these mutants corresponds to a surface on ADF/cofilin that binds both G- and F-actin, the G/F-site. A fourth mutant was defective specifically in debranching, demonstrating that nucleation inhibition and debranching are separable activities. Interestingly, the debranching-specific mutant is located on a different surface of GMF, analogous to the F-site in ADF/cofilin. Chemical cross-linking suggested that GMF interacts with the Arp2, Arp3, and p40/ARPC1 subunits of Arp2/3 complex, and anisotropy revealed that GMF binds with high affinity to Arp2/3 complex ($K_d = 7$ nM). Based on these data and structural modeling, we propose that the G/F-site of GMF interacts with the barbed ends of Arp2 and/or Arp3, and that the F-site may interact with the first actin subunit in the daughter filament. This mode of interaction is similar to ADF/cofilin binding to actin filaments, in which it uses its G/F- and F-sites to interact with two different actin subunits along the long-pitch of a filament. Thus, the actin severing mechanism of ADF/cofilin may have been adapted in GMF to specifically sever Arp2/3 complex generated branch junctions.

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Actin-regulated feedback loop based on Phactr4, PP1 and cofilin maintains the actin monomer pool.

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Phactr proteins are actin binding proteins (ABPs) required for several key steps during multicellular development such as angiogenesis. The four members of this family vary in their expression pattern and little is known of their exact functions in cell biological context. Interestingly, they bind actin with their RPEL domain, which has previously been shown to regulate the nucleocytoplasmic transport of MRTF-A in response to actin dynamics. Our work describes an entirely new adaptation for the RPEL domain in Phactr4 in the regulation of actin monomer levels. The RPEL domain does not regulate the subcellular localization of Phactr4. We show that protein phosphatase 1 (PP1) and actin compete for binding to Phactr4 RPEL domain. Therefore, at low actin monomer levels Phactr4 can bind to and activate PP1, which will then dephosphorylate its downstream target cofilin, a small ABP. Cofilin then acts to restore actin monomer levels by severing and disassembling actin filaments. Consequently, this final step closes the self-regulating feedback loop, where actin monomers regulate their own number through coordinated action via Phactr4.

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Using Bio-Layer Interferometry to determine affinities between ADF and a family of putative ADF regulators.

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The actin cytoskeleton is required by all eukaryotic cells to carry out key functions such as cellular motility and intracellular trafficking of cellular components. One of the most important actin-binding proteins responsible for actin remodeling is Actin Depolymerizing Factor/Cofilin (ADF). Higher plants have moderately-sized ADF families. The *Arabidopsis* genome encodes 11 ADF genes (*ADF1-ADF11*). Current work in our lab on ADF regulation in *Arabidopsis*, led to the identification of a novel regulator of ADF1 called Irregular Trichome Branch 3 (ITB3). In addition to *ITB3*, *Arabidopsis* has 21 additional *ITB3-Like* genes (*ITB3L1-ITB3L21*). The role of these ITB3 family proteins is still largely unknown. Based on our previous results, we suspect that the

remaining ITB3 family members also function in regulating ADF1 and/or other ADF family members. Here we will report the results of determining the binding affinities of ITB3 family members to the ADF family members. The central hypothesis being tested is that the ITB3 family members will show binding specificity with respect to specific ADF family members. We will use a label free assay called Bio-Layer Interferometry to determine if different ITB3 family members bind to specific ADF family members. In addition to testing the binding of one protein to another, Bio-Layer Interferometry will allow us to characterize binding affinity (K_d), association rates (k_a) and dissociation rates (k_d) in real time. Initially, we will test four different ITB3 family proteins, ITB3, ITB3L-3, ITB3L-6, and ITBL-11 (representing all three ITB3 family clades) to ADF1. We will then extend our analysis to other ADF family members representing the four characterized ADF subclasses in Arabidopsis. The results of our analyses will help define a function for a large conserved gene family in plants, and extend our knowledge about the regulation of ADF.

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Cofilin regulation of actin realignment is essential for vascular endothelial barrier integrity during shear stress.

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Fluid shear stress (FSS) induces vascular endothelial cell and actin microfilament realignment in the direction of FSS in vitro and in vivo, yet the molecular mechanisms underlying this process are not completely understood. At least one mechanosensing mechanism has been identified involving a complex of PECAM-1, VE-cadherin, and VEGFR2. While PECAM-1 and VEGFR2 are reported to be responsible for downstream signaling, VE-cadherin functions as an endothelial cell-specific component of adherens junctions which is essential for maintaining endothelial barrier integrity. The cytoplasmic domain of VE-cadherin is known to associate with p120-, β -, γ -, and α -catenin to mediate connections to the actin cytoskeleton. Regulation of actin microfilament turnover depends in part upon the Actin Depolymerizing Factor (ADF) family of proteins, of which cofilin is a prominent player. We have determined that FSS induces p-cofilin accumulation in nuclei of vascular endothelial cells and decreased p-cofilin in the cytoplasm. Two cofilin mutants (S3A and S3D) both disrupt multiple stages of the actin realignment process indicating that continued modulation of cofilin phosphorylation is important for actin realignment. Vascular endothelial cells expressing either of the cofilin mutants were utilized to study the role of actin realignment during FSS in maintaining endothelial barrier integrity. Inhibition of dynamic changes in cofilin phosphorylation through cofilin mutants decreased barrier integrity as determined by immunofluorescent staining for VE-cadherin and β -catenin. In similar experiments, inhibition of stress kinases, JNK and p38, also interfered with actin realignment and maintenance of barrier structure. These results identify the importance of actin realignment in maintaining the endothelial barrier during FSS and the necessity of cofilin phospho-regulation in control of the actin realignment. Ongoing research in the lab is funded by NIH grant award HL54269 to LJK.

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Identification of novel nuclear actin regulating proteins using genome-wide RNAi screening.

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Actin is an essential protein that functions in various cellular activities including cell motility and cytokinesis. Actin exists either as monomers or filaments and the regulated assembly and

disassembly of actin filaments is important in the numerous functions of actin in the cytoplasm. In the nucleus, actin is central in the gene expression machinery and serves as an essential component of many nuclear complexes including the chromatin remodelling, RNA polymerases and mRNA processing and export complexes. Recent data have shown an active nuclear export and import mechanism for actin. However the dynamic regulation of nuclear actin is unclear. For example, the relationship between actin monomers and filaments in the nucleus is not known. Moreover, the form of actin involved in these critical nuclear functions remains a subject of debate. In the cytoplasm, the functions of actin are highly regulated by actin-binding proteins most of which also localize to the nucleus but the relations between actin and actin-binding proteins in the nucleus have not been studied. Here we employ RNA interference in cultured *Drosophila* cells to identify novel nuclear actin regulators and to understand the regulatory mechanism of actin in this compartment. Results from our genome-wide screen reveal several novel pathways that impinge on nuclear import of actin.

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Properties and dynamics of nuclear actin.

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Historically described as cytoplasmic, actin is also a nuclear protein that plays important roles in various steps of gene expression. Chromosome movements, transcription by the three types of polymerases but also messenger RNA processing in the nucleus are now widely recognized as actin-dependent events. Several actin-regulating proteins are located in the nucleus and some nuclear effectors of actin have been described. These include MRTF-A, a coactivator of the transcription factor SRF. Of importance, the nuclear import and export mechanisms of actin were recently clarified. However, many questions remain open about the organization of actin in the nucleus. For instance, its polymerization state, the variety and organization of actin-containing protein complexes, or the dynamics of actin interactions at the level of chromatin during gene expression processes. We have used molecular biology techniques and confocal microscopy to study the localization and the dynamics of actin in the nucleus of mammalian cells. Through these approaches, we collect clues to assess the mobility properties of actin in the nucleus and to identify the precise steps in nuclear events that involve actin. These observations aim ultimately to understand how this versatile protein performs its nuclear functions at the molecular level.

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RanGTP regulates the Arp2/3 complex to nucleate actin on chromosomes in starfish oocytes.

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Chromosome congression and segregation are known to be coordinated by the dynamic spindle microtubules in dividing cells. However, we recently showed that microtubules do not have sufficient length to capture all chromosomes scattered in the large nucleus of starfish oocytes. An actin-driven mechanism is therefore essential to deliver chromosomes to the “reaching distance” of spindle microtubules. Although this mechanism is essential to prevent aneuploidy of the egg and thus for the survival of the embryo, the underlying molecular mechanisms are completely unknown.

We tested actin nucleators that may be involved in this process, and we found the Arp2/3 complex to show a striking localization: right at nuclear envelope breakdown the Arp2/3 was recruited to the contact surface of chromosomes and nuclear envelope membranes. This

localization was confirmed by expressing a GFP-tagged Arp2/3 subunit, Arpc1-GFP, as well as by immunofluorescence using a starfish Arpc1 specific antibody. Concomitant with Arp2/3 recruitment we also observed the formation of large patches of F-actin that surrounded individual chromosomes. Treatment with an Arp2/3 inhibitor, CK-666 blocked the formation of the F-actin patches, evidencing that Arp2/3 complex nucleates these structures. In the contrary, Arp2/3 inhibition did not affect the overall structure of the F-actin the meshwork present in the cytoplasm. Therefore, we conclude that the Arp2/3 complex functions specifically to nucleate actin filaments on chromosomes and thereby forming a direct linkage between chromosomes and cytoplasmic actin filaments.

Next, we investigated how chromosomes may promote recruitment and activation of the Arp2/3 complex on their surface. The small GTPase Ran is known to form a RanGTP gradient around chromosomes during M-phase that serves as a positional marker to guide processes as such microtubule dynamics. Therefore, we tested whether RanGTP was also involved in regulating the Arp2/3 complex by injecting a large excess of the RanGTP binding protein, Importin-beta. In these oocytes Arp2/3 no longer localized around chromosomes and F-actin patches did not form. These results indicate that RanGTP generated on chromatin recruits and activates the Arp2/3 complex that nucleates dense F-actin patches directly linking chromosomes to the actin cytoskeleton. This also evidences that RanGTP not only regulates microtubule dynamics but also the actin cytoskeleton.

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CD2AP, Capping Protein and Cortactin Coordinate to Regulate Dynamic Actin Assembly at the Cell Periphery.

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Understanding the physiology of complex relationships between components of signaling pathways and the actin cytoskeleton is an important challenge. CD2AP is a membrane scaffold protein implicated in a variety of physiological and disease processes. The physiological function of CD2AP is unclear, but its biochemical interactions suggest it may have a role in dynamic actin assembly. Here we report that a critical physiological function of CD2AP is to recruit actin capping protein (CP) to the cell periphery, and that this is necessary for formation of the short branched filaments that characterize lamellipodia formation and are required for cell migration. Furthermore, we discovered that recruitment of CD2AP to the cell periphery is mediated by cortactin which can stimulate actin assembly and Arp2/3 activity. We provide the first evidence of physiological relevance for the biochemical interaction between cortactin and CD2AP and the novel conclusion that recruitment of CD2AP is a key element of cortactin function in cells. These results demonstrate how the interplay between specialized actin regulatory molecules can shape the actin cytoskeleton.

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The Role of The Capping Protein – CARMIL-1 Interaction in Migrating Cells.

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Regulation of the creation and capping of actin filament barbed ends by Rho family GTPases, is central to the control of actin cytoskeletal architecture and cellular migration. Recent work in this area has revealed a class of proteins, which can generate free, barbed ends, by uncapping capped filaments. The CARMIL family, (Capping Protein, Arp2/3 and Myosin I Linker), falls into

this class of actin filament uncapping proteins. Knockdown of CARMIL-1 leads to a reduction of cell migration speed, impaired actin assembly at the leading edge, and a loss of fibronectin-induced Rac1 activation. Here we examined the function of the CARMIL-1- Capping Protein, CP, interaction in these regulatory functions of CARMIL-1. In these studies we employed the KR987/989AA CARMIL-1 mutant, which does not interact with CP. Our results indicate CARMIL-1 's role in leading edge actin assembly is dependent on its interaction with Capping Protein. In contrast, its role in Rac1 activation and cell migration is not dependent on the CARMIL-1-CP interaction. This finding is of particular interest given that the assembly of branched actin networks, impaired in the CARMIL-1 knockdown, is believed to play an integral role in generating force for cell migration. Future studies will focus on discovering CP independent mechanisms of cell migration regulation by CARMIL-1.

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A CARMIL and V-1/Myotrophin-Dependent Regulatory Cycle for Capping Protein May Potentiate Actin Polymerization at the Plasma Membrane: Cytosol Interface.

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Cells focus robust actin assembly at the interface between the plasma membrane and the cytoplasm and suppress assembly throughout the rest of the cytoplasm. Actin polymerization is focused at the plasma membrane: cytosol interface by the site-specific recruitment/activation at this interface of the major protein complexes that promote nucleation (i.e. Arp2/3, formins). Actin polymerization at this interface is also supported by the site-specific recruitment/activation of factors (e.g. VASP, formins) that promote filament elongation by antagonizing the action of the major cellular barbed end capper, Capping Protein (CP). Another leading edge elongation factor may be CARMIL, as it exhibits two robust anti-CP activities: weak barbed end capping by the CARMIL: CP complex and the ability to uncap CP-capped filaments. Finally, the cell may also use proteins that globally sequester CP in an inactive form to promote leading edge polymerization. One such sequestering protein may be V-1/myotrophin, an abundant (cellular concentration ~8 μ M, versus ~1 μ M for CP) protein that binds CP 1:1 with an affinity of about 20 nM, resulting in a complex that has no affinity for the barbed end. Here we have explored at the biochemical level the mechanism of an exchange reaction that drives the transfer of CP from its sequestered state (CP: V-1) to its weak barbed end binding state (CP: CARMIL). These efforts yielded direct evidence of a CP: CARMIL: V-1 ternary complex, as well as an estimate of the relative contributions of this ternary complex pathway and a competitive binding pathway to the overall exchange reaction. Using both bulk actin polymerization assays and single filament imaging, we then provide evidence for the conversion of CP from its sequestered state to its weak barbed end binding state following the addition of CARMIL to assays containing the CP: V-1 complex. Finally, we show that CARMIL is recruited to the plasma membrane: cytosol interface *in vivo* and only during periods of active polymerization/edge advance. Together, these results suggest that a CARMIL-driven exchange reaction funnels inactive CP sequestered in freely-diffusing, CP: V-1 complexes into weak barbed end capping CP: CARMIL complexes specifically at advancing edges. This mechanism, together with VASP, which shields the barbed end from CP at advancing edges, may serve to enhance the growth of newly-nucleated actin filaments in a narrow zone at the plasma membrane: cytosol interface.

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The Over Expression of Myotrophin/ V-1, a Negative Regulator of Capping Protein, Enhances Actin Polymerization and Filopodia Formation in *Dictyostelium*.

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Myotrophin/V-1 is a ubiquitously expressed, ~13 kDa, ankyrin-repeat protein that binds Capping Protein (CP) 1:1 with an affinity of ~20 nM, resulting in a complex that has no affinity for the barbed end. This “CP sequestering” activity may allow V-1 to play a major role in regulating the barbed end capping activity of CP in *Dictyostelium* (*Dd*), especially given our estimates of the cellular concentrations of CP and V-1 in *Dd* (1 μM and 8 μM, respectively). Consistent with biochemical studies of mouse V-1, endogenous *Dd* CP is pulled down by GST-tagged, wild type *Dd* V-1 (WT *Dd* V-1) and is co immunoprecipitated by Flag-tagged, WT *Dd* V-1. Moreover, these interactions are abrogated when using a version of *Dd* V-1 (FBM *Dd* V-1) containing four closely-spaced point mutations that in mouse V-1 greatly attenuate its interaction with CP. Consistent with V-1’s ability to inactivate CP, the major cellular terminator of actin assembly, the over expression of WT *Dd* V-1 results in a significant elevation in total cellular F-actin content. Moreover, this increase scales positively with the degree of V-1 over expression. As expected, the over expression of FBM *Dd* V-1 does not alter cellular F-actin levels. The over expression of WT *Dd* V-1 (but not FBM *Dd* V-1) also induces the formation of actin-rich, filopodial-like structures, and this effect once again scales positively with the degree of V-1 over expression. WT-*Dd* V-1 over expression leads not only to a significant increase in the number of filopodia, but also to a significant increase in their length. Moreover, time lapse images of cells co expressing a live-cell reporter for F-actin reveal that the filopodia in WT-*Dd* V-1 over-expressing cells are less dynamic than filopodia in control cells. Together, these over expression studies suggest that V-1 regulates actin polymerization and filopodial formation *in vivo* by buffering the level of active CP (where, in the case of V-1 over expression, actin polymerization and filopodia formation are enhanced because more cellular CP is sequestered). These results are consistent with previous studies showing that CP knockdown leads to the explosive formation of filopodia in both B16F1 melanocytes and *Dictyostelium*, and that V-1 over expression enhances actin polymerization and induces finger-like surface structures in PC12D cells. Efforts to create a *Dictyostelium* cell line that lacks V-1, which should provide further confirmation of the protein’s role in regulating CP *in vivo* (these KO cells are expected to exhibit a profound decrease in cellular F-actin content because the bulk of cellular CP will now be active) are underway.

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The effects of antigen mobility and actin dynamics on B cell signaling activation and BCR cluster movement.

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B lymphocytes are an important part of the adaptive immune system and provide humoral immunity for an organism. Upon encountering a foreign antigen presented on the surface of an antigen presenting cell (APC), which demonstrates a sign of infection, the B cell rapidly spreads on the surface of the APC, binding and gathering antigen via the B cell receptor (BCR). This initiates the activation of the B cell, which then triggers changes in gene expression that enable the B lymphocyte to produce antibodies to fight infection. Although spreading is necessary for B cell activation, the mechanics of cell deformation during this process are poorly understood. It is clear that much of the change in morphology is driven by rapid rearrangement of the actin cytoskeleton, but many questions remain on how this is accomplished and how this process is dependent on the physical properties of antigen. Herein, we investigate the dependence of B

cell spreading and activation on the mobility of surface ligands. Specifically, we compare a ligand tethered to surfaces offering no lateral mobility (glass) with the same ligand tether to surfaces providing lateral mobility (planar lipid bilayers). We find that ligand lateral mobility modulates the spreading rates, the final areas, and the actin organization of spreading B cells. Spreading rates on the supported lipid bilayer are faster than on glass, however the final areas are significantly smaller. Furthermore, B cells spreading on ligand-tethered lipid bilayers exhibit more well-defined and persistent BCR clusters. This is concurrent with a much higher level of surface signaling, as assayed using phosphotyrosine staining as compared to cells spreading on antigen tethered glass surfaces. Finally, using simultaneous live cell imaging of actin and BCR during spreading, we find that the movements of BCR clusters and actin patches are highly correlated with each other during translocation of BCR clusters from the periphery towards the center of the B cell contact zone. Our results suggest that the lateral mobility of ligand regulates BCR signaling by modulating B cell spreading and BCR lateral movement and that actin dynamics play an active role in driving BCR cluster movement.

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The role of Mo25 for cytokinesis in *Dictyostelium discoideum*.

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Mo25 (morula protein 25) is a highly conserved 40 kDa scaffolding protein with 60% identity from amoeba to man. It facilitates and activates a complex of a Ste20-like kinase with downstream kinases. In humans for example, Mo25 is part of a complex with STRAD (STE20-related adaptor protein) and the kinase LKB1. Disruption of this complex causes Peutz-Jeghers-syndrome (Boudeau et al., EMBO J. 22: 5102, 2003), an inherited disease with high disposition to develop cancer (Jansen et al., Physiol. Rev. 89: 777, 2009). We generated for the first time a stable Mo25-minus cell line, using the social amoeba *Dictyostelium discoideum*. The knockout of the *mo25* gene in *D. discoideum* results in very large, multinucleated cells unable to complete cytokinesis. Growth as well as development are severely delayed in the Mo25-minus strain. Furthermore, in phototaxis assays performed with multicellular aggregates, the Mo25-minus was unable to migrate towards the light source. These findings imply that Mo25 plays an important role in cytokinesis, growth and cell polarity. By using GFP-Trap resin we identified as a binding partner of Mo25 the Ste 20-like kinase Severin kinase (SvkA) (Eichinger et al., J Biol Chem. 273: 12952, 1998), a homolog of the human MST3, MST4 and YSK1 kinases. To further elucidate the interaction of Mo25 with SvkA and other possible interactors as well as their role in cytokinesis or polarity signaling, we generated a series of GFP-Mo25 rescue constructs with distinct point mutations in its interaction surfaces and transformed these into the Mo25-minus background. With these mutations in Mo25 we hope to reduce the complex multilayer phenotype to its basic modules and identify the different downstream signaling pathways that branch off from the Mo25/SvkA kinase complex.

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Analysis and Comparison of the Effects of the Deafness-Causing D51N Actin Mutation in Yeast and Human Gamma Actin.

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The beta and gamma nonmuscle actin isoforms differ by only four biochemically similar amino acids, making separation of the two isoforms for in vitro assays exceedingly difficult. Conventional protein purification methods, including affinity chromatography methods using

tagged proteins, are not possible, as any alteration to the actin monomer interferes with the protein's activity. To circumvent this difficulty, we have used budding yeast as a model from which to isolate pure mutant actins, as yeast contain only a single actin isoform and separation of multiple isoforms is not necessary. Nevertheless, although yeast actin is 89% identical to human gamma actin, the question remains as to how closely the yeast model system can reproduce the behavior of human gamma actin. To address this question, we have expressed and purified deafness-causing D51N mutant actin from both the yeast model system and insect cells expressing human gamma actin. In vitro analysis of both actins indicates that yeast actin very accurately represents the behavior of human gamma actin at a fraction of the cost and time of production. Furthermore, characterization of D51N mutant actin reveals that the mutation increases flexibility in actin subdomain 2, increases the rate of polymerization, and reduces the rate of ATP hydrolysis and subsequent nucleotide release. In yeast cells, the D51N mutation also causes mitochondrial defects, reduced cable formation, and depolarized actin patches, strongly suggesting that the mutation causes significant regulatory defects within an in vivo system in addition to directly affecting polymerization dynamics.

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Quantitative Analysis of Approaches to Measuring the Cooperativity of Phosphate Release in Polymerized Actin.

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We use simulations of actin polymerization/depolymerization and phosphate cleavage/release to treat several experimental probes of actin dynamics that are affected by the cooperativity of phosphate release. The simulations treat both actin and phosphate dynamics stochastically using finite ensembles of filaments. The correlations between release on nearest-neighbor sites can be varied continuously between the random and vectorial limits. Using this model, we simulate phosphate release time-courses from polymerization and co-polymerization experiments of ATP- and ADP-actin, including the effects of variations in filament number concentration. We also treat single-filament depolymerization time-courses. For each value of the cooperativity parameter, we use rate constants which optimize the fit to experiment. We find that highly cooperative models are consistent with the experimental data. We also find that some types of experiments that are believed to provide strong constraints on the cooperativity of actin hydrolysis models provide much weaker constraints.

Microtubule Dynamics and Its Regulation I

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De novo centriole emergence does not initiate canonical interphase microtubule organisation in the mouse blastocyst.

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Centrioles form the central core of the centrosome, the major microtubule organising centre (MTOC) in most animal cells. In interphase, the centrosome organises a polarized microtubule (MT) array in which MT minus ends are proximal to the centrosome and plus ends are oriented towards the cell periphery. In contrast, oocytes of most species lack centrioles. In mouse embryos, new centrioles are generated by *de novo* formation during late pre-implantation development. How centriole emergence impacts MT organisation in this setting is not known.

Here we use a GFP-CETN2 transgenic mouse to study the dynamics of centriole emergence, and confirm that centrioles are first assembled *de novo* at the blastocyst stage in both the trophoectoderm, and the inner cell mass - the two major cell lineages of the blastocyst. Centriole biogenesis is preceded by the emergence of γ -tubulin foci at the morulae stage of embryogenesis.

To investigate the impact of centrosome and centriole emergence on MT organisation we performed detailed analyses of MT layout throughout embryogenesis. In early pre-implantation embryos, we find that MTs extend throughout the cytoplasm and are enriched at the cortex and around the nucleus. These early embryos do not possess an obvious dominant site of MT nucleation, which was expected given the absence of centrioles and centrosomes at this developmental stage. Unexpectedly however, later embryos also appear to lack a dominant MTOC, and MTs are similarly enriched at the cortex and nuclear membrane as opposed to at the centrioles. This arrangement is in stark contrast to the distinctive astral MT array which emanates from the centrioles in somatic cells.

To further analyse MT architecture in embryos we used EB1-GFP, which binds to growing MT plus ends and therefore allows MT growth trajectories to be visualised and mapped. Kymograph analysis revealed no difference in MT growth velocity between acentriolar and centriolar embryos. Furthermore, whereas EB1-GFP comets predominantly radiate from a single central focal point in cultured somatic cells, a dominant centre of MT organisation is not evident in embryos of any developmental stage, including centriole-containing blastocysts. Therefore, our experiments reveal that embryos adopt an atypical mode of MT organisation throughout pre-implantation development, despite the emergence of centrosomes and centrioles in morulae and blastocysts respectively. The mouse embryo is thus a rare example of a mammalian cell in which centrioles fail to serve as the dominant MTOC.

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Gamma-tubulin is required for *Drosophila* neuronal microtubule organization.

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The polarization of neurons into axons and dendrites is essential in building functional neuronal circuits. The arrangement of microtubules is an important factor in neuronal polarity, as microtubules are used for long-range trafficking of vesicles and other cargo, cytoskeletal structure, and synaptic growth. *Drosophila* provides a good model for the study of neuronal microtubule polarity due to its simple microtubule arrangement. In *Drosophila* neurons, microtubules have opposite orientation in axons and dendrites: axons have microtubules with plus ends distal to the soma, while dendrites have minus ends distal to the cell body. We have recently determined that the centrosome, an organelle that is the primary microtubule nucleator and organizer in dividing cells, does not contribute to neuronal microtubule organization in *Drosophila*, and are now looking at other factors that may be involved. A likely candidate is gamma-tubulin, as it is the core nucleation protein that is essential for acentrosomal microtubule nucleation. Thus, the objective of this study is to determine whether gamma-tubulin plays an integral role in establishing the differing microtubule orientations seen in axons and dendrites. To examine the function of gamma-tubulin, immunostaining and fluorescence localization experiments were performed, and microtubule dynamics were analyzed in gamma-tubulin mutants using the plus-end tracking protein EB1. Our preliminary studies indicate that gamma-tubulin is localized at dendrite branch points, which have previously been shown to house

proteins that affect microtubule polarity. Meanwhile, in axons, gamma-tubulin surprisingly showed distinct localization at the presynapse. Additionally, we saw that gamma-tubulin mutant animals had disrupted microtubule dynamics as well as altered microtubule orientation in axons and dendrites. In loss-of-function mutants, microtubule polarity became more mixed in dendrites, and the number of growing microtubules was decreased. In gain-of-function mutants, microtubule polarity was mixed in both axons and dendrites, and microtubule dynamics were increased in the axon. Based on these results, we predict that overactive gamma-tubulin contributes to the changes in microtubule orientation by nucleating extra microtubules in the wrong orientation. Also, dendrite microtubule polarity may require local microtubule nucleation, while axon microtubule polarity may require proper regulation of gamma-tubulin. We conclude that gamma-tubulin is a prime player in neuronal microtubule polarity, and that tight regulation of microtubule nucleation is crucial for correct neuronal microtubule polarity.

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Specific in vivo labeling of tyrosinated alpha-tubulin and measurement of microtubule dynamics using a GFP-tagged and cytoplasmically expressed recombinant antibody.

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GFP-tagged proteins are used extensively as biosensors for protein localization and function, but the size of GFP (27 kD) could interfere with protein-protein interactions within the cell. An alternative to direct protein labeling is to use intracellular expression of recombinant antibodies (scFvs), but these antibody fragments are typically insoluble in the reducing environment of the cytosol. We developed a large synthetic human scFv library that is optimized for stable recombinant antibody expression in mammalian cytosol (Philibert et al. 2007) and isolated an anti-tubulin scFv, 2G4, which is soluble when expressed as a GFP-fusion protein. Here we report the use of this GFP-tagged scFv to label microtubules (MTs) in living cells. GFP-alpha tubulin is a useful tracer for study of MT dynamics, allowing comparison between GFP-tubulin and 2G4-GFP as MT labels. We found that 2G4-GFP localized uniformly along the lengths of MTs and did not disrupt binding of EB1, a protein that binds MT ends and serves as a platform for binding by a complex of proteins regulating MT polymerization. MT dynamic instability, measured by tracking 2G4-GFP labeled MTs, was nearly identical to that measured previously in cells expressing GFP-tubulin or microinjected with rhodamine-tubulin (Rusan et al., 2001; Ringhoff and Cassimeris, 2009). Fluorescence recovery after photobleaching demonstrated that 2G4-GFP turns over rapidly on MTs ($t_{1/2} \sim 4$ s), similar to the turnover of photobleached tau-GFP ($t_{1/2} \sim 7$ s) and to turnover rates published previously for several other MT-associated proteins. These data indicate that 2G4-GFP binds relatively weakly to MTs, and this conclusion was confirmed in vitro. Purified 2G4 partially co-pelleted with MTs but a significant fraction remained in the soluble fraction, while a second anti-tubulin scFv, 2F12, was almost completely co-pelleted with MTs. In cells, 2G4-GFP localized to most MTs, but did not co-localize with MTs composed of de-tyrosinated alpha-tubulin, a post-translational modification associated with non-dynamic, more stable MTs. Immunoblots probing bacterially expressed tubulins confirmed that 2G4 recognized alpha-tubulin and required tubulin's C-terminal tyrosine residue for binding. Thus, a recombinant antibody with weak affinity for its substrate can be used as a specific intracellular tracer that can differentiate between unmodified and post-translationally modified forms of a protein.

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Mechanistic Significance of Phospho- α -tubulin in Microtubule Dynamics.

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Protein kinase C α (PKC α) serves diverse biological functions through phosphorylation of its various cellular substrates. A role for PKC α in the etiology of breast cancer metastasis arises from elevated PKC activity that has been observed in some metastatic human breast tumors. Previous work from this laboratory showed that engineered over-expression of PKC α in non-tumorigenic, non-motile human breast epithelial MCF-10A cells resulted in aggressive cell movement. A recently discovered PKC α substrate in these cells, α -tubulin, which is a structural component of microtubules (MTs), was shown to undergo intracellular phosphorylation by PKC α and consequently to engender cell motility. The present work addresses the impact of α -tubulin phosphorylation on MT dynamics in MCF-10A cells. Live-cell imaging of cells expressing EGFP-EB1, a MT plus-end binding protein, showed a significant increase in the number of MT growing ends near the cell periphery following treatment with DAG-lactone, a cell-permeable PKC activator. Quantitative analysis of MT dynamic parameters in DAG-lactone-treated cells transfected with EGFP- α -tubulin showed a dramatically increased MT growth rate and duration of the growth phase, as well as increased frequency of rescued catastrophes, all indications of enhanced MT stability. Further analysis was conducted in MCF-10A cells expressing an α 6-tubulin mutant in which the PKC phosphorylation site (Ser-165) was rendered as pseudo-phosphorylated (Ser \rightarrow Asp) or phosphorylation-resistant (Ser \rightarrow Asn). Analysis of MT dynamics in cells expressing the pseudo-phosphorylated mutant showed a significant increase in the average growth rate and dynamicity, similar to parental cells treated with DAG-lactone. However, MT dynamics recorded for cells expressing the phosphorylation-resistant mutant were similar to untreated parental cells. Immunocytochemistry performed with MCF-10A cells transfected with myc-tagged wildtype α -tubulin and treated with DAG-lactone showed enhanced incorporation of the myc signal into MTs, especially near the cell periphery, as compared with untreated cells. This enhancement was reproduced in untreated cells expressing myc-tagged pseudo-phosphorylated α 6-tubulin. These findings support a model in which PKC engenders motile behavior of human breast cells by phosphorylation of its substrate α -tubulin, thereby leading to altered MT dynamics that favor MT elongation. This work provides a foundation for exploring phosphorylated α -tubulin as a biomarker for metastatic breast disease and as a strategic target for chemotherapeutic intervention. [Funded by NIH CA125632].

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Regulation of microtubule mechanics by intraluminal acetylation.

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Nearly all studies of microtubules to date have focused on their outer surface. Currently, the only molecular event known to take place inside the microtubule lumen is acetylation of alpha-tubulin on lysine 40, a major post-translational modification with unknown biochemical consequences. In this context, our recent identification of the tubulin acetyltransferase TAT1 provides a molecular handle on the biology of the microtubule lumen. Interestingly, TAT1 is encoded by the mec-17 gene in nematodes and mec-17 mutants are defective for mechanosensation, thus linking intraluminal acetylation to the sensing of mechanical perturbations.

Surprisingly, we find that TAT1 can acetylate microtubules at any point along their length in vitro. Previously, Atomic Force Microscopy of unfixed microtubules had uncovered openings in bent but not straight microtubules. Bending may thus generate enough tensile stress on the

outer edge of the tube to overcome the rather weak lateral interactions between protofilaments and create an opening.

Intriguingly, long-lived microtubules in mammalian cells are both highly acetylated and highly bent. The very short radius of curvature found on long-lived microtubules is paradoxical when one considers the low flexural strength and high rigidity of pure microtubules. This disconnect suggests the existence of cellular mechanisms that locally decrease the flexural rigidity of long-lived microtubules. To address whether acetylation may be part of such a mechanism, we visualized long-lived microtubules in fibroblasts by treating them with nocodazole for 40 min. Surprisingly, nocodazole-resistant microtubules appeared highly fragmented in TAT1-depleted cells treated when compared to control-treated cells. Furthermore, time-resolved imaging of nocodazole-resistant microtubules shows that the frequency of microtubule breakage in TAT1-depleted cells is significantly elevated when compared to control-depleted cells. Based on these findings, we posit that intraluminal acetylation protects microtubules from breakage by locally decreasing flexural rigidity. In support of this model, physical or pharmacological release of cell tension leads to the recovery of long-lived microtubules in the absence of TAT1. Thus, acetylation may modify the mechanical properties of microtubules to prevent rupture under flexure.

We propose a two-step adaptive model for the mechanical stabilization of microtubules where bending triggers sidewall breathing to let TAT1 enter the lumen. Subsequent acetylation locally modifies the mechanical properties of the microtubule to protect it against flexural breakage.

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Posttranslational Incorporation of 3-Formyltyrosine in α -Tubulin Inhibits Cell Proliferation without Altering Microtubule Morphology.

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The C-terminus of α -tubulin undergoes a cyclic posttranslational modification of tyrosination/detyrosination. The genetically encoded C-terminal tyrosine is removed by a specific enzyme, tubulin tyrosine carboxypeptidase, and is reintroduced by another specific enzyme, tubulin tyrosine ligase. This cycle generates pools of tyrosinated and detyrosinated tubulin and regulates a number of microtubule functions. Here, we report that endogenous enzymes can reversibly incorporate a tyrosine derivative, 3-formyltyrosine (3fY), in the C-terminus of α -tubulin instead of tyrosine. Cellular incorporation of 3fY is completely restricted to α -tubulin even after extended periods of time. The reactivity of 3fY with hydrazine fluorophores then permits specific labeling of α -tubulin. The present study explores the implications of this phenomenon in human prostate cancer (PC3) cells. We find that 3fY inhibits PC3 cell proliferation in a cell cycle independent manner. This inhibition can be partially reversed by withdrawal of 3fY from the culture medium. Interestingly, despite significant growth inhibitory effects, no alteration in the cell morphology or microtubule network was observed during cycles of 3fY ligation and removal. Thus, incorporation of 3fY permits the simple fluorescent labeling of α -tubulin, but also interferes with some microtubule function essential for progression throughout the cell cycle. Identification of this microtubule function will be the focus of future experiments.

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Microtubule-associated proteins control microtubule nucleation from templates*M. Wieczorek¹, S. Bechstedt¹, G. Brouhard¹; ¹Biology, McGill University, Montreal, QC, Canada*

Cells nucleate new microtubules continuously, but the kinetics of nucleation is arguably the least understood aspect of microtubule dynamics. While microtubules can form spontaneously in vitro, γ -tubulin ring complexes (γ -TURCs) serve as template structures for microtubule nucleation in cells. Many microtubule-associated proteins (MAPs) have also been implicated in microtubule nucleation, but their direct role in the templating process is unclear. To determine the role of MAPs in templated nucleation, we have developed a quantitative assay that allows us to study the kinetics of microtubule nucleation from centrosomes (γ -TURCs), axonemes, and single microtubule templates using purified components. We found that templated nucleation is highly cooperative and that this cooperativity is independent of the type of template used. Performing the assay in the presence of different MAPs indicates that both catastrophe frequencies and growth rates influence the ability of a template to nucleate microtubules. Certain MAPs also changed the strength of cooperativity in the nucleation process. We directly demonstrate that MAPs can both enhance and inhibit templated nucleation by distinct mechanisms. These findings provide a means by which cells can control the numbers and locations of their microtubules throughout development and the cell cycle.

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The importance of Augmin in microtubule generation beyond cell division.*J. W-C. Chen¹, J. G. Wakefield¹; ¹University of Exeter, Exeter, United Kingdom*

Augmin, a conserved hetero-octomeric microtubule (MT) associated protein complex originally characterized in *Drosophila*, has recently been shown to be at least as important as centrosomes in generating MTs that contribute to the mitotic spindle. The current model is that it does so by recruiting the MT nucleator γ -TuRC to pre-existing MTs, where it can facilitate the formation of additional MTs. However, whether Augmin has a role in generating MTs outside of cell division is currently unknown.

During *Drosophila* oogenesis, extensive MT networks are generated for inter- and intracellular transport of materials. It is with these networks that various maternal mRNA are transported to specific areas within the oocyte. Centrosomes, although playing a role in generating a portion of these MTs, have been shown to be dispensable. We hypothesized that Augmin, being a generator of MTs, has a role in facilitating the oocyte MT network. To test this, we have been analyzing MT organization in oocytes carrying a null mutation in the Augmin subunit, *Wac*.

At stage 6 of *Drosophila* development, the MT-dependent transport of *gurken* mRNA to the posterior pole, followed by its translation into Gurken protein, signals the destabilization of posterior MT organizing centres (MTOCs). The drastic re-organization of the MTs releases the anchored nucleus, facilitating its re-distribution to the dorsal-anterior corner. Consequently, during stage 8-9, the MT network is arranged to an anterior-posterior gradient. By stage 10, distinct MT bundles are formed.

We show that *wac* mutant oocytes fail to anchor, and properly localize Gurken protein at stage 6. Interestingly, the nucleus is still released from the posterior pole, and migrates to the anterior-dorsal corner by stage 8. Although MTs are present at stages 8 and 9, the gradient normally formed at this time is absent. However, those MTs present still show bundling at stage 10. One interpretation is that Augmin generates additional MTs during stages 6, 8, and 9, contributing to the overall MT network formed by other mechanisms. We are currently investigating this further by analyzing MT dynamics during oogenesis in α -Tubulin-GFP expressing oocytes lacking Augmin, and those lacking both Augmin and other MT nucleators.

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Linker scanning mutagenesis of yeast microtubule nucleating proteins Spc97 and Spc98.

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The mitotic spindle is a large structure responsible for the regulation and organization of chromosome segregation. Microtubules are a major component of the mitotic spindle and play several key roles during mitosis—mediating chromosomal movements, stabilizing spindle structure, and positioning the spindle within the cell. It has been shown that γ -tubulin is highly conserved and essential for microtubule nucleation in all organisms. Characterization of γ -tubulin revealed that it is part of a larger complex. In yeast, the γ -tubulin small complex is sufficient for nucleation and is comprised of two γ -tubulin molecules and one copy each of Spc97 and Spc98. Both Spc97 and Spc98 are essential, suggesting they play supplementing roles to γ -tubulin during microtubule nucleation. Despite our advances in understanding microtubule function, dynamics and nucleation, purified γ -tubulin complexes fail to efficiently recapitulate nucleation *in vitro*, suggesting that additional regulatory processes activate the nucleating complex. The cryo-EM structure of the yeast γ -tubulin complex reveals 13 γ -tubulins in a ring as expected for a template for the 13-protofilament microtubule found *in vivo*. However, the spacing between γ -tubulins is offset from a perfect template. We have performed a saturating linker-scanning mutagenesis to define the regions of Spc97 and Spc98 required for the small complex to function *in vivo* and promote the formation of a microtubule. *In vitro* transposition reactions generated a library of random 15 base pair insertions in each gene, with approximately 89% coverage. Approximately 60,000 transposition events were screened for function. For both Spc97 and Spc98, about 4% of the screened colonies contained lethal mutations and 0.02% contained temperature-sensitive mutations. Sites of lethal insertions were identified and mapped by high-throughput sequencing. Temperature sensitive mutants were inspected by fluorescence microscopy and *in vitro* nucleation assays to reveal several nucleation hyperactive and deficient mutants. When combined with high-throughput sequencing, linker-scanning mutagenesis was a powerful approach to identify essential regions of the γ -tubulin complex and provide novel temperature-sensitive mutants with specific defects in microtubule nucleation.

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Depletion of the β III-tubulin isotype increases the affinity and activity of ³H-eribulin for brain tubulin and microtubules: a role for β III-tubulin in reduced neuropathy?

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To explore the role of β III-tubulin in the reduced incidence of peripheral neuropathy associated with eribulin mesylate as compared with other microtubule (MT) targeted drugs in mice, we analyzed ³H-eribulin binding to purified unfractionated (~ 25% β III-tubulin), and β III-depleted bovine brain tubulin and to MTs composed of unfractionated and β III-depleted tubulin. β III tubulin was depleted to undetectable levels after passing unfractionated tubulin through a column of CNBr Sepharose 4B covalently coupled to anti- β III tubulin antibody TuJ1. Eribulin binding was determined with ³H-eribulin (0.5 μ M - 80 μ M, 25 min, 30 °C) after separation of bound from free drug by Zeba spin columns. The ³H-eribulin bound to both unfractionated and to β III-depleted tubulin (1 mol eribulin/mol tubulin). However, the affinity of ³H-eribulin for β III-depleted tubulin (K_D , 1.4 μ M) was ~ 2-fold stronger than its affinity for unfractionated tubulin (K_D , 2.5 μ M). The stoichiometry and affinity of ³H-eribulin binding per MT to unfractionated MTs and

to β III-depleted MTs was determined by assembling MTs to steady state (30 μ M tubulin plus 3 μ M tau to stabilize the MTs, 35 °C, 30 min), incubating them with 3 H-eribulin (0.1-14 μ M), and separating 3 H-eribulin-bound MTs from unbound 3 H-eribulin by sedimentation through 30% glycerol cushions (2h, 32 °C, 200,000 x g). 3 H-eribulin bound to β III-depleted MTs ~ 2-fold more strongly ($K_D = 1.5 \mu$ M; ~ 7 (\pm 6) molecules of eribulin/MT) than to MTs prepared from unfractionated tubulin ($K_D = 3.9 \mu$ M, ~10 (\pm 5) molecules of eribulin/MT), indicating that the presence of β III-tubulin diminishes eribulin binding to brain MTs. In agreement with these results, eribulin suppressed the growth rate and dynamicity of unfractionated MTs, but did not appreciably affect any other dynamic instability parameter. However, eribulin more strongly suppressed growth of β III-depleted MTs as well as other parameters, including the shortening rate and the catastrophe/rescue frequencies. The reduced activity of eribulin on tubulin/MTs containing β III-tubulin supports a hypothesis that the normal presence of β III-tubulin in neuronal cells may play a role in the reduced peripheral neuropathy with eribulin as compared with other MT-targeted agents in mouse models. Supported by a grant from Eisai Inc.

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TubZ filament morphology is regulated by nucleotide state.

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Low copy number plasmids have developed complex systems to ensure that the plasmid DNA faithfully segregates during bacterial cell division, including the use of actin-like or tubulin-like cytoskeletal elements. Several bacterial tubulin homologs involved in plasmid segregation have been discovered through the aid of phylogenetic analysis. One such homolog, TubZ-Bt, is found on the pBtoxis virulence plasmid in *Bacillus thuringiensis*.

To gain insight into the mechanism by which TubZ-Bt is able to segregate plasmids, we sought to understand the impact of different nucleotide states on TubZ filament formation, structurally and kinetically, using electron microscopy and biochemistry.

In vitro, we observe that TubZ-GTP forms almost exclusively four-stranded filaments, whereas TubZ filaments formed with catalytically compromised protein or non-hydrolyzable GTP analogs are predominantly two-stranded. These results show that the filament conformation is linked to nucleotide state.

In order to further understand this morphological change, we have determined the cryo-EM structure of TubZ-GTP filaments to ~7Å resolution by iterative helical real space reconstruction (IHRSR), as well as the structure of TubZ-GTP γ S 2-stranded filaments to ~15Å. Mutational analysis of interfacial residues suggested by these structures implicate that an early form of the two-stranded filaments are necessary for the formation of the four-stranded filaments.

Additionally, the extended C-terminal tail of TubZ-Bt appears to be necessary for the formation of both filament morphologies. This extended C-terminus has previously been shown to interaction with the TubRC complex which connects TubZ-Bt with the pBtoxis DNA (Ni, L., et al. PNAS 2010). This suggests that *in vivo* TubZ-Bt kinetics and morphology may be regulated by interaction with this complex.

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Mitotic motor CENP-E cooperates with PRC1 in temporal control of central spindle assembly.

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Mitotic motor CENP-E plays key roles in chromosome congression and spindle checkpoint satisfaction. We have recently identified and characterized syntelin, a novel selective CENP-E inhibitor (Ding et al., 2010. Cell Res. 20, 1386-1390). Cells treated with syntelin progress through interphase, enter mitosis normally with a bipolar spindle and lagging chromosomes around the poles. Syntelin is an allosteric inhibitor which tightens CENP-E-microtubule interaction by slowing inorganic phosphate release. To delineate the role of CENP-E in reorganization of interpolar microtubules into an organized central spindle, metaphase synchronized cells were exposed to syntelin and other mitotic motor inhibitors. Syntelin does not perturb interpolar microtubule assembly but abrogates the anti-parallel microtubule bundle formation. Real-time image shows that CENP-E inhibited cells undergo central spindle splitting and exhibit chromosome instability phenotypes. Interestingly, inhibition of CENP-E did not alter the interaction between CENP-E and PRC1 but perturbed temporal assembly of PRC1 to the midzone. Surprisingly, inhibition of CENP-E perturbs the temporal control of PRC1 dephosphorylation which led to a persistent phosphorylation of PRC1 and an inhibition of central spindle assembly. These findings reveal a previously uncharacterized role of CENP-E motor in temporal control of central spindle assembly.

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Knockdown of microtubule polymerase XMAP215 can increase microtubule polymerization in *Xenopus laevis* neuronal growth cones.

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Proper neural connections, essential to nervous system function, depend upon precise navigation by the neuronal growth cone. A fundamental problem in growth cone cell biology is how guidance pathways are integrated to coordinate cytoskeletal dynamics, thus driving accurate steering. To address this question, we focus on the plus-ends of microtubules (MTs), which explore the growth cone periphery and play a key role in growth cone steering. MT plus-end dynamics are regulated by a conserved family of proteins called 'plus-end-tracking proteins' (+TIPs). Yet, it is still unclear how +TIPs interact with each other and with plus-ends to control MT behavior, especially in the developing nervous system. Here, we address this question by investigating the function of the traditional MT polymerase, XMAP215, during neurite outgrowth. When XMAP215 function is reduced, neurite outgrowth is severely compromised. By using automated, quantitative imaging analysis following acquisition of high-resolution live-imaging data of tagged +TIPs within cultured *Xenopus laevis* growth cones, we were surprised to discover that partial knock-down of XMAP215 leads to a 30% increase in MT plus-end velocity. This is unexpected given that XMAP215 is known to have MT polymerase activity. There are at least two possible mechanisms by which this increase in MT plus-end velocity may occur in growth cones, either by affecting the polymerization/depolymerization rate directly, or by changing MT translocation rates through the regulation of MT interaction with other cellular structures. We have tested between these two models using quantitative fluorescence speckle

microscopy, and we find that MT translocation rates are indistinguishable between controls and XMAP215 knockdown. Thus, reducing the function of the traditional MT polymerase XMAP215 leads to increased rates of MT polymerization in cultured *Xenopus* growth cones. We are currently investigating the mechanism by which this occurs, as well as how these changes in growth cone MT polymerization correlate with the reduced axon outgrowth phenotype that we also observe.

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p150^{Glued} regulates neuronal microtubule assembly through tandem tubulin-binding domains.

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Regulation of microtubule assembly is critical for cellular survival, particularly in large, post-mitotic cells such as neurons, where disruption of microtubule dynamics has been shown to contribute to neurodegenerative disease. We have previously suggested that the dynactin complex, best known as an activator of cytoplasmic dynein, may also participate in the regulation of microtubule dynamics through its p150^{Glued} subunit. Here, using solution biochemistry and direct visualization of microtubule assembly dynamics with TIRF microscopy, we demonstrate *in vitro* that p150^{Glued} promotes microtubule formation via multiple activities; it catalyzes nucleation, increases the microtubule polymerization rate, and inhibits catastrophe. We show that although either the N-terminal CAP-Gly or basic domains of p150^{Glued} are sufficient for binding to microtubules, both are necessary in tandem to stably bind soluble tubulin dimers and promote assembly. Splicing of p150^{Glued} is tissue-specific and only the longest p150^{Glued} spliceform, expressed primarily in the nervous system, includes both the full CAP-Gly and basic domains. To test the hypothesis that this isoform modifies microtubule dynamics in cells, we first depleted p150^{Glued} in a non-polarized cell line, and found no effect on microtubule dynamics. In contrast, knockdown of p150^{Glued} in primary neurons leads to a dramatic increase in microtubule catastrophe. Strikingly, a single point mutation in p150^{Glued}, causal for an early-onset Parkinson syndrome, is sufficient to completely abolish activity *in vitro* and in neurons. Together, our data reveal that dynactin plays a crucial role in promoting microtubule stability in neurons, and that disruption of this activity may lead to neurodegenerative disease.

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Kinesin 13s are key regulators of neuronal microtubule polarity.

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Neurons are highly specialized cells that transmit information over long distances by way of polarized processes known as axons and dendrites. One of the major cell biological differences between axons and dendrites is the arrangement of microtubules. In *Drosophila* dendritic arborization (da) neurons, which are multipolar neurons with a single axon and many dendrites, dendritic microtubules are minus-end-out and axonal microtubules are plus-end-out. While microtubule polarity is likely to play a key role in all aspects of neuronal polarity, it is not known how a single cell can establish two compartments with completely different arrangements of microtubules. In a screen to identify regulators of microtubule polarity in da neurons, we identified Kinesin 13s, which are known to be important for spindle organization in mitosis, as regulators of neuronal microtubules.

Kinesin 13s are a family of microtubule-based motors that are known to be microtubule depolymerases in mitotic and interphase cells¹; however, there have been very few studies of their roles in neurons. In *Drosophila* there are three Kinesin 13s, and we found that two of these,

Klp59C and Klp10A, play important roles in da neurons. Reduction of Klp59C caused severe mixing of microtubule polarity in dendrites as well as dendrite branching defects. Reduction of Klp10A had a comparably mild effect in dendrites, and instead showed the greatest phenotype in axons, changing both microtubule orientation and overall number of polymerizing microtubule plus ends. We therefore propose that Klp59C is a major regulator of dendritic microtubules and Klp10A is a major regulator of axonal microtubules. In keeping with this idea, antibodies that recognize Klp10A show more intense staining of axons than dendrites. We conclude that Klp59C and Klp10A act differentially on dendritic and axonal microtubules, and their localized function may be one mechanism that allows neurons to establish different arrangements of microtubules within axons and dendrites.

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Microtubule Length Regulation by the Kinesin-8 Motor Protein: Capping and Polymerizing Enzymes.

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In the cell, size and dynamics of filaments are tightly controlled. One important player in microtubule length regulation is the microtubule depolymerizing motor protein kinesin-8. The accumulation of this protein along microtubules enables a differential regulation of microtubule length. Cellular mechanisms of length regulation however, are still obscure due to the complicated mechanochemical dynamics of microtubules and the presence of a multitude of microtubule associated proteins. We extend a simple mathematical model based on what is known for the microtubule depolymerizing motor kinesin-8, to account for microtubule growth factors, like the microtubule polymerizing enzyme XMAP215. We predict that microtubule polymerizing enzymes significantly enhance the range over which microtubule dynamics can be regulated. Further, our findings shed light on the functioning of microtubule depolymerization by kinesin-8. We show that depending on microscopic details of kinesin-8's depolymerization mechanism, it's length regulatory function is reminiscent of dynamic instability.

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The novel Joubert syndrome protein KIF7 is involved in the regulation of microtubular dynamics, cellular polarity and cell cycle progression.

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Joubert syndrome (JBTS) is a rare mostly autosomal recessively inherited developmental disorder characterized by a specific brain malformation with various additional pathologies. JBTS results from mutations in at least 17 different genes, all of which play a role in the formation or function of primary cilia. Primary cilia are essential for vertebrate development, and mutations affecting this organelle underlie a large group of diseases referred to as ciliopathies. We recently identified mutations in *KIF7* as a cause of JBTS in a consanguineous family. *KIF7* belongs to the family of kinesin motor proteins and is a known regulator of Sonic Hedgehog signaling. Knockdown of *KIF7* in retinal pigment epithelial (RPE) cells affects the structure of

cilia, centrosomes and the Golgi network, and is associated with alterations in cell shape, rendering the cells larger and no longer able to align in a parallel manner as wild type RPE cells do, suggesting defects in cell polarity. Consistently, KIF7 was found to interact with the polarity complex protein PAR3. We have now subsequently analyzed the cell biological function of KIF7 in more detail. In migration assays over 48 hours *KIF7* knockdown cells presented with impaired directed cellular growth likely due to a defect in cell cycle progression. FACS analysis revealed a considerable G2/M arrest in U2OS cells. Additionally, we found KIF7 to interact with the histone deacetylase HDAC6 and observed increased tubulin acetylation after knockdown of *KIF7*, which may impair correct transport of polarity complex proteins and could be causative for the additional cellular phenotypes observed in *KIF7* knockdown cells. Changes in microtubule stability might function as underlying disease mechanisms impacting ciliary function, cellular polarity and cell cycle progression.

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Effect of Laulimalide on Microtubule Dynamics during Cell Division.

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Small molecule inhibitors of microtubules are valuable both as tools for studying the biology of microtubules and in the development of anti-tumor agent. Laulimalide is one such recent molecule isolated from the sea-sponges and has taxol like properties. It inhibits cell-cycle proliferation and is highly effective in taxol resistant cells. In contrast to taxol, it binds to a different site at the exterior of β -tubulin near the charged c-terminal tail and stabilizes the microtubules in vitro. Cells treated with laulimalide show no evidence of microtubule bundling in interphase. Live cell analysis reveals that cells treated with laulimalide enter mitosis but chromosomes scatter from the metaphase plate. HeLa cells incubated with low dose of laulimalide have high mitotic index and lead to multiple ring shaped spindle poles along with defects in chromosome alignment and segregation. In order to understand the molecular pathways that are affected by laulimalide treatment, we did an extensive literature search looking for proteins, depletion of which creates multiple ring shaped spindle poles. We found that cells lacking microtubule associated proteins (MAPs), ch-TOG or HAUS, have multiple spindle poles that are ring-shaped. We found that ch-TOG and HAUS were both mislocalized in HeLa cells treated with laulimalide. This result implicates the involvement of ch-TOG and HAUS in the laulimalide phenotype. Immunofluorescence and RNAi studies reveal that these proteins are localized at the spindle poles and required for their maintenance. Also, we show that in cells treated with laulimalide, Eg5 kinesin motor protein is required to generate the multipolarity. Currently, we are using quantitative immunofluorescence and fluorescence recovery after photobleaching (FRAP) to study the differences in spindle microtubule organization caused by laulimalide versus taxol in interphase and mitotic PtK2 cells. This will allow us to understand the effect of laulimalide on microtubule dynamics in cells.

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New Insights into the Inhibition of Microtubule Dynamics and Spindle Formation by the Novel Microtubule Stabilizer Taccalonolide AJ.

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Taccalonolide AJ causes cancer cells to arrest in mitosis due to the formation of abnormal, multipolar mitotic spindle asters. The morphology of these structures differs from those initiated

by paclitaxel. At the concentration that causes maximal G₂/M accumulation, paclitaxel initiated the formation of 2-3 relatively large diffuse spindle asters, while 5-7 compact spindle asters were observed in taccalonolide AJ-treated cells. Studies were conducted to determine if the mechanism of aberrant spindle aster formation differed between taccalonolide AJ and paclitaxel. High-content live-cell microscopy was used to investigate the initial formation and the fate of cells entering mitosis after treatment with each microtubule stabilizer. Two distinct mechanisms of multipolar spindle formation were observed: the slow fragmentation of an initial bipolar spindle or the initial formation of multiple spindle asters that seem to be nucleated from the cell cortex. While paclitaxel and taccalonolide AJ shared similar patterns of initial aster formation, dramatic differences were observed in the fate of these asters. Paclitaxel-treated cells contained multiple asters that appeared to fuse into 2-3 larger more diffuse asters. This is possibly the cell's attempt to restore a bipolar spindle morphology. In contrast, taccalonolide AJ-treated cells contained multiple spindle asters that did not merge and instead persisted as more numerous and compact asters. Co-localization studies showed that while many microtubule associated proteins were eventually localized to mature taccalonolide AJ-induced asters, NuMa was unique in that it associated with microtubules very early in mitosis, even localizing to the asters nucleated at the cell cortex. Depletion experiments showed NuMa was not required for the cortical localization of microtubules, but that it may play an important role in aster maturation after drug treatment. Further mechanistic studies were conducted to determine whether taccalonolide AJ inhibited microtubule dynamics in a similar way to paclitaxel. These experiments demonstrated that sub-stoichiometric concentrations of taccalonolide AJ cause a dose-dependent suppression of microtubule dynamics. While taccalonolide AJ had no effect on the growth rate of microtubules, it significantly suppressed the shortening rates and catastrophe and rescue frequencies. We hypothesize that the ability of taccalonolide AJ to suppress microtubule dynamics is responsible for the aberrant spindle aster formation and resolution observed.

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Neuropathic effects of eribulin, ixabepilone, paclitaxel, and vincristine on peripheral sensory neurons in vitro.

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Peripheral neuropathy can be a serious dose-limiting toxicity associated with microtubule-targeting anticancer drug treatment. We examined the neuropathic effects of four microtubule-targeting drugs in vitro, eribulin mesylate (ERB), ixabepilone (IXA), paclitaxel (PAC), and vincristine (VCR) and using an immortalized rat peripheral sensory neuronal cell line, 50B11. The goal is to investigate a possible correlation between drug effects on microtubule assembly dynamics and neuronal cell damage. As an assay for cell viability we determined the ability of each drug to reduce total cellular ATP levels. We found that IXA, which suppresses microtubule dynamics and enhances microtubule polymer mass, was 20-fold weaker than PAC, which has a similar effect on microtubules (EC₅₀, IXA, 6.7 nM; PAC, 0.33 nM). VCR and ERB, which suppress microtubule dynamics but decrease microtubule mass, induced cell death with EC₅₀ values of 0.36 nM and 0.82 nM, respectively. Thus, the order of potency for induction of 50B11 cell death is PAC=VCR>ERB>IXA. After 24 h incubation, the drugs induced a loss of neurites (neurite retraction) with potencies of: VCR>ERB>IXA> PAC. At 10 nM VCR, neurite number was reduced from 0.79 neurites/cell in control to 0.09 neurites/cell. Nearly complete neurite retraction occurred with ERB only at ≥ 100 nM (to 0.08 neurites/cell). Neither PAC nor IXA induced complete loss of neurites at ≤ 1000 nM. The microtubule-depolymerizing effects of VCR and ERB may be responsible for their stronger effects on neurite retraction. We measured drug effects on α-tubulin protein expression and tubulin acetylation by in-cell western.

Increased acetylation of α -tubulin is a correlate of increased microtubule stability. VCR strongly reduced α -tubulin protein levels, in agreement with Huff et al., 2010. α -tubulin was reduced by 60% at 100 nM VCR and by 38% at 100 nM ERB. No reduction in α -tubulin protein occurred with PAC or IXA, and at high concentrations both drugs increased tubulin expression, by 15% and 28% for 100 nM PAC and IXA, respectively. All four drugs increased the acetylation of tubulin at low nanomolar concentrations; at 4 nM, tubulin acetylation was increased by 107% for PAC, 75% for IXA, 70% for ERB and 61% for VCR. However, VCR uniquely reduced tubulin acetylation at high concentrations (by 88% at 100 nM, possibly due to microtubule destabilization). The relative effects on tubulin expression and acetylation by the four drugs correlate well with their relative potencies on neurite retraction, indicating that changes in microtubule stability may play a significant role in drug-induced neurite retraction. These results indicate that the specific functional perturbations of 50B11 sensory neurons by microtubule-targeting anticancer drugs vary substantially from one drug to another. Experiments to further assess effects of these drugs on sensory neuron damage in relation to their effects on microtubule assembly dynamics are underway. This work is funded by Eisai Inc.

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Novel method for examining the effects of beta tubulin mutations on drug resistance or sensitivity by creating isogenic U2OS cell lines expressing codon-customized mutant TUBB ORFs.

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Tubulin is one of the oldest, most highly validated targets for cancer chemotherapy. A large number of mutations in tubulin genes have been identified from cancer patients. However, the effects of such mutations on drug susceptibility have not been adequately characterized. This is because there is no appropriate assay system, due to high expression levels and isoform complexity of endogenous tubulins. To overcome this problem, we have established a new method of tubulin mutation analysis. Our system is comprised of 3 parts, 1) a codon-customized synthetic type 1 beta-tubulin (TUBB) ORF, which is resistant to siRNA, 2) siRNA targeting of the endogenous TUBB sequence, and 3) integration of mutant TUBB ORF-expression cassettes into the specific genomic locus of the host cells using the Jump-In™ TI™ Gateway® Targeted Integration System (Invitrogen). Several drug-resistant mutations of the TUBB gene were tested in our system, and the effects of each TUBB mutation upon sensitivity to or resistance against tubulin inhibitors, such as paclitaxel, vinblastine, vincristine and eribulin, were successfully determined. In conclusion, our versatile assay system is useful for characterizing the effects of TUBB gene mutations on drug resistance or sensitivity.

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Effects of the Chemotherapeutic Agent Taxol at Nanomolar Concentrations Observed by TIRF Microscopy.

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Microtubules are intracellular polymers that assemble from heterodimeric $\alpha\beta$ -tubulin subunits. Polymerizing microtubules exhibit dynamic instability, a GTP-hydrolysis-driven phenomenon in which they alternate abruptly between phases of growth and relatively rapid shortening. The kinetics of tubulin subunit exchange at the microtubule tip, which are crucial to processes such

as mitosis, are affected by the chemotherapeutic drug taxol, but the precise mechanism of action at therapeutic concentrations remains unclear.

We observe microtubules polymerized at 4.5 micromolar tubulin and at 1-480 nanomolar taxol using total internal reflection fluorescence microscopy (TIRFM), achieving improvements in spatial and temporal resolution of, respectively, 1 and 2 orders of magnitude compared to previous taxol studies. We measure microtubule length changes to within 25 nm at 8 Hz, and we detect changes in the structure of the microtubule tip. We find that taxol potently affects microtubule growth at concentrations as low as 10 nM. At these concentrations we observed abrupt changes in the growth rate not observed under control conditions, with phases lasting on the order of 1 – 2 minutes, suggesting multiple tip states and kinetic rate constants. In many of these cases, microtubules switched between a state of normal growth and a “paused” state, where the net growth rate is small or negligible. Further, we have found that 10 nM taxol almost completely suppresses rapid shortening, and beginning at 100 nM taxol, the tubulin on and off rates gradually decrease, though the microtubule net growth rate (excluding pauses in taxol-microtubules) remains constant.

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Fluorescent Probes for Carbonyl-Containing Proteins.

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Carbonyl groups can be introduced into proteins deliberately for bioorthogonal labeling or by endogenous processes such as oxidative stress. Chemical probes for detection and labeling protein aldehydes are primarily hydrazides or 2,4-dinitrophenylhydrazine, but the kinetics of the labeling reactions are slow at neutral pH. Aromatic hydrazines and amines are better candidates for performing the labeling reactions under biocompatible conditions. We have synthesized fluorophores that possess aromatic amino and hydrazinyl groups and studied their reactive and photochemical properties with a model aldehyde-containing protein. Of particular interest are fluorescein, rosamine and BODIPY-based fluorophores in which the nitrogen is bonded to the pendant aromatic ring. In most cases, the parent amine or hydrazine is weakly fluorescent due to intramolecular photoinduced electron transfer from the nitrogen. Imine or hydrazone formation can remove the intramolecular quenching, and therefore the fluorescence can “turn on” as a result of covalent bond formation. The reversible imine bond can be hydrolyzed to regenerate the carbonyl or reduced to form a stable covalent bond. Applications of these fluorophores to protein and cell biochemistry vary with the specific fluorophore. For example, cell impermeable fluorophores can label oxidized proteins on the cell surface, while cell permeable probes can react with proteins on the interior of the cell. Some of these reactions occur specifically in live cells.

Centrosomes I

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How Flies Build a Mitotic Centrosome

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Centrosomes are the main microtubule organising centres in many cells. They comprise a pair of centrioles, which can recruit a large number of proteins collectively known as the Pericentriolar Material (PCM). The PCM harbours the complexes that facilitate microtubule nucleation and large amounts of PCM are recruited during mitosis in a process known as centrosome maturation. Several proteins have been implicated in this process, but a clear understanding of how the mitotic PCM is assembled has remained elusive. Here we show that two PCM components, Cnn and DSpd-2, are constantly recruited into a zone of PCM immediately surrounding the centrioles by the centriolar protein Asl, and then move outwards to fill the rest of the PCM. Thus, they exhibit a 'central to peripheral flow'. In contrast, all of the other PCM components we have analysed are recruited evenly throughout the PCM, suggesting that they bind to a preformed PCM 'scaffold'. Mutant analysis in somatic cells shows that centrosomes can partially mature and nucleate MTs in the absence of either Cnn or DSpd-2. In the absence of both proteins, however, centrosome maturation and MT nucleation is completely abolished. We propose that Asl, Cnn and DSpd-2 cooperate to form a PCM scaffold around the centrioles onto which other PCM components can bind. Without this scaffold, other PCM components are unable to accumulate around the centrioles and the centrosomes cannot function to nucleate microtubules.

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Quantitative Mass Spectrometry-based Proteomics of Human Centrosomes after Cullin-RING E3 ligase and Proteasome Inactivation.

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Ubiquitin-ligases are key regulators of centrosome function during the cell cycle. The identity and substrates of these regulators are, however, not well established. To identify novel ubiquitin-ligases and substrates with a role in centrosome biology, we applied mass spectrometry-based proteomics to quantify proteins differentially associated with the centrosome before and after treatment of cells with the proteasome inhibitor bortezomib and the NEDD8-activating enzyme inhibitor MLN4924. Upon proteasome inhibition, we observed that a large number of proteins accumulate at the centrosome. This is consistent with previous reports where fluorescence microscopy experiments have revealed that proteins accumulate at the centrosome, most likely due to a failure of degrading poly-ubiquitylated proteins. We were able to quantify those changes for the majority of known centrosomal proteins and observed major differences in the degree of accumulation of proteins involved in distinct processes such as centriole duplication, elongation and cohesion. Inactivation of Cullin-RING E3 ligases by the NEDD8-activating enzyme inhibitor MLN4924 followed by quantitative proteomic analysis revealed more subtle and selective changes in stabilization of proteins at the centrosome. To identify novel proteins associated with centrosomes after proteasome inhibition, we applied our PCP-SILAC method (Jakobsen, EMBO J., 2011) to measure enrichment profiles of proteins in fractions collected after sucrose gradient centrifugation of centrosomes. Cluster analysis of

profiles for 3822 proteins revealed a distinct cluster with 150 known centrosomal proteins and 60 additional components representing novel candidate proteins and possibly ubiquitylated substrates associated with the centrosome. This group of proteins comprised several E3 ligases of interest for further analysis. Initial immunostaining experiments confirmed centrosome localization for 12 proteins not previously shown to be associated with the centrosome. Changes in the localization patterns indicated cell cycle dependent function for these proteins.

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Dissecting the interactions of the centrosome.

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The major microtubule-organizing (MTOC) center of many eukaryotic cells is the centrosome, a non-membrane bound organelle that consists of the centriole and surrounding peri-centriolar material (PCM). As cells enter mitosis, centrosomes accumulate PCM and become highly active MTOCs, nucleating and/or anchoring microtubules. Proteomics have identified the constituent parts of the centrosome and RNAi screens have identified proteins critical for centrosome function and maintenance. However, understanding how specific proteins function in the construction, maturation and function of centrosomes has been hindered by the fact that knockdown of many centrosomal proteins can cause a failure in centrosome duplication or in its structural integrity. As a result, how the many proteins of the centrosome interact and function together remains unclear. A critical next step is to understand the relationships among the proteins within the centrosome, and how these relationships change as the centrosome becomes active. To identify the specific interactions between centrosomal proteins, we have carried out a yeast two-hybrid screen to identify all of the interactions among 34 centrosomal proteins including proteins in the centriole and PCM, as well as proteins with the potential to regulate centrosome function. By analyzing known and predicted protein structures, we divided many of these proteins into smaller fragments representing likely functional domains. In total we analyzed all of the possible interactions among 88 polypeptides. This analysis has revealed a multitude of connections between centrosomal proteins, perhaps providing insight into why loss of a single protein is so detrimental to the entire organelle. We have now expanded this analysis to include 42 additional proteins that proteomics and RNAi have suggested may play a role at the centrosome. To begin understanding the significance of these interactions, we are now generating mutations to disrupt specific interactions within the centrosome. Our goal is to identify mutations that perturb aspects of centrosome maturation and function, but do not affect the overall stability or maintenance of structure. The effect of mutations that disrupt specific interactions will be analyzed in S2 cells and in live *Drosophila* neural stem cells to determine their effect on the recruitment of PCM, the MTOC capacity of centrosomes and asymmetric cell division. This system allows us not only to determine the consequence of these mutations at the cellular level, but also how these small perturbations in centrosome function affect the larger tissue's architecture. We have started this type of analysis with Asterless, which we found had 23 interactions with 14 centrosomal proteins.

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Centrosome maturation starts at centrioles: Implications from a clear cytoplasmic separation of key players of centrosome maturation in *C. elegans*.

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Centrosomes are the major microtubule organizing centers of the cell. Mature centrosomes consist of a two centrioles, surrounded by a proteinaceous matrix called pericentriolar material (PCM). Prior to mitosis centrosomes grow by accumulating PCM, a process called centrosome maturation. In *C. elegans* we know that SPD-2, SPD-5, AIR-1 and PLK-1 are essential for this process. Existing yeast-two-hybrid data suggests a direct interaction between SPD-5 and SPD-2 as well as AIR-1. Other data shows that the interaction between SPD-2 and PLK-1 is essential for proper centrosome growth. It was shown in *D. melanogaster* that centrosome proteins form pre-assembled cytoplasmic complexes that are tethered to the centrosome via SAS-4. Even though we know the key proteins and their molecular interactions the exact steps required for PCM accumulation in *C. elegans* remain unknown. Do pre-assembled complexes also exist in *C. elegans* or does the PCM accumulate in a step-wise manner? To differentiate between the two scenarios we ask the question whether SPD-5, SPD-2, AIR-1 and PLK-1 form pre-assembled complexes in the cytoplasm.

By using a combined approach of single molecule microscopy and proteomics on the cytoplasmic level we analyzed interactors that associate with SPD-2 and SPD-5 inside the cytoplasm of *C. elegans* embryos. We find that SPD-5 forms a complex with PP2A related proteins RSA-1 and RSA-2 but without AIR-1. Our analysis of SPD-2 indicates that it exists freely in the cytoplasm and does neither interact with SPD-5 nor with PLK-1.

With the use of Fluorescence correlation spectroscopy(FCS) we then measured the diffusion of the SPD-5 complex and the free SPD-2 in a spatial and cell cycle dependent manner. We report that the diffusion for both proteins stays constant throughout the cell cycle and is independent of the cytoplasmic location inside the embryo.

Here we show that SPD-2 and SPD-5 move separately throughout the cytoplasm in a localization and cell cycle independent manner. Combined with the absence of an interaction with AIR-1 and PLK-1 these results suggest that PCM assembly is initiated at the centrioles rather than in the cytoplasm.

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Quantitative Mass Spectrometry-based Proteomics and Electron Microscopy Reveals Cep128 as a Core Component of the Subdistal Appendages.

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The centriole is a microtubule based barrel-shaped multifunctional organelle serving as a common scaffold for the centrosome, mitotic spindle and the basal body of cilia. Each structure is tightly coupled with distinct cell cycle stages and associates with a large number of proteins that change dynamically during these stages. The identity of most centrosomal proteins has been revealed, but the macromolecular composition of substructures such as the distal and

subdistal appendages and how they interact with the centrioles and the pericentriolar material remain unknown. Detailed localization studies may provide clues to the function of individual proteins, but the overall cellular pool of centriole-associated proteins could represent alternative functions. To further investigate the localization/function relationship we use quantitative mass spectrometry-based proteomics on centrosomes isolated from human cells to characterize the spatial organization and the biochemical properties of centriolar proteins. We have developed two methods profiling: 1) the depletion of proteins from the centrosomal scaffold before and after incubation in different salt concentrations, and 2) the distribution and exchange of proteins between two cellular compartments when incubating isolated human centrosomes with cytoplasm from cycling or growth-arrested human cells. We have correlated data from these two approaches and performed hierarchical cluster analysis to reveal proteins with similar functional properties. The protein Cep128 was identified as a core component of the centrosomal scaffold, supporting our previous finding that Cep128 localizes specifically to the mother centriole (L. Jakobsen et al., 2011). By performing immunogold electron microscopy on cultured human cells and purified centrosomes, Cep128 was localized specifically to the subdistal appendages. We depleted Cep128 in cultured human cells using esiRNA and analyzed the effect in growth arrested cells by immunofluorescence- and transmission electron microscopy. Cep128-depleted cells were not affected in their ability to form primary cilia, but displayed a higher number of pericentriolar dense bodies or satellites as well as of non-exocytotic vesicles in line with the cilium. This implies that Cep128 may play a role in the trafficking of satellites and vesicles to and/or from the centrosome. Our data also provide a system-level framework for further analysis of the molecular mechanisms controlling the assembly and function of the centrosome.

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Sub-diffraction-resolution fluorescence microscopy reveals a novel domain of the centrosome critical for pericentriolar material organization.

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As the main microtubule-organizing center in animal cells, the centrosome has a fundamental role in cell function. Surrounding the centrioles, the Pericentriolar material (PCM) provides a dynamic platform for nucleating microtubules. While the PCM's importance is established, its amorphous electron-dense nature has made it refractory to structural investigation. By using SIM and STORM sub-diffraction resolution microscopies to visualize proteins critical for centrosome maturation, we demonstrate that the PCM is organized into two major structural domains: a layer juxtaposed to the centriole wall (Asl and Plp), and proteins extending further away from the centriole organized in a matrix (Spd-2, Cnn and γ Tub). Analysis of Pericentriolar-like protein (Plp) reveals that its C-terminus is positioned at the centriole wall, it radiates outward into the matrix and is organized in clusters having quasi-nine-fold symmetry. By RNAi we show that Plp fibrils are required for interphase recruitment and proper mitotic assembly of the PCM matrix.

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STED super-resolution microscopy of multiciliated respiratory epithelial cells reveals structural organization of centriole and cilia components.

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Centrioles are microtubule-based organelles that template the formation of cilia. Motile cilia are involved in cellular motility and movement of extracellular fluid in specialized tissues. Non-motile primary cilia mediate signaling events in vertebrates. While the ultrastructure of centrioles and cilia has been well characterized by electron microscopy (EM), the structural organization of the molecular components of centrioles and cilia has not. A limitation has been that the relevant size scale of this organization is at, or below, the diffraction limit of light microscopy. We have used stimulated emission depletion (STED) super-resolution immunofluorescence microscopy to image the structural organization of centriole and cilia components in multiciliated respiratory epithelial cells derived from murine trachea. Imaged by STED at a resolution of 50-70 nm, the distal appendage protein Cep164 localizes with 9-fold symmetry at centrioles (Biophys. J. 102(12):2926-2935). The inner dynein arm component, Dnah2, localizes as puncta along ciliary axonemes with a ~100 nm periodicity. These findings are consistent with EM-based studies of centriole and cilia morphology, and suggest that this unique combination of technologies should facilitate the determination of the molecular anatomy of centrioles and cilia. Finally, super-resolution imaging of the ciliary axoneme with appropriate markers could facilitate diagnosis and classification of primary ciliary dyskinesias.

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Cryo-Electron Tomography of Centriole Assembly and Ciliogenesis in *Naegleria gruberi*.

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Centrioles and cilia/flagella direct many cellular functions, from organizing the mitotic spindle to mediating cell signaling and motility. Despite the importance of these organelles, the mechanisms of their construction are not thoroughly understood. The single-celled eukaryote *Naegleria gruberi* undergoes a remarkable synchronized differentiation from amoeba to flagellate, assembling centrioles, flagella, and a complete microtubule cytoskeleton *de novo*. We aim to gain structural insights into the mechanisms of centriole assembly and ciliogenesis via cryo-electron tomography and sub-tomogram averaging of intermediate structures isolated at successive time points throughout the amoeboflagellate transition.

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Elucidating the pathway of centrosome formation in *Drosophila*.

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Centrosomes are cellular organelles that comprise two centrioles surrounded by a more amorphous pericentriolar material (PCM) that is the site of microtubule nucleation. A conserved "core" of proteins that are required for centriole duplication and PCM recruitment have been identified in *Drosophila* (comprising Sak, DSas-6, Ana2, DSas-4, Asl, DSpd-2 and Cnn). Little is known, however, about how these proteins cooperate to drive centriole and centrosome assembly. This is partly because mutations in any one of the 5 core duplication proteins (Sak, DSas-6, Ana2, DSas-4 and Asl) result in the complete absence of centrioles, making it difficult

to analyse the pathway of centriole and centrosome assembly. Here we begin to elucidate this pathway using DSas-6/Ana2 particles (SAPs), which form rapidly and at high frequency in laid *Drosophila* eggs that moderately overexpress the centriolar proteins GFP-DSas-6 and GFP-Ana2. We show that SAPs are good models of centrosomes, as all tested centriolar and PCM markers exhibit very similar localisation and dynamics at centrosomes and at SAPs. Importantly, however, unlike centrosomes, SAPs can form even in the absence of certain essential centriole duplication proteins. This has allowed us to monitor the recruitment of various centrosome proteins to the SAPs in different mutant backgrounds. This analysis has allowed us, for the first time, to propose a detailed molecular pathway for centriole and PCM assembly in *Drosophila*.

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Mdm1, a gene linked to age associated retinal degeneration in mice, encodes a novel centriolar protein.

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Mutations in genes encoding centrosome and cilia proteins are implicated in human diseases including cancer and a class of disorders known as ciliopathies. Thus, identifying and understanding the function of centrosome and cilia proteins can shed light on the mechanisms underlying the development of these diseases. We identified Mdm1 as one of the most highly upregulated genes in a microarray study that examined gene expression during differentiation in a mouse tracheal epithelial culture (MTEC) system. Mdm1 was originally identified as a gene that is amplified on mouse double minutes in transformed NIH 3T3 cells, and it was previously linked to retinal degeneration in a mouse model of age-related retinal degeneration. We show that Mdm1 localizes to the basal body layer in MTECs, consistent with it being a centrosomal protein. We used deconvolution microscopy to show that it is present at the proximal end of centrioles, a region known to be important for centriole duplication and centrosome cohesion. Ongoing work is focused on determining the centrosomal function of Mdm1.

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Characterization of eta-tubulin: the final member of the vertebrate tubulin family.

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Tubulins are a well-conserved superfamily of proteins that play vital roles in cytoskeletal, centrosomal, and ciliary biology. Superfamily members alpha, beta, and gamma are found in all eukaryotes, and are the structural elements of the microtubule polymer, and the nucleator of that polymer, respectively. Two other members of the tubulin family, delta and epsilon, are present in many, but not all, eukaryotes and are associated with centrioles. We have characterized another member of the vertebrate tubulin family, which most closely resembles eta-tubulin, previously described in *Paramecium*. Based on this similarity we will refer to this as the eta-tubulin subfamily. Eta-tubulin is present in a variety of organisms from single-celled eukaryotes, chordates, and marsupial mammals, but is absent in flies, worms, zebrafish, and placental mammals. Analysis of genome sequences indicates that eta-tubulin is the last remaining tubulin family member to be characterized in vertebrates. Eta-tubulins are the most divergent family within the tubulin superfamily, with frog and lizard eta-tubulins sharing 59%

identity compared to 70% identity between frog and lizard delta-tubulins, the next most divergent tubulin family. Comparison of the sequence of eta-tubulins with other tubulin family members suggests that it might have unique properties with respect to interaction with other tubulins. We present an *in vivo* and *in vitro* characterization of the *Xenopus laevis* eta-tubulin, including localization relative to the centrosome and cilium in cells and tissues, a preliminary functional assessment of its role *in vivo*, and biochemical determination of its interactions with other members of the tubulin superfamily.

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Sas-4-mediated PCM assembly is regulated by Tubulin Dimers.

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A centrosome consists of a pair of centrioles surrounded by a protein network of pericentriolar material that is essential for centrosome function. Sas-4 was reported to be essential for both centriole and PCM recruitment in *Caenorhabditis elegans* and it was suggested that PCM recruitment defects are a consequence of structurally compromised centrioles. This idea was reinforced by the observation that in *Caenorhabditis elegans*, Sas-4 is a stable component of the centriole that is incorporated only during its initial formation. We find that, in *Drosophila*, Sas-4 has distinct characteristics, suggesting that Sas-4 may have in addition to its role in centriole formation, a specific role in PCM function. *Drosophila* Sas-4 is not only found in the centriole but can also be found in the PCM, and Sas-4 levels change along the cell cycle. Sas-4 forms complexes with multiple PCM proteins such as γ -tubulin, Asl, Cnn, Dplp, and CP190. Eliminating the conserved PN2-3 segment of Sas-4 leads to a reduction in PCM recruitment without blocking centriole duplication. In contrast, mutating Sas-4 tubulin binding site results in an increase in PCM recruitment without effecting centriole number. Additional evidence for the role of Sas-4 in PCM formation has come from biochemical studies of the interaction between Sas-4, tubulin dimers, PCM proteins, and whole centrosomes. Sas-4 scaffolds complexes of PCM proteins and can tether them to purified centrosomes that have been stripped of PCM. This function is regulated by a guanine nucleotide that binds to the tubulin dimer attached to Sas-4. Tubulin-GDP favors binding to Sas-4 and promotes PCM complex formation, while the binding of tubulin to a GTP analog blocks this process. Similarly, the binding of tubulin-GDP to Sas-4 promotes Sas-4-PCM complex tethering to the centrosome while the tubulin-GTP analog blocks this tethering activity. These studies suggest a model in which tubulin dimers regulate Sas-4-mediated PCM recruitment to the centrosome.

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PCM involvement in the formation of a centrosome-like particle that functions in basal body formation.

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The typical metazoan centrosome is a membrane-less cytoplasmic zone containing 2 centrioles surrounded by a lattice-like pericentriolar material (PCM). A main function of the centrosome is to serve as the MTOC, the primary organizer of the microtubule cytoskeleton in the cell. The centrosome is also the site of centriolar replication. The PCM consists of regulatory and structural proteins and changes in size and composition through the cell cycle. Plant cells lack centrioles, and accordingly, they do not possess organized centrosomes. Nevertheless, they organize microtubules in ordered arrays, usually from diffuse zones of cytoplasm. We are interested in knowing if plant cells regulate the assembly of their microtubule cytoskeletons with PCM-like arrays. Only a few select cells in a restricted group of plants possess basal bodies, and they form these 9+2 structures *de novo*, in a particle known as a blepharoplast. Though it

does not contain or consist of centrioles, the blepharoplast can serve as an MTOC, and as such, it behaves like an organized centrosome. The male gametophyte of *Marsilea vestita* provides a unique opportunity to study the recruitment of PCM components and their roles in the formation and maturation of the blepharoplast. The blepharoplast arises ~3.5 hours into gametophyte development and then reappears at 4 hours and serves as centrosome-like MTOC for the final division. It then functions as the site for *de novo* assembly of 140 basal bodies in each spermatid. RNAseq and transcriptome assembly enabled us to identify a series of likely PCM proteins present in the gametophyte. Centrin is essential for blepharoplast formation and serves as a key component in the particle. Its distribution serves as an assay for both blepharoplast and basal body formation. In functional assays, RNAi knockdowns of transcripts encoding several PCM proteins revealed that a striking number of structural proteins found in the PCM are required for proper formation and function of the blepharoplast, including Sfi1p, several CEPs, and gamma tubulin ring complex proteins. Silencing of certain PCM components blocked blepharoplast formation altogether. If the blepharoplast formed after a PCM component knockdown, it usually failed to complete its maturation for the assembly of functional basal bodies. This silencing approach should allow us to identify a variety of proteins that are essential for the *de novo* formation of the blepharoplast and basal bodies. This research was supported by NSF grant 0842525 to SMW.

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Control of centriole replication by centrosomin proteins.

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Centrosomins are conserved centrosome proteins. In *Drosophila*, centrosomin (Cnn) is required for PCM assembly and for the MTOC activity of mitotic centrosomes. In humans the centrosomin homologue *Cdk5rap2* is mutated in MCPH, a neural stem cell disease that effects brain growth. We recently reported that in *Cdk5rap2* mutant mouse cells the mitotic centrosomes have apparently normal MTOC activity but have amplified centrioles. The mechanism appears to involve loss of centriole engagement. Consistent with the role for *Cdk5rap2* in centriole replication control, we found mutations in *Drosophila cnn* that also cause unrestricted centriole duplication. One cluster of mutations causes centriole amplification in testis. These mutations knock out the expression of one of two centrosomal isoforms of Cnn in testes. By expressing a set of rescue constructs, we show that the developmental timing of Cnn expression, rather than a specific role(s) for Cnn isoforms, is responsible for restricting centriole replication. Moreover, we show that a conserved domain, the CM2 domain, is necessary for centriole replication control, while another domain required for microtubule assembly, CM1, is not required. The CM2 domain is also necessary and sufficient for targeting Cnn to the centriole, where it appears to be enriched at the proximal base of the daughter centriole. Therefore, Cnn restricts centriole replication to once per cell cycle via a conserved domain. The functional participation for a partner of the CM2 domain is under investigation.

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Centriole duplication licensing in *C. elegans*: the role of separase.

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Centrioles are cytoplasmic structures that recruit pericentriolar material to form centrosomes, the major microtubule organising centers in animal cells. Given the central role of centrosomes in mitotic spindle assembly, centriole duplication has to be tightly controlled to ensure the

formation of a bipolar spindle in mitosis. New centriole assembly initiates during S phase and depends on an initial licensing step involving the separation of the previous mother-daughter centriole pair in anaphase/telophase, also referred to as disengagement. The cysteine protease separase, best known for its role in chromosome segregation in anaphase, has been suggested to play an additional role in several other events occurring in late mitosis, including the disengagement of centrioles. We therefore sought to characterize the role of separase in centrosome dynamics in the one-cell embryo of *C. elegans*. Consistent with reports in vertebrate cells, we found that depletion of the *C. elegans* separase ortholog SEP-1 resulted in a failure of centrosome separation and consequently formation of monopolar spindles in the first embryonic mitosis. However, immunofluorescence microscopy with PCM and centriole markers showed that depletion of SEP-1 did not prevent centriole disengagement. Nevertheless, centriole duplication was impaired in the majority of embryos, resulting in improper numbers of centrosomes in the subsequent division. Measurement of inter-centrosome distance across different cell cycle stages revealed a correlation between centrosome proximity and failure in centriole duplication, more consistent with a diffusible inhibitor model for regulating centriole duplication than a model based on a physical tether between mother and daughter centrioles as currently hypothesized. By analogy with chromosome segregation, separase-mediated cleavage of cohesin has been posited to underlie centriole disengagement in vertebrates. We are currently examining whether cohesin plays a direct role in centriole separation in *C. elegans*. Additionally, we are testing whether microtubule-dependent forces are involved in disengagement of centrioles in the *C. elegans* embryo. This question is particularly relevant as most previous experiments suggesting a role for separase in centriole disengagement were conducted in cell-free contexts where the role of the cytoskeleton could not be assessed.

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RNA-binding Proteins ATX-2 and PAB-1 Interact with SZY-20 To Regulate Centrosome Assembly in *C. elegans* Embryos.

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Centrosomes are critical sites for controlling microtubule dynamics, and exhibit dynamic changes in size, promptly responding to changing cellular demands during the cell cycle. As cells progress to mitosis, centrosomes recruit more microtubules to form bipolar spindles. The *szy-20* gene encodes a novel centrosome-associated RNA-binding protein that negatively regulates ZYG-1. *szy-20* mutants possess enlarged centrosomes, leading to abnormal microtubule processes and embryonic lethality. Thus, SZY-20 limits centrosome size by negatively regulating the recruitment of centrosome components. SZY-20 contains putative RNA-binding domains; mutating these domains perturbs RNA-binding by SZY-20 in vitro and its capacity to regulate centrosome size in vivo. Recent studies showed a number of RNAs and RNA-binding proteins associate with centrosomes and microtubules, and that they function to assemble mitotic spindles.

We used proteomics, and identified known RNA-binding proteins such as ATX-2 and PAB-1 that are reproducibly immunoprecipitated with SZY-20. In this study, we aim to understand the roles of SZY-20 and RNA-binding in regulating centrosome assembly and size, in particular, to understand how ATX-2/PAB-1 RNA-binding protein complex interacts with SZY-20 to achieve proper centrosome assembly and size. Depleting ATX-2 or PAB-1 leads to abnormal cell divisions, including cytokinesis failure, abnormal spindle positioning. Knockdown of these proteins enhances cell division defects and the embryonic lethality of *szy-20*(*bs52ts*) embryos, while restoring centrosome duplication in *zyg-1*(*it25ts*) embryos. Quantitative western analyses show that ATX-2 knockdown leads to significantly reduced levels of SZY-20, and vice versa.

Our findings suggest that ATX-2 and PAB-1 positively regulate SZY-20 by enhancing protein levels, and negatively regulate centrosome assembly which is mediated by SZY-20 via their physical and genetic interaction.

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A Quantitative Assay for Soluble Protein Entry into Primary Cilia.

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The primary cilium is a signaling organelle with a distinct complement of membrane and soluble proteins that is required for its assembly, maintenance, and function. However, the mechanisms that allow specific proteins to be concentrated within cilia or excluded from cilia remain poorly characterized. We have developed and validated a method for selective permeabilization of the plasma membrane that leaves the ciliary membrane intact, thus enabling quantitative analysis of protein entry into primary cilia. Specifically, our assay monitors the capture of soluble GFP-binding proteins (GBPs) by GFP molecules displayed on the cytoplasmic face of the ciliary membrane. As expected, we find that GBP probes first enter at the base of the cilium and spread distally until all binding sites are occupied. Using this system, we have characterized a size-dependent diffusion barrier at the base of mammalian primary cilia that restricts entry of inert proteins. The entry rate decreases for GBPs of increasing size, and entry is undetectable for proteins with diameter greater than 8.5nm. We further used this system to test the potential role of nucleoporins in forming this barrier; however, we find that reagents that either block or loosen the nuclear pore barrier do not affect ciliary entry. Similarly, no effect is seen for actin depolymerization, which disrupts the diffusion barrier at the axon initial segment of neurons. Thus the ciliary diffusion barrier appears to operate by a novel molecular mechanism. Lastly, we have used the permeabilized cell system to measure diffusion rates inside primary cilia for soluble and membrane proteins. Fluorescence recovery after photobleaching reveals that membrane protein diffusion is rapid for the ciliary GPCR somatostatin receptor 3 (Sstr3), but is blocked completely when Sstr3 is crosslinked by the lectin wheat germ agglutinin (WGA). Quantitative modeling of protein entry into cilia of WGA-treated cells enabled us to determine diffusion coefficients for soluble proteins within the ciliary lumen. Here we find diffusion coefficients similar to those reported for diffusion in the cytoplasm. Thus, diffusion may permit rapid protein movement within cilia in the absence of active transport processes such as intraflagellar transport. In summary, a permeabilized cell system enables quantitative analysis of protein entry into cilia and reveals a diffusion barrier that underlies physical compartmentalization of the primary cilium.

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Kinetics of ciliary protein transport.

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Primary cilia are specialized sensory signaling organelles with diverse functions, many of which have been implicated in human disease. Critical to these functions is the correct distribution of proteins to the ciliary membrane. Rhodopsin (RHO) is a GPCR that is targeted and restricted to the specialized cilium of retinal photoreceptors. The purpose of the current study is to compare

the kinetics of RHO movement along cilium with that of entry into the cilium. To do so, we have used live-cell imaging and fluorescence recovery after photobleaching (FRAP) on the cilium of human retinal pigmented epithelial cells (hTERT-RPE1) that have been transfected with RHO-EGFP. We have found that the recovery of RHO-EGFP, following photobleaching of the distal region of the cilium is dependent upon heterotrimeric kinesin- 2, supporting previous studies suggesting a role for this motor in the movement of RHO along the cilium. When different regions of the cilium were photobleached, we found that the recovery of RHO fluorescence in the cilium is faster (as measured by the $t_{1/2}$ of the recovery) when the distal half of the cilium is photobleached versus when the entire cilium is photobleached. This observation is consistent with the hypothesis that the entry of RHO into the cilium is distinct from its movement along the cilium, and presents a rate-limiting step in the ciliary transport of RHO.

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The Architecture of the Ciliary Partitioning System.

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A flurry of recent advances in cilia biology has revealed much evidence for a structure at the base of cilia and flagella that serves as a barrier to isolate both the membrane and the interior of the cilia from the rest of the cell and regulate traffic between these regions. However, structural information about the cilia pore complex and the membrane diffusion barrier in cilia has so far been limited. Recently, remarkable mechanistic homology between nuclear and ciliary transport has been recognized, including common targeting sequences and Ran-GTP-dependent, importin-mediated transport. Just as visualization of the nuclear pore complex (NPC) has set the stage for understanding architecture and function in nuclear transport, characterizing this ciliary structure would provide substantive insights on ciliary trafficking. In this study, we have identified and isolated a putative cilia pore complex from *Tetrahymena pyriformis*. As seen by electron microscopy using negative stain and cryo tomography, the complex, which we call the ciliary partitioning system, is tightly associated with the basal body and comprises three components: a disc-shaped ciliary pore complex (CPC), a ring complex and a detergent-resistant periciliary membrane. EM of plastic sections of intact and deciliated *Tetrahymena* cells reveals that the complex forms a barricade layer at the cilia opening, confirming its role in cilia development and homeostasis. MS analysis indicates strong involvement of the ring structure and detergent-resistant periciliary membrane domain in membrane trafficking, suggesting their roles as a diffusion barrier. The CPC contains 9 pores; each pore comprises a distinct region through which a microtubule doublet passes and a larger area associated with the doublet B-tubule which appears to be well suited for passage of the IFT complexes, suggesting role of the CPC as a cytosolic filter. The association of the CPC and the ring seals the ciliary opening. Our structure offers novel insights into the mechanism of ciliary trafficking.

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Proteins of the ciliary axoneme are found on cytoplasmic membrane vesicles during growth of *Chlamydomonas* flagella.

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The cilium is a specialized extension of the cell where many specific proteins are admitted and retained, while many others are excluded or expelled. In order to maintain the organelle, the cell

must possess mechanisms for the targeted delivery of proteins there, as well as for the selective gating of protein entry and exit. We hypothesize that the cell employs vesicular trafficking as a means not only to transport integral proteins destined for the ciliary membrane, but also as a vehicle for the transport of axonemal proteins to the cilium by virtue of peripheral associations with these vesicles. Thus, we think axonemal proteins, synthesized on free polysomes in the cytoplasm, "piggy-back" on vesicles already directed toward the pericentriolar region at the base of the cilium, where they are then moved up through the transition zone by intraflagellar transport (IFT). To test this hypothesis we are using the flagellated algal model system, *Chlamydomonas*, to isolate and analyze vesicles from the cytoplasm at the time of flagellar assembly. Using step-gradient procedures to fractionate cytoplasmic extracts, we have enriched for a specific class of membrane vesicles that carry integral flagellar membrane protein polycystin-2 (PKD2), as well as protein components of intraflagellar transport (IFT) and radial spokes (RSP); all showing increased levels when the vesicles are isolated during formation of flagella. Using immunogold labeling and electron microscopy, we have documented the localization of an axonemal protein, RSP1, on the outer surface of cytoplasmic vesicles in situ during regeneration of flagella. Flagellar membrane protein, PKD2, and IFT proteins were also observed to localize to cytoplasmic vesicles during flagellar regeneration. Complete biochemical and proteomic analyses of these membrane vesicle preparations will reveal key components of the mechanisms by which cells form and maintain cilia. Supported by NIH grant GM14642

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Ectosome-like vesicles released from the cilium/flagellum carry specific biologically-active proteins.

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It is now well established that primary and motile cilia/flagella can act as antennae, *receiving signals* from the environment, and that the activated signaling pathways are involved in normal cell functions and in ciliopathies. Here we report that ectosome-like vesicles pinch off from the flagella and have a unique protein composition, enriched in certain flagellar membrane proteins and small GTPases. To determine a function of flagella-derived vesicles, we corroborated and extended the original findings by Matsuda and colleagues (*Plant Cell Physiol.* 50:572, 2009), showing that *Chlamydomonas* cells require a serine protease (sporangin), *released from the flagella*, to digest the mother cell wall, allowing the daughter cells to be released. Sporangin is synthesized just prior to daughter cell release, transferred to the flagellum and, following daughter cell release, is down-regulated. We used negative staining with silver-enhanced immunogold labeling to show that this serine protease is carried by the vesicles released from the flagella, and we have isolated the active vesicles. Mutants that lack flagella are usually not released from the mother cell wall, but addition of isolated sporangin-carrying vesicles will permit their release. These results demonstrate that membrane vesicles originating from the flagellum have a specific and necessary bio-reactive function. Other membrane vesicles released from *Chlamydomonas gamete* flagella have been shown in the past to have an in vitro function in promoting flagella:flagella agglutination, which initiates the signaling pathways required for mating of plus and minus cells. *We hypothesize that all ciliated cells release ectosome-like vesicles from the ciliary membrane and that these vesicles are bio-active at other places.* Supported by NIH grant GM 14642-042 to JLR.

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The Exocyst subunit Exo70 and the Biogenesis of Lysosome-Related Organelles Complex-1 (BLOC-1) subunit Pallidin Interact with IFT20.

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Cilia are conserved organelles present on most eukaryotic cells, excluding yeast and higher plants. Almost all human cells possess a single non-motile primary cilium, which is a sensory organelle that perceives the extracellular environment due to the concentration of signaling receptors in the ciliary membrane. The intraflagellar transport (IFT) system is required for assembling primary cilia and it is dependent on two protein complexes: IFT complex A and B. The IFT particles are composed of about twenty proteins and these proteins are highly conserved across ciliated species. The complex B subunit IFT20 is the only IFT protein known to localize at both the primary cilium and Golgi apparatus. Previously we showed that IFT20 interacts with the golgin GMAP210 and we suggested that these proteins are important for sorting and/or transport of ciliary membrane proteins, but the step(s) at which they function remains to be defined. It is likely that IFT20 interacts with other proteins but other binding partners have not been identified. Published large-scale yeast-two hybrid screens have identified new candidate IFT20-interacting proteins. Three proteins of interest include the exocyst subunit Exo70, the biogenesis of lysosome-related organelles complex-1 (BLOC-1) subunit Pallidin and a KxDL motif containing protein, KXD1. We find that both Exo70 and Pallidin co-immunoprecipitate with IFT20. Additionally, overexpression of Exo70 and Pallidin in IMCD cells displaces IFT20 from the Golgi apparatus. This evidence suggests that Exo70 and Pallidin interact with IFT20. Ongoing research is focused on determining the role of IFT20 and binding partners such as Exo70 and Pallidin in ciliary membrane protein trafficking.

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Exchange of IFT proteins between flagella and cytoplasm in *Chlamydomonas reinhardtii*.

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Intraflagellar transport plays a key role in determining the length of flagella in *Chlamydomonas*. Extensive studies have shown that the quantity of IFT protein in the flagellum is roughly independent of the length of the flagellum, which leads to a simple model that can explain the regulation of flagellar length. However this model depends on the quantity of IFT protein per flagellum being constant, and the mechanisms that regulate IFT quantity in the flagellum are still unclear. One possible mechanism is the "initial bolus" model, in which a fixed quantity of IFT protein is injected into a flagellum when it is first assembled, and then that fixed set of proteins circulates back and forth inside the flagellum without ever leaving or any new protein being injected. This simple scheme would guarantee that the quantity of IFT protein is length independent. We tested the initial bolus model using FRAP (fluorescence recovery after photobleaching) on *Chlamydomonas* cells expressing GFP-tagged IFT proteins grown in microfluidic chambers. We find that IFT proteins exchange rapidly with a pool of reserve IFT protein at the basal body, but exchange with the rest of the cytoplasm was not observed on the time scale of our measurements. These data rule out the initial bolus model but suggest that IFT dynamics involve multiple pools that exchange at vastly different rates.

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***Chlamydomonas* nephrocystin-4 functions at the transition zone to regulate ciliary trafficking of membrane proteins and large soluble proteins.**

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Mutations in human *NPHP4*, encoding the protein nephrocystin-4, cause nephronophthisis and retinitis pigmentosa. To learn more about the function of nephrocystin-4 and why defects in it cause ciliopathies, we are studying its homologue in *Chlamydomonas*. Immunofluorescence microscopy using antibodies against *Chlamydomonas* nephrocystin-4 indicated that it is located at the base of the flagellum. Immuno-gold electron microscopy in which *Chlamydomonas* cells expressing HA-tagged nephrocystin-4 were probed with anti-HA antibodies revealed that nephrocystin-4-HA is specifically localized to the distal transition zone, closely associated with the Y-shaped microtubule-membrane links seen in transition zone cross sections but more distal than the transition zone protein CEP290. It has been reported that the *Caenorhabditis elegans* homologue of nephrocystin-4 likewise is located in the transition zone; therefore, the location of nephrocystin-4 is conserved among species, suggesting that the function of nephrocystin-4 also is conserved. We have identified a *Chlamydomonas* insertional mutant (*nphp4*) in which the nephrocystin-4 gene is completely deleted. The mutant has normal length flagella, normal IFT, normal phototaxis, and normal basal body and axonemal ultrastructure. Some cells have ectopic transition zones and other transition zone abnormalities, but the Y-shaped links are still present. The mutant phenotype is rescued by transformation with constructs expressing wild-type or HA-tagged nephrocystin-4. CEP290 is present in the *nphp4* transition zone and nephrocystin-4 is present in the transition zone of a mutant lacking CEP290, consistent with a report that these two transition zone proteins are in different complexes. SDS-PAGE of flagellar fractions indicated that the protein compositions of wild-type and mutant axonemes are nearly identical, but that there are numerous differences between the wild-type and mutant membrane-plus-matrix fractions, especially among proteins >60 kDa. Mass spectrometry revealed that several membrane proteins are reduced in amount while the amounts of a few other membrane proteins and many housekeeping proteins are increased in the mutant flagella. This result suggests that *Chlamydomonas* nephrocystin-4 functions at the transition zone to control traffic of membrane and large soluble proteins between the flagella and cell body.

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TTC26/DYF13 undergoes intraflagellar transport and is required for cilia/flagella formation and transport of inner dynein components into flagella.

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Cilia and flagella are hair-like organelles that protrude from the surface of cells and are important for making driving forces and sensing extracellular signals. Ciliary defects have been implicated in a wide spectrum of diseases including retinal degeneration, polycystic kidney disease, left-right asymmetry defects and hydrocephalus. Cilia/flagella are assembled and maintained by the process of intraflagellar transport (IFT), a highly conserved mechanism in almost all eukaryotes. IFT is composed of at least 20 proteins and motor proteins. However, the functions of individual IFT proteins are mostly unclear. To help address this issue, we

focused on a putative IFT protein TTC26/DYF13, which is conserved in various ciliated organisms and is a homologue of *Caenorhabditis elegans* DYF-13 and *Trypanosoma brucei* PIFTC3. TTC26/DYF13 localizes to cilia/flagella in mammalian cultured cells and *Chlamydomonas reinhardtii*. GFP-labeled mouse TTC26 moved bi-directionally along the length of cilia in mammalian cells. TTC26/DYF13 was also biochemically confirmed as an IFT complex B protein in mammalian cells and *C. reinhardtii*. Knockdown of TTC26 in zebrafish embryos produces short cilia within the Kupffer's vesicle and pronephric ducts and produces typical cilia-related defects including pronephric cysts, hydrocephalus, and the randomization of left-right asymmetry. Moreover, we identified a *dyf13* mutant in *C. reinhardtii*, which has shorter flagella and motility defects. Surprisingly, IFT behavior seems normal in *dyf13* mutant flagella. However, we identified that specific inner dynein arm components were reduced in flagella of *dyf13* mutants. Thus, TTC26/DYF13 is an IFT complex protein, which is required for transporting specific cilia/flagella proteins into flagella.

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Functional Analysis of FAP12, a Gliding-Associated Flagellar Protein in *Chlamydomonas reinhardtii*.

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In the biflagellate green alga, *Chlamydomonas reinhardtii*, gliding motility is a flagella-dependent whole-cell motility that occurs upon flagellar surface adhesion to a substrate. This adhesive function is mediated by the major flagellar membrane glycoprotein, FMG-1B. Essential for the assembly and function of cilia is the bidirectional transport of protein particles known as intraflagellar transport (IFT). In a search for IFT-associated cargo, pull-down assays of *Chlamydomonas* flagellar extract with the retrograde IFT motor, cytoplasmic dynein 1b/2 heavy chain (DHC1b), yielded a set of proteins, among which the most prominent were the major flagellar surface glycoprotein, FMG-1B and a flagellar-associated putative lipase of unknown function, FAP12. Reverse pull downs with anti-FMG-1B specifically co-purified FAP12 and a small fraction of the DHC1b. To address the potential functional role of FAP12 in gliding motility, artificial micro RNA interference was used to reduce expression of the *FAP12* gene. Western blot analysis of RNAi transformants showed the FAP12 protein level was significantly reduced in multiple strains that were then assayed for gliding phenotype. Cells with the greatest depletion of FAP12 protein were strongly compromised in their ability to adopt the characteristic gliding configuration upon contact with a surface where the two flagella are straight and oriented 180° from one another. Once attached in this fashion, however, the gliding velocity was unaffected. Upon electroporation of purified recombinant His₆-FAP12 protein into the FAP12-deficient cells, the frequency of cells adopting the 180° gliding configuration was doubled. These results indicate that FAP12 is involved with an early stage of gliding motility in the transition from the initial surface adherence by the flagella to the adoption of the characteristic 180° gliding configuration. In contrast, the loss of FAP12 did not affect the velocity of the gliding motor nor the average distance traveled by gliding cells. Such an early role suggests that FAP12 may participate in events leading to activation of the gliding signaling pathway.

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The role of septin in ciliogenesis.

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Primary cilia are non-motile solitary protrusions found on the surface of nearly every cell in the human body. They are composed of a cylindrically organized microtubule axoneme that stems from a centriole-derived structure called the basal body. Primary cilia function as a multisensory antenna of the cell, capable of detecting environmental inputs and regulating key signaling pathways. Recently studies have suggested a connection between primary cilia formation and a family of protein called septins. There are a total of 13 human genes encoding septins, namely SEPT1-12,14. Septins form oligomeric complexes that can join end-on-end to form filaments. Work in our lab has shown that SEPT9 binds and activates a guanine nucleotide exchange factor for Rho, SARhoGEF, to promote Rho activation. Interestingly, SARhoGEF is localized to the basal body, raising the possibility that Rho activation may be important in ciliogenesis.

We therefore examined septin distribution in RPE cells by immunofluorescence microscopy and determined the localization of SEPT2, SEPT7, SEPT9 at the primary cilium. All three septins were found at the base, as well as in the axoneme of the cilium; in contrast, SARhoGEF was concentrated at the base, but excluded from the axoneme. To investigate whether SEPT9 is required for cilium formation, we used a lentivirus to stably deplete ~70% of SEPT9 expression in RPE cells. Consequently, the average length of cilia decreased from 2.49 μ m to 1.74 μ m. We confirmed the result using transient transfection with small interfering RNA. SEPT9 expressed was reduced by ~85% upon transfection, and the average length of cilia decreased to 1.48 μ m. Similar inhibition was obtained by depletion of the SARhoGEF or inhibition of Rho.

Since SARhoGEF was restricted to the basal body, while septins were also found along the axoneme, we investigated whether Rho activation at the base of the cilium could rescue the ciliary length defect caused by SEPT9 knockdown. To do so we transfected SEPT9-depleted cells with a fusion construct between centrin, a basal body localized protein, and constitutively active SARhoGEF. This construct exclusively localized to the base of the cilia where it can activate Rho locally. The fusion protein was able to rescue the phenotype resulting from SEPT9 depletion and significantly increased ciliary length to 2.77 μ m. This indicates that the major function of SEPT9 in ciliogenesis is to activate SARhoGEF and Rho at the basal body.

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Superresolution STED Microscopy Reveals Differential Localization in Primary Cilia.

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The primary cilium is an organelle that serves as a signaling center of the cell and is involved in the cAMP, Wnt and hedgehog signaling pathways. Adenylyl cyclase type III (ACIII) is a primary cilia marker that is involved in cAMP signaling, while also playing an important role in regulating ciliogenesis and sensory functions. Ciliary function relies on the transportation of molecules between the primary cilium and the cell, which is facilitated by intraflagellar transport (IFT). The detailed localization and interactions of these important proteins remain unclear due to the limited resolution of conventional microscopy. We conducted superresolution imaging of immunostained ACIII and IFT88 in human fibroblasts using stimulated emission depletion (STED) microscopy. Instead of a homogeneous distribution along a primary cilium, our STED

images revealed that ACIII formed a periodic punctate pattern with a roughly equal spacing between groups of puncta. These puncta occupied less than 50% of the area, with an average width of 153 ± 27 nm. Superresolution imaging of IFT88, one important protein of the IFT complexes, demonstrated two novel distinct distribution patterns at the basal end not resolvable by confocal microscopy: a triangle of three puncta with similar fluorescence intensities, and a Y-shaped configuration of a bright punctum connected to two branches. Our results demonstrate the ability of superresolution STED microscopy to reveal novel structural characteristics in primary cilia. This project was supported by NSF CHE-0641523 and CMMI-1125760.

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The kinases MRK and MOK regulate cilia length and intraflagellar transport in renal epithelial cells.

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Primary cilia are small cellular extensions used to sense cues from the environment. A wide variety exists in cilia length and morphology, probably reflecting their specific functions in different tissues. Cilia length and morphology are regulated by several signalling pathways. Cilia are build and maintained by a specialized transport mechanism, known as intraflagellar transport (IFT). During IFT, motor proteins transport multimeric protein complexes along the microtubule axoneme, delivering proteins to the distal tip or returning them to the cell body. We identified *C. elegans*' DYF-5, a conserved kinase that regulates cilia length and IFT.

Here we analyze the functions of MRK and MOK, mammalian homologues of DYF-5 and members of the ros cross-hybridizing kinase family (RCK). MRK and MOK GFP fusions localized to the cilia of IMCD3 cells. Knockdown of MRK or MOK resulted in longer cilia, while overexpression of MRK resulted in short cilia. These results indicate that also MRK and MOK regulate cilia length.

To visualize IFT we have generated IMCD3 clones that stably express fluorescently tagged components of the IFT machinery: Kinesin-II (KIF3B), Kif17, complex A (IFT43) and B (IFT20), and the BBSome (BBS8). We found that these proteins all moved at ~ 0.45 $\mu\text{m/s}$ in anterograde and retrograde direction. Knockdown of MRK increased anterograde IFT velocity of all constructs to ~ 0.6 $\mu\text{m/s}$ but did not affect retrograde IFT, correlating with delivery of more axonemal precursors at the distal tip, and cilium lengthening.

Previous studies have shown that the mTOR pathway modulates cilia length, and that MRK phosphorylates Raptor, a subunit of mTORC1. We found that the mTOR inhibitor rapamycin can block all effects of MRK and MOK depletion on cilia length and IFT, indicating that MRK and MOK interact with the mTOR pathway.

In summary, we show that MRK and MOK play important roles in regulating cilia length, IFT, and possibly cilia function.

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Analysis of photoreceptor outer segment development in a mutant KIF17 zebrafish model.

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The outer segment (OS) of the vertebrate photoreceptor is a sensory cilium, which serves as a phototransduction organelle. Similar to other sensory cilia, photoreceptor OS's lack a protein synthesis machinery, and their assembly and maintenance depends on intraflagellar transport (IFT). Recent studies have suggested a role for two kinesin-2 family motors, heterotrimeric kinesin-II and the homodimeric KIF17, in vertebrate OS development. However, data on the role of KIF17 in vertebrate photoreceptor OS assembly is currently limited to antisense

knockdown or dominant negative overexpression strategies. To further elucidate KIF17's role in OS formation, we have utilized a line of zebrafish carrying an ENU generated mutation (*kif17^{sa0119}*) causing a UGA stop codon in exon 10 that is predicted to ablate KIF17 function. We show that animals homozygous for the mutation are viable and do not show any morphological defects; even in photoreceptors. However, analysis of protein levels revealed that KIF17 expression is reduced by only about 50% in the mutant animals indicating that the mutation is not a null allele. This hypomorphic mutation has provided an ideal background for analysis using a morpholino knockdown strategy. Mutant animals treated with very low concentrations of several KIF17 specific morpholinos exhibit complete knockdown of KIF17 protein levels and severe ablation of OS formation at 3 dpf, which is consistent with previous findings from our laboratory using wild type animals. This appears specific for photoreceptors as cilia defects were not observed in other tissues. These findings show that the first available zebrafish mutation for KIF17 is a hypomorph that, nonetheless, is a valuable model when combined with additional tools that further reduce protein level. Our results strengthen the idea that KIF17 plays an important role in vertebrate OS assembly, and also emphasizes the need to carefully evaluate hypothetical loss of function ENU mutations at the level of protein expression.

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The role of the D1bLIC in retrograde IFT, flagellar assembly, and motility.

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Eucaryotic cilia and flagella play critical roles in sensory signaling and cell motility in a variety of organisms. Assembly of these organelles depends on a bidirectional, intraflagellar transport (IFT) system. Anterograde IFT is driven by Kinesin-2, and retrograde IFT is driven by a cytoplasmic dynein known as DYNC2H1 in humans and DHC1b in *Chlamydomonas*. Mutations in either motor lead to defects in ciliary assembly and signaling and are associated with human genetic diseases known as ciliopathies (reviewed in Ishikawa and Marshall, 2011). Each motor is associated with intermediate and/or light chain subunits that contribute to cargo binding and motor regulation. We previously identified a light intermediate chain subunit (D1bLIC) as a component of the retrograde IFT motor (Perrone et al., 2003). *d1blic* mutations are associated with flagellar assembly defects and the accumulation of IFT particles (Hou et al., 2004), but the phenotype is not as severe as that observed with *dhc1b* or *dll1(LC8)* mutations (Pazour et al., 1998, 1999; Porter et al., 1999). To better understand D1bLIC function, we analyzed IFT in a *d1blic* null mutant, a GFP-tagged rescued strain, and several strains in which D1bLIC expression was reduced by RNA interference. Knockdown to ~20% of wild-type levels reduced both the frequency and velocity of retrograde IFT with minimal effects on anterograde IFT or flagellar length. Knockdown below 10% (RNAi-A) resulted in more severe defects in retrograde IFT and flagellar length, similar to the *d1blic* null. iTRAQ analysis indicated that several membrane plus matrix polypeptides were elevated. Transformation of the *d1blic* mutant with *D1bLIC-GFP* rescued the assembly defects and increased the velocity and frequency of retrograde IFT. Thus D1bLIC stabilizes the retrograde motor and increases the velocity and frequency of IFT, but it is not absolutely essential for cDHC1b function. Transformation of other strains further demonstrated that D1bLIC-GFP is expressed at levels comparable to the endogenous subunit and co-purifies with it and other subunits of the retrograde motor during dynein extraction, sucrose density gradient centrifugation and immunoprecipitation. The D1bLIC-GFP construct will be a useful reagent for the characterization of mutations in several genes that affect retrograde IFT, flagellar assembly, and ciliary signaling. (supported by the NIH).

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Regulation of intraflagellar transport in *Caenorhabditis elegans* cilia by ubiquitination.A. van der Vaart¹, S. Rademakers¹, G. Jansen¹; ¹Department of Cell Biology, Erasmus MC, Rotterdam, Netherlands

Cilia are cellular extensions that can discern several environmental cues. Many of these cues are detected by G-protein-coupled receptors and relayed by heterotrimeric G-proteins to produce a cellular response. We recently found that a dominant active mutation in the G α -subunit *gpa-3* (*gpa-3QL*) affects cilia development and function. This is likely caused by changes in intraflagellar transport (IFT), the protein transport machinery in cilia. In wild type *C. elegans* anterograde transport is mediated by two kinesin motor complexes; kinesin-II and OSM-3 (mammalian KIF17). Together they transport particles in the middle segment of cilia, while only OSM-3 enters the distal segment where it moves at a higher speed. In *gpa-3QL* mutants the two motors move at different speeds and thus seem uncoordinated. We are setting up dual-colour live imaging of fluorescently-tagged IFT proteins to determine the IFT particle composition in *gpa-3QL* mutants. Using transgenic animals that express fluorescently-tagged IFT proteins specifically in ASI neurons, we found that the dynamics of the IFT proteins are affected by their expression levels. This indicates that the stoichiometry of the IFT proteins is important for their coordination. We are currently determining the optimal conditions for dual-colour live imaging.

In addition, we have performed a genetic screen for mutants that suppress the *gpa-3QL* induced ciliary defects. We have identified the E2 ubiquitin-conjugating enzyme variant UEV-3 as a suppressor of *gpa-3QL*. UEV-3 belongs to the highly conserved UBC family of proteins, however it lacks the cysteine necessary to conjugate ubiquitin. Although the UEV proteins lack catalytic activity, it has been suggested that these proteins play a role in the ubiquitination pathway. *gpa-3QL* animals that lack *uev-3* show wild type cilium length, while *gpa-3QL* animals have short cilia. Mutation of *uev-3* on its own does not affect cilium length. Furthermore, IFT measurements in the suppressor strain shows that OSM-3 and kinesin-II move at different speeds compared to both wild type animals and *gpa-3QL* mutants. Future experiments are directed to determine the precise role of UEV-3 in cilia formation and the regulation of IFT.

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The *Chlamydomonas* NIMA-related kinase CNK2 regulates flagellar resorption.L. K. Hilton¹, K. Gunawardane¹, J. Kim¹, L. M. Quarmby¹; ¹Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

Cilia and flagella are resorbed prior to cell division and in response to certain environmental stimuli. *Chlamydomonas* flagella also resorb when cells are transferred from liquid culture to agar media. We have uncovered a relationship between resorption in response to environmental stimuli and the regulation of flagellar length. Both RNAi knockdown of CNK2 (Bradley and Quarmby, 2005) and a null mutation in the *CNK2* gene cause an increase in flagellar length. This *cnk2-1* mutant also fails to resorb its flagella in response to growth on solid media, or exposure to sodium pyrophosphate (NaPPi) or IBMX. In contrast, other *Chlamydomonas* mutants with long flagella (*If3* and *If4*) resorb their flagella more quickly than wild-type in response to these same stimuli, suggesting that *If* mutant length defects arise from dysregulated flagellar assembly. Double mutants between *cnk2-1* and *If3* or *If4* have flagella that are considerably longer than any of the single mutants, which indicates that the disassembly pathway limits flagellar length in *If* mutants. We are currently studying the CNK2 pathway in order to understand the signals that stimulate resorption and their role in flagellar length control.

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Protein Methylation During Flagellar Resorption in *Chlamydomonas*.R. Werner-Peterson¹, R. D. Sloboda¹; ¹Biological Sciences, Dartmouth College, Hanover, NH

We are studying protein methylation during flagellar resorption in *Chlamydomonas*. Previously (Mol. Biol. Cell 19:4319) we showed that resorbing flagella contain increased levels of the enzyme Met E, plus several polypeptides containing asymmetrically methylated arginine residues. Met E produces methionine, and methionine is then converted to S-adenosyl methionine (SAM), which serves as the methyl donor in protein methylation reactions. Met E in resorbing flagella is phosphorylated, and in full-length flagella it is dephosphorylated. When cells are induced to resorb their flagella by treatment with 3-isobutyl-1-methylxanthine (IBMX) and the axonemal proteins are resolved on 2-D gels and immunoblotted, seven polypeptides can be identified containing asymmetrically methylated arginine residues. Polypeptides with this modification are not detected in samples from full-length flagella. Tandem mass spectrometry has been used to identify these seven polypeptides and the position(s) of their methylated residues in the primary sequences: four of these polypeptides are radial spoke proteins 1, 2, 5, & 6, a fifth is tektin, a structural component of the outer doublets, and the remaining two are as yet uncharacterized flagellar associated proteins. The enzyme PRMT 1 (protein arginine methyl transferase 1) is a type I PRMT; this class of enzymes produces asymmetric dimethyl arginine by transferring two methyl groups to one of the two terminal nitrogens of the guanidino moiety of the R-group. A peptide antibody specific for *Chlamydomonas* PRMT 1 labels full-length flagella in a punctate pattern along the length of the axoneme. When cells are induced to resorb their flagella by treatment with IBMX, PRMT 1 localization changes. In the presence of IBMX, PRMT 1 localizes to the flagellar tip complex (FTC), the site of net disassembly of the flagellar axoneme during resorption. These results, when considered in combination with relevant data from the literature, are consistent with a model in which resorbing conditions induce the activity of CALK, an Aurora-like kinase. CALK phosphorylates Met E stimulating production of methionine and subsequent conversion to SAM. PRMT 1 then uses SAM as the donor to methylate asymmetrically these seven axonemal polypeptides in the region of the FTC. These specific methylations induce axonemal disassembly directly and/or enhance the interaction of the disassembled polypeptides with intraflagellar transport particles to move these axonemal components in the retrograde direction to the cell body for degradation and/or recycling. We are currently using cloned enzymes to recapitulate the methylation pathway *in vitro* to study further the effect of protein methylation on the stability of the axoneme. Supported by NSF MCB 0950402 to rds.

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A CDK-like protein kinase is involved in flagellar shortening in *Chlamydomonas*.Z. Hu¹, J. Pan¹; ¹School of Life Sciences, Tsinghua University, Beijing, China

Primary cilia assemble in interphase, and disassemble prior to mitosis. The disassembly of primary cilia is implicated in controlling cell cycle progression from in G1 to S transition. Protein phosphorylation plays a critical role in ciliary disassembly. By using *Chlamydomonas* as a model system, we are studying the mechanism of ciliary disassembly. Here, we report that a CDK-like protein kinase is involved in flagellar disassembly. By using insertional mutagenesis, we have generated a mutant fs1 (3-1) that was defective in flagellar shortening. Upon induction of flagellar shortening by NaPPI or IBMX, fs1 exhibited slower kinetics compared to wild type cells. Prior to mitosis, wild type cells shortened their flagella rapidly within 1 hr, whereas flagellar shortening in fs1 (3-1) took 1 to 2 hrs. Biochemical study showed that CALK phosphorylation, which occurs immediately upon induction of flagellar shortening, was delayed. Molecular cloning identified the mutated gene encoding a CDK-like protein kinase, named CrCDKL1C.

Transformation of HA-tagged CrCDKL1C gene complemented the mutant phenotype, confirming the identity of the mutated gene. CrCDKL1C is localized in the flagella as well as in the cell body. Upon induction of flagellar shortening, CrCDKL1C became phosphorylated. Defective CALK phosphorylation in *fs1* (3-1) mutant upon induction of flagellar shortening indicates that CrCDKL1C acts upstream of CALK activation. Taken together, CrCDKL1C is a new player of the protein phosphorylation pathway in regulation of flagellar disassembly. This work is partly supported by the National Natural Science Foundation of China (Grants 30988004, 30830057) and Tsinghua university (2010THZ0).

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Rootletin is required for intraflagellar transport and ciliary maintenance.

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Primary cilia are microtubule based sensory organelles anchored by a basal body that is closely associated with rootletin, a protein often found as striated rootlets. Rootletin is known to function in centriole cohesion, and its disruption causes ciliary photoreceptor degeneration. However, it remains unresolved whether rootletin is universally required for ciliary maintenance, and if so, by what mechanism. Here we identify *C. elegans* chemosensory gene, *che-10*, as a rootletin homolog. CHE-10/rootletin localizes proximally and distally to basal bodies of cilia and maintains the structural and/or functional integrity of the cilium. In *C.elegans*, cilia extend from the dendritic tip of 60 sensory neurons, 3 of which possess conspicuous striated rootlets. In cilia that lack conspicuous rootlets, we demonstrate that CHE-10 is required for the assembly and sustained function of intraflagellar transport (IFT) particles, a process known to be critical for ciliary maintenance. In the absence of CHE-10, ciliogenesis occurs but cilia degenerate over time. In these cilia, CHE-10 is also required for the stability of the ciliary transition zone, basal body and the periciliary membrane compartment. In cilia that possess striated rootlets, we show that CHE-10 is required for its functional maintenance. Although the cilia remain structurally intact, in the absence of CHE-10/rootletin, their ability to function is altered (observed as a prolonged response to stimuli). Our findings provide mechanistic insights into how rootletin, either as rootlets or non-filamentous forms, supports the continued function of cilia by modulating IFT.

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Actin polymerization and turnover are required for normal flagellar assembly in *Chlamydomonas*.

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Chlamydomonas actin has 90% identity with mammalian skeletal muscle actin. It is known to be required for inner dynein arm assembly in flagella and the formation of fertilization tubules in mt+ gametes. In vegetative cells during G1, total actin labeling is seen in perinuclear regions. Localization shifts to the anterior of the cell below flagella early in mitosis and is at the cleavage furrow during cytokinesis. It was previously thought that vegetative *Chlamydomonas* cells contain very little F-actin due to inability of fluorescent phalloidin to label this population but successfully label gametic fertilization tubules. However, here we show functional consequences of filament disruption. Acute disruption of actin polymerization using a variety of small molecule inhibitors at non-toxic concentrations results in flagellar shortening. In a null mutant of actin and when disrupting actin filament formation, flagellar elongation and regeneration are impaired. Stabilization of actin filaments also resulted in impaired flagellar

assembly. These data suggest that normal actin polymerization dynamics are required for flagellar length maintenance through regulation of flagellar assembly.

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Primary Cilia Respond to Uniaxial Strain by Reorienting and Elongating Along the Axis of Stretch.

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Primary cilia, the cellular 'antennae' found in nearly every cell type, are critical chemosensory organelles that transduce external signals into the cell interior. These signaling centers are essential for maintaining normal cellular activity and tissue integrity, as genetic mutations affecting ciliary function can cause debilitating diseases such as polycystic kidney disease and Bardet-Biedl syndrome. Primary cilia are key sensors of extracellular chemical cues, exemplified by their established role in sonic hedgehog signaling, however their role in mechanosensation remains elusive. In this study, we seek to determine whether primary cilia are involved in mechanosensation by testing their response to mechanical strain across an epithelial cell sheet. Epithelial cells are thought to coordinate and dissipate responses to mechanical stresses through robust intercellular junctions that are critical for maintaining sheet integrity. Using a PDMS stretching device, we test the effect of uniaxial strain on cilia behavior in a confluent sheet of retinal pigment epithelial cells. We find that primary cilia rotate and elongate along the axis of stretch. In addition, we find that this behavior is dependent on actin network integrity and myosin activity. This interesting result suggests a potential mechanism of primary cilia in regulating and coordinating epithelial cell behavior, namely through sensing and transducing mechanical signals. Our novel observations propose a potential link between primary cilia behavior, cellular mechanosensation and actomyosin contractility.

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Smad2/3 activity is induced by fluid shear stress in proximal tubular epithelial cells, which is more prominent in *Pkd1*^{-/-} cells.

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Autosomal dominant polycystic kidney disease (ADPKD) is characterized by formation of many fluid-filled cysts, leading to loss of renal function in adulthood. ADPKD is caused by mutations in the *PKD1* or *PKD2* genes, encoding polycystin-1 (PC-1) and polycystin-2 (PC-2), respectively. PC-1 and PC-2 co-localize at cell-cell interactions and the primary cilium of renal epithelial cells, where PC-2 functions as Ca²⁺ channel in a complex with PC-1. Lack of this complex in cilia is one of the mechanisms of cyst formation. Moreover, mutations or deletions of other ciliary proteins can also cause polycystic kidneys in mouse models and patients, indicating the role of cilia during cystogenesis. Primary cilia are essential in organizing various signaling systems that sense environmental cues, triggered by fluid flow and growth factor stimulation, and transmit signals to the cell interior.

To study flow dependent signaling in ADPKD, we make use of an *in vitro* conal flow system in which mouse *Pkd1* wild-type and knockout proximal tubular epithelial cells (PTEC) are exposed to laminar shear stress. Smad2/3 activation of the TGF- β signaling pathway was analyzed using the CAGA luciferase-reporter assay, using qRT-PCR and RT-MLPA to measure expression of down-stream targets, and immunofluorescence microscopy to study nuclear localization of pSmad2.

PTEC exposed to shear stress showed increased Smad2/3 activation and nuclear localization of pSmad2. In addition, expression of the Smad2/3 targets *Pai1* and *Fibronectin* was dramatically increased. An inhibitor of the upstream receptors Alk4/5 completely blocked Smad2/3 activity, indicating that increased autocrine TGF- β or activin signaling is involved. Strikingly, this response is more pronounced in *Pkd1* knockout PTEC compared to wild-type cells.

Our data suggest that *Pkd1* knockout cells are more responsive to fluid shear stress than wild-type cells with respect to TGF- β signaling activation. We hypothesize that *Pkd1* disruption sensitizes cells to increased Smad2/3 dependent signaling, leading to processes that are involved in cyst formation, like dedifferentiation, proliferation, and fibrosis.

Cytokinesis I

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Role of Cytokinetic Ring in Coordinating Septum Formation.

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Cytokinesis in fungal cells requires both a contractile actin-based ring and the assembly of a cell wall septum. In *S. pombe*, ingression of the plasma membrane at the cleavage furrow is accompanied by polymerization of the cell wall fibers on the extracellular side of the membrane, and pulling from contractile ring on the cytoplasmic side. Previous work in the lab suggests that a large force is needed from the division apparatus to counter large internal turgor pressure, and that most of this force is from pushing by the growth of the cell wall, not from pulling forces of the contractile ring (Proctor et al, Curr Biol 2012). Ingression of the septum continues even after removal of the ring by Latrunculin A treatment. However, the rate of septum ingression is decreased when the ring is absent or defective. Here, we examine the function of the contractile ring in coordinating septum assembly. We find that the ring keeps the ingressing membrane in a circular shape. In the absence of F-actin or in various ring mutants, the septum ingresses, but in a disorganized and asymmetric manner, so that the furrow is non-circular. If we physically manipulate the cell to distort the division site to an oval, the septum grows asymmetrically to reform a circular shape. We propose that the glucan synthases responsible for septum assembly at the plasma membrane may be mechanically sensitive to forces exerted by the ring. In this manner, the ring globally coordinates cell wall assembly to keep the closing furrow in circular shape.

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Phosphorylation of Iqg1 by Cyclin Dependent Kinase (CDK), Cdc28, Temporally Regulates Actin Ring Formation.

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Cytokinesis is the final step in cell division when the cell separates the cytoplasm by contracting a ring composed of filamentous actin (F-actin) and type II myosin. Iqg1, an IQGAP family member, is an essential scaffolding protein in budding yeast (*S. cerevisiae*) required for actin recruitment to, and contraction of, the actomyosin ring. Actin is recruited by the calponin homology domain (CHD) late in anaphase after Iqg1 is localized to the bud neck. Four perfect consensus sites for the cyclin-dependent kinase (CDK) Cdc28 were identified flanking the CHD, which lead to the model that Cdc28 phosphorylation of Iqg1 negatively regulates F-actin binding to prevent actin ring formation before anaphase. To test this model the four consensus sites

were mutated into a non-phosphorylatable form (4A) and a phosphomimetic form (4E). Morphological analysis of mutant cells showed that preventing phosphorylation resulted in 40% chained cells, while mimicking phosphorylation caused 60% chained cell phenotype, indicating that phosphorylation of Iqg1 by CDK is important for cytokinesis. Immunofluorescence data on synchronized cells indicates that actin ring formation occurs 20 minutes early in cells that express Iqg1-4A. We are currently analyzing the timing of ring formation in Iqg1-4E mutant cells, and we plan to determine if the mutants have altered binding to F-actin. Our data so far supports the hypothesis that phosphorylation of Iqg1 by CDK inhibits actin ring formation.

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Genetically separable roles of IQGAP Rng2p in the formation and constriction of the cytokinetic contractile ring in *S. pombe*.

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Cytokinesis by the fission yeast *Schizosaccharomyces pombe* requires formation and constriction of a contractile ring of actin and myosin. Contractile rings normally form by condensation of precursor protein assemblies, called nodes, but contractile rings form from strands of actin filaments and myosin-II if the node based mechanism is compromised. We report a systematic analysis to determine why fission yeast IQGAP Rng2p is required for cytokinesis. Rng2p is required for ring formation by both the primary, node-based mechanism or the alternative strand-based mechanism. Rng2p is also required for efficient ring constriction. Complementation experiments with deletion constructs lacking each of the four predicted domains of Rng2p revealed how each domain contributes to cytokinesis. The N-terminal actin binding calponin homology domain is not required for viability, ring formation or ring constriction. The IQ motifs are required for normal ring constriction, and the GAP related domain is required for node based ring formation. The C-terminal domain of Rng2p is the only domain essential for viability.

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Assembly and Architecture of Interphase Contractile Ring Precursor Nodes in *S. pombe*.

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During cytokinesis, cells form a contractile ring of actin and myosin to split the cell in two. In the fission yeast *S. pombe*, the contractile ring forms from an equatorial band of ~75 discrete protein structures called nodes. Understanding how these nodes assemble is necessary to understand the mechanism of contractile ring assembly and constriction. We used quantitative confocal microscopy to measure the times of appearance and numbers of seven node proteins long before mitosis, during interphase. We identified two distinct classes of nodes. One class of nodes tracks with the nucleus as it divides, appearing as new bands of nodes around daughter nuclei least 5 min before the daughter cells separate. The second class of nodes appears during the disassembly of the contractile ring at the old cell's equator, corresponding to the new tip of each daughter cell. These nodes then disperse through the cell cortex until they join the first class of nodes at the equators of the daughter cells, within 50 min after the cells separate. Quantitative fluorescence measurements indicate that proteins from each class of nodes exist in stoichiometric ratios. Based on time-lapse microscopy, particle tracking, and simulations of a mathematical model, we propose a mechanism by which the two classes of

nodes rejoin after cell birth. These measurements account for the location and movement of contractile ring precursor nodes throughout the course of the cell cycle.

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Cytokinesis in fission yeast: the pulling force behind ring assembly.

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The physical separation of a cell during cytokinesis requires spatial and temporal regulation of specialized cytoskeletal machinery to form and constrict a contractile ring. The cytokinetic ring of the fission yeast *Schizosaccharomyces pombe* assembles by coalescence of ~65 protein assemblies named precursor nodes distributed in a broad, cortical band around the equator of the cells. Different actin regulating proteins localize to nodes including myosin II and formin Cdc12p. The search-capture-pull-release hypothesis proposed that myosin II in one node captures and pulls on actin filaments polymerized by Cdc12p from a neighboring node, thus bringing the nodes closer together. This model assumed that myosin II is the sole source of force necessary to coalesce the nodes into a ring. Numerical simulations of this model reproduce the temporal and spatial features of contractile ring formation.

To test directly the force generating potential of myosin II during node coalescence, we analyzed ring formation in cells with different alleles of the myosin II heavy chain *myo2* (*myo2-E1*, *myo2-S1*, *myo2-S2* and *myo2-ΔIQ1*) and expressing a fluorescently-tagged node marker. As predicted by the search-capture-pull-release mechanism, cells with defective Myo2p took longer for nodes to coalesce than wild-type cells. However, a detailed analysis of node movements showed that their velocities were similar in *myo2* mutants and wild-type cells, indicating that the total force exerted on motile nodes was the same. Unconventional myosin II Myp2p is dispensable for cytokinesis under most conditions, but deletion of *myp2+* exacerbated the slow formation of contractile rings in *myo2* mutant cells, suggesting that Myp2p compensates for Myo2p during node coalescence when Myo2p function is compromised. Consistent with this hypothesis, Myp2p localized to nodes in cells with *myo2* mutations rather than concentrating only in fully formed rings and not in nodes.

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F-BAR protein Cdc15p is required for equatorial retention of the cytokinetic contractile ring in fission yeast.

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Cytokinesis is the final stage of cell division that involves partitioning of the mother cell into two equal daughter cells. The machinery required for cytokinesis is conserved in from fungi to humans. The fission *Schizosaccharomyces pombe* is an excellent model system to investigate the mechanism of cytokinesis. Cytokinesis in *S. pombe* begins by recruiting proteins that determine the position of the contractile ring followed by those that are required for the ring assembly. The contractile ring undergoes maturation for a short period of time before it begins to constrict. *S. pombe* recruits three different F-BAR proteins during different stages of cytokinesis: Cdc15p, Imp2p and Rga7p. Cdc15p is a phosphoprotein that is recruited before the assembly of the contractile ring and its localization depends in part on dephosphorylation by a Cdc14 phosphatase, Clp1p. We investigated the role of Cdc15p during cytokinesis by depleting the protein from *41xnm1cdc15* mutant cells by repressing expression of the gene. Cells

depleted of Cdc15p exhibit multiple cytokinesis defects at 25°C including misplaced and multiple septa. Contractile rings assemble normally in cells depleted of Cdc15p, but most of these rings slide along the cortex away from the cell center where they constrict or disassemble similar to cells depending on a truncated construct lacking the SH3 domain *cdc15 Δ SH3* (Roberts-Galbraith et al., 2009). The temperature sensitive allele *cdc15-140* interacted negatively with mutations in many cytokinesis genes and was synthetically lethal with deletion of unconventional myosin-II, *Δ myp2*. Myp2p concentrates in contractile rings at approximately the time when contractile rings begin to slide in cells depleted of Cdc15p and in *cdc15 Δ SH3* cells. In cells also lacking Myp2p contractile rings slide slowly and none constricted. Contractile rings assembled normally in cells lacking Myp2p but became irregular during constriction and septum deposition. In some *Δ myp2* cells the primary septum advanced ahead of the irregular contractile ring, suggesting a lack of coordination between ring constriction and septation. Contractile rings in *41xnm1cdc15* and *cdc15 Δ SH3* cells stopped sliding when Bgs1p, a transmembrane enzyme that synthesizes the primary septum, appeared in the membrane next to these rings. Timely appearance of Bgs1p next to the contractile ring depended on the Clp1p phosphatase.

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Anillin phosphorylation by Polo-like kinase 1 promotes a stable cleavage furrow in cytokinesis.

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Cytokinesis is the final step of cell division, partitioning the genetic information evenly into daughter cells. Failure in cytokinesis causes polyploidy and genetic instability, which can lead to cancer. Polo-like kinase 1 (Plk1) plays essential roles at multiple stages of cell cycle, yet little is known about its role in cytokinesis. Two screens identified Anillin as a possible Plk1 substrate. Anillin is a crucial organizer for furrow initiation that is highly concentrated at cleavage furrow and anillin depleted cells fail to complete cytokinesis. Here, we show that the levels of anillin phosphorylations peak in mitosis and then drop as cells exit from mitosis. We also found that Plk1 directly phosphorylates anillin in vitro and identified eight Plk1 phosphorylation sites on Anillin using tandem mass spectrometry. To investigate physiological significance of those phosphorylations, we generated stable HeLa cells expressing phosphorylation-deficient Anillin (Anillin 8A). Interestingly, Anillin 8A causes asymmetric furrow localization, anillin aggregation and membrane blebs. In concordance with these defects, Anillin 8A results in higher fraction of binucleate cells, indicative of complete cytokinesis failure, compared to its wild type counterpart (Anillin WT). These results demonstrate that Plk1 phosphorylates Anillin and stabilizes the cleavage furrow to enable successful cytokinesis.

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Anillin patterns the cortex by localizing to astral microtubules during cytokinesis.

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Animal cell cytokinesis occurs by the ingression of an actin-myosin contractile ring that bisects the parental cell. The anaphase spindle, comprised of polar astral microtubules and central spindle microtubules, spatially and temporally regulates the contractile ring to ensure that the cell cleaves after sister chromatids have segregated to their respective poles.

Cues from the central spindle activate RhoA, the upstream regulator of contractile ring assembly and ingression. However, additional cues from the astral microtubules also reinforce the

localization of active RhoA in a tight zone. Using cultured human cells, we show that astral and central spindle microtubules independently regulate the localization of contractile proteins during cytokinesis. Astral microtubules prevent the accumulation of contractile proteins during metaphase and early anaphase, and restrict their localization during ingression. The central spindle helps these proteins form a discrete ring, likely by directing the activation of RhoA in the equatorial plane. Anillin is a scaffold that crosslinks actin and myosin to the overlying membrane, and is required for cytokinesis. Previously, we found that co-depletion of anillin and MKLP1 (a central spindle component) blocked furrowing, supporting that anillin functions redundantly with the central spindle for determining the division plane. Similar to *C. elegans* anillin, we found that human anillin localizes to astral microtubules, and this localization competes with its recruitment to the cortex. Over-extension of astral microtubules restricts the localization of contractile proteins, and this is overcome by depleting anillin. The sequestration of anillin by microtubules may alter the organization of contractile ring proteins at the cortex to help establish the division plane.

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Anillin mediates feedback between the cortex and microtubules to define the division plane.

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Cytokinesis separates a cell into two daughter cells by the ingression of an acto-myosin contractile ring. The mitotic spindle, which is comprised of central spindle and astral microtubules, dictates the site of contractile ring assembly. Central spindle microtubules provide positive cues to the overlying equatorial cortex, while astral microtubules negatively regulate the recruitment of contractile proteins at the cell poles. Anillin is a scaffold protein that interacts with several components of the division machinery and is a key regulator of cytokinesis. In human cells, these components include acto-myosin filaments, their upstream regulators, RhoA and Ect2 (GEF for RhoA), and membrane-associated septins. Recently, the *C. elegans* anillin homologue, ANI-1, was shown to interact with astral microtubules. This interaction removes anillin from the cortex, causing a change in the organization of contractile proteins. In this manner, anillin functions as a component of the astral pathway. Therefore, anillin crosslinks the contractile ring to the plasma membrane and relays signals from the mitotic spindle to maintain the division plane.

The mitotic spindle can provide signals to the cortex, but it is not known if there are also signals that feedback from the cortex to the mitotic spindle. An attractive hypothesis is that anillin, which scaffolds the acto-myosin cytoskeleton to the membrane and associates with microtubules in *C. elegans* embryos, could be part of this feedback pathway. To address this, we are investigating possible anillin-microtubule interactions in cultured human cells. Preliminary results show that anillin co-localizes with microtubules at different stages of mitosis. Upon disruption of signals that normally recruit anillin to the cortex, such as decreasing RhoA activation, anillin's association with microtubules is enhanced. Anillin's localization to microtubules is also enhanced after increasing microtubule stability. Depletion of anillin causes a decrease in the proportion of central spindle microtubules and in monopolar cells, causes a complete loss of central spindle microtubules. These phenotypes are similar to those that arise after depletion of central spindle regulators, such as the centralspindlin components MKLP1 or MgcRacGAP/Cyk-4. This data suggests that anillin stabilizes or bundles central spindle microtubules, which could reinforce the division plane by ensuring that the cues that promote formation of the contractile ring are maintained throughout cytokinesis.

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Anillin couples polarity- and spindle-directed cleavage furrows during *Drosophila* neuroblast asymmetric cell division.

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Neuroblasts divide to generate a large daughter cell that maintains the neuroblast fate and a smaller one that differentiates. Asymmetric cell division results from two contractile actomyosin populations-the mitotic spindle directs contraction at the equatorial cortex (as in symmetrically dividing cells), whereas polarity proteins regulate myosin at the basal pole (unique to asymmetrically dividing cells). The link between polarity proteins and basal actomyosin contraction is poorly understood. It is also unclear how the two myosin populations collaborate to generate a single asymmetric furrow. We surveyed polarity factors for their ability to regulate basal myosin and identified the heterotrimeric G-protein subunit G β as a critical downstream component. We also examined which proteins from the canonical furrow pathway might be important. Although centralspindlin localizes with myosin at the neuroblast basal cortex, it is not required for basal furrowing. However, the Rho nucleotide exchange factor Pebble is required, suggesting that the polarity pathway may regulate basal furrowing by impinging with the canonical pathway at this node. The scaffold protein Anillin does not control basal myosin, but instead regulates the connection between the spindle and polarity furrows. Reduction of Anillin function leads to formation of a basal furrow, followed by a spatially separate equatorial furrow. Our results indicate that asymmetric cell division in this system results from the coupling of two distinct actomyosin populations that are regulated by related pathways.

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The transition from contractile ring to midbody ring is controlled by distinct mechanisms of retention and removal of Anillin.

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Background: During cytokinesis, closure of a dynamic actomyosin contractile ring (CR) is followed by formation of a stable midbody ring (MR). Towards understanding the architecture of these two structures and their relationship to one another, we have analyzed the behavior and regulation of the scaffold protein, Anillin, known to stabilize the furrow during CR closure and to allow formation of the MR.

Results: Time-lapse microscopy of *Drosophila* S2 cells revealed that the MR forms and thins via a maturation process lasting approximately 1 hr from CR closure. During this time, membrane-associated Anillin-GFP was progressively removed from the nascent MR by mechanisms that included extrusion, shedding and internalization, until only a mature MR remained. Depletion of the Citron kinase, Sticky, led to complete removal of Anillin-GFP and MR disintegration, resulting in either premature abscission or furrow regression. Sticky depletion also blocked the ability of an Anillin N-terminal fragment to prevent furrow oscillations during CR closure and to form persistent MR-like structures. Conversely, depletion of the septin, Peanut, or deletion of the C-terminal domains of Anillin, blocked Anillin extrusion and perturbed the normal maturation and thinning of the MR. Co-depletion of Sticky and Peanut led to cleavage furrow oscillations and disrupted both the normal retention and removal mechanisms of Anillin-GFP during MR formation.

Conclusions: These data show that the transition from dynamic CR to mature MR occurs via a gradual maturation process involving Sticky-dependent retention of a select pool of Anillin N-termini at the midbody that protects against septin-dependent removal mechanisms acting on the Anillin C-terminus. Proper coordination of these antagonistic mechanisms of retention and removal is important for furrow stability, MR maturation and the fidelity of cytokinesis.

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PAR-5/14-3-3 Inhibits Cortical Association of Centralspindlin to Limit the Site and Extent of the Cytokinetic Furrow.

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During cytokinesis, a cell furrows at a unique position between segregating chromosomes to successfully divide into two daughter cells. The mechanism by which a single, well-focused cleavage furrow is generated is inadequately understood.

The small GTPase RhoA promotes the cortical recruitment and organization of actomyosin for furrow ingression. RhoA is activated by signals from the mitotic spindle. In particular, the centralspindlin complex, a heterotetramer of a kinesin-like protein ZEN-4 and a RhoGAP protein CYK-4, associates with the RhoGEF ECT-2 at the central spindle, an interaction that is required to activate RhoA. However, because the spindle is not adjacent to the cortex, it is unclear how RhoA activation is achieved and fine-tuned at the cell cortex.

To gain insight into this process, we examined early *C. elegans* embryos deficient in PAR-5/14-3-3 which were previously shown to have dramatically increased contractility during polarization, through an unknown mechanism (Morton et al., 2002). Recent studies demonstrate that contractility during cytokinesis depends on the combined action of a recently characterized protein, NOP-1, and CYK-4 (Tse et al., unpublished results). During polarization of wild-type embryos, all contractility requires NOP-1 and centralspindlin has no role. We find that PAR-5 depletion results in a hypercontractile phenotype during both polarization and cytokinesis. The hypercontractile phenotype is CYK-4 dependent and is associated with increased accumulation of CYK-4 and ZEN-4 on the cortex. This demonstrates that PAR-5 is an inhibitor of centralspindlin-dependent contractility. CYK-4 and ZEN-4 are interdependent for their cortical localization, though surprisingly they are not microtubule dependent. It has previously been shown in HeLa cells, that 14-3-3 binding to ZEN-4 prevents centralspindlin clustering, thereby inhibiting its stable localization to the central spindle (Douglas et.al., 2010). This inhibition is released upon phosphorylation of ZEN-4 by Aurora B kinase. Intriguingly, we have found that while Aurora B is required for centralspindlin-mediated furrowing in wild-type embryos, it is not required in PAR-5-depleted embryos. These results are congruent with the pathway shown to regulate centralspindlin oligomerization in HeLa cells. Thus, both central spindle and cortical localization of the centralspindlin complex may be modulated through its clustering activity. Centralspindlin oligomerization may promote cortical activation of RhoA, and spatial regulation of this activity could help focus the position of cleavage furrow formation to a single site.

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FIP3-Endosome Dependent Activation and Delivery of p50RhoGAP to the Cleavage Furrow Regulates Actin Depolymerization and ESCRT-III Recruitment to the Abcission Site During Cytokinesis.

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The last step of cell division is a physical separation of two daughter cells via process known as cytokinesis. After replication of the genetic material, mother cell divides by the formation of the cleavage furrow that constricts cytoplasm leaving two daughter cells connected by a thin intracellular bridge (ICB). The resolution of this bridge (abscission) results in separation of two daughter cells. Although mechanisms that govern abscission are not fully understood, recent evidence suggest that Rab11/FIP3-containing recycling endosomes (FIP3-endosomes) and the ESCRT protein complex play critical role in this process. The focus of this study was to dissect the roles of endosomes and ESCRT complex during cytokinesis and investigate the cross-talk between the ESCRT, cytoskeleton and endocytic membrane transport. First, we used correlative high-resolution 3D tomography and time-lapse microscopy to study the dynamics and localization of FIP3-endosomes and various ESCRT complex components. As the result of these studies, we demonstrate that FIP3-endosome fusion with the furrow plasma membrane mediate the initial thinning of the ICB to ~100 nm, known as the secondary ingression. In contrast, ESCRT-III complex components are only recruited after the formation of the secondary ingression and appear to only mediate the final scission event. Consistent with that, inhibition of FIP3-endosome targeting to the ICB and fusion block the recruitment of the ESCRT-III to the abscission site.

To further understand the role of endosomes during cytokinesis, we analyzed the proteome of immuno-isolated FIP3-endosomes. As the result of this analysis, we identified p50RhoGAP as a protein that is delivered to the furrow by FIP3-endosomes. We also demonstrate that p50RhoGAP is activated by association with FIP3-endosomes and is required to inactivate RhoA at the furrow during late telophase. Finally, we demonstrate that p50RhoGAP accumulatation at the ICB is required for localized actin depolymerization, secondary ingression formation and ESCRT-III targeting to the abscission site. Thus we propose that FIP3-endosomes regulate the disassembly of the ICB cortical actin network during the formation of the secondary ingression and are required for ESCRT-III recruitment to the secondary ingression site and subsequent abscission. These results provide a novel framework for the coordinated efforts of actin, FIP3-endosomes and ESCRT complexes to mediate abscission.

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Dissection of the temporal requirement for cytokinetic proteins.

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The physical division of one cell into two, or cytokinesis, is highly regulated both spatially and temporally. Separation of the chromosomes during anaphase must be coordinated with the assembly and constriction of a contractile ring at the division plane. Many of the proteins necessary during division also play crucial roles during oogenesis and development, making them difficult to study by traditional forward and reverse genetic approaches. The use of fast-acting (<30s), temperature-sensitive (*ts*) cytokinesis-defective mutants allows one to bypass any developmental requirements in order to study the role of these proteins specifically during cytokinesis. The proteins encoded by these alleles are functional at low, permissive temperatures and are inactivated at high, restrictive temperatures. A number of fast-acting *ts*

mutants in the nematode worm *Caenorhabditis elegans* have been identified that affect crucial functions in the division process, including myosin-II motor activation, actin filament nucleation, Rho GTPase-mediated signal transduction, and chromosomal segregation. Using novel, rapid temperature-shifting technology in conjunction with time-lapse confocal microscopy, we have achieved precise temporal control over these *ts* mutants. We have found that they are rapidly reversible by shifting single-cell embryos from permissive to restrictive temperature for a short period following anaphase onset, before returning the embryos to permissive temperature and scoring for cytokinesis completion. We are currently dissecting the precise temporal requirement for the proteins affected by each *ts* mutation relative to furrow initiation, contractile ring constriction, and contractile ring closure, by shifting embryos from permissive to restrictive temperature at various time points following anaphase onset. As has been published (Liu et al., *Dev. Biol.*, 2010, 339:366–373), we have found myosin motor activity is required for furrow initiation, constriction, and for a brief time following furrow closure. However, we have also found other cytokinetic proteins that are necessary during furrow initiation but dispensable during furrow closure. The determination of these proteins' temporal requirements will aid in better understanding the precise roles they play throughout division.

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Vertebrate Cytokinesis: Nonmuscle Myosin II Exerts Tension but does not Translocate Actin.

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In vertebrate cells, nonmuscle myosin II (NM II) and cytoplasmic actin compose two major contractile proteins at the contractile ring mediating cytokinesis. It is generally thought that contractile ring constriction is driven by NM II translocation of antiparallel actin-filaments, similar to the sliding filament model in muscle contraction. Using a combination of in situ (COS-7 cells treated with siRNA and blebbistatin), kinetic (stopped flow experiments with baculovirus-expressed protein), as well as in vivo (genetically altered cardiomyocytes in mouse hearts) approaches we provide evidence that NM II translocation of actin is not essential for vertebrate cytokinesis. The three NM II paralogs (II-A, II-B and II-C), similar to skeletal muscle myosin, are composed of catalytically active heads, which bind to and translocate actin in an ATP-dependent manner, and bipolar filament-forming rods. NM II-B is the major paralog expressed in cardiac myocytes and COS-7 cells. Ablation of NM II-B in mouse cardiac myocytes in vivo or siRNA depletion of NM II-B in cultured COS-7 cells results in cytokinesis defects indicating the requirement of NM II-B for cytokinesis. Graded knockdown of NM II in cultured COS-7 cells reveals that the amount of NM II limits the rate of ring constriction. Restoration of the constriction rate with motor-impaired NM II mutants shows that the ability of NM II to translocate actin is not the rate-limiting factor for cytokinesis. Blebbistatin inhibition of cytokinesis further reveals the importance of myosin strongly binding to actin thereby exerting tension during cytokinesis. This role is substantiated by transient kinetic experiments showing that the mechanochemical properties of mutant NM II support efficient tension maintenance despite loss of the ability to translocate actin. Under loaded conditions, mutant NM II exhibits a prolonged actin attachment in which a single mechanoenzymatic cycle spans most of the time of cytokinesis. This prolonged attachment promotes simultaneous binding of essentially all NM II heads to actin, thereby increasing tension and resisting expansion of the ring and cell cortex. Importantly, in the three dimensional context of the mouse heart in vivo, mutant NM II-B R709C that cannot translocate actin filaments also rescues multinucleation in NM II-ablated cardiac myocytes. We propose that the major roles of NM II in vertebrate cell cytokinesis are to bind and crosslink actin-filaments and to exert tension on actin during contractile ring constriction.

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Characterizing cortical myosin mini-filament length and its macroscopic implications in cytokinetic dynamics.

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Non-muscle myosin II (here called myosin) is the sole motor implicated in closing the contractile ring during cytokinesis. While myosin has long been assumed to slide actin filaments in a motor capacity, several recent papers propose that myosin's crosslinking activity plays more prominently in cytokinesis. Myosin holoenzyme dimers can assemble into oligomeric bipolar mini-filaments. The size and abundance of myosin mini-filaments likely dictates the relative contributions of myosin's activities to ring closure. However, mini-filaments have never been directly observed or characterized in animal cell cytokinesis.

To determine the composition of individual myosin-containing cortical features, we use a novel TIRF microscopy-based imaging assay, specifically illuminating cortical GFP-tagged myosin heavy chain (NMY-2) in transgenic *C. elegans* embryos. Cortical NMY-2-GFP is immobilized by fixation and completely photobleached. A custom image analysis algorithm calculates bleaching events from the decrease in intensity throughout photobleaching, therefore counting GFP molecules per diffraction-limited feature. Based on the ratio of endogenous to functional GFP-tagged myosin, the total number of NMY-2 dimers per feature is calculated.

Control cells possess two populations: mini-filaments with an average of 17 dimers and those with 25 dimers. Statistical analysis of the distributions around these averages indicates that mini-filaments incorporate variable amounts of labeled myosin, but are built to consistent specifications. Depletion of the myosin activator Rho-Kinase increases the relative abundance of larger myosin mini-filaments.

To test the macroscopic implications of perturbations that alter cortical myosin assembly, we depleted myosin kinases and measured myosin recruitment and organization, and contractile ring kinetics and geometry (concentricity within the division plane). Rho-K depletion decreases myosin cortical recruitment and organization, slows ring closure and makes it less concentric. Our unpublished work suggests that ring concentricity is dictated by the relative efficiency of cytoskeletal filament sliding and alignment. Thus, we predict that larger mini-filaments participate more in actomyosin sliding, perhaps via load-dependent enhancement of motor activity. Depletion of MRCK-1, a less well-understood conserved myosin kinase, increased myosin cortical recruitment but had little effect on furrowing kinetics. Following simultaneous depletion of both kinases, cortical myosin organization and recruitment were drastically reduced and, as expected for a much weaker cortex, a unique concentric phenotype emerged. Thus, while Rho-k is the more important kinase for myosin activation, MRCK-1 contributes to myosin organization and contractile ring dynamics.

We conclude that myosin is recruited to the cortex as multi-headed mini-filaments whose assembly is tightly regulated and which impacts several aspects of contractile ring function.

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A novel protein Rng8 binds type V myosin Myo51 and is involved in cytokinesis.

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Cytokinesis, the final stage of cell-division cycle, partitions a mother cell into two daughter cells. More than 100 proteins have been identified to be involved in cytokinesis in the fission yeast *Schizosaccharomyces pombe*. However, more proteins were implicated in cytokinesis. Rng8 is a novel coiled-coil protein that has been localized to the division site in a global analysis of protein localization in fission yeast. We found that Rng8 colocalizes with type V myosin Myo51 in the contractile ring during cytokinesis as well as in the cable and particle structures during interphase. rng8 deletion phenocopies myo51 deletion in cytokinesis defects, especially in the contractile-ring assembly. Surprisingly, localization of Myo51 is dependent on Rng8, whereas myo51 deletion partially compromises Rng8 localization. Moreover, Rng8 interacts with Myo51 in a co-immunoprecipitation assay. Thus we hypothesize that Rng8 is an adaptor protein for Myo51 and regulates Myo51's localization and function. To test the hypothesis, we identified the domains involved in the Rng8 and Myo51 interaction. Rng8 is predicted to contain three coiled-coil domains and an EF-hand motif. Our data suggest that the third coiled-coil domain (CC3) is required for binding to Myo51, while the second coiled-coil domain (CC2) is critical for Rng8 localization. Through domain analyses of Myo51, we found that the rod region in the tail is necessary for Rng8 association, and the globular tail domain may stabilize the interaction.

Interestingly, we discovered that rng8 deletion but not myo51 deletion suppressed the contractile-ring assembly defects in rlc1 deletion, suggesting Rng8 may have roles other than recruiting and regulating Myo51. Genetic evidence further suggested that Rng8 may negatively regulate the motor activity of type II myosin Myo2, although the mechanism is currently unknown. Taken together, our data suggest that the novel protein Rng8 is involved in cytokinesis through regulating Myo51 and probably also Myo2.

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Chromatin mediates MLC contraction during cytokinesis by regulating Aurora B kinase.

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Typical cell division requires coordinated chromosomal separation, cleavage furrow ingression, and abscission. The Saunders' lab is interested in how defects in mitosis, including lagging chromosomes and anaphase bridges, contribute to aberrant cytokinesis. This cortical contraction is dependent upon the dynamics of the acto-myosin cytoskeleton. Myosin light chain (MLC) is phosphorylated by a dedicated kinase (MLCK) and initiates contraction following movement of the chromosomes away from the metaphase plate. This process is regulated by the Aurora B kinase, which moves away from segregating chromosomes to the site of cell division as part of the chromosomal passenger complex (CPC). Our current model is that Aurora B acts to inhibit MLCK to regulate the timing of cortical contraction. We are focused on the role of chromatin in activating Aurora B to prevent ingression of the cleavage furrow until the chromosomes have moved away from the divisional plane. After instigating lagging chromatin with gamma irradiation cells overexpress Aurora B, show decreased levels of MLCK activity and pMLC, and have a significantly larger propensity for cytokinesis failure. Cytokinesis failure from lagging chromatin can be mostly prevented by inhibiting Aurora B, phosphomimetic MLC or constitutively active MLCK. We propose a pathway by which Aurora B, which is activated by chromatin, inhibits MLCK and thereby prevents phosphorylation of MLC to prevent premature contraction until all chromosomes have cleared from the site of cell division. Furthermore, we show that cancer cells become tetraploid, a precursor of genomic instability, by initial DNA

damage causing lingering chromatin and Aurora B activation, leading to inhibition of MLCK and furrow regression.

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Identifying the role of Cin85 and CD2AP in Septin mediated cytokinesis.

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Cytokinesis is the last step of cellular division where the mother cell is split into two daughter cells. During telophase an actomyosin ring constricts until the two daughter cells remain attached by a thin, microtubule-stabilized intercellular bridge. The center of this is referred to as the midbody and the last step of cytokinesis, abscission, is the physical breakage of the bridge at the midbody. We have previously shown that Septin 9 (SEPT9) is required for abscission in HeLa cells, and depletion of SEPT9 results in the accumulation of cells connected by midbody bridges. Structure-function studies have revealed that overexpression of the N-terminus of SEPT9 also causes cell division defects at the abscission stage, raising the possibility that we may be titrating out a critical binding partner of SEPT9 important in cell division. Perusal of the sequence of the SEPT9 N-terminus revealed the presence of two proline-rich motifs that would be predicted to serve as binding sites for Src Homology Domain 3 (SH3). In particular, these sequences match the consensus binding sequence of the SH3-containing Cbl- Interacting Protein of 85kDa (Cin85) and CD2-Associated Protein (CD2AP) adaptor proteins. These proteins belong to a family of adaptor proteins and are the human homologs of Cindr which has been implicated in Drosophila cytokinesis. We therefore hypothesize that the proline-rich motifs at the N-terminus of SEPT9 serve as binding for Cin85 and CD2AP. Preliminary immunofluorescence results from HeLa cells revealed that both Cin85 and CD2AP are localized to the intercellular bridge and midbody, respectively. In vitro pull down assays show an interaction between both adaptors with the Sept9 N- terminus and this interaction is dependent on the two proline rich motifs of Sept9. In addition, these studies have revealed specificity of the among the SH3 domains in each protein. Current studies are now investigating the individual roles of Cin85 and CD2AP in cytokinesis and the importance of SEPT9 binding in this process.

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Single molecule analysis of septin assembly.

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Septins are conserved GTP-binding proteins that can self-assemble into filaments with an affinity for phospholipid-containing membranes. Septin filaments are knit together into cortical assemblies where they act as protein scaffolds for diverse events such as cytokinesis, membrane remodeling, and cell polarization. We have combined TIRF and polarization microscopy to analyze the kinetics and mechanisms driving septin assembly in vitro on supported membrane bilayers and in vivo in fungal cells. In vitro, septin filaments are initiated from paired protofilaments that extend by diffusion and annealing. The presence of PIP2 enhances the rate of assembly. Similarly, in vivo, septin-puncta are diffusing in two-dimensions on the plasma membrane. Puncta that have not assembled into a higher order structures in vivo contain minimally a paired protofilament but in some cases puncta contain higher numbers of labeled septin molecules. This suggests that septins are in extended complexes before the initiation of a higher-order structure at a specified location. Orientation analysis using polarized light has revealed that transient septin complexes always align relative to the growth axis even while diffusing and not committing to an assembled state. This indicates that there is either a

membrane or some other cytoskeletal-based template that may spatially restrict annealing-driven septin polymerization in vivo.

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Mechanism of F-actin assembly during cytokinesis in fission yeast.

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In many eukaryotes, cytokinesis requires the assembly and constriction of an actomyosin-based contractile ring. Despite the central role of this ring in cytokinesis, the mechanism of F-actin assembly and accumulation in the ring is not fully understood. Here we investigate the mechanism of F-actin assembly during cytokinesis in *Schizosaccharomyces pombe* using lifeact as a probe to monitor actin dynamics. Previous work has shown that F-actin in the actomyosin ring is assembled de novo at the division site. Surprisingly, we find that a significant fraction of F-actin in the ring is recruited from formin-Cdc12p nucleated long actin cables that are generated at multiple non-medial locations and incorporated into the ring by a combination of myosin-II and myosin-V activities. Our results together with findings in animal cells suggest that de novo F-actin assembly at the division site and directed transport of F-actin cables assembled elsewhere can contribute to ring assembly.

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Cooperation between the formins Cdc12 and For3 for cytokinesis in fission yeast.

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Cytokinesis is the final step of the cell-division cycle, resulting in formation of two daughter cells. The fission yeast *Schizosaccharomyces pombe* is an ideal model organism to study eukaryotic cytokinesis, because the components, mechanisms, and regulatory pathways are well conserved. Cells from yeast to humans assemble the contractile ring of actin, myosin-II, formin, and other proteins, which constricts to divide the cells. Formins nucleate long, unbranched actin filaments. The fission yeast has three formins, but only Cdc12 is essential for contractile-ring assembly. Cells lacking Cdc12 fail to divide and become long multi-nucleated cells before dying. Cdc12 localizes to cytokinesis nodes, precursors of the contractile ring, where it nucleates actin filaments that interact with myosin-II in neighboring nodes to pull them together into a ring. Two temperature-sensitive alleles of *cdc12*, *cdc12-112* and *cdc12-299*, lack the ability to nucleate actin filaments in vitro but localize normally to nodes. However, the nodes condense into clumps rather than rings at the restrictive temperature. Clump formation in the absence of Cdc12 nucleation activity suggests the presence of other actin nucleators. Another formin, For3, nucleates ~10% of the actin filaments at the division site, although its primary role is nucleating actin cables in interphase for polarized growth. For3 depends on F-BAR protein Cdc15 for its localization to the division site during anaphase A in puncta that resemble cytokinesis nodes but do not overlap with myosin-II. Observation of node movements in *cdc12-112 for3* deletion double mutant at the restrictive temperature suggests that actin filaments nucleated by For3 might be responsible for pulling nodes together into clumps in *cdc12-112* single mutant. Consistently, overexpressing For3 could rescue *cdc12-112* at the restrictive temperature, suggesting that For3 might assist Cdc12 in contractile ring assembly. Amino-terminal truncations of Cdc12 can replace the endogenous copy, but they compromised cell viability in combination with other cytokinesis mutants. Moreover, such truncations depended on actin to localize to the division site and were synthetic lethal with *for3* deletion or the actin-binding

mutant *for3-1930A*. Both *for3* mutants were also synthetic lethal with ring assembly mutants *rng2-D5* and *cdc4-8*. Together, these results reveal a novel role for For3 at the division site and cooperation between two formins for successful completion of cytokinesis.

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Interaction of Iqg1 with formins in budding yeast cytokinesis.

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In budding yeast, both the IQGAP Iqg1 and the formins Bni1 and Bnr1 are required for actin ring formation, either cooperatively or through parallel pathways. Evidence that IQGAPs can interact with formins in mammalian cells and *C. albicans* suggests that these proteins act together to organize F-actin. Our goal is to determine how these proteins function to assemble the actin ring. The possible mechanisms, which are not mutually exclusive, are 1) Iqg1 is required for formin localization to the bud neck, 2) binding keeps one or both proteins in an active conformation, 3) Iqg1 bundles F-actin nucleated by formins. To determine if Iqg1 is required to localize the formins, we examined Bni1-GFP localization after cell synchronization and *GAL1-IQG1* shut-off. Quantitative analysis of fluorescence intensity indicates that there is no loss of Bni1 at the bud neck when Iqg1 is depleted. We are currently performing similar experiments with Bnr1-GFP. In human cells, the C-terminus of Iqg1 was shown to be necessary and sufficient for binding to the FH3 region of formin Dia1, and the authors termed this region Dia1 binding region (DBR). We created GST fusions with the N terminus of Bni1 and Bnr1, which contain the FH3 regions. These GST-formin-N-terminus proteins pull down full length Iqg1 from yeast extracts. Surprisingly, we found that the DBR region of Iqg1 is not required for this interaction. We are currently using Iqg1 deletion mutants to determine the region of Iqg1 that mediates binding to the formins. Once this interaction domain has been mapped, we can use Iqg1 deletion mutants in yeast cells to determine the effects on actin ring formation.

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Biomechanics of actomyosin ring contraction in budding yeast.

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In this work we constructed a quantitative biomechanical model of actomyosin ring constriction mediated by filament sliding driven by actin depolymerization coupled with crosslinkers and myosin motor activity. Model simulations using the parameters constrained by experimental measurements confirmed that actin depolymerization is the predominant force for ring constriction. The model predicts the proportionality of the contraction speed to cell size, which is validated by measurements for rings with different initial sizes in yeast cells with different ploidies.

Mitosis I

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17 β -estradiol-induced apoptosis is mediated by impaired bipolar microtubule array causing prometaphase arrest, Cdk1-dependent phosphorylation of Bcl-2 family proteins, and activation of Bak and the mitochondrial caspase cascade in human Jurkat T cells

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In Jurkat T cell clones (JT/Neo), G2/M arrest, apoptotic sub-G1 peak, mitochondrial membrane potential ($\Delta\psi$) loss, and TUNEL-positive DNA fragmentation were all induced following exposure to 17 β -estradiol (17 β -E2), whereas all these events except for G2/M arrest were not induced in JT/Bcl-2 cells overexpressing Bcl-2. Under these conditions, phosphorylation of Cdc25C at Thr-48, dephosphorylation at Tyr15 and phosphorylation at Thr161 of Cdk1, upregulation of the cyclin B1 level, histone H1 phosphorylation, Bcl-2 phosphorylation at Thr-56 and Ser-70, Mcl-1 phosphorylation, and Bim phosphorylation were detected, regardless of Bcl-2 overexpression. However, 17 β -E2-induced the upregulation of the Bak level, Bak activation, activation of caspases-9, -8, and -3, and PARP degradation were abrogated by Bcl-2 overexpression. In the presence of G1/S blocking agent hydroxyurea, 17 β -E2 failed to induce G2/M arrest and all apoptotic events including activation of Cdk1 and phosphorylation of Bcl-2, Mcl-1, and Bim. The 17 β -E2-induced phosphorylation of Bcl-2 and Bim, and the mitochondria-dependent apoptotic events were suppressed by Cdk1 inhibitor, but not by Aurora A and Aurora B kinase inhibitors. Analysis by immunofluorescent microscopy showed that an aberrant bipolar microtubule array, incomplete chromosome congression at the metaphase plate and prometaphase arrest, which appeared to be reversible, were the underlying factors for 17 β -E2-induced mitotic arrest. The in vitro microtubule polymerization assay demonstrated that 17 β -E2 could directly inhibit microtubule formation. These results demonstrate that the apoptogenic activity of 17 β -E2 was attributable to the impaired mitotic spindle assembly causing prometaphase arrest and prolonged activation of Cdk1 and resultant phosphorylation of Bcl-2, Mcl-1 and Bim, and the activation of Bak and the mitochondria-dependent caspase cascade in Jurkat T cells.

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Cell death after mitotic arrest: the role of Bub1 and Survivin.

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The Spindle Assembly Checkpoint (SAC) is a surveillance mechanism that halts mitosis until all kinetochores are attached to microtubules, by inhibiting the Anaphase Promoting Complex (APC). Drugs that target microtubules can activate the SAC and induce a protracted mitotic arrest. After a prolonged arrest cells can die in mitosis, in the subsequent G1 phase or survive. However, mechanisms dictating the cell fate after a prolonged mitosis are unknown. Bub1 and survivin are proteins involved in the SAC and in chromosome alignment. Moreover, both proteins have been proposed as regulators of cell death in some models. As previously reported, we observed cells dying in mitosis or interphase in time lapse experiments with paclitaxel treated HTC116 cells. Bub1 is localized to the kinetochore from the beginning of mitosis until metaphase, and it is diffusely localized during interphase. Immunofluorescence experiments revealed that Bub1 nuclear foci can be found in cells released from a prolonged mitosis. We did not observe changes in Bub1 protein levels during the mitotic arrest as were evaluated by immunoblotting. Survivin localization is unmodified during a prolonged mitosis, but

levels of this protein are diminished. We hypothesize that Bub1 foci in treated cells could be complexes of Bub1 coupled with other proteins and may be playing a role in functions not linked to mitosis. On the other hand, low levels of survivin after a mitotic arrest could be related to cell death in cells treated with antimetabolic drugs. Grants: PAPIIT (IN213311-3), CONACYT (83959)

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Investigating the biochemical properties of the Chromosomal Passenger Complex *in vitro*.

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The chromosomal passenger complex (CPC) is a major regulator of mitosis in eukaryotes. It is comprised of four essential and conserved proteins known in mammals/yeast as Aurora B/Ipl1, INCENP/Sli15, Survivin/Bir1 and Borealin/Nbl1. These subunits act together in a highly regulated fashion. Two activities important for CPC function are the regulation of its kinase activity and its localization. Although kinase activity regulation at centromeres and regulation of localization have been investigated extensively, studies on the CPC's basic biochemical properties are still in their infancy. Here we describe the biochemical characterization of purified *S. cerevisiae* CPC. Our *in vitro* findings show that the Sli15/INCENP microtubule-binding domain (MTB) is not necessary for CPC binding to microtubules (MTs), but only provides regulatory fine-tuning of the CPC/MT interaction. Furthermore, the Sli15/INCENP linker domain, comprised of residues E91 to I631, is not necessary for physical association of Ipl1/Aurora B with Bir1/survivin and Nbl1/borealin, suggesting that a direct interaction between Ipl1 and Bir1 exists and is strong enough to ensure CPC's integrity and stability. The CPC interaction with microtubules appears to be electrostatic in nature and depends on the C-terminal tail of tubulin. The Ipl1 kinase activity requires activation by auto-phosphorylation. Microtubules activate CPC kinase activity, suggesting a cooperative model for activation.

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Regulation of the chromosomal passenger complex and cell cycle checkpoints during early embryogenesis.

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Following fertilization most metazoans undergo a characteristic stage typified by rapid, transcriptionally silent cell cycles that oscillate between replication and mitoses without G1 and G2 phases. Cell volume halves at each cleavage without cell growth until the mid-blastula transition (MBT) occurs at a threshold nuclear to cytoplasmic ratio (N:C). The MBT is characterized by activation of zygotic transcription, cell cycle lengthening, acquisition of gap phases and checkpoint function. Mechanisms underlying this cell cycle remodeling at the MBT and the regulatory burdens of the enormous pre-MBT cells are poorly understood. We first addressed the absence of cell cycle checkpoints before the MBT in zebrafish embryos. We find that changes in checkpoint function are not explained by activation of zygotic transcription or cell cycle lengthening at the MBT. Additionally, we find that pre-MBT cells can recognize DNA damage and initiate the DNA damage response, based on histone H2AX phosphorylation, yet remain unable to arrest the cell cycle. We propose that while nuclear checkpoint function is intact and checkpoint components are maternally provided, the N:C ratio achieved at the MBT is required for proper checkpoint function. Second, we addressed regulation of the chromosomal passenger complex (CPC) in large pre-MBT cells. The CPC, composed of Aurora B kinase and regulatory components INCENP, Borealin and Survivin, modulates many events during mitosis.

The CPC is concentrated at structures such as chromatin and spindle microtubules, where Aurora B is activated by autophosphorylation, and can reach distant substrates by diffusion. Because soluble CPC will be diluted in the cytoplasm, changes in cell size may have significant consequences for Aurora B function. Zebrafish have two forms of survivin, birc5a and birc5b. Birc5b is a maternal effect gene, highlighting its early embryonic importance. We demonstrate using *birc5b* maternal effect mutant embryos that birc5b is required for the efficient phosphorylation of soluble but not chromatin-bound Aurora B substrates. We propose that birc5b contributes to the generation of diffusible Aurora B activity that can handle the spatial demands of large pre-MBT cells.

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The transition from prometaphase to metaphase in mitosis is determined by a switch-like cellular state change.

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The cell cycle is an orderly sequence of events governed by switch-like changes in cellular biochemistry to ensure unidirectionality. For example, the transition from G1 to S phase and from metaphase to anaphase in mitosis is driven by switch-like cellular changes regulated by threshold levels of cyclin/Cdk activities. In contrast, the transition from prometaphase to metaphase in mitosis is not viewed as a switch-like transition because it has historically been defined only by chromosome congression which occurs progressively as chromosomes interact with spindle microtubules. However, we show that the stability of kinetochore-microtubule (k-MT) attachments undergoes a switch-like change in RPE-1 cells increasing from an average $t_{1/2}$ of 1.8 ± 0.5 min in prometaphase to 3.8 ± 0.5 min in metaphase. Similar switch-like changes in k-MT stability also occur in PTK-1 and U2OS cells. To rule out that the variability in our measurements arise from a progressive stabilization of k-MTs during prometaphase, we performed repeated measurements on the same cells using photoactivation of GFP-tubulin. Equivalent k-MT stabilities were obtained for each measurement when the same cell was photoactivated twice during prometaphase (1.7 ± 0.5 min and 1.7 ± 0.4 min). In contrast, photoactivating the same cell twice, once in prometaphase and once in metaphase, yielded different k-MT stability (2.0 ± 0.5 min in prometaphase and 3.9 ± 0.5 min in metaphase). Strikingly, the difference in k-MT stability between prometaphase and metaphase on these single cell analyses was consistently 1.9 ± 0.2 min, in line with the differences obtained from averaging populations of prometaphase and metaphase cells. Thus, the variability in our data using populations of cells reflects cell-to-cell variation and not the progressive stabilization of k-MT during prometaphase. Furthermore, the stabilization of k-MT attachments in metaphase was abolished by inhibition of proteasome activity with either epoximicin or high concentrations of MG-132, but only if the inhibitor was added before prometaphase onset. Thus, by examining the stability of k-MT attachments during mitosis, our data reveal that the transition from prometaphase to metaphase cannot be defined solely by morphological criteria (i.e. chromosome positioning) and that it is governed by a switch-like change in the cellular state akin to other defined cell cycle transitions.

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SPISO is a novel *Pik1* substrate involved in spindle orientation.

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For accurate placement of the cleavage plane, the mitotic spindle needs to be precisely oriented. Spindle positioning is mediated through a cortical machinery by capturing astral microtubules thereby generating pushing/pulling forces at the cell cortex. These cortical cues

are spatially defined by retraction fibers modulating the positioning of actin regulators and therefore force generation. Moreover, astral MTs are engaged with these cortical structures through so-called +TIPs including adenomatous polyposis coli (APC), CLASPs and the dynein/dynactin complex, which have been shown to regulate spindle orientation and positioning. The cortically localized dynein/dynactin complex is believed to provide pulling forces on astral MTs and is recruited by heterotrimeric G proteins/LGN/NuMA during spindle positioning in *C. elegans* embryos, *Drosophila* and human cells. However, the detailed mechanisms underlying the correct positioning and orientation of the mitotic spindle are still not fully understood and further investigation is needed to identify potential missing components involved in this process.

Here we identified a previously uncharacterized protein - SPISO (C19orf21) as a substrate of Plk1 and Cdk1 that is required for correct positioning of the mitotic spindle. SPISO is broadly conserved among metazoans down to *Drosophila* with homology to human AKAP2. We demonstrate that SPISO is an actin-associated protein throughout the cell cycle. Depletion of SPISO leads to a Plk1-dependent block in mitosis at the metaphase to anaphase transition with fragmented centrosomes, scattered chromosomes and misoriented, rocking spindles. Loss of SPISO induces mitotic defects including spindle misorientation that is accompanied by shortened astral microtubules. Furthermore, we find that SPISO forms a complex with and regulates the cortical distribution of the +TIP binding protein p150glued, a subunit of the dynein/dynactin complex. Our findings implicate that Plk1 targets an actin binding protein to regulate spindle orientation. We propose that SPISO establishes a link between astral microtubules and the cell cortex required for proper mitotic spindle positioning.

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Cell elongation- an adaptive response clearing long chromatid arms from the cleavage plane.

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Chromosome segregation must be coordinated with cell division to ensure proper transmission of the genome to daughter cells. Studies in budding yeast and mammalian cells revealed an Aurora B-dependent pathway that prevents complete closure or regression of the cleavage furrow when chromatin is present at the cleavage plane. In addition, Aurora-B increases the compaction of trailing chromatid arms during sister chromatid segregation. Here we identify a novel mechanism by which *Drosophila* larval neuroblasts coordinate chromosome segregation with cell division. Cells adapt to a dramatic increase in chromatid arm length by transiently elongating during anaphase/telophase. This cell elongation is concomitant with a slight increase in spindle length and the spreading of cortical myosin rings without compromising cytokinesis. Rho Guanine-Nucleotide Exchange Factor (Pebble)-depleted cells failed to elongate during segregation of long chromatids. As a result, Pebble-depleted adult flies exhibit morphological defects likely due to cell death during development. These studies reveal a novel pathway between trailing chromatid arms and cortical myosin that ensures the clearance of chromatids from the cleavage plane prior to completion of cytokinesis.

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REEP proteins are new microtubule membrane linkers required for positioning the endoplasmic reticulum during mitosis.

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The spatial internal organization of cells is determined in part by interactions of organelle membranes with the microtubule cytoskeleton. For cell division, organelles undergo essential morphological changes and need to be cleared from the spindle area. These behaviors might be based on altered contacts of organelles with microtubules during mitosis. However, a complete inventory of the molecular linkers between cell organelles and microtubules and an understanding of their regulation during the cell cycle are lacking. With a purification approach to isolate new candidates for organelle-microtubule linker proteins we identified REEP4, a previously uncharacterized member of the DP1-family of endoplasmic reticulum (ER) morphogenic proteins. We found that REEP4 and the closely related REEP3 act redundantly to ensure nuclear envelope homeostasis. REEP3/4 depletion from HeLa cells results in irregularly shaped nuclei, displaying deep invaginations of the nuclear envelope. Remarkably, during mitosis the endoplasmic reticulum associates aberrantly with the mitotic chromatin in REEP3/4-RNAi cells and these contacts between ER and chromatin lead to morphologically altered interphase nuclei. Moreover, late stages of cell division are impaired upon REEP3/4 depletion with daughter nuclei separating insufficiently. These observations suggest that REEP3/4-mediated mitotic positioning of the ER away from the chromatin promotes correct progression of mitosis and proper nuclear envelope formation and that previously unappreciated microtubule-membrane interactions contribute to the generation of proper intracellular architecture during the cell cycle.

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Human Mio is required for progression through metaphase and completion of cytokinesis.

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The Nup107 complex is an evolutionary conserved nucleoporin subcomplex that plays a crucial role in nuclear pore complex assembly. A fraction of the Nup107 complex localises to kinetochores from early prophase to late anaphase (*Belgareh et al., 2001*). Efficient depletion of the Nup107 complex causes chromosome congression defects and a spindle checkpoint-dependent delay in mitosis via control of the localisation state of the chromosome passenger complex (*Platani et al., 2009*). We used Seh1, a member of the Nup107 complex to identify interacting partners by co-immunoprecipitation in mammalian cells. We identified an interaction between Seh1 and human homologue of *missing oocyte (mio)*, a drosophila protein required for proper meiosis (*Iida and Lilly, 2004*). We depleted Mio in human cells and observed pro-metaphase arrest, chromosome bridges in anaphase, and severe defects in mitotic progression. We are currently characterising the pathway that leads to these chromatin and cell cycle defects.

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Kinase crosstalk: interactions between the Chromosomal Passenger Complex and Polo kinase at the centromere.

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Crosstalk between Aurora and Polo kinases is essential for cell cycle progression. On mitotic entry, Aurora A is the kinase responsible for the activation of Plk1 at centrosomes, but the identity of the kinase(s) that perform this function later in mitosis and at other locations had remained elusive.

The coordinated activities at centromeres of Polo and Aurora B kinases are critical for ensuring that the two sister kinetochores of each chromosome are attached to microtubules from opposite spindle poles. In early stages of the attachment process errors occur frequently as this process is largely based on chance interactions between kinetochores and dynamic microtubules. The balance between microtubule binding and error correction (e.g. release of bound microtubules) requires the activities of Polo and Aurora B kinases. Current evidence indicates that Polo promotes stable attachments and Aurora B promotes detachment. Here we present our recent studies concerning the coordination of the activities of these two kinases in vivo.

We have shown that Aurora B (but not Aurora A) is responsible for Polo kinase activation at the centromere in *Drosophila* cultured cells. These results were validated in vivo in larval neuroblast mitoses. The CPC component INCENP plays an essential role in Polo kinase regulation: it interacts with Polo and it is required for its activation by T-loop phosphorylation. This activation happens at the inner centromere where both proteins colocalise early in mitosis. This mechanism of activation of Polo kinase by the CPC at the centromere is conserved in human cells.

Here we further demonstrate that Polo activity is required for the stable localisation and activity of the CPC at the centromere in cultured *Drosophila* cells. Analysis of *polo* alleles revealed that this mechanism is important in mitosis in vivo and also in the meiotic divisions.

We propose that INCENP acts as a platform for the coordination of the activities of Polo and Aurora B kinases at the centromere.

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Activation of the membrane/actin linkers ERM guides the orientation of cell division axis.

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Accurate control of spindle orientation during mitosis is crucial for the determination of animal cell division axis, thus for embryogenesis, morphogenesis and adult tissue homeostasis. Spindle orientation depends on the localization of cortical landmarks which ultimately enable proper interactions between microtubules of the mitotic spindle and the polarized cellular cortex in metaphase. Several reports showed that cortical F-actin depolymerization impinged on spindle orientation. ERM (Ezrin, Radixin, Moesin) proteins are attractive candidates for

organizing the cell cortex in metaphase as, upon activation, they directly link F-actin to integral plasma membrane proteins. We have proposed that ERM activation could provide inputs in spindle orientation (1,2) but direct evidence is still lacking.

We observed that the three ERM proteins expressed in mammalian cells are strongly activated upon mitotic entry, and we identified the major and direct kinase responsible for mitotic ERM activation. Taking advantage of fibronectin micropattern technology to reproducibly control spindle orientation in culture cells, we found that ERM proteins are essential for the proper orientation of cell division axis. Of note, both the levels and the asymmetric (polarized) localization of activated ERM impinge on spindle orientation and dynamics. Consistent with this observation, we similarly found an asymmetric localization of activated ERM *in vivo*, in mouse apical progenitors that are known to divide in a remarkably stereotyped manner within the embryonic neuroepithelium. Importantly, specifically impairing ERM activation in apical progenitors strongly disturbed spindle orientation. In addition, ERM activation was essential for the correct cortical localization of well-established molecules at the interface between the plasma membrane and the spindle microtubules.

We propose that activated ERM behave as polarized cortical marks that are important for guiding mitotic spindle orientation.

(1) Théry M, et al. *Nat.Cell.Biol.* 2005; 7(10):947-53

(2) Carreno S, et al. *J.Cell.Biol.* 2008; 180(4):739-46

C. Alvarez and J. Fink: Equal contribution.

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Disruption of Arf1 GTPase Activity Can play A Role In Mitotic Chromosome Segregation in *Drosophila* Embryos.

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Proper assembly of the microtubule based bipolar spindle is a prerequisite step for a faithful segregation of sister chromatids during mitosis. Although dynamics of mitotic chromosomes have been well studied, a defined model of spindle assembly and chromosome segregation is not fully understood. Previous studies have suggested a role for membrane-associated proteins in major mitotic events including spindle assembly and cytokinesis. Recently, it has been proposed that the small GTPase Arf1, a key regulator for Golgi structure and retrograde transport, may play a potential role in chromosome segregation. Here, we report a role for Arf1 in regulating spindle dynamics and morphogenesis during the early syncytial divisions of the *Drosophila melanogaster* embryo. RNA inhibition of Arf1 in transgenic embryos containing GFP-tubulin / RFP-histone showed a mass defect in chromosome segregation and spindle organization, with a prolonged mitotic delay. A similar phenotype was also observed with microinjections of Brefeldin A (BFA), a small molecular inhibitor of Arf1, upon mitotic entry in GFP-tubulin / RFP-histone embryos. In addition, we show that the Arf1-dependent metaphase delay is independent of the spindle assembly checkpoint (SAC). RNA inhibition of Arf1 and BFA microinjections were performed in transgenic embryos containing a GFP tagged Mad2, a reporter of SAC activity. Mad2 localization was unaffected by inhibition of Arf1 or treatment with BFA. Specifically, Mad2 was not present at the kinetochores during the Arf1 / BFA induced metaphase delay indicating that the SAC is not active. Therefore, we conclude that Arf1 GTPase activity may play a distinct role in normal patterns of spindles and chromosomes, and metaphase-anaphase transition. Thus, our findings may explain a novel pathway that further determines the mechanism behind the coordination between organelles inheritance and functional regulation of spindles.

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A SxIP motif in the yeast kinesin Kar3/Cik1 is necessary for anaphase spindle elongation.E. Davidson¹, T. Davis¹; ¹Biochemistry, University of Washington, Seattle, WA

Cells carrying out mitosis must maintain and regulate the distance between the spindle poles in order to successfully deliver a full set of chromosomes to each daughter cell. This pole separation is established by interpolar microtubules (ipMTs) that emanate from either pole and are bundled together in the spindle midzone. Several microtubule-binding proteins, including the budding yeast kinesin-14 Kar3/Cik1, have important roles in ipMT bundling, but their exact functions remain unclear. Cik1's N-terminus contains a widely conserved motif (SKIP), that resembles the established animal EB1-binding motif SxIP, suggesting that Cik1 may function in a complex with the yeast EB1 homolog Bim1. However, that interaction has not been shown to occur.

To investigate what role Cik1's SKIP motif plays on the mitotic spindle, we have mutated the motif to alanines in a *CIK1-GFP* background. Cik1 spindle fluorescence is reduced but not eliminated in strains carrying this allele (*cik1-4A-GFP*), and Cik1's movement across the spindle appears unaffected. The strain does, however, suffer from a clear defect in anaphase spindle elongation: wild-type yeast spindles often reach 7 μm in length, but elongating anaphase *cik1-4A-GFP* spindles slow sharply at 3 μm and rarely surpass 4.5 μm . The spindle elongation phenotype is specific to mutation of the SKIP motif and does not appear in all Cik1 mutants. Interestingly, the phenotype also does not appear in Bim1 mutants.

To determine whether the SKIP motif recruits Cik1 to microtubule plus ends, we have introduced a SKIP motif into the Cik1 paralog Vik1, which normally accumulates at the spindle poles. However, introduction of the SKIP motif had no discernible effect on Vik1 localization, even in Cik1 mutant backgrounds.

Together, these data suggest that Cik1's SKIP motif is a significant part of the protein and plays a role in spindle length regulation. However, the exact mechanism of that regulation, as well as whether the motif functions as a Bim1 binding site, are still under investigation.

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Kinetochore association of the Ska complex enhances degradation of mitotic targets to promote anaphase and mitotic exit.S. Sivakumar^{1,2}, G. J. Gorbsky^{1,2}; ¹Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, ²Cell Cycle and Cancer Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK

The Spindle- and kinetochore-associated (Ska) protein complex is a heterotrimeric complex that accumulates on microtubules and at kinetochores after nuclear envelope breakdown. The Ska complex becomes most enriched at kinetochores at metaphase. Previously we showed that the major phenotype seen after siRNA-mediated depletion of the Ska3 protein in HeLa cells and other cell lines was metaphase arrest or delay with many cells exhibiting chromosomes that eventually undergo asynchronous chromatid separation, a phenomenon we term cohesion fatigue. The strong pulling forces required to induce cohesion fatigue indicate that Ska3-depleted cells in mitosis have robust kinetochore-microtubule attachments. In support of this conclusion we find that Ska3 depleted cells have cold stable kinetochore fibers and metaphase levels of checkpoint proteins at kinetochores suggesting that the spindle checkpoint may be satisfied in cells depleted of Ska complex. Further we found that anaphase onset induced by depletion of checkpoint proteins is slowed in cells co-depleted of Ska3 indicating that the Ska complex may have a direct role in promoting anaphase onset. Treatment of cells with a

chemical inhibitor of Cdk1 at any point after nuclear envelope breakdown induces rapid degradation of cyclin B and biochemical progression to G1 even in cells arrested in mitosis with microtubule drugs. Thus treatment of cells with Cdk1 inhibitor allowed us to assay rates of cyclin B degradation in the presence or absence of microtubules and in the presence or absence of Ska complex. The persistence of kinetochore-associated tubulin in cells treated with taxol or with low levels of nocodazole correlated with accelerated Cyclin B breakdown induced by Cdk1 inhibitor. The concentration of Ska complex at kinetochores is partially dependent on kinetochore-associated tubulin. High concentrations of nocodazole deplete tubulin from kinetochores and significantly reduces kinetochore-associated Ska complex. Forced localization of Ska complex to kinetochores in cells treated with high nocodazole resulted in accelerated cyclin B degradation after addition of Cdk1 inhibitor. We propose that Ska complex-tubulin-kinetochore association enhances degradation of mitotic targets to sharpen the metaphase-anaphase transition and promote mitotic exit. Supported by the National Institute of General Medical Science of the National Institutes of Health under award number R01GM50412.

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The DNA damage checkpoint triggers autophagy to regulate the initiation of anaphase.

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Budding yeast cells suffering a single unrepaired double-strand break (DSB) trigger the Mec1 (ATR)-dependent DNA damage response that causes them to arrest prior to anaphase for 12-15 h. Hyperactivation of the cytoplasm-to-vacuole autophagy pathway causes the permanent G2/M arrest of cells with a single DSB that is reflected in the nuclear exclusion of Esp1 and partial degradation of Pds1. Arrest persists even when the DNA damage-dependent phosphorylation of Rad53 diminishes. Permanent arrest can be overcome by blocking autophagy, by deleting the vacuolar protease Prb1 or by driving Esp1 into the nucleus with a SV40 nuclear localization signal. Autophagy in response to DNA damage can be induced in three different ways: by deleting the Golgi-Associated Retrograde Protein complex (GARP), by adding rapamycin or by overexpression of a dominant-negative ATG13-SA8 mutation.

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Anaphase B induction by Patronin-mediated repression of Kinesin-13-dependent Poleward Flux.

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Chromosome segregation during mitosis involves chromosome-to-pole motility (anaphase A) and spindle elongation (anaphase B). While most attention is currently focused on anaphase A, the equally important process of anaphase B spindle elongation, in which the spindle poles themselves separate pulling the chromosomes along with them, has received less attention. We propose that anaphase B is driven by a kinesin-5-generated interpolar (ip) microtubule (MT) sliding filament mechanism that engages when poleward flux is turned off. In pre-anaphase B spindles the outward sliding of ipMTs is balanced by depolymerization of their minus ends at the poles producing flux. Following cyclin B degradation, depolymerization ceases, allowing ipMT minus ends to push apart spindle poles as their plus ends grow, building a robust midzone where the sliding motors act. In the current study, we have used live cell imaging combined with biochemical and genetic manipulations to illuminate the molecular mechanism of anaphase B induction, using the *Drosophila* embryo as a model system. We have developed an

experimental system for arresting spindles at their characteristic pre-anaphase B spindle length in the presence of cyclin B, whereupon poleward flux persists and the spindle does not elongate. We show that when the kinesin-13 depolymerase (KLP10A) is experimentally dissociated from spindle poles, poleward flux ceases and the spindle elongates pulling apart the chromosomes, in a manner similar to normal anaphase B spindles. Conversely, inhibiting a recently-discovered minus end capping protein, Patronin, causes flux to persist and inhibits wild type anaphase B while chromosome-to-pole motility proceeds. This suggests that Patronin induces anaphase B spindle elongation by protecting MT minus-ends against kinesin-13-induced depolymerization and that this cessation of depolymerization is sufficient to induce anaphase B. Significantly, the anaphase B-like spindle elongation induced by kinesin-13 inhibition was not accompanied by the redistribution of ipMT plus ends to the spindle equator whereas Patronin inhibition had no effect on MT plus end redistribution during anaphase B. Thus the anaphase B switch in *Drosophila* embryos involves two, probably independent mechanisms, namely: i) Patronin mediated suppression of kinesin-13-dependent MT minus end depolymerization, which is sufficient to convert MT poleward flux to anaphase B spindle elongation; and ii) spindle midzone re-organization including the redistribution of ipMT plus ends, which is neither sufficient nor required for anaphase B.

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Dynamics of kinesin motors throughout the anaphase B switch in *Drosophila* syncytial embryos.

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The mitotic spindle is a dynamic protein machine. In *Drosophila* syncytial embryos, Fluorescence Recovery after Photobleaching (FRAP) experiments have shown that tubulin exhibits turnover on the order of a few seconds during all stages of mitosis. However, the extent of recovery changes at anaphase B onset: before anaphase B, recovery is complete all along the spindle, whereas during anaphase B, recovery near the poles is lower. Microtubule based motor proteins are important for spindle assembly, maintenance and elongation. Here, we present our studies of motor dynamics using FRAP, Fluorescence Speckle Microscopy (FSM) and quantitative mathematical modeling using a computational model that describes the dynamics of the MTs and the mitotic kinesins' binding and detachment from the MTs. The bipolar kinesin-5 KLP61F, required during mitosis, and kinesin-14 Ncd, which acts antagonistic to kinesin-5, have turnover rates of 3-4 seconds and recover 80 to 90% of fluorescence intensity. KLP61F-GFP exhibits a decrease in recovery near the poles during anaphase B similar to that seen in tubulin FRAP. Fluorescence Speckle Microscopy (FSM) of these proteins shows that a fraction of KLP61F-GFP motors is stationary on the spindle, while interpolar microtubules (MTs) are slid apart. The kinesin-8 KLP67A also has a turnover rate of approximately 4 seconds and recovers 70 to 90% with the lower recoveries near the poles. KLP10A, a kinesin-13, exhibits a full recovery with rates of 1 to 2 seconds, the slowest recovery occurs near the poles during anaphase B. Finally, the kinesin-4 KLP3A exhibits a complete recovery with a halftime of approximately one second all along the spindle. The motors' data conform to a reaction-diffusion model in which a fraction of the motor is bound to spindle MTs and the remainder diffuses freely.

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PP2A^{Cdc55} opposes Cdk1 activation of anaphase onset by inhibiting the APC^{Cdc20} and Separase.

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Deletion of *CDC55*, which encodes a B-subunit of Protein Phosphatase 2A (PP2A) causes defects in both the morphogenesis and spindle checkpoints. Checkpoint bypass in *cdc55Δ* cells differs from most checkpoint mutants in that the anaphase promoting complex (APC) is only partially active, and APC substrates are slowly degraded as cells bypass the mitotic arrest caused by checkpoint activation. We have explored the possible mechanisms for how PP2A^{Cdc55} inhibits activation of the APC and anaphase onset. First, we have tested if PP2A^{Cdc55} opposes Cdk1 activity and inhibits the activation of the APC^{Cdc20}. We have shown that *cdc55Δ* cells have increased APC phosphorylation on previously mapped Cdk1 sites on the APC subunits Cdc16, Cdc23 and Cdc27. Blocking Cdk1 phosphorylation by mutating these sites (the *APC-12A* mutant) partially suppresses the checkpoint defects of *cdc55Δ*. However, this suppression is not complete, as *cdc55Δ APC-12A* mutant cells still initiate anaphase after activation of the morphogenesis checkpoint. Additionally, deleting multiple APC substrates (including *PDS1*, the budding yeast Securin) does not bypass the morphogenesis checkpoint-dependent block to anaphase. These data suggest that PP2A^{Cdc55} dephosphorylates and inhibits a second substrate that regulates anaphase downstream or independently of the APC. Our preliminary data suggests that Esp1 (the budding yeast Separase), like the APC, is dephosphorylated and inhibited by PP2A^{Cdc55}, and Cdk1 phosphorylation opposes PP2A^{Cdc55} dephosphorylation. Phosphorylation of Esp1 in vivo depends on Cdk1 activity, and purified Cdk1/Cyclin can phosphorylate Separase on six potential Cdk1 sites. We are currently testing if preventing phosphorylation of Esp1 will suppress *cdc55Δ* checkpoint defects, and if mimicking constitutive phosphorylation increases Esp1 activity in vitro, and causes premature anaphase onset.

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Aurora B-dependent phosphorylation of condensin is required for telomere disjunction in mitosis.

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It is now well established that the spatial organization of telomeres is non random within the nuclear space. However, how is this organization controlled throughout the cell cycle including mitosis is still elusive. The segregation of centromeres and telomeres is coordinated in space and time in order to prevent the formation of aneuploid cells, a phenotype frequently observed in cancer. One potential source of mitotic instability is chromosomes with dysfunctional telomeres, which give rise to chromatin bridges at anaphase, leading to chromosome loss. The mechanisms leading to these bridges are just beginning to be elucidated.

Aurora B kinase is one of the key regulators of mitotic events such as chromosome condensation, chromosome bi-orientation and cytokinesis in a wide range of organisms, including fission yeast. Here, we have investigated the spatio-temporal control of telomere positioning throughout the cell cycle in live fission yeast cells. We have uncovered a new mechanism leading to telomere disjunction in early mitosis, which requires the activity of the Aurora kinase Ark1. Our study identifies the condensin subunit Cnd2 as a specific substrate of Aurora B, which controls telomere disjunction in early mitosis to prevent the formation of anaphase chromatin bridges in cells.

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Cell Cycle Regulated Chs2p Endocytosis during Mitotic Exit.

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Cytokinesis is the final step in cell cycle in which a dividing cell undergoes physical separation to form two progeny cells. In budding yeast, cytokinesis is accomplished by the spatio-temporal coordination of acto-myosin ring (AMR) constriction and primary septum (PS) formation. The PS is synthesized by a transmembrane protein, chitin synthase II (Chs2p) and it plays a crucial role in stabilizing the AMR throughout cytokinesis. During metaphase-anaphase transition, Chs2p is retained in the endoplasmic reticulum due to the phosphorylation on clusters of Cdk1 sites at its N-terminus by mitotic cyclin dependent kinase, Cdk1p. At the end of mitosis, loss of mitotic kinase Cdk1p activity and reversal of Chs2p phosphorylation by cytoplasmic Cdc14p triggers Chs2p ER export. Chs2p then translocates to the neck where PS is laid. After completion of cytokinesis, Chs2p will be removed and internalized through endocytosis. Clathrin Mediated Endocytosis (CME) is a major route of endocytosis in budding yeast. The dynamic of key CME machineries at the plasma membrane is well characterized in interphase cells. However, the cell cycle specific regulation of CME at the cytokinesis site during mitotic exit remains unexplored. Here, we employ Chs2p as an internal endocytic cargo to study the relationship between Cdk1 activity reduction, cytokinesis and endocytosis at the end of mitosis. By utilizing time-lapsed fluorescence microscopy, we characterized the dynamic localization of CME proteins during cell division cycle. We also investigated the timing of neck localization of endocytic proteins relative to Chs2p neck localization and AMR constriction.

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A maternal effect screen in *Drosophila* to map PP2A-dependent pathways in mitotic exit.

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Mitotic progression is largely governed by kinases, phosphatases and ubiquitin ligases. Whereas high levels of CycB-Cdk1 kinase activity trigger mitotic entry, mitotic exit requires the inactivation of this kinase and the activation of a phosphatase at the onset of anaphase. It has recently been demonstrated that PP2A plays a major role in CycB-Cdk1 substrate dephosphorylation in mitotic exit in higher eukaryotes. PP2A in complex with adaptor subunits of the B55 subtype (Tws in *Drosophila*) is required for this process. The substrates that must be dephosphorylated by PP2A-B55/Tws to allow mitotic exit events and other regulators that could promote this process are unknown.

Here, we used a genetic approach to identify genes that collaborate with PP2A-B55/Tws in mitotic exit. We exploited the fact that meiosis and early embryonic mitoses are particularly sensitive to cell cycle perturbations. We conducted a second-site non-complementation, maternal effect lethal screen. Large genetic deletions altogether covering over 60% of the fly genome were screened against both the catalytic Mts and regulatory Tws PP2A subunit genes. Further mapping identified several genes that collaborate with PP2A-B55/Tws for cell cycle progression. Phenotypic examination reveals a variety of specific cell cycle defects in meiosis and mitosis.

We suggest that PP2A-B55/Tws regulates multiple events during mitotic exit, including chromosome segregation and centrosome-nuclear envelope cohesion. Our results also identify

specific kinases and nuclear transport factors as potential co-regulators of these processes. Future work will be aimed at the elucidation of these mechanisms.

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Clathrin Mediated Endocytosis of Chs2p at the End of Mitosis.

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Chs2p (Chitin Synthase II) is a transmembrane protein that lays down the primary septum (PS) during cytokinesis in budding yeast. At the onset of mitotic exit, activation of Mitotic Exit Network (MEN) and release of Cdc14p into the cytoplasm trigger Chs2p ER export through COPII secretory pathway. Upon its arrival at the neck, Chs2p synthesizes chitin, which forms the PS and stabilizes the acto-myosin ring constriction during cytokinesis. Subsequently, Chs2p is removed from the cytokinesis site. Previous study from our lab demonstrated that Chs2p endocytosis during cytokinesis is regulated in a Sla2p-dependent manner. Nonetheless, the underlying mechanism for Chs2p endocytosis remains poorly understood. In this study, we establish that Chs2p is indeed a cargo of clathrin mediated endocytosis (CME) and we determine which key endocytic components are needed for Chs2p internalization during cytokinesis. We examined if post-translational modifications on Chs2p are required for its endocytosis. We also investigated the relationship between endocytosis and cytokinesis. Interestingly, Chs2p endocytosis at the neck is not affected in MYO1 null cells that failed to perform cytokinesis. Taken together, we propose that Chs2p is a cargo of CME and the endocytic pathway that functions at the plasma membrane also functions at the mother-bud neck during cytokinesis.

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Feedback control of anaphase spindle elongation by Aurora B spatially regulates nuclear envelope reassembly.

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Faithful chromosome segregation during anaphase requires the physical and synchronous separation of two sets of sister-chromatids that will give rise to genetically identical daughter cell nuclei. Critically, nuclear envelope reassembly must therefore enclose each and entire set of sister-chromatids, implying spatiotemporal coordination with the mechanisms that govern anaphase. Using *Drosophila melanogaster* S2 cells as model system, we identified a novel feedback control mechanism that delays chromosome decondensation and nuclear envelope reformation in response to slow spindle elongation during anaphase. We show that this mechanism is dependent on Aurora B activity since its inhibition at anaphase onset causes premature chromosome decondensation and nuclear envelope reformation before complete separation of two sister-chromatid sets. We show that an anaphase Aurora B phosphorylation gradient is also present in S2 cells, accounting for the observed spatial regulation of chromosome decondensation and nuclear envelope reformation. We further obtained evidence that PP1 phosphatase counteracts the effect of this gradient, whose inhibition originates super-elongated spindles, highly condensed DNA and impaired nuclear envelope reformation. Finally, we show that Barren, the *Drosophila* homolog of human Cap-H subunit of Condensin I whose loading into chromosomes is Aurora B-dependent, is required for the observed feedback mechanism. We propose that an Aurora B phosphorylation gradient at the spindle midzone is part of a post-anaphase checkpoint, which provides the necessary spatial cues that prevent

premature chromosome decondensation and nuclear envelope reassembly before effective separation of sister chromatids is reached.

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Regular spacing is not required for nuclear autonomy in multinucleate cells.

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In mononucleate cells, it is known that cell cycle progression is regulated by the presence of cytoplasmic factors. In some syncytia, however, individual nuclei are able to progress through mitosis independently of their neighbors despite bathing in the same cytoplasm. In order for these two models to be consistent, limitations must exist on exchange of gene products between nuclei. In the multinucleate filamentous fungus *Ashbya gossypii*, inter-nuclear spacing and the overall number of nuclei per unit area is tightly regulated, resulting in minimal variation in the distances between adjacent nuclei. In order to test the role of spacing in nuclear division autonomy, we utilized a strain lacking the dynein-interacting protein dynactin. This mutation results in the formation of large clusters where 10-30 nuclei are <0.5 μm apart. Individual clusters are far from each other in the cell, resulting in large regions lacking nuclei. Remarkably, despite the close proximity of nuclei to each other in these clusters, asynchronous division still occurs. We investigated cell cycle activity at the level of single nuclei by analyzing the fluorescence of single molecule FISH probes against cyclin transcripts using an automated MATLAB based quantification program. Using this technique for transcript visualization yields fluorescent peaks of homogenous size and intensity in the cytoplasm. Within nuclei, however, signals of brighter intensity and larger size are detectable which correspond to sites of gene expression. In wild type cells, only a subset of nuclei produces any specific cell cycle transcript. Remarkably, this also holds true for clustered nuclei in dynactin mutants, despite their being greater than five times closer to each other than wild type nuclei and presumably being bathed with the exact same cytoplasmic signals. A small proportion of nuclei can be found on their own and not part of a cluster in these cells. These lone nuclei are more likely to produce some of the transcripts investigated than nuclei in clusters or wild type, regularly spaced, nuclei. These data provide tantalizing clues into how nuclei sense their local environment and respond depending on the proximity of neighboring nuclei. These multinucleate cells are able to maintain nuclear autonomy in gene expression and cell cycle progression despite the extremely abnormal nuclear localization.

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Separase biosensor reveals that cohesin cleavage timing depends on phosphatase PP2A^{Cdc55} regulation.

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In anaphase, sister chromatids separate abruptly and are then segregated by the mitotic spindle. The protease separase triggers sister separation by cleaving the Scc1/Mcd1 subunit of the cohesin ring that holds sisters together. Polo-kinase phosphorylation of Scc1 promotes its cleavage, but the underlying regulatory circuits are unclear. We developed a separase biosensor in *Saccharomyces cerevisiae* that provides a quantitative indicator of the rate and timing of cohesin cleavage in single cells. Separase is abruptly activated and cleaves most cohesin within one minute, after which anaphase begins. Cohesin near centromeres and telomeres is cleaved at the same rate and time. Protein phosphatase PP2A^{Cdc55} inhibits cohesin cleavage by counteracting polo-kinase phosphorylation of Scc1. In early anaphase, the previously described separase inhibition of PP2A^{Cdc55} promotes cohesin cleavage. Thus, separase acts directly on Scc1 to cleave it, and also indirectly, through inhibition of PP2A^{Cdc55}, to

stimulate its phosphorylation and cleavage. This may provide a feedforward loop that could contribute to a robust and timely anaphase.

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Cohesion fatigue: chromatid separation in the absence of Cohesin cleavage.

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During chromosome duplication in S phase, the cohesin complex is loaded upon and provides cohesion between homologous sister chromatids until the onset of anaphase in mitosis. Cohesion resists the pulling forces generated by the mitotic spindle and allows the cell to orient paired sister chromatids such that each individual chromatid will form interactions with microtubules emanating from opposing centrosomes of the mitotic spindle. At the transition from metaphase to anaphase, cohesion is lost and individual chromatids proceed to opposite poles of the cell ensuring that both daughter cells receive the appropriate complement of chromosomes. During this transition, the loss of cohesion is accompanied by the cleavage of SCC1/RAD21, a component of the cohesin complex. Accordingly, the strict regulation of cohesion by the cohesin complex is critical for the maintenance of ploidy. Recently, we described a phenomenon termed cohesion fatigue that occurs when cells are delayed at metaphase wherein paired sister chromatids separate from each other prior to the regulated cleavage of SCC/RAD21 and the onset of anaphase. We isolated chromosomes from mitotic cells that had undergone cohesion fatigue and from cells with intact sister chromatid cohesion. Surprisingly we found little difference between levels of SCC1/RAD21 and other cohesin complex members associated with paired sister chromatids and the amounts associated with separated individual chromatids. Therefore it is possible to lose sister chromatid cohesion without cleaving SCC1/RAD21 and without observable loss of chromosome-associated cohesin complex. Cohesion fatigue proceeds asynchronously within cells and evidence of fatigue, noted by abnormal increases in distances between paired sister centromeres, is apparent in a subset of chromosomes when the duration of metaphase is increased only two to threefold. This condition can arise spontaneously in a subset of untreated cells as they attempt to form appropriate centromere-microtubule interactions. We show that additional recruitment of the centromeric protein, BUB1, is an early indicator of cohesion fatigue. We postulate that the earliest elements of cohesion fatigue may induce inappropriate centromere-microtubule attachments by spatially altering the intracentromeric components responsible for forming and maintaining correct interactions. Cohesion fatigue may cause additional delays in mitotic progression, lagging chromosomes during anaphase, centromere fission, or the generation of micronuclei. These errors are implicated in the failure to maintain ploidy during mitosis and meiosis and in carcinogenesis.

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Induction of Premature Chromosome Condensation without Histone H1 Phosphorylation.

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We have found that calyculin A, a specific inhibitor of Protein Phosphatases 1 and 2A, induces prematurely condensed chromosomes (PCC) in interphase HeLa cells at any point in the cell cycle. However, under conditions where Cdk1 is inhibited or at points in the cell cycle where Cdk1/Cyclin B cannot be activated, histone H1 does not become phosphorylated in the calyculin A-induced PCC. These results indicate that H1 phosphorylation and Cdk1/cyclin B activity are

not essential for PCC. They also suggest that when Cdk1/cyclin B triggers mitosis, it may exert its effects at least in part by inactivation of protein phosphatases, and this role can be mimicked by calyculin A. We propose that in the onset of mitosis, inactivation or sequestration of protein phosphatases may be just as important as activation of protein kinases.

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Aurora B function depends on dissociation kinetics at the inner centromere.

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The Chromosomal Passenger Complex (CPC), localizes predominantly to inner centromeres during mitosis. Aurora B kinase, the enzymatic component of the CPC, phosphorylates spatially distant substrates, particularly microtubule-binding proteins at the outer kinetochore, to destabilize erroneous kinetochore-microtubule attachments. How the CPC physically accesses its outlying substrates, and how this access is regulated to achieve proper Aurora B function are unknown. We propose a model in which the CPC is fully activated by autophosphorylation at high concentration in the inner centromere, and this activated form of the complex accesses distant substrates by dissociation and diffusion from the inner centromere. This model implies that Aurora B function depends on the dissociation rate of the CPC from the inner centromere. To test this hypothesis, we sought to experimentally decrease the rate of dissociation of the CPC by increasing its affinity for the inner centromere. We constructed a mutant version of INCENP, the CPC scaffolding protein, with an extra copy of the domain that binds Borealin and Survivin, the CPC members which direct localization to the inner centromere. This “two-footed” or “bipedal” INCENP localizes normally to the inner centromere but dissociates more slowly than wild-type INCENP, as measured by FRAP. In addition, bipedal INCENP is retained at centromeres following anaphase, consistent with increased affinity for centromeres. We compared wild-type to bipedal INCENP in functional assays for correction of chromosome attachment defects. Cells expressing bipedal INCENP exhibit increased frequency of chromosome attachment errors following recovery from either a monopolar spindle (monastrol washout) or from microtubule depolymerization (nocodazole washout). These errors are characteristic of defective Aurora B function. We conclude that Aurora B-mediated error correction depends on its localization to the inner centromere and its dissociation rate.

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Phosphoregulation of Ndc80 and Dam1 subcomplexes promotes release of kinetochores from microtubules via multiple mechanisms.

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During mitosis, multiprotein complexes called kinetochores orchestrate chromosome segregation by forming load-bearing attachments to dynamic microtubule tips, and by participating in phosphoregulatory error correction. Aurora B kinase phosphorylates the major microtubule-binding subcomplexes, Ndc80 and (in yeast) Dam1, to promote release of erroneous attachments, thereby giving another chance for proper attachments to form. It is unknown whether Aurora phosphorylation promotes release directly, by increasing the rate of kinetochore detachment, or indirectly, by destabilizing the microtubule tip or disrupting the kinetochore structure itself. Moreover, the relative importance of phosphorylation of Ndc80 versus Dam1 in the context of whole kinetochores is unclear. To address these uncertainties we isolated native yeast kinetochore particles carrying phosphomimetic mutations on Ndc80

and Dam1, and applied advanced laser trapping techniques to measure the strength and stability of their attachments to individual dynamic microtubule tips. Composition of the purified particles was unaffected by the phosphomimetic mutations, suggesting that phosphorylation at these sites does not disrupt kinetochore structure. Rupture forces were reduced by phosphomimetics on both subcomplexes, in an additive manner, indicating that both subcomplexes make independent contributions to attachment strength. Likewise, phosphomimetics on either subcomplex reduced attachment lifetimes under constant force, primarily by accelerating detachment during microtubule growth and shortening. Phosphomimetics on Dam1 also increased the likelihood of switches from microtubule growth into shortening, further promoting release in an indirect manner. Together our results suggest that, *in vivo*, Aurora B releases kinetochores via at least two mechanisms – by directly weakening the kinetochore-microtubule interface, and also by destabilizing kinetochore-attached microtubule tips.

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Mad2 is essential in the absence of centrosomes for cell cycle progression.

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Centrosomes act in eukaryotic cells as the major microtubule organizing centers (MTOCs). In addition, many important regulatory and signalling proteins localise to the centrosome. In light of this it is somewhat surprising that flies get to adult stages of life in the absence of centrosomes (Basto et al.). Here, we have taken a genetic approach to elucidate whether compensatory mechanisms exist to enable cell division in the absence of centrosomes. We observe that cells lacking centrosomes and the protein Mad2, essential for proper spindle assembly checkpoint function, arrest in the cell cycle. This phenotype appears to be non mitotic and microtubule independent suggesting an interphase role for Mad2 which facilitates non-centrosomal cells to divide.

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Characterizing Laulimalide using *C. elegans* embryo.

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Marine organisms have served as an important source for chemicals that target eukaryotic microtubules and hence they have been used to isolate new anti-cancer molecules. In one such screen done using the marine sponge, *Cacospongia mycofijiensis*, a crude lipophilic extract yielded the compound laulimalide (Mooberry S.L., et al 1999, Pryor D.E., et al 2002). Laulimalide is known to disrupt the normal microtubule assembly and cause bundling of microtubules. It is known to inhibit cell proliferation and initiate mitotic arrest. Studies done using mammalian cell lines suggests that laulimalide leads to failure in proper chromosome segregation and cytokinesis. In some cases it has been observed that, similar to paclitaxel, it induces microtubule stabilization. Studies done so far using mammalian cell lines show that laulimalide causes cytotoxicity however, its precise effect on cytoskeletal structures and mode of action is unclear. Mammalian cells can take up to 20 hours to divide; therefore acute effects of the drug are often not readily apparent.

In the present study we have used the first mitotic division in the *Caenorhabditis elegans* embryo to characterize laulimalide. The early cell division events in *C. elegans* have been studied in detail. The *C. elegans* embryo serves as an excellent system for the preliminary characterization of microtubule-targeted drugs because morphological changes to the cytoskeleton occur rapidly and are highly stereotypical. In *C. elegans* embryo the use of small molecule inhibitors is limited by eggshell impermeability. We have used an RNAi-based

approach to create a permeable eggshell (Carvalho A., et al 2011). We have observed dose-dependent phenotypes for laulimalide, whereby different concentrations lead to stabilization or destabilization of microtubules. We have also carried out experiments to look for synergistic effects of laulimalide and paclitaxel on microtubules. In the present study, results will be presented to characterize the similarities and differences between paclitaxel and laulimalide with respect to their effects on the microtubule cytoskeleton in the one-cell *C. elegans* embryo.

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SKAP forms a link between kinetochore core complex KMN and dynamic spindle microtubules.

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Chromosome segregation in mitosis is orchestrated by the dynamic interactions between the kinetochore and spindle microtubules. Our recent study shows that mitotic motor CENP-E cooperates with SKAP to orchestrate an accurate chromosome movement in mitosis (J. Biol. Chem. 287, 1500-9). However, it remains elusive as how kinetochore core microtubule-binding activity KMN (KNL-MIS12-NDC80) regulates microtubule plus-end dynamics. Here, we identify a novel interaction between MIS13 and SKAP which orchestrates accurate interaction between kinetochore and dynamic spindle microtubules. SKAP physically interacts with MIS13 and specifies kinetochore localization of SKAP. Suppression of MIS13 by small interfering RNA abrogates the kinetochore localization of SKAP. Total internal reflection fluorescence microscopic assays demonstrate that SKAP exhibits an EB1-dependent, microtubule plus-end loading and tracking in vitro. Importantly, SKAP is essential for kinetochore oscillations and dynamics of microtubule plus ends during live cell mitosis. Based on those findings, we reason that SKAP constitutes a dynamic link between spindle microtubule plus ends and mitotic chromosomes to achieve faithful cell division.

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Molecular mechanism of EB1 family proteins in regulating microtubule dynamics.

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Microtubule dynamics at the plus end, called dynamic instability, is important for proper cellular functions, such as migration, differentiation and cell division. Plus-end tracking proteins (+TIPs), which accumulate selectively at growing microtubule plus ends are considered to play an important role in regulating of stability of microtubules. Among them, EB1 is highly conserved across eukaryotes. The recent studies demonstrated that most of the other +TIPs, which can directly interact with microtubules, are able to bind to EB1, suggesting that EB1 may function as a central hub in the network of +TIPs.

Mutational analysis of *mal3*, a fission yeast homolog of EB1, showed that Mal3 was phosphorylated at a cluster of serine/threonine residues in the linker connecting the CH domain and EB1-like C-terminal motif. The phosphorylation reduced the binding affinity of Mal3 to microtubules, thereby regulating microtubule dynamics. In human cells, we noticed that

treatment of cells with mitotic arrest-inducible drugs results in the phosphorylation of EB2, but not of EB1 and the phosphorylation occurred in mitosis, but not in interphase. Furthermore, phosphorylation of EB2 was diminished by inhibition of aurora B kinase. The aurora B kinase has an important role in the mitotic progression, especially in correcting spindle-kinetochore attachments during chromosome segregation. To evaluate the significance of EB2 phosphorylation, we established cell lines expressing a RNAi-resistant EB2-EGFP. Expression of EB2 nonphosphorylatable form led to chromosome alignment defect in metaphase. Currently, we are attempting to elucidate the biological significance of EB2 phosphorylation.

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Psr1p interacts with SUN/sad1p and EB1/mal3p to establish the bipolar spindle.

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During mitosis, interpolar microtubules from two spindle pole bodies (SPBs) interdigitate to create an antiparallel microtubule array for accommodating numerous regulatory proteins. Among these proteins, the kinesin-5 cut7p/Eg5 is the key player responsible for sliding apart antiparallel microtubules and thus helps in establishing the bipolar spindle. At the onset of mitosis, two SPBs are adjacent to one another with most microtubules running nearly parallel toward the nuclear envelope, creating an unfavorable microtubule configuration for the kinesin-5 kinesins. Therefore, how the cell organizes the antiparallel microtubule array in the first place at mitotic onset remains enigmatic. Here, we show that a novel protein psr1p localizes to the SPB and plays a key role in organizing the antiparallel microtubule array. The absence of psr1+ leads to a transient monopolar spindle and massive chromosome loss. Further functional characterization demonstrates that psr1p is recruited to the SPB through interaction with the conserved SUN protein sad1p and that psr1p physically interacts with the conserved microtubule plus tip protein mal3p/EB1. These results suggest a model that psr1p serves as a linking protein between sad1p/SUN and mal3p/EB1 to allow microtubule plus ends to be coupled to the SPBs for organization of an antiparallel microtubule array. Thus, we conclude that psr1p is involved in organizing the antiparallel microtubule array in the first place at mitosis onset by interaction with SUN/sad1p and EB1/mal3p, thereby establishing the bipolar spindle.

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Branching microtubule nucleation in *Xenopus* egg extracts mediated by augmin and TPX2.

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Mitotic and meiotic spindles are formed by local nucleation of microtubules. Nucleation occurs at centrosomes in animal mitotic spindles. It is also thought to occur within metazoan spindles, stimulated by signals from chromatin, but this has not been observed directly. Here, we demonstrate microtubule nucleation from existing microtubules in meiotic *Xenopus* egg extracts. Daughter microtubules grew with the same polarity as mother filaments, preserving local polarity. Branching microtubule nucleation reaction required g-tubulin and augmin and was stimulated by RanGTP and its effector Tpx2, factors previously implicated in chromatin-stimulated nucleation. A low branch angle leading to local preservation of polarity make microtubule-dependent microtubule nucleation well suited for spindle assembly and maintenance.

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Differences in Ran regulation and the microtubule-associated protein TPX2 contribute to interspecies spindle scaling in *Xenopus* egg extracts.

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A bipolar, microtubule (MT)-based spindle performs the crucial and universal function of segregating chromosomes during eukaryotic cell division, but spindle size, assembly mechanism and architecture vary depending on the organism and cell type. We previously established an egg extract system that recapitulates size differences between meiotic spindles of the frog *Xenopus laevis* and its smaller relative *Xenopus tropicalis*, and identified differential phosphorylation of the MT severing factor katanin as a spindle length scaling mechanism. However, katanin activity does not fully account for meiotic spindle differences between the two species. Interestingly, we have found that the small *X. tropicalis* spindles are resistant to inhibition of RCC1, the chromatin-bound nucleotide exchange factor that generates a gradient of RanGTP, whereas the large *X. laevis* spindles are disrupted with concomitant mislocalization of downstream factors including the spindle MT-associated protein TPX2. Interestingly, TPX2 is present at three-fold higher levels in *X. tropicalis* extract and is missing a 7 amino acid insertion found in *X. laevis* TPX2 that introduces two consensus Polo kinase phosphorylation sites. When added to *X. laevis* extract in equal amounts, both *X. tropicalis* TPX2 and an *X. laevis* TPX2 mutant lacking the 7 amino acid insertion induce dramatic MT nucleation compared to wild-type *X. laevis* TPX2. We are testing the hypothesis that phosphorylation inhibits TPX2 activity in *X. laevis* extract, causing a decrease in MT nucleation that affects spindle size. Consistent with this model, addition of excess TPX2 to *X. laevis* extract reduced spindle length. Higher levels and activity of TPX2 may decrease average MT length in the *X. tropicalis* spindle, and could also act to decrease kinesin-5 dependent MT sliding. Both of these mechanisms would limit the distance that a spindle MT slides poleward in its lifetime, thereby decreasing steady state spindle length. Thus, differences in meiotic spindle size in the two *Xenopus* species are accompanied by changes in MT nucleation and stability that reflect differences in spindle architecture and assembly pathway.

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Incremental phosphorylation of competing NDC80 complexes tunes kinetochores for diverse mitotic functions.

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The stability of kinetochore-microtubule (KMT) attachments is finely tuned to drive different mitotic processes. This dynamic regulation is essential for accurate chromosome segregation, and involves phosphorylation of NDC80, a major component of the MT-binding interface at kinetochores. The fundamental question of how the number and stability of KMT attachments is regulated by phosphorylation remains unanswered. To address this question and create an integrated view of the KMT interface, we used a combination of quantitative approaches in vivo, in vitro and in silico. To determine the molecular characteristics and dynamic range of Hec1 phosphorylation in human cells we replaced Hec1 with a series of mutants that prohibit its phosphoregulation. Although chromosome segregation was perturbed, some mitotic processes, including release of erroneous kinetochore-microtubule attachments and chromosome oscillations, proceeded normally. However, they required different numbers of phospho-mimetic

substitutions in Hec1. With single molecule methods in vitro we then determined the molecular characteristics of MT binding of the Hec1-containing NDC80 complexes, and how these interactions depend on Hec1 tail phosphorylation. We found that Hec1 tail phosphorylation leads to a graded increase in NDC80 diffusion and shortening of residency time, but cooperativity of NDC80-MT binding is only weakly affected by Hec1 tail phosphorylation. Using four independent approaches we conclude that phosphorylation of the Hec1 tail results in the incremental decrease in NDC80-MT binding energy. To seek a quantitative understanding of regulation of KMT attachments by NDC80 phosphorylation the data from single molecular and cellular scales were then linked with advanced computational approaches. We show that modeling the KMT interface with “repetitive sites” of NDC80 complexes greatly amplified the phosphorylation-dependent tuning of single NDC80 complexes, but a “dynamic lawn” interface of un-clustered NDC80 complexes produced an excellent fit to the phosphorylation-driven KMT affinity both in prometaphase and metaphase. We conclude that incremental phosphorylation of Hec1 drives large-scale tuning of kinetochore-microtubule affinity.

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Integration of kinase/phosphatase activities at kinetochores by the mitotic pseudokinase BUBR1.

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Maintenance of chromosomal stability depends on error-free segregation of chromosomes. The pseudokinase BUBR1 is essential for this, as it is a core component of the mitotic checkpoint and it is required for the formation of stable kinetochore-microtubule attachments. The mechanism by which BUBR1 regulates stability of attachments is unknown, but it is controlled by PLK1 activity and involves Aurora B-mediated error-correction. We have identified a conserved and highly phosphorylated domain (KARD) in BUBR1 that is crucial for formation of kinetochore-microtubule attachments. Deletion of this domain or prevention of its phosphorylation abolishes formation of kinetochore microtubules, which can be reverted by inhibiting Aurora B activity. Tension-sensitive phosphorylation of the KARD by PLK1 promotes direct interaction of BUBR1 with the PP2A-B56 α phosphatase that counters excessive Aurora B activity at kinetochores. As a result, removal of BUBR1 from mitotic cells or inhibition of PLK1 reduces PP2A-B56 kinetochore binding and elevates phosphorylation of Aurora B substrates on the outer-kinetochore. We propose that PLK1 and BUBR1 cooperate to stabilise kinetochore-microtubule interactions by regulating PP2A-B56-mediated de-phosphorylation of Aurora B substrates at the kinetochore-microtubule interface.

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Loss of the cohesin subunit STAG2 induces chromosomal instability by disrupting kinetochore-microtubule attachments during mitosis.

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The cohesin complex is vital for proper chromosome segregation in mitosis. Mutations in cohesin genes have been found in colon cancers as well as cell lines originating from different cancers. Recently, loss of function mutations in the STAG2 gene, which encodes a cohesin subunit, have been described in a variety of tumor types. Interestingly, STAG2-deficient cells do not display a complete loss of sister chromatid cohesion or gross alterations in gene expression. Instead, they display a slight loss of centromeric cohesion and a significant increase in the frequency of lagging chromosomes during anaphase. Lagging chromosomes are a hallmark of chromosomal instability (CIN) – the persistently high rate of chromosome loss

and/or gain – and arise from persistent errors in the attachment of microtubules to kinetochores. To investigate the cause of increased lagging chromosomes in anaphase upon loss of STAG2 we are using RNA interference in human diploid RPE-1 cells. STAG2-depleted RPE-1 cells maintain a spindle assembly checkpoint response. Furthermore, they display no change in the frequency of formation of multipolar spindles indicating no increased propensity for forming erroneous kinetochore-microtubule attachments. However, we demonstrate that STAG2 depletion increases kinetochore-microtubule attachment stability from 1.8 ± 0.6 min to 4.6 ± 2.5 min in prometaphase and 3.8 ± 1.1 min to 8.5 ± 3.0 min in metaphase. This decreases the efficiency of correction of kinetochore-microtubule attachment errors and accounts for the increased frequency of lagging chromosomes in anaphase. Additionally, STAG2 depletion induces a 20% increase in centromere length as well as a mislocalization of the CPC components Aurora B and INCENP on chromosomes. Thus, loss of the cohesin subunit STAG2 induces CIN in cancer cells by disrupting the appropriate regulation of kinetochore-microtubule attachments through the inner centromere signaling pathway.

Spindle Checkpoints

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Mechanical constraints induce mitotic arrest in multicellular tumor spheroid.

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Modification of the extra cellular matrix stiffness has been reported to affect tumor growth. As several studies performed on monolayer cell-based model suggest that mechanical cues impair cell division, we wondered whether environmental constraints might also affect cell cycle control within a tissue.

To address that issue, we used MultiCellular Tumor Spheroid (MCTS), a 3D model recapitulating the microenvironment, the proliferative gradient and cell-cell interactions found in a tumor. We first designed, produced and used dedicated polymer microdevices in which MCTS engineered to express fluorescent biomarkers were confined to apply mechanical constraints. We observe that under constraints, MCTS display a higher proportion of proliferative cells in low compression regions. Strikingly, this modification of the proliferation gradient is associated with accumulation of mitotic cells (pHistone H3 +) in compressed regions. We show that these cells remain arrested at mitosis for at least 24 hours (EdU incorporation neg.) and that mitotic arrest is not caused by impairment of rounding.

We next used live SPIM (Selective Plane Illumination Microscopy) 3D imaging to monitor mitosis progression in isotropically constrained MCTS. We show that constraints impair bipolar spindle assembly and delay progression toward metaphase-anaphase transition.

Our data indicate that in a multicellular structure mechanical constraints are responsible for a defect in cell cycle progression associated with a mitotic arrest.

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The spindle checkpoint senses spindle asymmetry.

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The alignment of chromosomes in the middle of the mitotic spindle is a very distinctive feature of all dividing metazoan cells. Even asymmetrically dividing cells such as *Drosophila* neuroblasts

first build symmetric spindles before elongating asymmetrically during anaphase. Such a centering mechanism is likely necessary for error-free chromosome segregation and precise positioning of chromosomes after division. According to our current understanding, the spindle checkpoint only monitors kinetochore-microtubule attachments and bipolar tension of kinetochores. Is the alignment of chromosomes in the middle just a consequence of this microtubule attachment, or is its reproducibility also ensured by a checkpoint mechanism? To investigate this, we generated asymmetric mitotic spindles in HeLa cells by depleting HsSAS-6, a protein required for centriole duplication. In severe asymmetry, where only 1 centrosome is present in the cell, a highly asymmetric, monopolar spindle is formed and the spindle checkpoint is strongly activated. However, in a milder asymmetry where both centrosomes are present but one centrosome lacks a daughter centriole, a bipolar spindle can be formed but the spindle is asymmetric: the metaphase plate is displaced from the middle, further away from the centrosome with 2 centrioles. These asymmetric spindles are still able to form normal metaphase plates with oscillating, bipolarly attached kinetochores that have normal attachment and tension (based on Mad2 status and inter-kinetochore distances). Strikingly, these asymmetric cells experience a delay in the metaphase-anaphase transition, spending twice the amount of time in metaphase compared to cells with symmetric spindles. Furthermore, prior to anaphase onset, the position of the metaphase plate is corrected to the middle of the spindle, suggesting that the additional time required by the cell is used to correct spindle asymmetry. Abrogation of the spindle checkpoint not only removes the anaphase delay, it also allows the cell to progress to anaphase without correcting spindle asymmetry. This suggests that the alignment of chromosomes in the middle is not just a consequence of symmetric spindle poles, but rather the result of active correction mechanisms and a control checkpoint. We are now trying to elucidate how the spindle checkpoint is able to sense spindle asymmetry. Our preliminary results show that asymmetric spindles have different microtubule stability at the poles and different microtubule flux in each half-spindle. Additionally, cells with asymmetric spindles have more kinetochores with reduced SKAP levels, indicating higher Aurora B activity at the kinetochores. We thus hypothesize that the spindle checkpoint monitors spindle symmetry by measuring microtubule occupancy or stability at the kinetochore.

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The Mad2-binding motif in Cdc20 contributes to activation of the Anaphase-Promoting Complex/Cyclosome.

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The spindle checkpoint prevents cell cycle progression in response to unattached kinetochores by regulating the activity of the Anaphase Promoting Complex/Cyclosome (APC/C), an E3-ubiquitin ligase. Cell Division Cycle 20 (Cdc20), a co-activator of the APC/C in mitosis, is targeted for inhibition, in part, by the spindle checkpoint component mitotic arrest deficient 2 (Mad2). Cdc20 has four known functional motifs and domains: the Mad2-binding motif, a WD40 domain, the C-box motif, and the IR motif. The IR and C-box motifs have been proposed to contribute to Cdc20's ability to interact with and/or activate the APC/C, and the WD40 domain is thought to interact with both target substrates and APC/C subunits to promote substrate ubiquitylation. Currently, the only proposed role for the Mad2-binding motif is to serve as the binding site for Mad2. How Mad2 interacting with the Mad2-binding motif leads to APC/C^{Cdc20} inhibition remains unknown. Previously isolated mutant alleles of Cdc20 that allow cells to bypass Mad2-dependent spindle checkpoint inhibition of cell cycle progression were found to contain mutations in the Mad2-binding motif, where these separation of function mutant alleles are still able to support APC/C activation *in vivo*. In order to investigate how Mad2 interacting with the Mad2-binding motif leads to APC/C^{Cdc20} inhibition, we have employed quantitative *in*

in vitro APC/C^{Cdc20} and APC/C^{Cdc20-127} enzyme assays to characterize one of these mutant by-pass alleles, namely Cdc20-127. As expected, the addition of Mad2 inhibited APC/C^{Cdc20} activity, but had no effect on APC/C^{Cdc20-127} activity. However, we have observed a significant difference in the ability of Cdc20-127 to function as an APC/C co-activator. *In vitro* transcription/translation in the presence of ³⁵S-Methionine revealed no difference in protein amounts or stability between Cdc20 and Cdc20-127 proteins. This suggested that the observed difference in co-activator activity was due to a change in the ability of Cdc20-127 to interact with or activate the APC/C. These observations have led us to hypothesize that the Mad2-binding motif in Cdc20 may have dual functions: one as the binding site for Mad2 with Cdc20, and a second function as an activator that promotes APC/C enzyme activity. To explore this hypothesis, we are currently engaged in investigating the co-activator activities of several other spindle checkpoint by-pass alleles of Cdc20, all of which contain mutations in the Mad2-binding motif.

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Mad2B is involved in the DNA damage response and not in the mitotic checkpoint.

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Mad2B (also called Rev7 or Mad2L2) is a regulatory subunit of DNA polymerase ζ that is involved in translesion DNA synthesis. Previous studies have implicated Mad2B in several cell functions including cell cycle regulation, where it was shown to inhibit the Anaphase Promoting Complex (APC)/cyclosome by binding to Cdc20/Cdh1. As Mad2B shares 26% identity and 48% similarity at the amino acid level with the human mitotic checkpoint protein Mad2, it has been suggested that Mad2B may also function as a mitotic checkpoint protein. However, the results of our studies indicate that Mad2B is not a mitotic checkpoint protein. Instead, our data show that Mad2B does bind to Cdc20, but only following DNA damage, and suggests that Mad2B has a role in the mammalian DNA damage response.

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Measuring aneuploidy by counting yeast artificial chromosomes in *mad2* spindle checkpoint mutant cells.

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Our goal was to investigate the degree of aneuploidy arising in response to loss of *MAD2* spindle checkpoint function in budding yeast. In the past, yeast artificial chromosomes have been employed to measure chromosome loss rates in both wild and *mad2* mutant cells. However, a corresponding direct measurement of the degree of aneuploidy in *mad2* mutants, as determined by counting the average number of artificial chromosomes per cell, has not been reported. Towards this end, the phenotypes and average copy number per cell for both low-copy and high-copy short-linear and circular artificial chromosomes was explored in haploid and diploid cells where Mad2 expression was either normal, reduced, or removed. As expected, in a previously developed genetically sensitized plate assay, where cell growth depends upon loss of *MAD2* function in response to loss of chromosome tension, cells carrying low-copy and high-copy circular artificial chromosomes do not display a phenotype. By contrast, and consistent with previous observations with low-copy short-linear chromosomes, both haploids and diploids carrying low-copy or high-copy short-linear chromosomes displayed a similar loss of *MAD2*-dependent growth phenotype. In this same sensitized genetic background, and consistent with previous observations on wild type cells, short-linear chromosomes also displayed an increase in average copy number per cell relative to circular chromosomes. Combined, the plate

phenotypes and the observed increases in aneuploidy both correlate with a unique feature short-linear chromosomes: namely that they undergo premature sister chromatid separation, leading to a loss in chromosome tension, whereas circular chromosomes do not. Wild type diploid cells accumulated high-copy short-linear chromosomes, with an average limit of 8-9 chromosomes per cell. Consistent with previous observations in wild type diploid cells, forcing *MAD2/MAD2* diploid cells in our genetic background to carry a high-copy number of short-linear chromosomes led to a decrease in the chromosome retention rate. By contrast, *MAD2* heterozygotes and *MAD2* homozygous delete diploid cells displayed a decrease in the average chromosome copy number per cell, but maintained normal chromosome retention rates. Our observations indicate that Mad2 function is required to maintain high levels of aneuploidy, but that Mad2 also contributes to a decrease in chromosome retention rate when chromosome numbers become aberrantly high.

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Catalytic Amplification of the BubR1-Cdc20 Mitotic Checkpoint Inhibitor by Mad2-induced Conformational Switch in Cdc20.

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The mitotic checkpoint acts to maintain chromosome content by generation of a diffusible “wait anaphase” inhibitor. Unattached kinetochores catalyze a conformational shift in Mad2, converting an inactive open form into a closed one that can capture Cdc20, the mitotic activator of the APC/C ubiquitin ligase. Mad2 binding is now shown to promote a conformational switch in Cdc20, exposing a previously inaccessible site for binding to BubR1’s conserved Mad3 homology domain. BubR1, but not Mad2, binding to APC/CCdc20 is demonstrated to inhibit ubiquitination of cyclin B. Closed Mad2 is further shown to catalytically amplify production of BubR1-Cdc20 without necessarily being part of the complex. Thus, the mitotic checkpoint is produced by a cascade of two catalytic steps, an initial one acting at unattached kinetochores to produce a diffusible Mad2-Cdc20 intermediate and a diffusible one in which that intermediate amplifies production of BubR1-Cdc20, the inhibitor of cyclin B ubiquitination by APC/CCdc20.

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Differential regulation of the mitotic spindle assembly checkpoint in stem and progenitor cells.

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Cell cycle checkpoints are crucial gatekeepers monitoring the fitness of the cell to generate new progeny. For this reason, they are considered indispensable to all eukaryotic cells. However, this isn’t universally true as there are reports of differential dependencies of stem and committed progenitors on cell cycle checkpoints. For example, embryonic stem (ES) cells and immature neural progenitor cells have dormant G1/S and G2 decatenation checkpoints, which become activated only upon differentiation.

The spindle assembly checkpoint (SAC) oversees chromosome-microtubule attachments and tension during mitosis to ensure fidelity of chromosome segregation. Although extensively studied in cultured somatic cells and cancer cell lines, very little is known about it in stem and progenitor cells.

Muscle satellite cells (MuSCs) are the sole source of tissue specific stem cells for adult muscle repair. Here, we investigate the effects of disrupting the SAC in MuSCs compared to progenitor

cells (myoblasts). To this end, we have used a conditional truncated form of the critical SAC kinase Mps1 (Mps1^{Δ ff}) that is unable to localize to the kinetochore to carry out its checkpoint function even though it has an intact kinase domain. To confer MuSC-specificity, we crossed Mps1^{Δ ff} with Tamoxifen (Tmx) inducible Pax7Cre^{ER} mice, which allow inducible expression of truncated form of Mps1 in MuSCs and their progeny. Induction of this truncated mutant in committed myoblasts leads to proliferative defects, which are not observed when the mutation is induced in MuSCs. Moreover, committed myoblasts with this mutation fail to differentiate, while mutant stem cells are able to differentiate normally. These results indicate that mutant stem cells are able to compensate and withstand proliferative arrest. Furthermore, when subjected to mitotic insult through nocodazole, we observe that myoblasts with the truncated Mps1 fail to arrest, suggesting an impaired SAC, but not mutant stem cells. These results indicate that stem and progenitor cells have distinct ways of regulating their mitotic spindle assembly checkpoint.

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Localization dynamics of the Survivin splice variants in mitosis.

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Survivin is a multifaceted member of the IAP family of proteins with functions in the Chromosome Passenger Complex that has been shown to be over-expressed in nearly every human tumor type. Survivin splice variants 2B and Deltaex3 have been strongly correlated to cancer cell death and survival, respectively. However, the study of their roles in mitotic progression has derived in contradictory results and the mechanisms by which they exert their death regulatory functions remain largely unexplored. Furthermore, much of the Survivin isoforms study has been achieved by exogenously expressing these proteins.

Our goal was to define the endogenous Survivin variants localization throughout the cell cycle in HCT116 dividing cells in order to gain insights in their potential roles in the cell cycle regulation.

We assessed Survivin variants expression with the use of RT-PCR and found that all three isoforms studied are consistently expressed in the cell line HCT116. According to previous reports, we observed that Survivin Deltaex3 is the least expressed transcript, while Survivin and Survivin 2B show comparable levels of expression.

We then proceeded to determine Survivin variants subcellular localization in different phases of the cell cycle by the use of confocal and ELYRA high resolution microscopy (Zeiss). In interphase cells, Survivin 2B was almost exclusively localized to non-heterochromatin pinpoint regions in the nucleus. Contrarily to previous reports, no fraction of the protein was found in mitochondria. In mitosis, Survivin 2B was confined to centrosomes in prophase, metaphase and anaphase cells and later re-localized to the contractile ring during cytokinesis. However, we did not find it at any other structure of the mitotic spindle. Staining with MitoTracker revealed that Survivin 2B does not co-localize with mitochondria at any phase of mitosis either.

On the other hand, Survivin Deltaex3 was mainly accumulated at non-heterochromatin regions in the nucleus. However, there was also a pool in the cytoplasm and mitochondria. During mitosis, Survivin Deltaex3 is found outside the chromosomes. Interestingly, Survivin Deltaex3 shows a sharp decrease in anaphase, revealing a potential Anaphase Promoting Complex-dependent degradation mechanism. The mitochondrial pool of Survivin Deltaex3 seems to decrease in mitotic cells.

Survivin isoforms have not been shown to contribute to chromosome segregation. However, the localization and degradation dynamics observed in this study might have some biological significance. The question is then raised whether Survivin 2B dynamic localization or Deltaex3 degradation play a role in mitosis, other than directly participating in chromosome segregation.
nts: PAPIIT (IN213311-3), CONACYT (83959)

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Structure Function Analysis of the Mitotic Checkpoint Kinetochores protein, hSpindly.

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Spindly was shown to recruit dynein to the kinetochores in *Drosophila* S2 cells and the dynein/dynactin complex in human cells. The dynein/dynactin complex is required for alignment of chromosome during prometaphase and silencing the mitotic checkpoint following the proper attachment of chromosomes by mediating the transport of the checkpoint proteins off the kinetochores. Recruitment of Spindly to the kinetochores is dependent on the RZZ (Roughdeal, Zeste-White10 and Zwilch) complex in *Drosophila*, *C. elegans* and human cells. Spindly is localized to kinetochores at prometaphase and once the chromosomes are aligned properly on the metaphase plate, it is translocated to the poles. Little is known about the interactions of Spindly or its mechanism of function. It has been shown that Spindly mediates the mitotic checkpoint signaling by acting as an adaptor between RZZ and the dynein complex but its interacting partners are not yet known. The aim of the study is to characterize the structural and functional domains of hSpindly. hSpindly consists of two coiled coil domains, which are separated by a conserved spindly box. We have made 18 insertion, 16 truncation, 15 site-directed, and 15 phospho hSpindly mutants (non-phosphorylatable and phospho-mimics). These mutants were transfected into HeLa cells followed by fluorescence microscopy analysis. My kinetochores mapping studies indicated that both coiled-coil domain II and far C-terminus of hSpindly are required for kinetochores localization, which correspond to the data, published by Barisic et al. The non-phosphorylatable mutants of hSpindly localized to the kinetochores but showed enhanced localization at the spindle poles in both prometaphase and metaphase. The phospho-mimics localized to the kinetochores and did not show enhanced spindle pole localization. Furthermore, we are interested if the phospho-mimics can be transported to the poles at metaphase. Also, we are looking into the potential interacting partners of Spindly using yeast 2-hybrid and pull down assays. We are interested in how hSpindly is recruited to kinetochores by RZZ complex and downstream dynein/dynactin complex recruitment by hSpindly.

Neuronal Cytoskeleton I

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Activity-dependent modulation of the interaction between CaMKII α and Abi1 regulates structural plasticity of dendritic spines.

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Remodeling of dendritic spines through regulation of actin dynamics is a key event in activity-dependent structural plasticity. However, the molecular mechanism underlying this process is poorly understood. Here, we show that activity-dependent modulation of Abi1-CaMKII α interaction, and thereby their activity, is important for regulation of spine morphology in cultured rat hippocampal neurons. Abi1 interacts with CaMKII α at resting conditions through its tSNARE

which harbors striking homology with CaMKII α regulatory domain. The interaction of the two proteins results in their simultaneous inhibition, inhibition of CaMKII α activity and also Abi1-dependent Rac activation. Their functional impediment is released when they dissociate from each other by Calmodulin binding through glutamate receptor activation. Prior to dissociation, Abi1 is phosphorylated by CaMKII α at serine 88 which is crucial for Rac activation and spine maturation. Our results suggest that modulation of the interaction between Abi1 and CaMKII α , through the glutamate receptor pathway, may be a molecular mechanism underlying activity-regulated structural plasticity.

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Asef2, a Rac and Cdc42 Guanine Nucleotide Exchange Factor, Regulates Dendritic Spine Formation in Hippocampal Neurons.

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The Rho family of small GTPases consists of molecular switches that are integral for the formation of dendritic spines – small, actin-enriched structures that establish excitatory synapses with presynaptic axons. Upon activation by guanine nucleotide exchange factors (GEFs), these GTPases are thought to regulate actin cytoskeletal dynamics in dendritic spines. While the functions of several key members of the Rho GTPase family have been described, the roles of many GEFs in spine development are not currently known. Here, we show that Asef2, a GEF that has been shown to activate Rac1 and Cdc42, is important for the formation of dendritic spines in hippocampal neurons. A short-hairpin interfering RNA (siRNA) targeting Asef2 causes a significant decrease in the number of spines and synapses. Conversely, expression of GFP-tagged Asef2 causes an increase in the density of spines and synapses. These experiments suggest that Asef2 is important for dendritic spine formation. We hypothesized that Asef2's GEF activity, which is facilitated by its DH domain, is responsible for the observed effects on spine formation. Thus, we generated DH domain mutations that greatly reduce GTPase activation. Disruption of Asef2's GEF activity results in a decrease in the number of spines compared to wild-type Asef2. To better understand the mechanism of Asef2-mediated spine formation, we altered the levels of activated Rac1 downstream of Asef2. Treatment with the Rac inhibitor NSC23766 causes a reduction in spine density in Asef2-expressing neurons. In addition, our data indicate that an siRNA targeting Rac1 abrogates Asef2-mediated spine formation. Collectively, our data show that Asef2 mediates dendritic spine formation via its GEF activity, potentially through a Rac-dependent mechanism.

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Myosin-X and its Motorless Isoform Play Distinctive Roles in Regulating the Development and Morphology of Dendritic Spines.

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Dendritic spines are small, actin-rich structures that serve as the postsynaptic component of most excitatory synapses. The formation of spines and synapses most likely begins with the extension of dendritic filopodia, which are dynamic, finger-like protrusions from the dendrite that make initial contact with axons. Alterations in the morphology and plasticity of dendritic spines and filopodia are mediated by actin remodeling and underlie cognitive processes, such as

learning and memory. Myosin-X (Myo10), an unconventional actin motor, and a headless isoform (HL), which lacks the motor head domain, are expressed in the brain. However, their role in regulating neuronal processes is not well understood. Here, we show that full-length (FL) Myo10 and its HL isoform differentially modulate the morphology and development of spines in hippocampal neurons. FL Myo10 localizes to the tips of dendritic filopodia, whereas HL Myo10 is enriched at the heads of dendritic spines. While most HL Myo10 puncta co-localize with presynaptic terminals, the majority of FL Myo10 puncta do not make stable contacts. Elevated expression of FL Myo10 results in a 2.3-fold and a 1.7-fold increase in the number and length of dendritic filopodia. A 1.5-fold increase in the speed of extension and a 1.6-fold increase in the speed of retraction of dendritic filopodia are also observed in neurons expressing FL Myo10. Conversely, knockdown of FL Myo10, using an siRNA approach, leads to an approximately 2-fold decrease in the density of dendritic filopodia. In contrast with FL Myo10, HL Myo10 expression increases spine number by 2.3-fold and promotes a 1.5-fold increase in spine head size. Collectively, these results indicate that expression of FL Myo10 increases the number, length, and dynamics of dendritic filopodia, while HL Myo10 induces the formation and expansion of spine heads. Thus, FL and HL Myo10 cooperatively modulate the formation and maturation of dendritic spines.

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Myosin 18A-alpha is highly concentrated in the dendritic spines of mouse cerebellar Purkinje neurons: possible implications for spine morphogenesis and function.

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Dendritic spines are small, actin-rich protrusions on the surface of neuronal dendrites that serve as sites of excitatory synaptic input and memory formation. Previous studies have implicated myosin II, the conventional non-muscle myosin, in spine organization and function. Here we have explored the localization within cerebellar Purkinje neurons (PNs) of myosin 18A (M18A)-alpha and M18A-beta, two spliced isoforms of M18A, a recently discovered member of the myosin super family that is similar to, and yet distinct from, class II myosins. These distinctions include a C-terminal PDZ-ligand motif in both spliced isoforms, and in the case of M18A-alpha, a 333-residue N-terminal extension that harbors an apparent ATP-independent actin-binding site, a Lys- and Glu-rich region (KE region), and a PDZ domain. The fact that M18A-beta lacks this N-terminal extension suggests distinct functions for these two isoforms. Using GFP-tagged versions of M18A-alpha and beta and a novel system for gene transduction, we find that M18A-alpha, but not M18A-beta, localizes dramatically to the dendritic spines of PNs in dissociated culture. Moreover, M18A-alpha's spine distribution overlaps extensively with that of F-actin, as revealed using a novel reporter for F-actin (F-Tractin). The postsynaptic, spine localization of M18A-alpha was confirmed by immuno-EM of cerebellar tissue sections using a highly specific anti-M18A antibody. Importantly, M18A-alpha's N-terminal extension by itself targets extensively to spines, arguing that it is largely if not entirely responsible for the myosin's spine localization. Function-blocking point mutations show that the KE region and the PDZ domain within M18A-alpha's N-terminal extension do not determine spine targeting. Rather, the interaction of the ATP-independent actin-binding site with actin filaments appears to drive the spine localization of M18A-alpha. Consistently, *in vitro* binding assays using M18A-alpha's N-terminal extension show that it binds F-actin with moderately high affinity (305 ± 105 nM). We suggest that M18A-alpha may play important roles in the development, organization, and/or function of PN spines. Future experiments using a conditional M18A knockout mouse and the PN-specific expression of Cre recombinase will be used to address the physiological significance of the myosin's spine localization in PNs.

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Regulation of Filopodia Dynamics in Outgrowing Neurites by Rho GTPase Signaling Programs.

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Early stages of neuronal differentiation involve extensive cell polarization, characterized by the outgrowth of long tubular structures called neurites from the neuron cell body. Since neurites possess a dynamic, actin-rich tip called the growth cone, it is not surprising that the RhoGTPase proteins, ubiquitous regulators of the actin cytoskeleton, are heavily involved in coordinating neurite extension, retraction, and turning events. However, the precise spatial and temporal pattern of RhoGTPase activity corresponding to these events, as well as how these patterns are maintained and manipulated by extracellular cues is not established. Important actin structures likely contributing to neurite outgrowth and turning are the growth cone filopodia, long filamentous actin bundles at the tip of an outgrowing neuron. Filopodia are generally thought to be “sensors” of the extracellular environment serving to effectively “steer” the outgrowing neurite in response to external cues. Therefore, understanding the signaling and dynamics that control filopodia is fundamental to effectively manipulating the processes associated with neuronal development.

Here, using a combination of live-cell imaging and computer vision, we are developing methods to comprehensively and quantitatively characterize how Rho GTPase signaling programs initiated specifically in the growth cone filopodia may be functionally linked to local filopodia dynamics and larger-scale growth cone outgrowth and turning. We present a novel method for fully automated detection and tracking of filopodia in the fluorescently labeled growth cone. This software provides a framework to systematically quantify and cluster filopodia dynamic behavior (ie filopodia protrusion, retraction, lateral movement etc) – a critical local morphological “output” of actin related signaling in the navigating neurite. To explore how different RhoGTPase signaling pathways may serve to maintain specific filopodia dynamic populations, we have used our approach to methodically compare filopodia dynamics upon knockdown of select RhoGTPase activity modulators and effectors recently pre-screened for robust and diverse neurite outgrowth/growth cone structural phenotypes. While here we focus on the responses elicited in N1E-115 neuroblastoma cells in the presence of the extracellular matrix protein laminin, our method for filopodia dynamics measurement is generic and provides a means to quickly quantify functionally relevant signaling outputs - a vital first step in future neurite outgrowth signal-pathway reconstruction studies.

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+TIP-Kinesin complexes steer microtubule growth in vitro.

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In neurons, proper microtubule orientation in axons and dendrites is essential for targeting intracellular cargo to their correct destinations. However, the mechanisms by which uniform microtubule organization is established and maintained are poorly understood. In vivo imaging in *Drosophila* dendrites showed that microtubules growing into branch points were consistently directed towards the cell body, thus maintaining their minus-end out orientation. RNAi knockdown experiments established that proper microtubule steering required EB1, APC and kinesin-2. Further two-hybrid assays indicated that APC interacts with both EB1 and the kinesin-2 subunit KAP3, forming a +TIP-kinesin complex. These findings led to the hypothesis that

kinesin, concentrated at the plus-tips of growing microtubules by EB1, bends the growing microtubule by walking along static microtubules present at junctions. However, this mechanical role of EB1 is not consistent with the rapid kinetics reported for EB1 dissociating from microtubule plus-ends. To elucidate the minimum requirements for microtubule steering and investigate the mechanical activities of EB1 and kinesin during steering, we immobilized microtubule seeds on glass surfaces in vitro, extended them with free tubulin and observed encounters between dynamic growing microtubule plus-ends and the lattice of stable microtubules. To reconstitute +TIP-kinesin complexes in vitro, purified EB1 and kinesin were linked together through the Rapamycin-induced heterodimerization of FKBP-FRB. From gel filtration, the binding ratio of EB1 to kinesin ranged from 1.5:1 to 2:1. Under TIRF microscopy, EB1-kinesin complexes moved along microtubules and preferentially accumulated at growing microtubule plus-ends, indicating that association with EB1 promotes the localization of kinesin to growing plus-ends. More importantly, when +TIP-kinesin complex was present, the growing microtubules were bent and directed toward the plus-ends of immobilized microtubules, demonstrating that the +TIP-kinesin complex is sufficient to steer microtubule growth. We conclude that EB1 can localize kinesin to growing microtubule plus-ends and EB1 binding to microtubule plus-ends is sufficiently strong to steer microtubule growth. The work is supported by NIH.

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Microtubule dynamics in neocortical neurons during distinct modes of migration and polarization in the developing mouse cerebrum.

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Neuronal migration and process formation require cytoskeletal organization and remodeling. Recent studies suggest that centrosome translocation is involved in initial axon outgrowth, while the role of centrosomal positioning is not clear. Here we study the relations between centrosome translocation, axonogenesis, and microtubule (MT) polarization in multipolar and bipolar neocortical neurons undergoing migration and axonogenesis. To monitor the dynamic movements of centrosomes and MT plus ends in migratory neurons in embryonic mouse cerebral slice, we expressed PACT-mKO1 and EB3-EGFP by using in utero electroporation. In the developing mouse cerebrum, young pyramidal neurons born in the ventricular zone migrate toward the brain surface through the intermediate zone and cortical plate. In multipolar migrating neurons in the intermediate zone, new processes emerged irrespective of centrosome localization, followed by centrosome movement toward the dominant growing process. In bipolar-shaped locomoting neurons in the cortical plate, the centrosome targeted the pia-directed leading process. We observed distal tip-directed movements of MT plus ends predominantly in multipolar neuron processes. MT plus ends were enriched in the leading process at the transition from multipolar to bipolar morphology. In bipolar neurons, distal-directed MT plus end movements were prominent in leading processes, while trailing processes showed bidirectional movements. We further observed that in multipolar neurons axons form by tangential extension of a dominant process and the centrosome orients toward the growing axon, while in locomoting neurons an axon forms opposite to the direction of migration and the centrosome localizes to the base of the leading process. Our observations suggest that MT organization between processes may alter centrosomal localization and that centrosomal positioning does not direct process formation.

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CSPR-1 stabilizes the microtubule cytoskeleton and presynaptic specializations of neurons in *C. elegans*.

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Lesions in cytoskeletal-associated proteins are associated with a variety of degenerative and excitatory neurobiological disorders in humans. Remodeling of synapses and changes in neuronal morphology are thought to underlie diseases as diverse as Alzheimer's and epilepsy. Here we report that the cytoskeletal-associated gene *cspr-1* is required for proper morphology and cytoskeletal stability of several distinct classes of *C. elegans* neurons. Remodeling of neuronal morphology including both the formation and retraction of branches and synapses occurs throughout development in *cspr-1* mutants. By contrast, in wild type animals, the same neurons exhibited virtually no morphological changes in the same time period. Our subcellular localization data indicates that CSPR-1 acts near the periphery of the soma and in neuronal processes. Because the phenotypes we see in *cspr-1* mutants mimic alterations caused by microtubule destabilizing mutations, and *cspr-1* mutant phenotypes are exacerbated by growing worms in conditions where microtubule polymerization is slowed, we suggest a paradigm whereby microtubule stabilization mediated by a functional CSPR-1 is necessary for both formation and maintenance of correct neuronal morphology. We speculate that the human homolog is likely involved in the prevention of disorders associated with changes in cellular morphology

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JNK phosphorylation of hnRNP K is required for axon outgrowth during nervous system development in *Xenopus laevis*.

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The RNA-binding protein hnRNP K is required for axon outgrowth during development. Its suppression in *Xenopus* embryos causes defects in the translation of mRNAs of multiple cytoskeletal genes. Studies in cell lines have established that hnRNP K shuttles between the nucleus and the cytoplasm to bind and regulate the fates of its target RNAs, from splicing to export and translation. At each step, hnRNP K is regulated through post-translational modifications that alter its nucleic acid and protein interactions, and subcellular localization. Precisely how this happens in developing neurons to coordinate cytoskeletal gene expression with the extracellular signals directing axon outgrowth is unknown. We have identified a JNK phosphorylation site within hnRNP K that is essential for its function during neuronal development. Treatment with SP600125, a pharmacological inhibitor of JNK, prevented formation of axons in primary neuronal cultures; a phospho-mimetic mutation of the JNK site on hnRNP K successfully rescued axon outgrowth in the presence of SP600125, implicating hnRNP K as a major substrate on which JNK acts to effect axonogenesis. Western analysis and qRT-PCR on extracts from SP600125-treated cultures revealed the involvement of JNK in the post-transcriptional control of the medium neurofilament (NF-M) and microtubule-associated protein tau, both mRNA targets of hnRNP K. Polysome profiling experiments further demonstrated involvement of JNK in translational regulation; a phospho-deficient mutation of the JNK site on hnRNP K caused a shift of hnRNP K into the non-translating, premonosomal fractions. We propose a mechanism whereby JNK controls translation of hnRNP K's target mRNAs, and by extension axon outgrowth, at the point of translation initiation through prevention of 80S ribosome assembly. JNK has long been implicated in the intracellular

signaling pathways that mediate effects of several receptors on axon outgrowth, although a mechanism of its action had not previously been described. These data suggest a role for hnRNP K as a central regulatory component linking extracellular signals that regulate axon outgrowth directly with the expressions of key axonal structural components. *Supported by NSF IOS-951043 (BGS), a Sigma Xi Grant-in-Aid (EJH), and an AAUW dissertation fellowship (EJH).*

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Kinesin-mediated microtubule sliding drives axon outgrowth in *Drosophila* neurons.

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How neurons extend axons is a key long-standing question in neuronal cell biology. Recently, our lab revealed a novel role of the microtubule motor kinesin-1 in driving microtubule-microtubule sliding and formation of long processes in tissue culture cells. Here, we use *Drosophila* embryonic neurons as a model system to study whether a similar mechanism operates in axon outgrowth. By using transgenic flies that carry a photoconvertible tubulin, EOS- α tub, we are able to track microtubule dynamics at different stages of embryonic neuron development in culture. We observe that microtubules are very actively sliding in young developing neurons. Within a few minutes, microtubules photoconverted within a small area are found to be distributed throughout the full neuron by sliding. Furthermore, these sliding microtubules push directly against the cytoplasmic membrane of the growing neurite tip and very often become looped, suggesting that sliding microtubules generate mechanical force for axon extension during neuronal development. In mature, fully-developed neurons, microtubules become very stationary, and very little sliding can be detected. The strong correlation between microtubule sliding and axon growth rate strongly suggests that microtubule sliding can contribute to axon growth. Consistent with these findings, we demonstrate that neither actin depolymerization nor inhibition of microtubule polymerization blocks axon extension at initial stages of neuronal development. Finally, we have data to show that kinesin-1 powers microtubule sliding, which is important for axon growth in young neurons. These results reveal a novel mechanism driving initial axonal outgrowth in *Drosophila* neurons. Due to the high evolutionary conservation of kinesin-1, a similar mechanism may function in other neurons.

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Cytoskeletal architecture and assembly of the axon initial segment in hippocampal neurons.

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The axon initial segment (AIS) is a special compartment of differentiated neurons that is essential for regulating action potential initiation and axono-dendritic polarity. For maintaining polarity, the AIS is thought to act as a diffusion barrier that prevents mixing of axonal and dendritic components, or as a sorting station that uses oriented cytoskeletal tracks to direct molecules to proper destinations. However, the cytoskeletal organization at the AIS is unknown, making it difficult to distinguish between these two models. Although the integrity of the actin cytoskeleton is known to be essential for the barrier functions of the AIS, it remains unclear whether actin filaments act by forming a molecular sieve or oriented tracks. Using platinum replica electron microscopy, we have determined the architecture of the AIS cytoskeleton in cultured hippocampal neurons and its development during neuronal maturation. We show that

as early as 3 days in vitro (DIV) the proximal axon has structural features distinguishing it from the distal axon or dendrites. The formation of the AIS begins with appearance of microtubule bundles (DIV 3-4), which then become covered with ankyrin G-positive fibrillar/granular networks, whose density progressively increases, while composition shifts from predominantly fibrillar (DIV 7-10) to mostly granular (DIV 21). Actin filaments of appreciable lengths are very rare in mature AISs (DIV 7-21). Accordingly, phalloidin staining reveals some F-actin enrichment in the AIS only up to DIV 4, but not at later time points. Decoration with myosin S1 showed that the existing actin filaments display no predominant orientation. Remarkably, the AIS architecture remains apparently intact even after depletion of microtubules (by calcium and cold treatment), or actin filaments (by gelsolin treatment), or both, from the cytoskeletal preparations of mature neurons. Collectively, we show that the mature AIS cytoskeleton is a self-maintained fibrillar/granular structure, which lacks oriented actin filament tracks for directional myosin-driven transport. Our results suggest that the AIS submembranous cytoskeleton predominantly functions as a molecular sieve, which likely represents a large supramolecular assembly of spectrin, ankyrin G, and very short gelsolin-resistant actin filaments, similar to the membrane skeleton of red blood cells.

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Cortactin functions as a clutch molecule to promote axon outgrowth.

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Axon outgrowth is essential for the formation of neuronal networks. The nerve growth cone is the migrating tip of growing axons and plays a central role in axon outgrowth and guidance. Linkage between the treadmilling actin filaments (F-actins) and cell adhesion molecules (CAMs) in the growth cone is thought to transmit the force of F-actin retrograde flow to extracellular substrates via CAMs, thereby providing traction force for neurite outgrowth ("clutch" mechanism) (Mitchison and Kirschner, *Neuron*, 1988; Suter and Forscher, *J. Neurobiol.*, 2000). Recently, we reported that shootin1 mediates the linkage between F-actin retrograde flow and L1-CAM to promote axon outgrowth as a "clutch molecule" (Shimada et al., *J. Cell Biol.*, 2008). However, components of the clutch modules in axonal growth cones are largely unknown and the molecular linkage between F-actin retrograde flow and shootin1 remains elusive.

Cortactin is an actin-binding protein and is known to be involved in growth cone formation in neurons. In this study, by co-immunoprecipitation (IP) assays, we found that cortactin interacts with shootin1. Cortactin was co-localized with shootin1 and F-actin in the peripheral domain of the axonal growth cone. *In vitro* binding assay and co-IP study showed that endogenous cortactin binds directly to endogenous shootin1. Fluorescent speckle imaging of EGFP-cortactin revealed that cortactin also interacts with F-actin retrograde flow in the axonal growth cones. Furthermore, we found that cortactin mediates the interaction between F-actin and shootin1 *in vitro* and *in vivo* and that the driving force of F-actin retrograde flow is transmitted to L1-CAM via cortactin. By impairing this linkage using cortactin RNAi, clutch engagement was attenuated and L1-dependent axon outgrowth was inhibited. These findings suggest that cortactin functions as a clutch molecule to promote axon outgrowth by mediating the linkage between F-actin retrograde flow and shootin1 in the axonal growth cones.

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JNK reduces SCG10 protein level to regulate axon formation.

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Axon formation in neurons depends on the microtubule dynamics, which is regulated by a variety of microtubule associated/regulatory proteins. We have shown that axon formation of neocortical neurons depends on the component of the MAP kinase cascade, dual leucine zipper kinase (DLK) and c-Jun N-terminal kinase (JNK), using mutant mice and *in vitro* mRNA knockdown systems. JNK phosphorylates some microtubule associated/regulatory proteins including MAP1b, MAP2, DCX, and SCG10. Among them, SCG10 forms a complex with tubulin tetramer and destabilizes microtubules, while others associate with polymerized tubulin, microtubules. It has been reported that MAP kinases including JNK phosphorylate Ser62 and Ser73 of SCG10 and reduce its activity as a microtubule destabilizer. These observations are consistent with our observation that treatment with taxol, a microtubule stabilizer, partially rescues the reduction of axon formation caused by the suppression of DLK-JNK pathway. However, how JNK inactivates SCG10 remains to be elucidated. In this paper, we show that JNK activation reduces SCG10 protein level: Endogenous SCG10 protein in primary cortical neurons increased upon DLK knockdown or JNK inhibitor treatment. The expression of exogenous DLK or active JNK in DLK knockdown cells reduced SCG10 protein level. The protein level of ectopically expressed SCG10 in COS-1 cells was also sensitive to JNK activation, which became insensitive when Ser62 and Ser73 were converted to alanine. These observations suggest that DLK-JNK pathway regulates SCG10 protein level via phosphorylation of Ser62 and Ser73. While SCG10 is one of critical factors for axon formation, spatiotemporal regulation of its quantity will be essential, which may, at least in part, depend on DLK-JNK pathway.

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Characterization of dendrite morphology defects caused by overexpression of DCX patient mutations in cortical neuronal cultures.

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Proper functioning of neuronal circuits depends critically on the correct wiring of large numbers of neurons. Dendrites play a crucial role in synaptic connections and any change in their morphology can affect neuronal connectivity and impact brain function. The growth of dendrites is a precisely regulated process. During development, dendrites extend and reach their final destinations, a process regulated by both extracellular and intracellular proteins. Doublecortin (DCX) has been implicated in multiple steps in brain development. Mutations in DCX underlie lissencephaly and subcortical band heterotopia in humans, caused by disrupted neuronal migration during development. These defects are phenocopied in rats by DCX downregulation whereas the defects in DCX null mice are more subtle. Patients with mutations in DCX also have other defects, which are not related to the disruption in neuronal migration. For instance, hypoplasia of the corpus callosum is likely caused by defects in axon growth. DCX is most studied as a microtubule associated protein. Thus, the various developmental roles of DCX are thought to be due to its essential role in microtubule organization, dynamics and function. In addition, DCX has been proposed to be involved in regulating protein transport, possibly by interacting and regulating the function of microtubule motors. Interestingly, rat DCX knock down neurons have aberrantly short dendrites as do neurons from the hippocampus of DCX ^{-/-} mice. It is not yet established if human patients carrying DCX mutations have defects in dendrite morphology as well. Most work on understanding DCX has centered on analyzing the loss-of-

function phenotypes using knock-out or knock-down approaches. To date, around 52 mutations have been mapped to the DCX gene. The disease severity is related to the location of the mutation in the DCX protein. We are studying a set of patient mutations in DCX, which are located in different regions of the protein and have different biochemical behaviors with respect to microtubule binding. We find that expression of wild type DCX in rat cortical neurons increases dendrite complexity. Similarly, the patient mutation DCX-G253D still increases dendrite complexity. In contrast, other patient mutations (patient mutation DCX-272X) are deficient in increasing dendrite morphology. Furthermore, a microtubule binding-deficient DCX (patient mutation DCXR89G) causes shortening of dendrites after 5 days. Therefore, different DCX patient alleles have separable phenotypes and not all patient mutations are loss of function mutations.

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Regulation of neuronal morphogenesis and intracellular transport by NudE.

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Dynein plays a role in a variety of cellular processes, yet it remains unclear how this motor complex performs such different tasks. Our previous studies revealed that within the developing fly nervous system, dynein is necessary for two fundamental aspects of neuronal polarity: the polarized localization of dendritic proteins and organelles, and the uniform plus-end distal organization of axonal microtubules. Dynein interacts with several different co-factors, including NudE. Similar to the loss of functional dynein, the loss of NudE disrupts axon and dendrite morphogenesis. Fly dendritic arborization (da) neurons typically extend unbranched axons into the ventral nerve cord, with axonal microtubules uniformly oriented with their plus-ends positioned distally. Unlike wild type, but similar to dynein loss-of-function, the axons of neurons lacking nudE split into multiple branches and contain microtubules of mixed polarity, suggesting these axons have acquired dendritic characteristics. To further probe the function of NudE in the transport of different cargo within neurons, we developed and are employing a fluorescently-tagged NudE protein to follow NudE dynamics live within da neurons. Through this combination of techniques, our results further elucidate the role of NudE in neuronal morphogenesis as well as its roles in intracellular transport.

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Branching Kinase (Branck) regulates arborization of neurites.

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Ramification of neuronal processes is essential for formation of elaborate neural network in the brain. During the screen to identify novel regulators of neuronal morphogenesis, we found a serine/threonine kinase whose over-expression strongly induces neurite branching of cultured hippocampal neurons. We tentatively named this molecule Branching Kinase (Branck). To examine if Branck plays a crucial role for neural development, Branck gene was disrupted in mouse ES cells by homologous recombination. In the hippocampi of the Branck-null mice, arborization of the pyramidal neurons is slightly impaired. Divergency of the axonal processes of the developing peripheral nervous systems is reduced as well. We are currently analyzing the molecular mechanism of Branck-mediated bifurcation by identifying physical binding partners

and functional interactors. The preliminary results of our yeast two-hybrid screen as well as in vitro examination of candidates for the downstream molecules will be presented.

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Microtubules promote filament formation from unmodified full-length Tau *in vitro*.

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Tau is a neuronal protein that stabilizes the microtubule network, but it also forms filaments associated with Alzheimer's disease. Understanding Tau-microtubule and Tau-Tau interactions would help to establish Tau function in health and disease. For many years, literature reports on Tau-microtubule binding behavior and affinity have remained surprisingly contradictory. Tau-Tau interactions have also been investigated, but whether microtubules might affect Tau filament formation is unknown. We have addressed these issues through binding assays and microscopy. We assessed Tau-microtubule interactions via cosedimentation and found that the measured affinity varies greatly depending on the experimental design and the protein concentrations used. To investigate this dependence, we used fluorescence microscopy to examine Tau-microtubule binding. Strikingly, we found microtubules promote Tau filament formation without characterized Tau filament inducers. We propose that these novel Tau filaments account for the incongruence in Tau-microtubule affinity measurements. Moreover, these filaments appear similar to the heparin-induced Alzheimer's model by electron microscopy. These observations suggest that the microtubule-induced Tau filaments provide a new model for Alzheimer's studies and support the possibility that microtubules play a role in the formation of Alzheimer's associated neurofibrillary tangles.

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Stably transfected PC12-TAU as a model to study Neuronal Toxicity associated with Extracellular Hyperphosphorylated TAU in Alzheimer Disease.

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Neuronal cell death in Alzheimer Disease damaged brain regions is associated with the presence of TAU tangles. At present, there is no disease described where TAU accumulates and is not hyperphosphorylated. Indeed, phosphorylation of some specific TAU sites is sufficient to induce cell death. Moreover, neurodegeneration associated with TAU has also been related to the spreading of its pathological form. Those neurons that are successful in keeping low levels of hyperphosphorylated TAU are able to survive longer. Our previous results indicated that cells stably transfected with TAU survived better against pathological agents such as TNF α and Staurosporine. This work aimed to understand if PC12 cells and PC12 cells stably transfected with human TAU (PC12-TAU) could be used as a model system to study the role of intracellular TAU phosphorylation balance on cell survival. In doing so, we built up a foundation to test if the presence of TAU stably transfected to the neurons could protect against substances such as the minimal TAU sequence inducing aggregation. PC12 and PC12-TAU cells were cultured in different cell culture media and plated with different coating protocols. This work demonstrated that PC12 and PC12-TAU exhibited different patterns of proliferation and survival in a different coating and medium conditions. Specifically, both types of cells are attaching and growing healthy in RPMI-1640 supplemented with 15% FBS and Penn 1/500 in wells previously coated either with Roche-collagen or Sigma-Poly-D-lysine. Other media such as RPMI-1640 supplemented with 10% Horse Serum, Fetal Bovine Serum and Penicillin/Streptomycin showed a healthy growth pattern for PC12-TAU cells but not for PC12 cells. Collagen-based coating also produced different growth patterns, with PC12-TAU cells

growing healthy in all conditions. While these results point to an impact of TAU in cellular growth, their interpretation is complicated by the observation that PC12 cells appear to be particularly sensitive to differences in the quality of serum among different suppliers and batches. This could lead to a selection of subclones different from the original cells influencing poor cell attachment to plastic. In order to circumvent these difficulties, we plan to use neuronal cell line B35/B50 as an alternative to PC12 cells to test the toxic effect of pseudophosphorylated TAU. All together, these results will help to establish a best model to study the cellular balance regarding TAU phosphorylation and its relation with neuronal commitment to death in pathological conditions. Indeed, the avenues for successful treatment for tauopathies may involve down regulation of the phosphorylation and up regulation of the clearance of the abnormal form of TAU.

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Expression of HDAC Enhances Mistargeting of Phosphorylated Tau to Soma of Hippocampal Neurons.

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Alzheimer disease (AD) is a devastating neurodegenerative disease that currently affects millions of people worldwide and has no cure. Extracellular amyloid plaques and intracellular neurofibrillary tangles, the two hallmark lesions in a typical AD brain, are comprised of aggregated beta-amyloid (A β) and hyperphosphorylated tau (p-tau), respectively. The accumulation of oligomers of A β and p-tau correlate with disease progression and deficits in learning and memory. Accumulation of these proteins, are associated with disrupted synaptic function and neuronal death. Here we focus on understanding the underlying molecular mechanisms leading to the accumulation of aggregated A β and p-tau. We investigate the role of a histone deacetylase (HDAC), which has been shown to have increased protein levels in the neural tissue of patients who have succumbed to the disease. Our experiments have focused on characterizing the biochemical interaction between this HDAC and Protein Phosphatase 1 (PP1), a protein that is known to play a key role in regulating tau pathology. By using standard biochemical techniques in a heterologous expression system, as well as confocal imaging using dissociated neurons, we have found that overexpression of this HDAC causes an increase in the amount of phosphorylated tau. Of particular interest is the enhanced level of p-tau in the soma of these neurons. This data suggest that this HDAC is an underlying molecular factor mediating tau pathology.

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Hyperphosphorylation of Tau.

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Tau is a microtubule associated protein that is expressed in neurons of the central nervous system. It regulates microtubule stability and axonal transport. Neurodegenerative diseases known as tauopathies are characterized by the abnormal hyperphosphorylation and subsequent assembly of tau protein. Normal tau contains ~ 2-3 mol of phosphate per mole of protein. Hyperphosphorylated tau contains ~7-10 mol of phosphate per mole of protein. Hyperphosphorylated tau aggregates into straight and paired helical filaments, the primary component of neurofibrillary tangles. It also binds to and sequesters normal tau, inhibiting its function to promote microtubule assembly. It is not proven whether neurodegeneration is the cause or a consequence of tau hyperphosphorylation. In vitro studies demonstrated that hyperphosphorylated tau promotes neurodegeneration and that the combination of

phosphorylation at Thr212, Thr231 and Ser262 produces toxic effect in the cells. To study these specific sites, pseudophosphorylated tau constructs were generated. The above-mentioned threonine or serine residues were switched by site directed mutagenesis of that specific codon to a glutamic acid, to mimic the negative charge carried to that site by a phosphate. This pseudophosphorylated tau is termed PH-tau. These same constructs were used to generate a transgenic *Drosophila* line expressing inducible PH-tau. Here, *Drosophila* were used to investigate the in vivo mechanism of tau toxicity induced by phosphorylation. They were controlled by yeast upstream activating sequence (UAS). PH-tau was expressed in a pan-neural pattern (Elav-Gal4) in the background of wild-type human tau (wt-tau), or tau carrying one of the point mutations associated with FTDP-17, R406W. Additionally, the lab has generated an inducible mouse model to study the effects of inducing degeneration in a mammalian system. Transgenic mice were generated to express PH-tau under regulation by the tetracycline-controlled transactivator protein (tTA) linked to the calcium-calmodulin dependent kinase II promoter (CAMk2a) to express PH-tau only in neurons. The expression of the target gene can be blocked by administration of the doxycycline feed (1g/kg). PH-tau expressing cells have shown tau no longer associated with the microtubule network, membrane blebbing, excessive intracellular vacuolization, protein aggregation and a breakdown of polymerized actin and increased caspase activation. To confirm that the toxic effect seen in cells was a product of phosphorylation and not the mutation, cells expressing wild type tau were treated with okadaic acid, which inhibited phosphatase activity and caused wild type tau to become hyperphosphorylated. These cells exhibited the same pathologies as those expressing PH-tau. Our results show that flies expressing hyperphosphorylation of tau had shorter lifespan and impaired motor function. This expression also interfered with their ability to learn. Flies kept at 23°C expressed lower levels of tau expression and flies kept at 29°C demonstrating the PH-tau expression is temperature dependent. Behaviorally, the expression of PH-Tau in mice caused a cognitive impairment in the Morris Water Maze. Pathologically, PH-tau expression in the mouse brain has shown an increase in silver stained cells and increased levels of fibers in the tissue, suggesting that presence of aggregated protein in the tissue. Additionally, immunohistochemical analysis has shown the presence of human tau in the brains of double transgenic mice off of the doxycycline food. In the presence of doxycycline, no human tau is detectable demonstrating that the inducible system is functional. Taken together our results suggest that hyperphosphorylated tau is an early event in the process of neurodegeneration.

Membrane Trafficking at the Synapse

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Expression of Glutamate Transporters are increased in the Prefrontal Cortex and Hippocampus of Rats Reared in Isolation from Weaning.

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There is growing evidence implicating the glutamate in the pathophysiology of schizophrenia. It has been reported that glutamatergic neurotransmission is decreased in the prefrontal cortex and hippocampal formation in schizophrenics. Rats reared in isolation from weaning have been used as an experimental model of schizophrenia. In this model it was reported a decrease in the expression of AMPA receptors in rat hippocampus. This study aimed at evaluating the changes in the expression of glutamate transporters, EAAC1 and GLT1, in hippocampus, amygdala,

prefrontal cortex (PFC) and entorhinal cortex (EC) of rats reared in isolation from weaning. Two groups of Wistar rats (n=11-12/each) were used. In both groups the pups remained with their mothers (6 pups per mother) until weaning (21 days - 40g) when they were allocated randomly to one of two conditions: 1) grouped, housed 3 per cage and handled 3 times a week; 2) isolated, housed individually and handled once a week for cleaning purpose. After 10 weeks all animals were anaesthetized, perfused and their brains removed. 40-um sections of the brain areas were used for immunohistochemistry. The number of immunopositive cells (IC) was quantified bilaterally in 3 sections/rat and the average for grouped and isolated compared by Student t-test ($p < 0.05$). Isolation rearing induced a significant increase on the expression of EAAC1 in the PFC (38%, $p = 0.017$), hilus of dentate gyrus (81%, $p < 0.01$) and CA3 (144%, $p < 0.05$). However, no difference was found in CA1 and EC. A non-significant increase in EAAC1 expression was induced by isolation rearing in basolateral (13%) and lateral (6%) amygdala. The number of GLT1-IC did not change in the PFC ($p < 0.05$) of rats reared in isolation when compared to grouped rats. However, immunofluorescent labeling for GLT1 was seen associated to glial and neuronal cells in the PFC and EC. These results contribute with additional experimental evidence for the reduction in the glutamatergic neurotransmission reported in schizophrenia. These results may also contribute for considering this pathology a syndrome of "hypofrontality" and for considering the glutamate transporters a future therapeutic target for the treatment of schizophrenia.
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The kinesin-3 family motor KLP-4 regulates anterograde trafficking of GLR-1 glutamate receptors in the ventral nerve cord of *C. elegans*.

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The transport of glutamate neurotransmitter receptors from the cell body to synapses is essential during neuronal development and may contribute to the regulation of synaptic strength in the mature nervous system. We previously showed that cyclin-dependent kinase-5 (CDK-5) positively regulates the abundance of GLR-1 glutamate receptors at synapses in the ventral nerve cord (VNC) of *C. elegans*. Here, we identify a kinesin-3 family motor *klp-4* in a *cdk-5* genetic suppressor screen for genes that regulate GLR-1 trafficking. KLP-4 is homologous to Drosophila kinesin Khc73 and mammalian KIF13. We found that *klp-4* mutants have decreased abundance of GLR-1 at synapses in the VNC. Genetic analysis of *klp-4* and the clathrin adaptin *unc-11/AP180* indicates that *klp-4* functions prior to GLR-1 endocytosis in the ventral cord suggesting that KLP-4 regulates anterograde trafficking. To test directly whether KLP-4 regulates GLR-1 transport, we performed time-lapse imaging of GLR-1 tagged with the photoconvertible fluorescent protein Dendra2. Kymograph analysis indicates that *klp-4* mutants exhibit decreased anterograde flux, run length and velocity of GLR-1 in the VNC. Genetic analysis of *cdk-5* and *klp-4* suggests that they function in the same pathway to regulate GLR-1 trafficking. Interestingly, GLR-1 accumulates in cell bodies of *cdk-5* but not *klp-4* mutants suggesting that GLR-1 might be degraded in the absence of KLP-4 motors. Consistent with this idea, we found that GLR-1 does accumulate in *klp-4* mutant cell bodies if receptor degradation in the MVB/lysosome pathway is blocked. In summary, this study identifies the kinesin KLP-4 as a novel regulator of anterograde glutamate receptor trafficking in *C. elegans*, thus implicating the KIF13 family of motors in GluR trafficking in mammals. Furthermore, we reveal an interesting cellular control mechanism where GLR-1 receptor cargo is targeted for

degradation in the absence of its motor KLP-4. Thus, the cellular fate of receptor cargo early in the secretory pathway, and consequently the abundance of GluRs at synapses, could be controlled by regulating the expression level or availability of KLP-4 motors.

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Inactivity up-regulates the levels of rabaptin-5 leading to enhancement of synaptic delivery of GluA1.

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The control of glutamate receptor delivery to the synapse is a mechanism that is critical for determining synaptic strength and potential of plasticity. but, it still remains unclear whether synaptic activity history controls receptor trafficking at the synapse. We focused on rabaptin-5, which is a putative mediator molecules for receptor trafficking. We found that rabaptin-5 was down-regulated by substantial neuronal circuit activity. Conversely, over-expression of rabaptin-5 promoted GluA1, not GluA2 surface expression at spines. We explored the role of rabaptin-5 in synaptic plasticity. Synaptic scaling in response to inactivity of neuronal circuit, a type of plasticity affected by chronic circuit activity, was prevented by rabaptin-5 siRNA treatment. It indicated preventing up-regulation of rabaptin-5, which would to be increased by inactivity, prevented GluA1 mediated expression of synaptic scaling. Our results demonstrate that synaptic activity controls rabaptin-5 level and that define the GluA1 surface delivery at synapse. A part of this work was supported by KAKENHI (20500304)

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Recycling endosomes undergo kiss-and-run exocytosis in hippocampal neurons.

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Membrane trafficking is essential for neuronal function: from growth of neurons and synapse formation to recycling of synaptic vesicles and receptors, questions concerning exocytosis and endocytosis are stimulating neurobiology research. In particular, trafficking of glutamate receptors present in recycling endosomes (REs) is necessary for the expression of synaptic plasticity. The localization and the characteristics of exo- and endocytic events are thus crucial determinants of synapse function and plasticity. To investigate these parameters, we have imaged cultured rat hippocampal neurons transfected with transferrin receptor (TfR), a classical marker of REs, tagged with superecliptic pHluorin (SEP), a green fluorescent protein which is not fluorescent at the low pH of acidic intracellular organelles. Single exocytosis events were of two types: in most cases, receptors diffuse quickly in the plasma membrane after exocytosis (discharge events), but in about 25 % of cases, receptors remain clustered (display events). Events observed with other markers, the AMPA receptor subunit GluA1 or the β 2-adrenergic receptor, had similar characteristics, as previously shown. However, their frequency was much lower than for events in cells expressing TfR-SEP. Using fast extracellular pH changes around the recorded cell, we show that display events are characterized by a short opening (median 2.6 s) followed by re-internalization, or kiss-and-run exocytosis. Moreover, using two color imaging of single exocytosis events with markers of neuronal compartments, we found that Rab11a-mCherry is enriched at the exocytosis site, confirming the endosomal origin of the vesicles. Rab11a-mCherry is released from the RE in a few seconds after discharge exocytosis, but not after display exocytosis. Overexpression of a dominant negative form of Rab11a, which largely blocks receptor recycling, decreases the frequency of discharge events and give rise to the formation of large (up to 11 μ m) tubular REs. In conclusion, the last steps of RE exocytosis and receptor release are controlled in neurons in a manner which could depend on Rab11a.

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Detecting the formation of endocytic vesicles in the soma and dendrites of live cultured neurons.

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Endocytosis is a fundamental process regulating the number of receptors at the surface of cells. In neurons, blocking clathrin mediated endocytosis (CME) leads to an increase of the number of postsynaptic AMPA glutamate receptors within minutes and blocks long term depression of synaptic transmission. Moreover, clathrin coated pits are concentrated near synapses, consistent with a specific role of CME in synaptic plasticity. To clarify the link between CME and synaptic plasticity, it would be important to directly detect the formation of clathrin coated vesicles (CCVs) in live neurons. To do so, we have adapted a method based on extracellular pH changes which detect the moment when transferrin receptors labeled with superecliptic phluorin (TfR-SEP) are isolated from these changes, i.e. the moment of CCV formation (Merrifield, Perrais & Zenisek, Cell 2005). We have imaged cultured hippocampal neurons (9-15 days in vitro), transfected with TfR-SEP, with TIRF (total internal reflection fluorescence) microscopy. When neurons were submitted to the so-called ppH protocol, we detected the formation of CCVs throughout the somato-dendritic compartment. CCVs were formed at clathrin coated structures labeled with CLC-DsRed, which are very stable in these cells. Consequently, CCV formation cannot be detected just by imaging the clathrin structures. We determined whether the ppH protocol affected CME in neurons. The frequency of detected CCVs was constant during the recording, and transferrin labeled with Alexa568 was normally internalized and trafficked within cells. However, application of low pH solution evokes large currents in these cells which are due to the opening of acid sensing ion channels (ASICs). Blocking ASICs with amiloride (500 μ M) did not affect the formation of CCVs. Moreover, when the low pH solution was replaced by a cell impermeable quencher of SEP fluorescence, Trypan Purple, we could monitor the formation of CCVs with similar frequencies, demonstrating that the activity of endocytic pits can be recorded in live neurons in basal conditions. We will now monitor the possible changes in rate of vesicle formation during protocols inducing synaptic plasticity.

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Investigating Nogo-A and its potential role with maspardin in Mast Syndrome.

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Hereditary spastic paraplegias (HSPs) are a family of inherited, heterogeneous neurological disorders that are defined by progressive spasticity and weakness of the lower limbs. One particular autosomal recessive form of HSP, known as Mast syndrome (SPG21; MIM 248900), is the result of a mutation in SPG21 gene (aliases MASPARDIN, ACP33, MAST, BM-019 AND GLO10). Though the normal functions of maspardin are still unknown, it is ubiquitously expressed and hypothesized to be involved in protein sorting and trafficking in the *trans*-Golgi network/endosomal pathway. Our investigation of maspardin function includes studying its potential interaction with a member of the reticulon family, RTN4. An isoform of RTN4 known as RTN4A (Nogo-A) is expressed in oligodendrocytes and localizes to the endoplasmic reticulum. Numerous studies have shown that Nogo-A acts to repress neuronal regeneration in the central nervous system, implying that Nogo-A may be a contributing factor in the pathology of certain HSPs. Maspardin-Nogo-A interaction has been suggested by others using yeast two-hybrid. Here we demonstrate *in silico* analyses suggest a direct interaction between maspardin and Nogo-A. We sought to confirm this interaction *in vivo* and *in vitro*. The correct orientation of a commercially available Nogo-A clone has been confirmed using the restriction digest technique. Successful transfection of the Nogo-A clone into Cos7 cells, confirmed through Western Blot

analysis, has been achieved and used to study any interactions in cells over-expressing the Nogo-A protein. Preliminary results suggest an interaction exists. Immunoprecipitation experiments have also been employed in brain homogenates extracted from SPG21 mouse models. Samples have been co-immunoprecipitated with either anti-Nogo-A or anti-masparidin antibodies and subjected to Western Blot analysis as a means for confirming interactions between masparidin and Nogo-A. Preliminary results indicate an *in-vivo* interaction. These results suggest disruption of masparidin and Nogo-A may contribute to pathogenesis.

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Granulocyte colony stimulating factor (G-CSF) positive effects on muscle fiber degeneration and gait recovery after nerve lesion in MDX mice.

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Duchenne muscular dystrophy causes degeneration of muscle fibers what results in retrograde changes that affect the CNS. In this sense, spinal motoneurons show the typical signs of chromatolysis and loss of synapses during the course of the disease. G-CSF is a neuroprotective drug that has also positive effects on muscle fibers after lesion, mostly by its anti-inflammatory capabilities. Such properties may positively act on both CNS and skeletal muscle fibers following peripheral nerve lesion, influencing the process of nerve regeneration and motor function recovery. Therefore, the present work investigated muscle fiber preservation, peripheral nerve regeneration, and motor function recovery after axotomy and treatment with G-CSF (200 µg/kg/day, subcutaneously). For this, six weeks old male MDX mice were treated for seven days before and twenty one days after unilateral sciatic nerve crush. C57BL/10 mice were used as the control strain. The recovery of motor function was monitored for three weeks using the walking track test (Catwalk system). The animals were sacrificed and the sciatic nerves processed for immunohistochemistry (neurofilament and pan-neurotrophin receptor antisera – p75NTR) and transmission electron microscopy. The soleus muscles were processed for H&E staining for histopathological analysis. The results showed that MDX mice present a significant forty percent ($p < 0.05$) motor deficit as compared to control animals even before injury. The motor recovery following crush injury was similar for both strains, although MDX mice showed a significantly decreased motor function after three weeks post lesion (MDX+G-CSF -18.71 ± 2.66 , C57BL/10+G-CSF -9.50 ± 3.70 ; sciatic nerve index, $p < 0.05$). Neurofilament and p75NTR immunolabeling increased in both strains after nerve crush and G-CSF treatment. Histological analysis showed an increased percentage of normal fibers (23%) in soleus muscles from MDX mice treated with G-CSF. Also, a decreased percentage of regenerated muscle fibers (24%) and a decreased percentage of degenerated fibers (46%) were obtained in MDX mice. The ultrastructural analysis showed an increased total number of axons (21%) in the dystrophic mice after treatment and a decrease of the total number of degenerated axons (8%). Overall, the results herein indicate that G-CSF treatment cannot reverse the decreased motor function in MDX mice. However, this pharmacological approach preserves postural muscle fibers and positively influences the regenerative potential of spinal motoneurons, what may in turn facilitate the reinnervation of regenerated fibers during the course of the disease.

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Investigating the contribution of Neurotrypsin and Agrin in Rett Syndrome's associated synaptic defects.

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Rett syndrome is a neurodevelopmental disorder primarily caused by mutations in the X-linked methyl CpG binding protein 2 (*MECP2*) gene. It is characterized by regressions of acquired skills, particularly in language and motor abilities, development of stereotypic hand movement, and seizures. However, the underlying molecular mechanisms causing the disease's neuronal dysfunction are not fully understood. Recently, we found that MeCP2 represses expression of the *Neurotrypsin* gene, which encodes a serine protease primarily expressed in neurons of the cerebral cortex, hippocampus, and lateral amygdala. Neuronal activity induces the release of neurotrypsin to the synaptic cleft and the neurotrypsin-dependent cleavage of the heparan sulfate proteoglycan agrin. Deficiency or excess in neurotrypsin-dependent cleavage of agrin leads to mental retardation and important synaptic defects, suggesting that appropriate levels of neurotrypsin and/or agrin are key for proper synaptic function. Therefore, our findings linking MeCP2 to the regulation of the neurotrypsin/agrin system suggest that alterations in neurotrypsin/agrin expression and function might contribute to the synaptic defects associated with Rett syndrome. Recently, synaptic defects associated with Rett syndrome have been recapitulated in Rett syndrome induced pluripotent stem cell (iPSC)-derived neurons, allowing us to study the underlying molecular mechanisms. Thus, we have determined the levels of *Neurotrypsin* and *Agrin* expression in fibroblasts and neuronal precursor cells (NPCs) derived from controls and Rett syndrome patients carrying the nonsense *MECP2* mutation Q83X (Q83X) or a *MECP2* duplication. qPCR analysis revealed increased *Neurotrypsin/Agrin* expression in Q83X fibroblasts and NPCs while no significant changes were observed in *MECP2* duplication cells, suggesting that a lack of MeCP2 has a larger effect over *Neurotrypsin/Agrin* expression than an excess of MeCP2 in this system. As a complementary approach, we analyzed *Neurotrypsin/Agrin* expression changes in different brain regions of *Mecp2*-deficient mice, a mouse model for Rett syndrome. We observed *Neurotrypsin* up-regulation in cortex and in hippocampus, but not in cerebellum, suggesting that *Neurotrypsin* expression regulation by MeCP2 might be brain region specific. These results suggest that MeCP2 loss of function leading to Rett syndrome alter *Neurotrypsin* and/or *Agrin* expression. Given that the appropriate levels of neurotrypsin-dependent cleavage of agrin are key for normal synaptic function, our findings suggest that an altered neurotrypsin/agrin system might contribute to the synaptic defects observed in Rett syndrome models.

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Unknown protein and GAP-43 expression in the thalamus in post-mortem schizophrenic brains.

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The symptom complex we term schizophrenia has been known for at least 3500 years, (Lehmann, 1967). Yet the circumstances that are believed to be the etiology of this disorder still remain a conundrum. Numerous studies have found cortical and subcortical abnormalities,

while some studies have suggested structural damage to the thalamus may play an important role in schizophrenia. Several of these studies have suggested that thalamic volume reduction characterizes patients with schizophrenia. Other studies suggest a lower metabolic rate in the thalamus of schizophrenic patients. Previous studies from our lab have shown that there is an increase level of dopamine in the thalamus of schizophrenic patients when compared to controls (Oke, Carver, & Adams, 1993). Our research also shows that the possibility of the failure to convert dopamine into norepinephrine is not related to the increased levels of dopamine. The theory proposed in this study is that the increased concentration of dopamine in the thalamus of individuals with schizophrenia could be due to synaptic disturbance and or an abnormal distribution of growth protein in the thalamus that could possibly stimulate aberrant growth of dopaminergic neurons from the dopamine enriched areas surrounding the thalamus. To test the hypothesis that synaptic disturbances are involved in the pathogenesis of schizophrenia, we looked at the levels of Growth-Associated Protein-43 (GAP-43), a neuro-specific phosphoprotein localized in the presynaptic terminals that is associated with the initial establishment, regeneration, and functional modulation of synaptic relationships. Western blots using both polyclonal and monoclonal antibodies were done to detect the levels of GAP-43 protein in post-mortem thalamic brain tissue of schizophrenic patients and controls. This data was controlled for confounding factors such as age, post-mortem time delay, and antipsychotic medication. Negative controls were also run with each blot. Quantitative analysis showed a significantly decreased level of GAP-43 protein expression in the thalamic brain tissue of schizophrenic compare to control. Furthermore it also showed an unknown protein of a molecular weight of approximately 100kDa in the thalamic tissue of schizophrenic patients, but not in the controls. Our data may indicate impaired synaptic plasticity in the thalamus of the schizophrenic brain. Our data also demonstrate an unknown protein that could be precursor/splice variant of GAP-43 or an aberrant form playing an important role along with reduced level of GAP-43 expression in schizophrenia. Further studies are needed to identify the unknown protein and learn if there is any connection with the increased levels of dopamine in the thalamus of schizophrenic patients.

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G protein signaling tunes R7BP activity by regulating its palmitoylation turnover.

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Signaling molecules in the nervous system are abundantly lipidated, including G protein coupled receptors (GPCR), channels, signal transducers and their regulators. Protein palmitoylation is the only reversible lipid modification. Cycles of palmitoylation-depalmitoylation alter protein localization and activity, which are critical for diverse cell functions. We have discovered that palmitoylation cycling occurs on an important neuronal protein called R7BP (R7-family binding protein). In neurons, R7BP forms obligatory complexes with R7 family of G protein regulators. The R7BP serves as membrane anchor and allosteric regulator of the R7 family of G protein signaling regulators. In cultured cells, palmitoylated R7BP is membrane localized, while depalmitoylated R7BP mutants accumulates in the nucleus. However, the molecular mechanism that regulates the palmitoylation and localization of the R7BP associated complexes are not known. Using a combination of biochemistry, cell imaging and cell signaling approaches, we identified distinct roles of palmitoylation and depalmitoylation processes in R7BP intracellular trafficking and GPCR signaling regulation. We identified protein acyl transferase DHHC2 to mediate de novo and turnover palmitoylation of R7BP. DHHC2 silencing redistributes R7BP from the plasma membrane to the nucleus. Furthermore, we found that R7BP depalmitoylation can be inhibited by Go signaling in cultured cells. Inhibition of depalmitoylation also significantly

alters R7BP membrane localization. These results indicate that the R7BP palmitoylation cycling machinery is adaptive to cellular signaling activity. Given the critical roles of R7 complexes in neuronal signal transduction regulation, our studies indicated the importance of palmitoylation cycling on regulated protein trafficking in signaling processes important for neuronal development and function.

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Major histocompatibility complex of class I (MHC I) gene silencing increases stability of synapses and reduces astrocyte pro-inflammatory response in vitro.

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The establishment of neuronal networks is a complex process that is still not well understood. Stability of synapses depends on different factors including the degree of reactive gliosis within the CNS microenvironment. The MHC I expression by neurons and glial cells and its correlation with synaptic plasticity revealed a molecular mechanism of interaction between such cells. Nevertheless, the correlation between MHC I expression and the degree of astrogliosis may be a key factor for the stability of newly formed synapses. Based on this fact, the present study aimed to analyze, in spinal neurons and astrocyte co-cultures, the influence of GFAP and MHC I gene silencing on the expression of major interleukins as well as on the synaptic marker synaptophysin. In this way, astrocyte primary cell cultures were obtained from newborn C57BL/6J mice cerebral cortices and transfected with siRNA for beta 2 microglobulin (B2M) using magnetic nanobeads. Also, MHC I upregulation was achieved by treating the cultures with interferon 1 beta (IFN1B). The levels of mRNA for GFAP, beta-2 microglobulin, BDNF, GDNF, IL-1, IL-6, TNF-alpha, IL-12 and IL-17 were analyzed by quantitative RT-PCR. For immunocytochemistry, the cultures were fixed and incubated with GFAP, OX18 (MHC I), Synaptophysin, NeuN and neurofilament primary antisera followed by Cy3-conjugated secondary antibodies. The results showed that B2M silencing lead to 50% reduction of MHC I and GFAP expression, indicating a reduction of astroglial reaction. In line with this, B2M silencing resulted in significant reduction of pro-inflammatory cytokines, namely IL-1, IL-6, TNF-alpha, IL-12 and IL-17 ($p < 0.05$). Also, the levels of neurotrophins were significantly downregulated. Interleukin gene expression was partially reversed ($p < 0.05$) and BDNF and GDNF mRNA levels were totally recovered after IFN1B treatment. Contrarily, IFNB treated control cultures presented decreased mRNA levels for all parameters, except for IL-12 and IL-17. The immunocytochemistry results were in line with the RT-PCR data and indicated a decreased astroglial reaction, coupled with decreased dendrite elongation and increased synaptic formation in the absence of MHC I. Overall the present data indicate that MHC I downregulation decreases astrogliosis as well as the transcript levels of pro-inflammatory cytokines. This may, in turn, contribute to the establishment of more complex neural circuits and increased number of synapses.

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Downregulation of the major histocompatibility complex of class I (MHC I) by COUP-TF II increases the synaptic circuits density and the size of dendrites in PC12 cells.

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The molecular mechanisms behind the establishment of complex neural circuits are still not well understood. It is already known, however, that the expression of MHC I by neurons and glial cells, influence the stability of newly formed synapses. In this sense, the identification of

molecules able to regulate MHC I transcription may lead to new therapeutic approaches following CNS lesion and disease. The present study aimed at studying the regulation of MHC I expression by COUP-TF II in PC-12 cells, that constitute a neuron-like cell line in the presence of nerve growth factor (NGF). For this purpose, PC-12 cells were transduced with viral vectors containing plasmids designed either to silence (sh-COUP-TF II) or overexpress COUP-TF II (rescue-COUP-TF II). The transfection effectiveness was obtained by Western-blotting and flow-cytometry and cell selection was achieved by treatment with hygromycin. All cell lines were cultured in 24 well plates with an initial density of 5×10^3 cells. In order to induce neuron-like differentiation, the cultures were treated with NGF, in a dose of 250 micrograms/ml. After 5 and 10 days of culturing, the cells were fixed in phosphate buffered 4% paraformaldehyde. Immunocytochemistry with primary antisera against synaptophysin and MHC I (OX-18) were used to evaluate the size of dendrites, synaptic boutons density as well as the levels of MHC I protein immunoreactivity. The results revealed that COUP-TF II silencing results in approximately 50% increased levels of MHC I coupled with significant reduction of dendrite branching and elongation. In this way, the size of dendrites did not reach more than 25 micrometers, so that the occurrence of long dendrites was absent. On the contrary, COUP-TF II overexpression results in downregulation (~30%) of MHC I and increased number of long dendrites, resulting in significantly more complex neural circuits. Long sized dendrites reached more than 250 micrometers, after 10 days of culturing. Overall, the present results indicate a connection between COUP-TF II activation and the downregulation of MHC I by neuronal cells. This finding may in turn lead to the better understanding of the mechanisms behind synaptic plasticity in the CNS, allowing future strategies to treat neural tissue damage and to reduce the pace of neurodegenerative diseases.

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***Fmr1* transcripts with shifted translational reading frame in the embryonic rat telencephalon.**

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Fragile mental retardation protein (FMRP), encoded by the Fragile Mental Retardation 1 (*FMR1*) gene, is an important mRNA translation regulator in the central nervous system (CNS), notably in the cerebral cortex, hippocampus and cerebellum. Alternative exclusion of *Fmr1* exon 14 may be combined to the choice among three splicing acceptor sites in the exon 15, shifting the reading frame and eliminating FMRP nuclear export signal and RGG-box, a domain that binds and regulates several mRNA species. Translation stop codons are prematurely found in *Fmr1* messages where exon 13 is directly ligated to exon 15, except if the third splicing site is used. It is currently unknown if those alternative splicing forms of *Fmr1* are functionally relevant. Therefore, we present here a developmental expression analysis of *Fmr1* exon 14 in the rat CNS, based on RT followed by qPCR.

We first studied the period around birth, E19 and P2, when *Fmr1* brain expression is known to increase. In the hippocampus, both *Fmr1* and specific exon 14 transcripts increased from E19 to P2. A ratio between specific exon 14 over total *Fmr1* transcripts was defined as an inclusion rate (IR) of that exon. In both ages, nearly all hippocampal *Fmr1* transcripts contained the exon 14, indicating an IR of about 100%. In the cerebellum, the exon 14 IR decreased between E19 and P2 from 84 to 66%. Conversely, we found an increased exon 14 IR from 56% (E19) to ~100% (P2) in the cerebral cortex, which was not due to an elevation on *Fmr1* total transcripts between time points. On E14, 20% to 45% of *Fmr1* transcripts lacked exon 14 in the telencephalic vesicle. Therefore, E14 telencephalic vesicle, E19 cerebral cortex and P2 cerebellum may indicate developmental time points, and CNS structures, where *Fmr1* exon 14

exclusion is relevant. Immunoprecipitation of the eukaryotic translation initiation factor 4E (eIF4E) was used to evaluate if the nonsense-mediated mRNA decay (NMD) system could remove *Fmr1* messages harboring premature stop codons in the telencephalic vesicle. Co-immunoprecipitated mRNA were reversely transcribed and analyzed by RT-PCR with a forward primer plus any of the three chimeric primers tagging *Fmr1* exons 13 and 15. Our preliminary results indicate the stability of *Fmr1* messages lacking exon 14 and using the third splicing site from exon 15 in the rat E14 telencephalic vesicle. Ongoing experiments are likely to provide a relative quantification of those results.

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Characterization of the aggregation the poly-glutamine domains of presynaptic proteins Piccolo and Bassoon: Potential determinants of the active zone assembly.

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Piccolo and Bassoon are two related multidomain presynaptic proteins suggested to play a crucial role in the formation of the active zone. The specific functions of these molecules at the synapse are poorly understood as well as their mechanism of synaptic recruitment. We have identified domains of poly-glutamine (PQ) in Piccolo (PQ 1, 2 and 3) and Bassoon (PQ 1 and 2). Some of these domains are similar to PQ domain of the CPEB dendritic protein of *Aplysia californica*, which has been shown to have prion-like behavior and aggregation properties suggested to be important for its function. Here, we evaluate the ability of aggregation and distribution of Piccolo and Bassoon PQ domains in different heterologous cell lines (HEK, COS-7) and their distribution in primary cultures of hippocampal neurons. Heterologous cells showed different levels of aggregation in the cytoplasm as well as nuclear localization. In neurons, PQ domains showed a similar localization with a juxtannuclear aggregation, in addition to clusters found at growth cones. The aggregation properties of these domains suggest that these motifs could be important for their clustering to active zones.

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Establishment and Maintenance of Polarity I

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Loss of Ciliary Polycystin-2 in Induced Pluripotent Stem Cells from Polycystic Kidney Disease Patients.

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We investigated the potential of induced pluripotent stem (iPS) cells to model polycystic kidney disease (PKD), a very common, multi-organ ciliopathy in which epithelial cysts and fibrosis replace the normal kidney architecture. Fibroblasts with characterized mutations from three autosomal dominant (ADPKD) and two autosomal recessive (ARPKD) patients were reprogrammed into iPS cells. Undifferentiated PKD iPS cells and healthy controls exhibited similar rates of proliferation, apoptosis, and ciliogenesis, and expressed the PKD disease genes polycystin (PC)-1 and -2. However, PC2 localization to the primary cilium was reduced ~75 % in ADPKD iPS cells, compared to ARPKD and control lines. A single PKD1 mutation was observed in ADPKD fibroblasts and iPS cells, suggesting that reduced ciliary PC2 occurred without

complete PC1 loss. Somatic epithelial cells and hepatoblast-biliary precursors differentiated from ADPKD iPS cells had ~50 % fewer cilia with detectable PC2, compared to controls. Reduced ciliary PC2 in PKD iPS cells may be a useful metric to better understand ADPKD pathophysiology and guide therapeutics.

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Planar Cell Polarity Orients Airway Cilia.

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Propulsion of contaminants out of the lungs by coordinated action of motile cilia is essential for airway function. Multiciliated airway epithelial cells undergoing ciliogenesis generate hundreds of basal bodies that traffic to the surface, elongate motile axonemes and align with the tissue axis for coordinated motility. The mechanisms of cilium polarization remain poorly explored. We showed that a conserved set of planar cell polarity (PCP) proteins polarize airway epithelial cilia: PCP proteins localize asymmetrically to the cell cortex at the level of the apical junctions, revealing that PCP signaling starts before ciliogenesis; molecular and morphological polarity are disrupted in PCP mutant epithelia; and PCP mutants display defective airway clearance, highlighting the requirement for ciliary alignment. Studying the cytoskeleton, a target of PCP signaling, we uncovered two temporally-distinct, cell type-specific, planar polarized microtubule (MT) networks. Prior to ciliogenesis, MTs are necessary in all cells for asymmetric PCP protein localization. Subsequently, after cilia appear, MTs extend from the polarized appendages of basal bodies toward the apical junctions present within an asymmetric PCP protein domain. These MTs are PCP-regulated, ciliated cell-specific, interact with the cortex via their plus ends, and are required for basal body distribution and alignment. Our data suggest that MTs have two separable PCP functions: MTs are first required in every cell for trafficking PCP proteins to the cortex, and later they interact with and align cilia. Our studies reveal strong conservation of PCP mechanisms initially described in *Drosophila*, and cilium alignment by PCP-oriented MTs provides the first mechanistic description of how asymmetric PCP proteins at the cortex orient cilia along the cell surface.

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MCC is a PCM/centrosome component that coordinates cilia formation.

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Mutated in colorectal cancer, MCC, is a putative tumor suppressor gene found somatically mutated in familial polyposis coli and Gardner's syndrome. Previous studies have identified MCC as molecular scaffold involved in numerous cellular processes ranging from cell cycle, DNA damage response, cell proliferation, cell migration to cell polarity. However, the function and mechanism of MCC as a scaffold remains largely unknown. Using mass spectrometry we identified novel protein interaction partners of MCC including centrosomal proteins CEP170, AZI1 (CEP131), NDE1 (NudE) and components of the pericentriolar material (PCM). Here we uncovered a new function of MCC in cilia formation as demonstrated by depletion of MCC by siRNA knockdown which result in a defect in proper cilia formation. MCC functions as a scaffold to recruit multiple GTPase regulators such as RasaL2, whereby knockdown of MCC disrupts localization of RasaL2 to the base of the cilium. We further demonstrate that MCC and RasaL2 function to play an important role in coordinating membrane and cargo trafficking at the ciliary base. Here we propose a model for MCC as a molecular scaffold during ciliogenesis and further suggest a broader role in planar cell polarity.

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Analysis of mouse forebrain lateral wall polarity: multi-scale implication of PCP genes.C. Boutin¹, A. M. Goffinet¹, F. Tissir¹; ¹DENE, UCL, Brussels, Belgium

Ciliated epithelia are essential for development and correct functioning of many organs. In the mouse forebrain, lateral walls of lateral ventricles are lined by multiciliated ependymal cells that participate to correct circulation of cerebro-spinal fluid (CSF). Ependymal cells display different levels of organization and polarization. At the cell scale, each ependymal cell bears a tuft composed of multiple cilia beating in a polarized and coordinated manner. Furthermore, this tuft is always positioned in anterior side of the cell relative to the anatomical axis. At the tissue scale, neighboring ependymal cells coordinate their polarity leading to a precisely polarized tissue that propels CSF unidirectionally. In the present study, we investigated the implication of planar cell polarity (PCP) genes in regulation of lateral wall organization. We combined genetic approach and confocal microscopy to analyze in vivo the consequences of PCP genes deletion on cell and tissue polarity. In *Celsr1* mutant mice, we found that while intra-cellular organization was preserved, the coordination of tissue polarity was lost. In contrast, *Vangl2* deficient lateral walls showed defect both at the cell and tissue scales. Both phenotypes have drastic consequences on the tissue function since mutants mice displayed impaired flow at the ventricular surface and developed hydrocephalus. Our results demonstrate that in ventricular walls establishment of tissue and cell polarities are distinct process sharing common regulators.

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E-cadherin is recruited to the apical membrane by Enteropathogenic *E.coli*.G. A. Pedersen¹, C. Toft¹, M. Amieva², L. N. Nejsum¹; ¹Department of Molecular Biology & Genetics, Aarhus University, Aarhus C, Denmark, ²Dept of Pediatrics and Microbiology & Immunology, Stanford University, Stanford, CA

E-cadherin is the major component of adherens junctions that connect neighboring cells in polarized epithelia. Adherens junctions are dynamic structures that are subject to regulation by controlling E-cadherin stability in the membrane. E-cadherin interacts with catenins that are involved in downstream signaling pathways that affect cell mobility and proliferation. We studied regulation of adherens junctions in epithelial cells infected with Enteropathogenic *E.coli* (EPEC), a foodborne pathogen that colonizes the epithelium of the small intestine. EPEC attaches to the apical side of epithelial cells where it injects several bacterial effector proteins that manipulate host cellular signalling pathways. EPEC effectors are known to disrupt tight junctions making the epithelium permeable and presumably allowing EPEC access to nutrients through paracellular diffusion.

We investigated whether EPEC infection of polarized epithelial cells also modifies the adherens junctions. We found that E-cadherin was dispersed from the adherens junctions in the infected cells and recruited to the infection site on the apical membrane where it colocalized with EPEC. The recruitment of E-cadherin proved to be specific since other members of the cadherin superfamily (N-cadherin, desmosomal cadherins, and protocadherins) were not recruited. Preliminary data indicate that E-cadherin is recruited by redirecting endosomal trafficking from the existing adherens junctions.

We also investigated the localization of catenins during EPEC infection. We saw that β -catenin dissociated from E-cadherin in the infected cells but was not recruited to the EPEC attachment site. In contrast, p120-catenin was found at the EPEC attachment site and was also observed in the nucleus of the infected cells suggesting activation of the p120-catenin signaling pathway. The role of E-cadherin recruitment for bacterial survival is unknown, however, we propose that

E-cadherin signaling takes place locally at the infection site through activation of the p120-catenin signaling pathway.

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Robust polarity establishment via an endocytosis-based cortical corraling mechanism.

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Formation of a stable polarity axis underlies many essential biological processes. Here, using high-resolution imaging and complementary mathematical modeling we find that cell polarity can be established via the spatial coordination of opposing membrane trafficking activities: endocytosis and exocytosis. During polarity establishment in budding yeast, these antagonistic processes become apposed. Endocytic vesicles corral a central exocytic zone, tightening it to a vertex that establishes the polarity axis for the ensuing cell cycle. Concomitantly, the endocytic system reaches an equilibrium where internalization events occur at a constant frequency. Mutants in which endocytosis was depolarized failed to initiate internalization events with normal periodicity within the corral, resulting in wide, unstable polarity axes. These results, predicted by novel *in silico* modeling and verified by high resolution *in vivo* studies, identify a requirement for endocytic corraling during robust polarity establishment.

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Control of Epithelial Polarization and 3D Morphogenesis by the APC Tumor Suppressor.

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Adenomatous polyposis coli (APC) is a multifunctional tumor suppressor that is frequently mutated or lost in most epithelial-derived tumor types, including colorectal, breast and renal cancer; however, the mechanisms by which APC suppresses tumorigenesis are incompletely understood. We have previously demonstrated that germline heterozygous *Apc* mutation disrupts mammary epithelial architecture and that this effect was likely to represent a Wnt-independent activity of APC. The objectives of the present study were to extend these observations to *in vitro* models and begin to identify the molecular mechanism by which APC regulates epithelial polarization and morphogenesis. Using lentiviral-mediated RNA interference, APC was stably suppressed in MDCK kidney and HC11 mammary epithelial cells. Although the APC-knockdown cells did not show any significant morphological or growth differences compared to control vector-only or scrambled shRNA-infected cells on plastic, they displayed a dramatic multi-layering phenotype when cultured on Transwell permeable supports. The polarized localization of basolateral markers, such as Dlg and Scrib tumor suppressor proteins, and apical proteins, including MUC1 and gp135, was severely perturbed in APC-knockdown cells. When these cells were cultured under three-dimensional (3D) conditions, APC-knockdown epithelial cells formed enlarged, non-spherical cysts or acini with filled-in lumens, many of which were invasive into the surrounding matrix, as compared to controls. Remarkably, inverted polarity was observed in the MDCK APC-knockdown cells such that gp135 localized to the periphery of the cysts. All of the polarization and morphogenesis phenotypes associated with APC-knockdown were specific since they were rescued by stable introduction of full-length APC, but, similar to our previous *in vivo* studies, the epithelial architecture defects associated with APC loss did not involve hyperactivation of the Wnt signaling pathway. APC depletion or mutation in colonic epithelial cells also resulted in 3D morphogenesis defects, suggesting that the control of epithelial architecture may be a major tumor suppressive activity of APC in the colon. Collectively, these data indicate that APC is a

potent regulator of epithelial polarization and morphogenesis and predict that one mechanism by which APC loss promotes tumorigenesis is by disrupting epithelial architecture to subsequently drive tumor cell growth, survival and invasion.

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Molecular control of apical polarization during endothelial lumen formation in 3D collagen matrices: Role for tubulin acetylation, Rac1-, Cdc42-, and Src-dependent signaling.

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Our laboratory has focused for many years on elucidating the molecular basis for endothelial cell lumen formation in 3D extracellular matrix environments. We have elucidated a key role for Cdc42 and Rac1 which act in conjunction with the p21-activated kinases (Pak)-2, and Pak-4 as well as protein kinase C epsilon and Src family kinases during these events. Here, we show that the microtubule cytoskeleton including post-translationally modified tubulins such as acetylated tubulin are distributed in a sub-apical domain which is highly polarized while F-actin is strongly distributed in a basal fashion. This distribution depends on the microtubule plus-end regulatory proteins, EB1, p150Glued and Clasp1, which control EC lumen formation as well as this asymmetric apical-basal cytoskeletal polarity which appears to be a fundamental requirement for the EC lumen formation process. We further expressed GFP-Rac1 wild-type or its V12 constitutively active form and also GFP-Cdc42 during lumen formation and observe a strong apical polarization of targeting particularly with Rac1V12, but also with wild-type Rac1 and Cdc42. This expression leads to increased tubulin acetylation in a polarized subapical location which appears to support or facilitate targeting of vesicles with apical membranes during this process. Blockade of Rac1 and Cdc42 interferes with tubulin acetylation which corresponds to inhibition of lumen formation. Finally, we also identify very strong apical targeting of phosphorylated Src which similarly localizes to the apical membrane as well as to vacuoles and vesicles which target this tubulin-rich region to fuse with the developing apical membrane surface to control EC lumen formation in 3D matrices.

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FIP5 Phosphorylation by GSK3 β Regulates its Interaction with SNX18 and Kinesin-2 and is Required for Epithelial Lumen Formation In Vitro and In Vivo.

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Proper sorting and targeting of endocytosed proteins is necessary for the maintenance of epithelial cell polarity and apical lumen formation. A leading model of epithelial lumen morphogenesis suggests that to form the apical lumen, apical proteins must be transcytosed via Rab11-containing recycling endosomes and fuse at the lumen formation site, also known as apical membrane initiation site (AMIS). While the importance of Rab11-endosomes in the formation of the apical lumen is now well established, we know little about the machinery that regulates formation, transport and targeting of these Rab11-endosomes during lumen formation. FIP5/Rip11, a Rab11 effector protein, is known to be involved in apical-directed transport in polarized cells and is thought to act as a scaffolding protein that recruits and binds effectors of endocytosis. It was recently demonstrated that FIP5/Rip11 is required for the formation and targeting of Rab11-endosomes to AMIS. Additionally, it was shown that FIP5/Rip11 functions by binding to Sorting Nexin 18 (SNX18) and kinesin-2 to mediate formation/scission and transport of endocytic carriers. In this study we demonstrate that during apical lumen formation

FIP5/Rip11 is phosphorylated by GSK3 β , and that this phosphorylation regulates FIP5/Rip11 binding to SNX18 and kinesin-2 and is required for apical lumen formation in vitro.

The following model of lumen formation in epithelial cells is largely derived from studies conducted in 3D tissue culture systems. It remains unclear whether the same molecular machinery is used during epithelial tissue morphogenesis in vivo. To test whether FIP5-dependent endocytic transport also mediates lumen formation in vivo, we used zebrafish intestine development model. Here we show that depletion of FIP5 inhibits the proper formation of zebrafish intestinal lumen and also leads to ectopic formation of microvilli-containing apical membranes. In summary, this study finds that FIP5 mediates the formation of the epithelial lumen via sequential interaction with SNX18 and kinesin-2, and the function of FIP5 during lumen initiation and expansion is regulated by GSK3 β phosphorylation.

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Regulation of epithelial apical polarity orientation by phosphorylation of the Podocalyxin complex.

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Formation of epithelial lumens requires the spatiotemporal co-ordination of apico-basal polarization in cells contributing to the lumen. In addition, a fundamental requirement for organogenesis is that the orientation of apical polarity is correctly formed away from the axis provided by the extracellular matrix (ECM). We previously demonstrated that this requires sensing of the ECM by beta1-integrins, regulating activation of a Rac1-dependent, RhoA-antagonized orientation pathway. Moreover, we demonstrated that correct polarity orientation involves endocytosis and transcytosis of the apical glycoprotein, podocalyxin (Podxl), initially from the ECM-abutting surface to the nascent lumen. This is dependent on a Rab11-Rab8 GTPase cascade, controlling such transcytosis, and resulting in formation of the Cdc42-Par3-aPKC polarity complex upon delivery of Podxl to the lumen. We here demonstrate that rather than being a passive apical cargo, podxl itself controls the orientation of apical polarity in three-dimensional MDCK cyst cultures. Podxl associates with the NHERF1/2 scaffold proteins, as well as Ezrin, and this multimeric complex controls subsequent apical polarity establishment. Dynamic spatiotemporal phosphorylation of the Podxl-NHERF-Ezrin complex controls the assembly-disassembly of the complex to ensure apical identity is excluded from the ECM-abutting surface, and occurs only at the lumen. We thus describe a molecular mechanism for correct formation and orientation of apical polarity by dynamic phosphorylation of the Podxl complex.

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Phosphatidylinositol Synthase regulates the polarized deposition of basement membrane components.

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Epithelial cells are characterized by their polarized architecture that enables them to exert their varied functions in embryonic and adult organisms. Epithelia exhibit a profound apical-basal polarity that is manifested in the cytoplasmic and surface organization of individual cells. Loss of their apical-basal cell polarity is often associated with carcinoma progression and tumor metastasis. The establishment and maintenance of polarity relies on the regulated transport of newly synthesized and recycled proteins to these specific domains. The basement membrane (BM), a specialized sheet of the extracellular matrix contacting the basal side of epithelial tissues, plays a major role in the establishment and maintenance of epithelial cell polarity.

However, little is known about how BM proteins themselves achieve a polarized distribution. An attractive model system for the study of epithelial structure and morphogenesis is the development of the follicular epithelium (FE), which envelops the germline during *Drosophila* oogenesis. To unravel the molecular mechanism regulating the polarized deposition of BM, we have previously performed a genetic screen in which we identified *Crag*, a DENN domain containing protein, as a regulator of polarized BM secretion (1). We recently isolated a new gene involved in this process, *pis*, encoding Phosphatidylinositol synthase, which plays a critical role in phosphatidylinositol 4,5-bisphosphate (PIP2) regeneration after its hydrolysis into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) by Phospholipase C (PLC). PIP2 regulates many cellular functions, such as intracellular trafficking and membrane and ion transport. Significantly, in follicular cells mutant for *pis*, we detect a strong accumulation of BM components apically. This defect is not generally observed in mutants affecting epithelial polarity. In *pis* mutant cells, BM components are secreted at the apical side of the epithelium leading to the formation of an "apical" BM. However, apical, junctional and basolateral polarity is not affected. Altogether, our data indicate a specific role for *pis* in the organization of epithelial architecture by regulating the polarized deposition of BM components.

(1) Deneff et al., 2008.

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The clathrin adaptor AP-1B generates an alternative recycling pathway for Transferrin Receptor in polarized and non polarized cells.

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In non-epithelial cells, such as CHO, the small GTPase rab11a mediates recycling of fast endocytic receptors from juxtacentriolar perinuclear endosomes. In contrast, in polarized epithelial cells (MDCK) many of these receptors utilize the clathrin adaptor AP-1B to recycle basolaterally from juxtacentriolar common recycling endosomes, whereas rab11a localizes to apical recycling endosomes clustered around the subapical centrosome. Here, we show that knock-down of AP-1B promotes re-routing of transferrin receptor (TfR), an AP-1B cargo, from its regular basolateral recycling route into a transcytotic route to the apical membrane that involves apical recycling and apical sorting endosomes. Trafficking of TfR along this route requires microtubules, the plus-end microtubule motor KIF16b, glycan signals and rab11a. Additional experiments show that AP-1B also mediates rab11a-independent recycling of TfR in subconfluent MDCK and CHO cells. Our experiments demonstrate a transcytotic route likely to be key in the organization and function of AP-1B deficient epithelia and identify a microtubule motor involved in transcytosis.

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Par6b is Required For Both the Membrane E-cadherin expression and the Apicobasal Polarity of *Xenopus* Ectoderm Cells.

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Epithelia is a sheet of highly polarized cells connected by Adherens Junctions(AJs). Loss of apicobasal polarity and AJs is an early hallmark of tumor progression. Par6b (partitioning defective 6 beta) is expressed in *Xenopus* ectoderm. Here we show that Par6b is required for

cadherins expressed by ectoderm cells. Depletion of Par6b in the non-neural ectoderm (the presumptive epidermis) causes a loss of membrane E-cadherin and skin cells dissociation at tailbud stage. Depletion of Par6b does not change the mRNA level but the protein level of E-cadherin, which indicates that this regulation is post-transcriptional. Further experiments suggest that Par6b depletion reduces the translational efficiency of E-cadherin. The changes of the apical markers Crumbs3 and aPKC caused by Par6b depletion suggest that Par6b controls the apicobasal polarity of deep ectoderm cells and outmost ectoderm cells differently. Depletion of Par6b reduces the protein level of the basolateral marker Lgl2, which may link to the loss of basolaterally expressed E-cadherin. In addition, tight junctions that structurally define the apical-basolateral border are also reduced by depletion of Par6b.

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Lipid and protein interactions coordinate aPKC polarization and activation during asymmetric cell division.

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The Par complex protein atypical Protein Kinase C (aPKC) polarizes cells through a cortical exclusion mechanism in which substrates are removed from the cortex by phosphorylation. However, aPKC is not catalytically active until it undergoes a dramatic conformational change in which an inhibitory “pseudosubstrate” is displaced from the kinase domain. While correct targeting and activation of aPKC determine Par-mediated polarity, little is known about the coordination of these processes. Using *Drosophila* neuroblasts as a model system we found that two pathways cooperate to ensure aPKC polarity and activity are properly regulated. We find that interactions with the Par complex protein Par-6, along with a newly identified lipid interaction, are both required for aPKC polarization. Although previously thought to completely specify aPKC polarity, the interaction with Par-6 fails to target aPKC to the cortex. However, interaction with membrane phospholipids leads to cortical but symmetric aPKC. Neither interaction on its own is sufficient to activate aPKC. Taken together, these data suggest that aPKC requires at least two separate interactions to become polarized: a general cortical targeting mechanism through phospholipid binding and an apical targeting mechanism mediated by PAR complex interactions. Finally, we show that proper control of these two signals is required to prevent neuroblast overproliferation. We hypothesize that a conformational change induced by these interactions activates aPKC’s catalytic activity, thereby coupling polarization and activation.

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Guanylate kinase domains of the MAGUK family scaffold proteins as specific phospho-protein binding modules.

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Membrane-associated guanylate kinases (MAGUKs) are a large family of scaffold proteins that play essential roles in organ developments, cell–cell communications, cell polarity establish and maintenance, and cellular signal transductions. Despite extensive studies over the past two decades, the functions of the signature guanylate kinase domain (GK) of MAGUKs are poorly understood.

Here we show that the GK domain of DLG1 binds to asymmetric cell division regulatory protein LGN in a phosphorylation-dependent manner. The structure of the DLG1 SH3-GK

tandem in complex with a phospho-LGN peptide reveals that the GMP-binding site of GK has evolved into a specific pSer/pThr-binding pocket. Phospho-LGN peptide binds to a hydrophobic cradle and phospho-site of the GK domain. Residues both N- and C-terminal to the pSer are also critical for the specific binding of the phospho-LGN peptide to GK.

We further demonstrate that the previously reported GK domain-mediated interactions of DLGs with other targets, such as GKAP/DLGAP1/SAPAP1 and SPAR, are also phosphorylation dependent. Finally, we provide evidence that other MAGUK GKs also function as phospho-peptide-binding modules which indicates that MAGUK scaffold mediated signalling complex organizations are dynamically regulated.

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PAR proteins regulate the localization of LET-99 during asymmetric division.

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Mitotic spindle positioning is essential for asymmetric divisions, where the spindle must be aligned with the axis of cell polarity. In many systems, PAR polarity proteins establish polarization of the cell and regulate spindle movements via a complex including Gα, GPR and LIN-5. We previously showed that LET-99 is a key regulator of GPR asymmetry in *Caenorhabditis elegans* one-cell embryos. LET-99, a DEPDC1 family protein, is asymmetrically localized at the cortex in a lateral-posterior band pattern, where it inhibits GPR localization. Analysis of LET-99 localization in mutant backgrounds showed that PAR-3 inhibits cortical LET-99 at the anterior cortex, while a gradient of PAR-1 inhibits LET-99 at the posterior-most cortex. In addition, PAR-1, a Ser/Thr kinase, was found to associate with LET-99 *in vitro*. To gain further insight in the mechanism of LET-99 localization, we tested LET-99 for association with the *C. elegans* 14-3-3 protein, PAR-5. In other systems, phosphorylation of targets by PAR-1 and the PAR-3 associated kinase PKC-3 generates binding sites for 14-3-3 proteins, which alters the targets' localization. We found that PAR-5 bound to His-LET-99 in wild-type embryo extracts, but PAR-5 binding was greatly diminished in extracts from *par-1*(RNAi) embryos. Computer predictions for 14-3-3 binding sites followed by yeast-two hybrid (Y2H) assays identified two LET-99 serine residues essential for PAR-5 binding. To determine the *in vivo* relevance of these sites, we introduced S-to-A mutations into an otherwise full-length rescuing LET-99 transgene (LET-99-AA). When transferred into a *let-99* deletion mutant background, the transgene-encoded LET-99-AA protein mislocalized to the entire posterior cortex of the one-cell embryo, similar to what was observed for LET-99 in *par-1* mutant embryos. These and other results support the model that PAR-5 binds to LET-99 to prevent association with the posterior-most cortex, and that this interaction is regulated by phosphorylation of LET-99 by PAR-1. To begin to determine how LET-99 localization is restricted from the anterior cortex, we analyzed LET-99 after depletion of anterior PAR components. We found that PAR-3 is not sufficient for normal LET-99 localization, but rather the PAR-3 associating proteins, PAR-6 and PKC-3, restrict LET-99 localization from the anterior. In the future, we will test the hypothesis that PAR-1 and PKC-3 directly phosphorylate LET-99 using *in vitro* kinase assays. Additionally, we are testing LET-99 interacting proteins for a role in anchoring LET-99 at the cortex.

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Dynamic polarization during asymmetric cell division by cell cycle control of the Inscuteable-Bazooka interaction.

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Asymmetric cell division is a highly dynamic process in which protein localization patterns dramatically change during the cell cycle to control daughter cell fate. During interphase, fate determinants are symmetrically distributed, but become highly polarized during mitosis so that they can be segregated by the cleavage furrow. For example, in *Drosophila* neuroblasts the Par complex protein Bazooka (Baz aka Par-3) is cytoplasmic during interphase, but is targeted to the apical cortex at metaphase. Although polarization dynamics are likely to be important for regulating fate determinant activity, little is known about the temporal aspects of this process. Using high-speed time-lapse imaging of larval brain neuroblasts we identified Inscuteable (Insc) as a key polarity landmark as it is restricted to the apical cortex throughout the cell cycle. Although Baz is known to bind directly to Insc, it is not recruited to Insc until prophase. Analysis of the primary sequences of Insc and Baz reveal several putative phosphorylation sites for mitotic kinases and we find that the *in vitro* Insc-Baz interaction is phosphatase sensitive. We hypothesize that the Insc-Baz interaction is regulated by a mitotic kinase, allowing for dynamic control of Par complex polarization. Thus, the Insc-Baz interaction is a key “regulatory node” that specifies neuroblast polarization dynamics and may link polarity to the cell cycle.

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Par1b orchestrates cell polarity with symmetric or asymmetric cell divisions in columnar and hepatic epithelial cells.

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Columnar epithelia establish their luminal domains at the cell apex parallel to the basal surface while hepatocyte lumina interrupt the lateral domain of neighboring cells perpendicular to two basal domains. The serine/threonine kinase Par1b promotes a switch from columnar to hepatic polarity in kidney-derived MDCK cells. Here we report that by concomitantly organizing the localization of a sub-luminal belt of the mitotic spindle anchoring complex Gai/NuMA/LGN/dynein Par1b regulates lumen polarity and mitotic spindle orientation in hepatic and columnar cells in a concerted manner. Thus, MDCK cell spindles attach both astral microtubule arrays at opposite regions of the lateral cortex parallel to the basal domain, which results in symmetric cell divisions. Par1b overexpressing MDCK cells and hepatic WIFB and HepG2 cells orient one spindle pole toward the lateral domain underlying their lateral lumen and attach the other set of astral microtubules randomly at the opposite cell surface resulting in spindles that are not aligned with the basal domains and that yield asymmetric divisions, which distribute the luminal domain to only one of the daughters. Par1b-inhibition, on the other hand, favors symmetric divisions and columnar polarity in WIFB and HepG2 cells. We determined that Par1b orchestrates both aspects of epithelial polarity by inhibiting cell-extracellular matrix signaling in a RhoA- and IRSp53-dependent manner.

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Mechanisms underlying the regulation of a Rho-family GTPase.

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During the cell cycle, cells undergo dramatic changes in cellular organization, requiring coordination between the cell cycle and proteins regulating cell polarity. Many polarity proteins are conserved; however, the regulation of these proteins during the cell cycle is poorly understood. In the budding yeast *Saccharomyces cerevisiae*, Cyclin Dependent Kinase 1 (Cdk1) directly phosphorylates regulators of Cdc42 during establishment of a polarity axis. Cdk1-dependent phosphorylation of Cdc42 regulators may link the establishment of cell polarity to the cell cycle, but the underlying mechanisms require clarification.

I am characterising the sole Cdc42 GEF, Cdc24. Cdc24 forms a complex with the scaffolding protein Bem1. Cdc42, Cdc24 and Bem1 are thought to function in a positive feedback loop, which may be crucial for maintaining polarised Cdc42. As the cell progresses through the cell cycle, Cdc24 activates Cdc42 at a specific site on the cell cortex, which recruits Bem1 that interacts with Cdc24, which in turn activates more Cdc42 molecules at the same site. This feedback loop may amplify the polarised Cdc42 signal at a specific site on the cortex to generate a robust polarity axis. We are testing how the affinity of Cdc24 and Bem1 may be regulated by Cdk1-dependent phosphorylation using biochemical and biophysical approaches.

In addition to the GEF Cdc24, Cdc42 is also regulated by the GAPs. In yeast, four GAPs have been identified for Cdc42: Bem2, Bem3, Rga1 and Rga2. We have generated strains expressing all four GAPs tagged with GFP from their endogenous promoter to provide a global view of GAP localization during the cell cycle. High-speed simultaneous imaging of the GAPs and active Cdc42 is providing a better understanding of how Cdc42 is activated and confined to a specific site during the polarisation process.

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Effects of Early Disruption in Polarity on Later Development in the Sea Urchin Embryo.

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Establishment and maintenance of cell polarity has become an increasingly interesting biological question in a diversity of cell types and has been found to play a role in variety of biological functions. Previously, it was thought that the sea urchin embryo remained relatively unpolarized until the first asymmetric division at the 16cell stage of development. However, there is mounting evidence to suggest that polarity is established much earlier. We analyzed roles of the cell polarity regulators, the PAR complex proteins, and how their disruption in early development affects later developmental milestones such as blastula and gastrula formation. We found that PAR6 along with aPKC and CDC42 localize to the apical cortex (free surface) as early as the 2-cell stage of development and this localization is retained through the blastula stage. Uniquely, in early cleavage stage embryos PAR1 and PAR6 also appear to colocalize in the apical cortex. PAR6, aPKC, and CDC42 are anchored in the cortex by myosin as disruption of myosin light chain kinase activity with ML-7 and ML-9 resulted in cytoplasmic pooling. Additionally, pulse treatments with ML-7 and ML-9 prevented the embryos from reaching the gastrula stage, while the Rho-kinase inhibitor, H1152, did not have this effect. This same pulse with ML-7 disrupted PAR6 localization at the fertilized egg and maintained this disruption through the first cleavage division. Interestingly, aPKC inhibition early in development prevented

blastula formation, but did not effect micromere formation. These observations suggest that disruptions of the polarity complex in the early embryo can have a significant impact on the ability of the embryo to reach later critical stages in development.

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The Role of the Discs Large Tumour Suppressor in the Regulation of Anoikis.

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The Discs Large (Dlg) tumour suppressor is involved in the regulation cell proliferation and cell polarity and its expression is frequently lost in diverse tumour types. Dlg is a target for a number of different viral oncoproteins, further indicating a role in tumour suppression. In order to more fully understand the effects of loss of Dlg upon cellular homeostasis we have made use of a panel of epithelial cell lines where hDlg expression has been stably ablated. An intriguing characteristic of these cells is an apparent increased resistance to anoikis, but normal levels of susceptibility to other forms of apoptosis induction. Using a series of transient siRNA knock-downs of Dlg expression provides a similar phenotype, indicating that this resistance to anoikis is not an off-target effect of the selection procedure. In order to more fully understand the role of Dlg in anoikis we have analysed the pattern of Dlg expression during anoikis. We obtained a marked redistribution of Dlg within the cells upon anoikis induction, with a significant accumulation within the nuclear fraction. Interestingly, in HPV positive cells where Dlg is targeted by the viral oncoprotein E6, this nuclear translocation of Dlg is not observed. Current studies are focused on determining whether HPV E6 is specifically inhibiting nuclear translocation of Dlg upon anoikis induction. Furthermore, in order to identify the signaling pathways being regulated by Dlg during anoikis, proteomic analyses are underway in order to identify the cellular proteins with which Dlg interacts post-anoikis induction.

Integrins and Cell-ECM Interactions I

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LFA-1 is endogenously inactivated by SHARPIN.

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Regulated activation of integrins is critical for cell adhesion, motility and tissue homeostasis. Talin and Kindlins activate integrins, but the counteracting inhibiting mechanisms are poorly defined. Previously we identified SHARPIN as an important inactivator of β 1-integrins (Rantala, Pouwels et al., Nature Cell Biology 2011). SHARPIN inhibited β 1-integrin activity and functions (affecting cell adhesion and migration) in several human cancer cell lines and primary cells from both human and mice, which was fully rescued by re-expression of SHARPIN. Mechanistically, SHARPIN directly bound to a conserved cytoplasmic region of integrin α -subunits and inhibited recruitment of Talin and Kindlin to the β 1-integrin. Therefore, SHARPIN binding to the cytoplasmic tail of α -integrins inhibits the critical switching of β 1-integrins from inactive to active conformations both in vitro and in vivo.

Unpublished data now show that SHARPIN is an endogenous inhibitor of integrin LFA-1 (α L/ β 2-integrin, CD11a/CD18) in leukocytes. Multiple immune functions, including leukocyte adhesion, are regulated by activity-dependent binding of LFA-1 to its cognate ligands such as ICAM-1/CD54. SHARPIN co-localizes with inactive LFA-1 in uropods in vitro and in vivo, regulates lymphocyte adhesion to and migration on ICAM-1 and affects constitutive homing in vivo. SHARPIN directly binds to the cytoplasmic tail of α L, and directly competes with talin for LFA-1 binding. The ubiquitin-like domain of SHARPIN binds in a mutually exclusive manner to LFA-1 and HOIP, the catalytic component of the linear ubiquitination complex, in vitro and in cells, showing that distinct pools of SHARPIN regulate integrin activity and linear ubiquitination. SHARPIN thus directly binds to LFA-1 and controls the dynamic balance between LFA-1 activation and inactivation during lymphocyte migration, which would explain the many aberrations of the immune system in SHARPIN null mice.

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Talin Activates Integrins by Altering the Topology of the β Transmembrane Domain.

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Talin binding to integrin β tails increases ligand binding affinity (activation). Changes in β transmembrane domain (TMD) topology that disrupt α - β TMD interactions are proposed to mediate integrin activation. Here, we used membrane-embedded integrin β 3 TMDs bearing environmentally-sensitive fluorophores at inner or outer membrane-water interfaces to monitor talin-induced β 3 TMD motion in model membranes. Talin binding to the β 3 cytoplasmic domain increased amino acid side chain embedding at the inner and outer borders of the β 3 TMD, indicating altered topology of the β 3 TMD. Talin's capacity to effect this change depended on its ability to bind to both the integrin β tail and the membrane. Introduction of a flexible hinge at the midpoint of the β 3 TMD decoupled the talin-induced change in intracellular TMD topology from the extracellular side and blocked talin-induced activation of integrin α IIb β 3. Thus, we show that talin binding to the integrin β TMD alters the topology of the TMD resulting in integrin activation.

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Kindlin-3 mediates integrin α L β 2 outside-in signaling and it interacts with the scaffold protein receptor for activated-C kinase 1 (RACK1).

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Integrins are heterodimeric ($\alpha\beta$) transmembrane receptors that are essential for many biological processes, including immunity, hemostasis, wound healing and the development of metazoans. Integrins are well known for their roles in cell adhesion and migration. Equally important, integrins allow communication between the cell's cytoplasmic compartment and its extracellular micro-environment. This is achieved by integrin cytoplasmic tails interacting with cytosolic proteins, many of which are signaling proteins. A hallmark of integrin activation, which is required for its ligand-binding, is the conversion of an integrin from a bent to an extended conformation. Studies have shown that the 4.1-ezrin-radixin-moesin (FERM)-containing cytosolic protein talin induces integrin activation by binding to the integrin β cytoplasmic tail. More recently, it has been reported that another family of FERM-containing proteins known as kindlins serve as co-activators with talin of integrins. Of the three kindlin paralogs, kindlin3 is expressed only in platelets, endothelial cells and hematopoietic cells. Although kindlin3 is known to be essential for β 2 integrins-mediated immune cell adhesion and migration as underscored

by the rare genetic disease leukocyte adhesion deficiency type III, other molecular partners of kindlin3 apart from integrins have not been reported. In this study, we show that kindlin3 can promote integrin α L β 2 micro-clustering that contributes to adhesion avidity. We also show that kindlin3 is required for integrin α L β 2-mediated cell spreading, suggesting a role of kindlin3 in propagating integrin α L β 2-derived downstream signaling. Notably, we identified receptor for activated-C kinase 1 (RACK1), a scaffold protein that folds into a seven-blade propeller, to be able to associate with kindlin3. Immunofluorescence analyses show that kindlin3 and RACK1 localize to the lamellipodia of migrating T cells on integrin α L β 2 ligand ICAM-1. Because RACK1 has been shown to be involved in modulating lamellipodium protrusions, our data suggest that the interaction of kindlin3 and RACK1 could play an important function in immune cell migration.

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Integrin α M β 2 Clustering Triggers Phosphorylation and Activation of Protein Kinase C δ that Regulates Transcription Factor Foxp1 Expression in Monocytes.

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Integrins are type I transmembrane and α/β heterodimeric adhesion receptors that attach cells to the extracellular matrix (ECM) or adjacent cells. They are present on most eukaryotic cells and participate in diverse biological functions, such as cell growth and differentiation, tissue development, neural functions, immune defense, hemostasis and bone resorption. Intracellular signals triggered by ligand-bound integrins are important for cell growth, differentiation, and migration. Integrin α M β 2 is mainly expressed on cells of the myeloid lineage, natural killer cells and $\gamma\delta$ T cells. It plays key roles in myeloid cell adhesion, phagocytosis and degranulation.

In this study, we show that protein kinase C (PKC) δ mediates α M β 2 signaling. In human monocytic U937 cells and peripheral blood monocytes, α M β 2 clustering induces PKC δ translocation to the plasma membrane, followed by Tyr311 phosphorylation and activation of PKC δ by the src family kinases Hck and Lyn. Analysis of the β 2 cytoplasmic tail showed that the sequence Asn727-Ser734 is important in α M β 2-induced PKC δ Tyr311 phosphorylation. We also show that α M β 2 clustering down-regulates Foxp1 expression in monocytes and this is dependent on PKC δ . Foxp1 expression was also reduced in monocytes that were allowed to adhere to human microvascular endothelial cells. These results demonstrate a role of PKC δ in α M β 2-mediated Foxp1 regulation in monocytes.

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β 1 integrin regulates Arg to promote invadopodial maturation and matrix degradation.

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Invadopodia are actin-rich invasive protrusions formed exclusively by tumor cells and are thought to facilitate basement membrane degradation and intravasation. β 1 integrin has been shown to localize to invadopodia, but the mechanism by which it regulates invadopodium function is poorly understood. We investigated the role of β 1 integrin in regulating invadopodium formation, maturation and dynamics in metastatic human breast cancer cells. Here, we show that β 1 integrin is required for invadopodial actin polymerization and matrix degradation, which are collectively referred to as invadopodial maturation. β 1 integrin knockdown with siRNA results in the formation of unstable invadopodia that cannot efficiently degrade extracellular matrix. β 1 integrin is activated in invadopodia during the maturation phase, and stimulating β 1 integrin-mediated adhesion accelerates invadopodial matrix proteolysis. Furthermore, FRET analysis revealed that β 1 integrin interacts with the Abl family kinase Arg in invadopodia and is essential for Arg-dependent cortactin Y421 phosphorylation, a key trigger for invadopodial

maturation. Cortactin remains dephosphorylated in $\beta 1$ integrin-depleted cells and sequesters cofilin, leading to a decrease in cofilin-mediated actin barbed end formation and Arp2/3-dependent actin polymerization. Thus, we provide evidence that $\beta 1$ integrin is a critical regulator of invadopodial maturation by stimulating the Arg-cortactin-cofilin pathway.

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Podosome Formation Requires Inhibition of Contractility and RhoA Activation.

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Clusters of matrix-activated integrins are important for the development of cell-matrix adhesions that in turn regulate cell growth and differentiation. Integrin clusters are sites of actin polymerization, and subsequent myosin contraction of the integrin clusters develops force and mature focal adhesions. Previously, we have utilized fluidic RGD-membranes, continuous supported bilayers with lipid-anchored RGD ligands, to activate integrins and investigate force dependence of initial integrin clustering during early adhesion formation (Yu et al., PNAS 2011). Here we demonstrated that cells, such as fibroblasts natively form podosomes within 45 minutes of adhesion on fluidic RGD-membranes where cell-matrix traction force cannot be stabilized. Podosome core assembly was initiated by Arp2/3-mediated actin polymerization within integrin clustering sites that were characterized by several integrin-associated proteins, such as paxillin, talin, and vinculin. In addition, myosin-1E and CARMIL1 were found as new markers at podosome cores. Interestingly, the same cells exhibited focal adhesions and actin stress fibers when adhering to identical RGD ligands immobilized on glass. We hypothesized that force on the integrin clusters produced a signal that caused matrix adhesion formation, and the absence of activated myosin contractility of integrins bound to an RGD-membrane resulted in the alternative activation of podosome formation. To test if RhoA signaling could be part of the force-activated signal, we measured RhoA-GTP levels with a FRET-based RhoA biosensor. RhoA-GTP levels were indeed low in cells forming podosomes on RGD-membranes, whereas RhoA-GTP levels were high in the cells on RGD-glass. Constitutively active RhoA-Q63L mutant blocked podosome formation on RGD-membranes. We also found that cells formed classic focal adhesions on RGD-membranes with dense line-partitions (1 μ m), but developed podosomes when plated on the same substratum with wider line-partitions (4 μ m). Dense 1 μ m-pitch line partitions in RGD-membranes provided mechanically stable sites for RGD-integrin immobilization thus stimulating adhesion maturation, but binding of the same cells to unpartitioned RGD membranes stimulated podosome formation. Maturation of focal adhesions requires local actomyosin contractility stimulated by RhoA; however, the signaling pathways downstream of activated integrins that cause podosome formation are largely unknown. From our data, we suggest that activation of myosin contractility at integrin clusters activates focal adhesion formation and inhibits podosome formation at integrin clusters, giving rise to a mutually exclusive behavior of focal adhesions and podosomes.

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Galectin-3 and phospho-caveolin-1 promote integrin-dependent EGF activation of RhoA, circular dorsal ruffles and matrix remodeling.

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In mammary epithelial cancer cells, galectin-3 binding to β 1,6-acetylglucosaminyltransferase V modified N-glycans forms a lattice that restricts EGF receptor mobility in the plasma membrane and also acts synergistically with phospho-caveolin-1 to promote integrin-dependent matrix remodeling and cell migration (Lagana et al., Mol Cell Biol. 26(8):3181-93, 2006; Lajoie et al., J Cell Biol. 179(2):341-56, 2008; Goetz et al., J Cell Biol. 180(6):1261-75, 2008). We then asked whether galectin-3 and caveolin-1 can promote EGF receptor and integrin crosstalk in motile cancer cells. We show that EGF signaling to downstream RhoA is galectin-3- and phospho-caveolin-1-dependent and is associated with circular dorsal ruffle formation and fibronectin fibrillogenesis. Circular dorsal ruffles are transient actin structures induced by growth factors in migrating cells, however their role is poorly understood. EGF-induced RhoA activation, circular dorsal ruffle formation and fibronectin fibrillogenesis are Src dependent and require expression of integrin linked kinase (ILK) and lipid raft integrity. Moreover inhibition of integrins by an RGD peptide prevents EGF induced RhoA activation and circular dorsal ruffle formation. Soluble fibronectin binding to the cell requires galectin-3 binding and galectin-3 is localized to fibronectin fibrils. This suggests that galectin-3 promotes EGF receptor-integrin crosstalk leading to phospho-caveolin-1 dependent RhoA activation, actin reorganization in circular dorsal ruffles and fibronectin remodeling. EGF-induced circular dorsal ruffles might represent a raft dependent signaling platform that recruits integrins and promotes their signaling to allow cell migration and matrix remodeling. This describes for the first time a common galectin-3/ phospho-caveolin-1 /RhoA signaling pathway that mediates EGF receptor and integrin crosstalk providing novel insight into the role of growth factor stimulated cancer cell motility.

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A novel mutation in β integrin reveals an integrin-mediated interaction between the extracellular matrix and cki-1/p27^{KIP1}.

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The cell-extracellular matrix (ECM) interaction plays an essential role in maintaining tissue shapes and regulating cell behaviors such as cell adhesion, differentiation and proliferation. Previous studies suggested that the cell-ECM interaction is particularly important for progression and arrest of cell cycle. However, the mechanism by which the ECM influences the cell cycle *in vivo* is poorly understood. Here we demonstrate that the β integrin PAT-3 regulates the localization and expression of CKI-1, a *C. elegans* homologue of the cyclin dependent kinase inhibitor p27^{KIP1}. In transgenic nematodes expressing wild type *pat-3* (+), CKI-1::GFP localizes primarily to nucleoli in hypodermal cells, whereas in transgenic animals expressing mutant *pat-3* (*sp*) carrying a defective splicing in intron 7, CKI-1::GFP appears clumped and disorganized in the outside of the nucleolus. In addition to the localization, the level of CKI-1::GFP protein was elevated in the splicing mutant, *pat-3* (*sp*) animal. Series of RNAi experiments linked cell adhesion genes to the regulation of CKI-1. RNAi of *unc-52/perlecan*, *ina-1/ α integrin*, *pat-4/ILK*, and *unc-97/PINCH* resulted in the *pat-3* (+)lines displaying the abnormal CKI-1::GFP localization, like that of *pat-3* (*sp*). Additional RNAi experiments revealed that the SCF E3 ubiquitin-ligase complex genes, *skpt-1/SKP2*, *cul-1/CUL1* and *lin-23/F-box*, are also required for the proper localization and expression of CKI-1, suggesting that integrin signaling and SCF E3 ligase work together to regulate the cellular distribution of CKI-1. These data also suggest that integrin plays a major role in maintaining proper CKI-1/p27^{KIP1} levels in the cell.

Perturbed integrin signaling may lead to the inhibition of SCF ligase activity, mislocalization and elevation of CKI-1/p27^{KIP1} protein. These results suggest that cell adhesion signaling is crucial for cell cycle regulation in vivo.

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Identification of protein synthesis and degradation genes associated with CKI-1/p27^{KIP1} localization patterns in the hypodermis of *Caenorhabditis elegans*.

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The interaction between a cell and extracellular matrix (ECM) is essential for maintaining tissue shape and regulating cell behaviors, which include cell adhesion, differentiation and proliferation. Studies have shown that cell adhesion affects cell proliferation by promoting cell cycle progression and arrest. However, the mechanism by which the ECM influences the cell cycle in vivo is poorly understood. Our previous analysis revealed that there is a link between β *pat-3* integrin and concentration levels of a cell cycle regulator. In the nematode *Caenorhabditis elegans*, CKI-1 is a homologue of the cyclin dependent kinase inhibitor p27^{KIP1}. Nematodes expressing wild-type β *pat-3* (+)integrin showed CKI-1 localization to the nucleolus of hypodermal cells, while nematodes expressing the splicing defective *pat-3* (*sp*) showed CKI-1 localization just outside and around the nucleolus. This mutant phenotype was further linked to cell adhesion and ubiquitin mediated protein degradation pathways.

To further analyze the genes involved in CKI-1::GFP localization, we have tested the contribution of protein synthesis and degradation genes to these prominent and significant CKI-1 localization patterns. Using the bacterial feeding method, RNA interference (RNAi) analyses of more than 60 genes were carried out on multiple worm populations. Following standard RNAi protocol, young adult *pat-3* (+)or *pat-3* (*sp*) worms were subjected to gene specific RNAi. Their F1 progeny were characterized for CKI-1::GFP localization. Genes that showed significant results for CKI-1 localization included *pbs-3*, *pbs-4*, *pbs-1*, *eif-3E*, *eif-6*, *rpn-1*, and *pas-5*. In brief summary, the knockdown of the protein synthesis genes *eif-3E* and *pas-5* in *pat-3* (*sp*) worms caused CKI-1::GFP localization to the nucleolus, while RNAi of *eif-6* in *pat-3* (+)worms displayed CKI-1::GFP being localized to the outside of the nucleolus. In addition, these genes also showed appreciable effects at different embryonic stages characterized by a significant delay in development. To better understand the cause/effect relationship of these genes on the expression and localization of the cell cycle inhibitor CKI-1, further analysis is currently underway.

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Phosphoinositide specificity determines which cytohesins regulate β 1 integrin recycling.

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Recycling of internalized integrins is a critical step in adhesion remodeling and cell movement. Recently, we determined that the ARF-GEFs Cytohesin 2/ ARNO and cytohesin 3/ GRP1 have opposing effects on adhesion and stimulated β 1 integrin recycling even though they are very closely related proteins (80% sequence identity). We have now determined the sequence differences underlying the differential actions of cytohesin 2/ ARNO and cytohesin 3/ GRP1. We find that the ability of cytohesins to promote β 1 integrin recycling and adhesion depends upon the presence or absence of a key glycine residue in their PH domains. This glycine residue determines the phosphoinositide specificity and affinity of cytohesin PH domains. Cytohesin PH domains with two glycines have a strong selectivity for PI-(3,4,5)P3, while those with three glycines have an equal affinity for PI-(3,4,5)P3 and PI-(4,5)P2. Switching the number of glycines in the PH domains of cytohesin 2 and cytohesin 3 is sufficient to reverse their effects

on adhesion and spreading and to reverse their sub-cellular locations. Importantly, we also find that a mutant form of cytohesin 3/ GRP1 that has 3 rather than 2 glycines in its PH domain rescues $\beta 1$ integrin recycling in cytohesin 2/ ARNO knockdown cells. Conversely, a mutant form of cytohesin 2/ARNO with 2 glycines in its PH domain fails to rescue $\beta 1$ integrin recycling. These data implicate the production of PI-(4,5)P2 as a key step in the formation of carriers during stimulated recycling.

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Fibroblast cluster formation on 3D collagen matrices requires cell contraction-dependent fibronectin matrix organization.

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Studies regarding cell behavior on 3D extracellular matrices provide a biologically relevant platform for understanding cell biology in tissue-like environments. Human skin fibroblasts moving on 3D collagen matrices exhibit two distinct behaviors-depending upon growth factor environment. In serum (or lysophosphatidic acid, LPA)-containing medium, cells undergo clustering, which depends on Rho kinase/myosin II-dependent contraction. However, in platelet-derived growth factor (PDGF) containing medium cells move as individuals. The objective of the present work was to determine the role of fibronectin (FN) in cell cluster formation. In serum or LPA but not PDGF or basal medium, cells organized FN (either serum and cellular) into a fibrillar, deoxycholate (DOC)-insoluble matrix with which the cells became associated. FN fibrils were found beneath the cell clusters and along the clusters borders in regions of matrix tension. Using siRNA silencing and function-blocking antibodies, we found that cell clustering and FN matrix organization required $\alpha 5\beta 1$ integrins and FN. Also, blocking Rho kinase or myosin II activity prevented FN matrix assembly and cell clustering. Conversely, if fibroblasts were incubated on detergent-extracted collagen matrices on which FN matrices had been pre-formed, partial cell clustering occurred in basal and PDGF-containing medium. Cell clustering was reversible if the growth factor when the growth factor conditions were switched from procontractile to promigratory. Dispersal of fibroblasts from the clusters was accompanied by disruption of the fibronectin matrix, which depended upon metalloproteinase function since dispersion was inhibited by broad spectrum MMP inhibitors. Based on these findings, we conclude that the function of cell contraction in fibroblast clustering on collagen matrices is to cause FN fibril assembly into cell cluster-organizing centers. Our findings provide new insights into how procontractile (serum/LPA) and promigratory (PDGF) growth factor environments can differentially regulate FN matrix assembly by fibroblasts on collagen matrices and thereby influence mesenchymal cell morphogenetic behavior under physiologic circumstances such as wound repair and development.

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Fibrin enables $\alpha V\beta 3$ -directed collagen gel contraction and upregulates the expression level of genes involved in extracellular matrix assembly.

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Fibrinogen in the concentration range 20 – 100 microgram/mL and thrombin were added to collagen type I (1 mg/mL) during collagen polymerization. Mouse myoblast C2C12 cells that lack collagen-binding $\beta 1$ -integrins contracted the resulting fibrin/collagen composite gels in the absence of extrinsic stimulators. Inhibition of $\alpha V\beta 3$ integrin by cyclic-RGD peptide repressed

this effect where thrombin alone had no effect on the contraction. Fibrinogen bound to immobilized native collagen type I, but not to denatured collagen in solid-phase assays. The binding was inhibited by the streptococcal protein CNE that blocks the site in collagen known to bind MMPs, fibronectin, DDR2 and SPARC. CNE suppressed the fibrin-directed collagen gel contraction. Scanning electron microscopy revealed a direct association of thin fibrin fibers to collagen fibers. This association was absent when CNE was present in the collagen gels. Expression level of several genes involved in matrix production and assembly, namely COL3A1, COL1A1, PLOD2, ITGB3, FN, POSTN, CTGF, and DECO was increased in C2C12 cells cultured in composite gels compared to C2C12 cells with a forced expression of the integrin $\alpha 2\beta 1$ that were cultured in pure collagen gels. Our data indicates that fibrin can link collagen fibers and cells. It also suggests that fibrin reprograms the cellular expression of genes associated with matrix network assembly. Such functions would be of relevance for the physiological properties of inflammatory lesions and in carcinomas, both characterized by the deposition of fibrin.

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The fibronectin synergy site promotes breast tumorigenesis by increasing cell tension and vinculin-dependent ERK signaling through a unique $\alpha 5\beta 1$ integrin catch-bond.

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Cancer cells are highly contractile and we showed that tumor tension induces extracellular matrix (ECM) remodeling and stiffening to drive tumor progression (Paszek et al., Cancer Cell 2005; Levental et al., Cell 2009; Samuel et al., Cancer Cell 2011). Integrins are mechano-transducers whose expression and activity is consistently modified in cancer, and integrin signaling promotes tumor progression by enhancing cell growth, survival and invasion and by facilitating actomyosin-dependent extracellular matrix (ECM) remodeling. Nevertheless, it is unclear whether specialized integrin heterodimers contribute to specific tumor behaviors such as force-dependent invasion, and if so how. Here, we explored the relationship between integrin specificity, cell tension and extracellular matrix-dependent malignant transformation. We determined that $\alpha 5\beta 1$ integrin and fibronectin were up-regulated following oncogene-induced transformation of the mammary gland in vivo, and during transition from nonmalignant to malignant mammary epithelial cells (MECs) cultured in vitro (Rizki et al., Cancer Res 2008). Importantly, we found that fibronectin-ligation of $\alpha 5\beta 1$ integrin but not αV integrin, or collagen-ligation of $\alpha 2$ integrin was necessary and sufficient for expression of the malignant phenotype of MECs in organotypic culture and in vivo. Interestingly, MECs with fibronectin-ligated $\alpha 5\beta 1$ integrin induced more ECM remodeling and contraction and exerted higher ROCK-dependent contractility than MECs with collagen-ligated $\alpha 2\beta 1$ integrin. Indeed, inhibiting cell contractility normalized the malignant behavior of MECs expressing high fibronectin-ligated $\alpha 5\beta 1$ integrin, consistent with our previous data showing that ROCK-induced cell tension per se can induce cell growth and survival, destabilize cell-cell adhesions, perturb tissue polarity and promote cell invasion (Paszek et al., Cancer Cell 2005; Miroshnikova et al., J Phys Biol 2011). Because $\alpha 5\beta 1$ integrin uniquely binds two domains in fibronectin, we asked whether the tension-induced tumor phenotype, induced through fibronectin-ligation of $\alpha 5\beta 1$ integrin, was mediated through its force-regulated catch-bond. Consistently, we noted that mammary epithelial cells with $\alpha 5\beta 1$ integrin ligated to a wild type, but, not to a synergy site deleted fibronectin, formed larger focal adhesions with abundant vinculin and exerted higher cell tension that destabilized adherens junctions, perturbed acini polarity, induced cell growth and survival and facilitated directed

cancer cell invasion. The high $\alpha 5\beta 1$ integrin-induced cell tension, mediated through ligation of the synergy site, potentiated vinculin-dependent epidermal growth factor stimulation of PI3 and ERK kinases to enhance cell growth and survival, disrupt tissue integrity and foster cell invasion in culture and in vivo. The findings demonstrate how extracellular matrix structure can engage specific integrin heterodimers to tune cell force, thereby providing a novel mechanism whereby fibronectin-ligated $\alpha 5\beta 1$ integrin could mediate tumor progression.

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Regulation of focal adhesion growth by external mechanical perturbations.

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The formation of adhesions between adherent cells and their microenvironment is crucial for various cellular processes, including migration and differentiation. Integrin-mediated adhesions, known as focal adhesions, are one of the primary mechanosensing structures that allow cells to sense the mechanical properties of their surroundings. Adhesion growth during spreading is thought to be coupled to the buildup of acto-myosin generated tension. Additionally, external forces applied to focal adhesions can modify them through a process of reinforcement, wherein more proteins are recruited to bear a higher load on these sites. Previous studies have suggested that reinforcement occurs rapidly when force on the adhesion increases, whereas slow disassembly occurs when internal cell tension dissipates. In a three-dimensional environment, however, cells can simultaneously experience forces of varying magnitudes from different directions with respect to the cell-extracellular matrix (ECM) interface plane. The response of focal adhesions to these variable mechanical inputs remains an active area of study. Here, we combine atomic force microscopy (AFM) and total internal reflection fluorescence (TIRF) microscopy to track the dynamics of focal adhesion assembly during cell spreading while simultaneously controlling cell forces. During cell spreading between a tipless cantilever on the top and a glass substrate on the bottom, we measured the intensity change in fluorescently-labeled zyxin, part of the focal adhesion complex, in response to changes in the AFM-generated tensile force that resisted cell spreading. We found that zyxin was not responsive to a broad range of applied forces, but that tensile forces above a threshold value halted spreading and induced growth of zyxin-rich adhesions within the main cell body, with a smaller effect on adhesions in the cell periphery. Interestingly, a subsequent decrease in tensile force reinitiated cell spreading and also promoted growth of both existing and new focal adhesions. These results suggest that the spatial location of focal adhesions determines their response to forces orthogonal to the cell-ECM interface plane.

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Live-cell super-resolution microscopy reveals early stages in adhesion formation and rigidity sensing.

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Rigidity of the extracellular matrix is a major determinant of many cellular aspects, including survival, proliferation, and differentiation [1]. Cells are able to sense rigidity and respond by modifying their adhesion, cytoskeletal organization, cellular forces, and gene expression patterns [2]. These effects are observed in many cell types, including fibroblasts, myocytes, and

neurons, and thus irregular rigidity signals underlie many medical disorders [3]. Recent studies show that rigidity sensing is an early event as cells spread on a surface that involves local lamellipodial contractions [4] and actin polymerization from nascent integrin adhesions [5]. However, we still don't understand the molecular mechanism of rigidity sensing upon initial contact with the surface. Here we show that the local nano-scale organization and kinetics of protein recruitment into nascent adhesion sites can be tracked in living cells using Bayesian analysis of fluorophores blinking and bleaching (3B microscopy [6]). We demonstrate a correlation between assembly of integrin clusters and force production, and show that talin changes its orientation upon lamellipodial retraction and pulling on the substrate. Furthermore, using nanofabricated surfaces with adhesion-permitting and non-permitting clusters of integrin ligands we distinguish which are the critical adhesion-related proteins that are required for the assembly of early adhesion sites that allow sensing matrix rigidity.

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Single piconewton forces at individual integrins support robust cell adhesion.

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Mechanical interactions between cells and the extracellular matrix (ECM) exert a profound influence on cell migration, proliferation, and stem cell differentiation. However, fundamental aspects of how cells detect and generate mechanical forces at the cell-ECM interface remain poorly understood. Here we describe a new technique, termed Molecular Force Microscopy (MFM), that measures the forces experienced by single cellular adhesion molecules with nanometer, piconewton, and sub-second resolutions. MFM uses a new class of FRET-based molecular tension sensors that bind to an avidin-coated glass coverslip at one end and present an integrin binding site at the other. Cellular integrins transmit force to the FRET pair, resulting in decreased FRET with increasing load. Unlike previously reported force sensors (1-3), MFM sensor molecules allow quantitative FRET imaging at the single molecule level. We found that human foreskin fibroblasts (HFFs) adhered to and spread on surfaces functionalized with the MFM probes, and developed mature focal adhesions as evidenced by paxillin localization and actin stress fiber formation. We observed a bimodal distribution of FRET efficiency values for MFM sensor molecules beneath HFFs, with one peak corresponding to zero load and the other indicating a distribution of forces between 1 and 4 pN. Despite evidence of robust adhesion, the forces we measured were ~10-fold lower than the force necessary to break individual integrin-ECM bonds (4). Our data provide the first direct measurement of the tension per integrin molecule necessary to form stable contacts with the ECM. The relatively narrow range of forces that we observed suggests that mechanical tension at individual adhesion molecules is subject to exquisite feedback and control. Ongoing work uses the unique capabilities of MFM to elucidate the mechanical signal transduction mechanisms that underlie cell migration and adhesion.

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Measure the molecular tension for integrin and Notch activation.*X. Wang¹, T. Ha¹; ¹Physics, University of Illinois at Urbana-Champaign, Urbana, IL*

Cells interact with extracellular matrix (ECM) or adjacent cells and regulate various cell functions using mechano-sensitive receptors on the cell membrane. Activation of these receptors is speculated to depend on molecular tension on the receptor-ligand bonds. To study the relation between the tension and receptor activation, we developed Molecular Tension Ladder (MTL). In MTL, ligand molecules are immobilized on a substrate through single rupturable tethers. Cells are cultured on such substrate and the target receptors bind with the tethered ligands. To activate the receptors, cells apply a tension on the receptor-ligand bonds. If the tether tension tolerance is lower than the threshold required by receptor activation, the tether would rupture and abolish the activation. Otherwise the receptor would be activated. A series of such ligand constructs with tension tolerances ranging from 12~56 pN were coated on different regions of one surface and therefore provides a ladder to test the tension threshold for the receptor activation. First, we applied MTL to study the molecular tension on integrins in the process of cell adhesion. MTL analysis of five different cell types showed that cells apply a universal, well-defined tension of about 38 ± 5 pN to single integrin-ligand bonds during initial cell adhesion and spreading. We also applied this method to Notch activation study and found that either no tension or a tension less than 12 pN is required to activate Notch receptor. More than a measurement tool, MTL can also provide a defined mechanical niche at the molecular level to regulate cell functions. We observed that focal adhesion and stress fiber well formed in the CHO-K1 cells adhered on 56 pN surface but not on 43 pN surface.

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Respective contribution of cell shape and matrix stiffness on the regulation of contractile forces.*T. Vignaud¹, F. Senger¹, Q. Tseng¹, L. Blanchoin¹, M. Thery¹; ¹iRTSV, CEA, Grenoble, France*

Matrix stiffness is known to be a key regulator of cell physiology. Matrix stiffness affects cell migration, growth, polarization and differentiation by modulating cell contractility. All reports so far led to the conclusion that matrix stiffness increases cell contractility. However, matrix stiffness has also been shown to increase cell spreading area, cell aspect ratio, focal adhesion size and alignment. Several evidences suggested that these geometrical parameters could also affect cell contraction level independently of matrix stiffness. Since cells are spatially confined in tissues, and can not freely extend their spreading area, this parameter may be highly critical in force regulation in physiological conditions. So the exact and specific contribution of matrix stiffness and cell shape to the regulation of cell contraction needs to be identified.

We distinguished the respective roles of cell shape and matrix stiffness by measuring the traction forces produced by micropatterned cells on deformable substrates. In accordance with previous reports, we found that an increase in cell spreading area, for a given matrix stiffness, promotes cell contraction. But, contrary to previous observations in which cell spreading area was not controlled, an increase in matrix stiffness did not promote cell contraction when cell spreading was limited.

A surprising absence of correlation between fiber width and the sustained forces prompted us to reconsider the structure-force relationship in stress fibers. We coupled laser nano-ablation, FRAP and traction force microscopy to quantify both actin filament dynamics and force production in micropatterned cells stress fibers. We measured the transport and renewal dynamics of fibers components in fibers of controlled size. The eventual severing and measurement of the corresponding substrate relaxation allowed us to measure the exact force production in the observed fiber.

Thereby we could draw a new framework for the production of mechanical force in stress fibers with respect to their size, sub-cellular location and internal dynamics.

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Effect of cytoskeletal tension on vascular smooth muscle cell adhesion.

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Cytoskeletal tension plays a central role in the development of contractile function in vascular smooth muscle cells (VSMC) and their adaptive responses to mechanical stimuli. The present study investigates the effect of cytoskeletal tension modulation by RhoA and Src on VSMC adhesion. Thus, an atomic force microscope probe functionalized with fibronectin was used to measure integrin $\alpha_5\beta_1$ -fibronectin adhesion force and local cell stiffness on cells expressing RhoA or Src wild-type or constitutively active mutants. Our results showed that both RhoA and Src activation increased $\alpha_5\beta_1$ integrin-fibronectin adhesion force and cell stiffness hence increased cytoskeletal tension. Fluorescence imaging experiments were performed to independently test the effect of RhoA and Src on stress fibers formation and focal adhesion activation. Actin area was quantified from confocal images of cells co-expressing RhoA-GFP mutants and actin-mRFP. The tyrosine phosphorylation at focal adhesion was quantified from total internal reflection fluorescence (TIRF) images of cells co-expressing Src-mCherry mutants and dSH2-YFP. The results showed increased actin fiber formation and focal adhesion protein activation for VSMC expressing constitutively active RhoA and Src, respectively. Moreover, constitutively-active Src translocates from cytoplasm to focal adhesions located mainly at cell edges, underscoring the role of Src pathway activation in cell adhesion and migration. To study the contribution of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, which regulate VSMC contractile function, to focal adhesion formation we performed TIRF imaging of cells expressing α_5 -GFP and β_3 -mCherry. Integrin $\alpha_5\beta_1$ was found mainly towards the center of the cell forming dotted streaks that followed a distribution similar to that of the endogenous fibronectin deposited by VSMC. In contrast, integrin $\alpha_v\beta_3$ was found at the cell edges forming small islands that showed similar location with the majority of tyrosine phosphorylation at focal adhesions. Due to this distribution, we hypothesize that integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ may have different roles in cytoskeletal tension development. The stress fibers oriented along the long axis of the cell may anchor preferentially at focal adhesions containing $\alpha_v\beta_3$ integrin, while the stress fibers in the center of the cell may anchor at focal adhesions containing integrin $\alpha_5\beta_1$. Taken together, these results show that actin cytoskeleton and focal adhesions contribute to cytoskeletal tension development, which in turn regulates cell stiffness and the strength of integrin binding to the matrix.

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The role of cell-intrinsic biophysical properties in enhancing glioma cell invasion.

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Adult brain cancer continues to be a significant challenge to both clinicians and patients with more than 20,000 new cases diagnosed yearly and incredibly poor mean survival of 12 to 14 months for individuals with glioblastoma multiforme (GBM), the most common type of adult brain cancer. The mortality associated with late stage GBMs can be largely attributed to their early dissemination and intrinsic death resistance. High grade GBMs are notoriously resistant to therapy and typically disseminate throughout the brain tissue, severely compromising patient treatment. Brain cancer environment is highly vascular, abundant in hyaluronic acid (HA) and

proteoglycans/linker proteins that are deposited by the tumor cells which mechanically reinforce the extracellular matrix and locally elevate the interstitial pressure from fluid buildup. Thus, the microenvironment of the most aggressive GBMs can be viewed as mechanically-challenged and the cells themselves are likely to have altered mechano-behavior. We identified a variety of cell-intrinsic GBM cell properties that, at least partially, are responsible for driving glioma cell aggressiveness, such as alterations in cellular compliance and HA-induced changes in the glycocalyx which drives integrin clustering and alters cell adhesion. Thereby, aggressive GBMs have unique physical properties which, if prove to be robust, can be used as means for identification of the cancer-initiating and propagating cells within tumor masses from the cells lacking the aggressive and metastatic potential.

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Regulation of Growth Factor Dependent Tumor Cell Proliferation by ECM Mechanics.

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The rapid progression of glioblastoma multiforme (GBM), the most common and lethal primary brain tumor, is driven by the diffuse infiltration of tumor cells into the brain. Recent genome sequencing efforts have revealed that many GBM tumors share lesions in the epidermal growth factor receptor (EGFR) pathway, which strongly promotes migration and proliferation. Previously, our laboratory has demonstrated that stiffening the extracellular matrix (ECM) can amplify the proliferation rate of human GBM tumor cells by more than a factor of five (Ulrich et al, Cancer Res 2009), raising the possibility that mechanical inputs can cross-talk with mitogenic signaling pathways to promote GBM tumor growth. The objective of this study was to directly explore this hypothesis by investigating the extent to which ECM biomechanics can regulate GBM cell cycle progression, chemotherapeutic sensitivity, and growth factor receptor (EGFR)-dependent signaling. We find that human glioma cells cultured on soft (80 Pa) fibronectin-coated polyacrylamide gels are more likely to be in G0/G1 and less likely to be in S phase of the cell cycle than cells cultured on stiff (119kPa) ECMs. Western Blot reveals that the expression and phosphorylation of EGFR and its downstream effectors, including Akt and PI3 kinase, depend strongly on ECM rigidity, with EGFR phosphorylation rising with increasing ECM stiffness. Furthermore, EGFR organization is highly rigidity-dependent, with EGFR co-clustering with focal adhesions on stiff substrates and receding into a diffuse distribution as matrix rigidity falls to physiological levels, suggesting that ECM stiffness may promote proliferation by spatially amplifying EGFR signaling. Together, these results support a model in which ECM stiffening acts through mechanotransductive pathways to trigger EGFR-based mitogenic signaling that promotes proliferation.

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Analysis of integrin organization and dynamics during focal adhesion formation and turnover using fluorescence anisotropy imaging.

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Integrins are transmembrane ECM receptors that link the extra-cellular matrix (ECM) and the cytoskeleton and play a crucial role in the immune response, cell migration and tissue morphogenesis. ECM-engaged integrins cluster together with proteins that mediate their signaling functions and linkage to the cytoskeleton to form focal adhesions that grow and turn

over in an actin and myosin II dependent manner. How actin and myosin mediate the clustering and organization of integrins during activation, focal adhesion growth and turnover is not known. We utilized fluorescence emission anisotropy imaging of cells expressing GFP-tagged integrins to analyze the evolution of integrin organization during focal adhesion dynamics in migrating fibroblasts and to test the role of integrin activation and actomyosin contractility in this process. Fluorescence anisotropy provides a measure of the rotational dynamics and/or degree of alignment of fluorophores. Our results show that integrins in nascent and mature adhesions have a higher value of anisotropy compared to integrins present diffusely on the cell membrane, indicating that upon clustering, integrins become more immobile and aligned in a polarized manner. Further, during the formation of a new lamellipodium, there is a relative increase in the polarization of integrins at the tip, suggesting a coupling between fast retrograde actin flow at the leading edge and integrin immobilization/polarized alignment irrespective of visible clustering. By employing specific perturbations we are attempting to understand how the association of focal adhesion components and the acto-myosin machinery affect the organization of integrins during the formation of mature adhesions.

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Proteomic analysis of integrin-associated complexes from multipotent and early differentiated mesenchymal stem cells.

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The interaction of the extracellular matrix (ECM) with mesenchymal stem cells (MSCs) controls stem cell fate. Factors such as cell-ECM adhesion, cell-cell interactions and soluble factors contribute directly to MSC differentiation and fate determination. Adhesion of cells to the ECM is mediated principally by integrin adhesion receptors. Following adhesion, integrins cluster at the cell membrane, and protein complexes called focal adhesions (FAs) assemble inside the cells. FA assembly is likely to induce signalling events in cells and affect cell fate.

In this study, a proteomics workflow has been developed to isolate, identify and quantify integrin-associated complexes in undifferentiated MSCs and MSCs induced to an adipocyte lineage (short-term induction, 3 hours). The aim of this study was to compare the composition of FAs and the abundance of their components to identify proteins that may be involved in MSC differentiation.

MSCs were allowed to adhere to surfaces coated with defined ECM proteins. Following incubation, equal populations of MSCs were subjected to two different treatments for 3 hours: 1) the adipogenic induction medium; or 2) adipogenic maintenance medium (control). Ligand-induced integrin-associated complexes were stabilized using crosslinker. Cells were lysed, and isolated complexes were subjected to downstream proteomic analysis.

Mass spectrometry (MS) analysis showed a higher abundance of FA proteins, such as integrin β 1, integrin α 5, vinculin, talin, α -actinin-4, zyxin, tensin, filamins, kindlin-2, integrin-linked kinase (ILK) and the actin-binding proteins PDZ and LIM domain proteins 1 and 7 (Pdlim1 and Pdlim7) in ECM-bound complexes from induced MSCs compared to non-induced MSCs. Bioinformatic analysis such as hierarchical clustering and ontological analysis showed a profile of enrichment of FA proteins in induced MSCs on defined ECM proteins. These data demonstrate that FA proteins can be isolated in a reproducible manner from induced and non-induced MSCs in a way suitable for MS analysis. They also indicate that the early induction of an undifferentiated cell line (MSCs) into a specific lineage (adipogenesis) increased the abundance of key FA proteins, specifically in integrin-associated complexes induced by ECM ligands.

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Vinculin-vinexin α interaction plays a key role in sensing extracellular matrix stiffness

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Extracellular matrix (ECM) stiffness influences cell behaviors such as cell migration, proliferation, and differentiation. Details of how cells feel ECM stiffness is still unclear although focal adhesions (FA) seem to play a key role. Vinculin, a FA protein linking actin stress fiber (SF) to the integrin-talin complex, is recruited to and stabilizes FA depending on intracellular tension. Thus, vinculin is thought to be a key molecule for mechanosensing through FA. In this study, we investigate mechanisms for sensing substrate stiffness through vinculin by focusing on vinexin, a FA protein binding to proline-rich linker region of vinculin.

We first analyzed the effect of substrate stiffness on vinculin status by using collagen I cross-linked acrylamide gel substrates with different stiffness. FRAP (fluorescent recovery after photo-bleaching) analysis revealed that immobile fraction (IF) of vinculin at FA was increased on rigid gels compared to on soft gels. It is known that exogenous expression of vinexin α accumulates vinculin at FA. Thus, we examined the effect of vinexin on vinculin behaviors depending on ECM stiffness. Interestingly, in vinexin knockout MEF cells, vinculin IF was decreased compared to WT MEF cells. Re-expression of vinexin α but not vinexin β rescued vinculin IF. Moreover, mutation in proline-rich linker region, which disrupts the binding to vinexin, impaired the stabilization depending on ECM stiffness. These results suggest that vinculin-vinexin α interaction is essential for stable localization of vinculin on rigid gels.

To test whether ECM stiffness or vinculin-vinexin α affects another FA protein, we examine the paxillin behavior, using FRAP analysis. Paxillin IF at FA was decreased on rigid gels compared to on soft gels. Vinexin α was necessary for the regulation of paxillin IF as well as vinculin IF. This indicates that vinculin-vinexin α interaction regulates the ECM stiffness-dependent behaviors of FA proteins.

Next we examined the effect of vinexin on vinculin status in vitro. We purified vinexin $\alpha\Delta N$, which lacks N-terminal region but retains a function in MEFs. FRET (fluorescence resonance energy transfer) assay and actin co-sedimentation assay showed that interaction with vinexin α induced the conformational change of vinculin and increased vinculin affinity to F-actin. These results suggest that interaction with vinexin α promotes vinculin activation and alters properties of FA depending on ECM stiffness.

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Vinexin-dependent distribution of vinculin to lipid rafts stabilizes vinculin at focal adhesions.

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Lipid raft, a plasma membrane domain enriched with cholesterol and sphingomyelin, is known to be required for focal adhesion (FA)-mediated signal transduction, such as Rac1 small GTPase and Src family kinases (SFKs). Signaling molecules localizing at FAs, including focal adhesion kinase, localize to and work in lipid rafts. However, it is not known whether cytoskeletal FA proteins localize to lipid rafts and whether lipid raft is involved in FA formation.

Here we investigated distribution of cytoskeletal FA proteins to lipid rafts and functions of lipid rafts in FA formation. Membrane fractions were extracted from mouse embryonic fibroblast cells

and treated with 1% Triton X-100 at 4°C, followed by density-gradient ultracentrifugation. FA proteins, including vinculin, vinexin, and paxillin, were separated into the fractions containing flotillin, a raft maker protein, but other proteins such as talin and integrin β 1 were not. Treatment with methyl- β -cyclodextrin (M β CD) disrupted the distribution of FA proteins into lipid rafts. We found that vinculin distribution to lipid rafts was regulated by cell adhesion. Detachment of cells from ECM removed vinculin from lipid rafts, and re-attachment to ECM rescued the localization to lipid rafts. Re-distribution to lipid rafts was impaired by blebbistatin, a myosin II inhibitor, suggesting that vinculin distribution to lipid rafts is regulated by cell adhesion and intracellular tension. Furthermore, we examined the effect of raft disruption on vinculin behavior. M β CD treatment promoted vinculin turnover at FA. Similarly, lipid raft disruption induced by ABCA1 expression promoted the turnover, indicating a role of lipid rafts in regulating FA. Vinexin is a protein interacting with flotillin as well as proline-rich linker region of vinculin. Thus, we examined whether vinexin is involved in vinculin distribution to lipid rafts. We found that vinculin was not localized to lipid rafts in vinexin knockout cells. Re-expression of vinexin α rescued vinculin localization. Together, these results suggest that the distribution of vinculin to lipid rafts involves the interaction with vinexin and stabilizes vinculin at FA.

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Mechanotransduction through Functional Interplay between Connexin Hemichannels, Integrins, and Signaling in Osteocytes.

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Mechanical loading induces the release of small bone anabolic factors, such as prostaglandins, from osteocytes, the principal mechanosensory cells with long dendritic processes. This release is mediated by opening of connexin 43 (Cx43) hemichannels (HC) at the cell surface. However, the mechanism for mechanotransduction that leads to the opening of these HC is unknown. Integrins expressed on the cell surface are thought to be mechanical sensors in the osteocytes. Our recent study showed that activation of PI3K/AKT induced the conformational activation of integrin α 5 upon mechanical stimulation in the form of fluid flow shear stress (FFSS), and led to the opening of Cx43 HC in osteocytic MLO-Y4 cells. Here, we show that integrin α v β 3 located in the dendritic processes is the primary mechanosensor that activates PI3K/AKT signaling. Activated AKT further phosphorylates both Cx43 and integrin α 5, enhances their interaction and increases Cx43 HC activity in the cell body. Integrin α v colocalized with integrin β 3 but not Cx43 on the dendritic side of the cells. Inhibition of integrin α v activation upon FFSS attenuated the activation of PI3K/AKT and blocked HC opening on the cell body. Interestingly, integrin α v β 3 present on the dendritic processes activated PI3K, which was required for the activation of integrin α 5 β 1 and opening of Cx43 HC on the cell body. FFSS activated AKT and facilitated HC opening. Cx43 and integrin α 5 were both directly phosphorylated by AKT and FFSS increased their phosphorylation due to activation of AKT. AKTi, an inhibitor specific to AKT, disrupted the interaction between Cx43 and integrin α 5, preventing the HC from opening. These results show that the interaction is essential for Cx43 HC to open. Taken together, these results suggest that integrin α v β 3 is the mechanical sensor that activates PI3K/AKT leading to the increased phosphorylation of integrin α 5 and Cx43 and enhanced their interaction, thereby inducing the Cx43 HC to open and release important bone anabolic factors.

Glycoproteins and Metalloproteases

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Characterization of Basigin gene expression in the mouse pineal gland.

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The Basigin gene expresses several products via splice variation. In the neural retina, two splice variants, named Basigin and Basigin-2 are expressed, with Basigin, the shorter variant, expressed by Müller glial cells and the retinal pigmented epithelium and Basigin-2, the longer variant, expressed by photoreceptor neurons. The two gene products associate with monocarboxylate transporters (MCTs) and are thought to form a metabolon that is critical for photoreceptor function. Since Basigin-2 expression has only been detected in photoreceptor neurons to date, the purpose of the present study was to determine if both Basigin and Basigin-2 are expressed in the pineal gland, which is a photoreceptive portion of the brain. Mouse pineal glands were harvested using established protocols and both RNA and protein were isolated using the TRI reagent protocol. Quantification of the relative expression of Basigin and Basigin-2 transcripts was performed through quantitative RT-PCR. Protein samples were subjected to immunoblotting analyses. The results indicate that although both transcripts were detected by quantitative RT-PCR, the longer transcript is expressed in trace amounts. Immunoblotting analyses indicate only the shorter form, Basigin, is translated into a protein in that tissue. The data suggest that while the longer transcript is generated, it is likely only a primary transcript that is used as a starting point for splicing of the shorter form. Basigin-2 protein expression remains a photoreceptor neuron-specific phenomenon. Future analyses will include quantification of MCTs in the pineal gland and comparison to their expression in the retina.

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Characterization of the ability of the photoreceptor-specific variant of the Basigin gene to induce expression of IL-6.

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The Basigin gene codes for two proteins in the neural retina by splice variation. Basigin and Basigin-2, members of the immunoglobulin superfamily (IgSF) are identical in amino acid sequence, with the exception of an additional Ig domain in the extracellular portion of the Basigin-2 molecule (Ochriotor et al., 2003, IOVS). The Basigin-2-specific loop is highly conserved throughout evolution, suggesting it has a specific, conserved role, most likely as a binding domain in the retina. A recent study suggests that Basigin-2 has the ability to induce expression of IL-6 from several cell lines (Redzic et al., 2011, JMB). The purpose of the present study was to determine which region of the Basigin-2-specific Ig domain is responsible for IL-6 expression. The monocytic cell line U937 was incubated with a recombinant form of the Basigin-2-specific Ig domain and IL-6 expression was assayed via enzyme-linked immunosorbant assay (ELISA). The results indicate the amino half of the Basigin-2-specific Ig domain has the ability to induce IL-6 expression in U937 cells. It is interesting to note that the amino half of the domain has also been implicated in binding to Basigin protein in the neural retina. It is not yet known if IL-6 expression is dependent on Basigin signal transduction. Future studies will be aimed at understanding the role that Basigin gene products play in IL-6 expression and its significance in the mammalian neural retina.

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Characterization of the role of Cyclophilin A in the Basigin-MCT1 complex in the neural retina.*K. M. Fletcher¹, J. D. Ochriotor¹; ¹Biology, University of North Florida, Jacksonville, FL*

It is well documented that the cell adhesion molecule Basigin and monocarboxylate transporter 1 (MCT 1) associate in the mammalian neural retina. It was suggested that Basigin acts as a molecular chaperone for the expression of MCT1 at the plasma membrane. However, a recent study from this laboratory indicated that Cyclophilin A expression overlaps that of Basigin and MCT1 in the neural retina, and may therefore chaperone the Basigin-MCT1 complex to the cell surface. The purpose of the current study was to determine whether Cyclophilin A interacts with Basigin gene products, specifically within the transmembrane domain of those molecules. Recombinant versions of the Basigin transmembrane domain were produced and used in an ELISA with endogenous mouse retina Cyclophilin A. In addition, immunohistochemical analyses for Cyclophilin A were performed on Basigin null mouse retina sections. The results indicate that Cyclophilin A binds to the central region of the Basigin transmembrane domain and is present at the plasma membrane of photoreceptors and Müller cells in Basigin null animals. These data suggest that Cyclophilin A binds to Basigin within the exocytic pathway and chaperones it to the plasma membrane. Basigin, however, is not needed for Cyclophilin A expression at the plasma membrane, as its expression was unaffected in the null animals.

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Characterization of the expression of Basigin and MCT1 in mouse reproductive systems.*P. J. Moran¹, J. D. Ochriotor¹; ¹Biological Sciences, University of North Florida, Jacksonville, FL*

Basigin and MCT1 are rather ubiquitously expressed transmembrane proteins that participate in cell adhesion and monocarboxylate transport respectively. Basigin null mice incur various abnormalities, including blindness and infertility, and research has suggested that a metabolic shuttling complex formed by these two proteins is necessary for proper maturation and function of photoreceptors. Without the construction of this metabolon in the neural retina, the mice are blind. This research group questions whether a similar mechanism exists in the in the uterus and testes that underlies the causes of infertility in Basigin null mice. Therefore, the expression of Basigin and MCT1 was assessed in normal mouse reproductive tissues. Immunoblotting and immunohistochemical analyses using antibodies specific for Basigin and MCT1 were performed on paraffin-embedded sections of mouse testis and uterus. Both immunofluorescence and a methyl green staining protocol were used to localize the proteins within the tissues. In the mouse testis, Basigin was found to be expressed primarily in the lumen of the seminiferous tubules and developing sperm, while MCT1 was expressed on the Leydig cells. In the uterus, preliminary analyses showed both Basigin and MCT1 to be expressed on the epithelial cells of the endometrium lining. The data suggest that it is unlikely that a faulty metabolic shuttle composed of Basigin and MCT1 is the cause of the arrest of spermatogenesis in Basigin null male mice, as the two proteins do not have overlapping expression. Conversely, both proteins were expressed in the same location within the mouse uterus. It is enticing to speculate what metabolic role Basigin and MCT1 play in the pre-placental phases of embryonic development, but additional experiments must be performed before one can truly know their role.

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Characterization of Basigin gene expression in mouse tissues.

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The Basigin gene expresses two major splice variants in the neural retina. The shorter form, named Basigin, is expressed on Müller glial cells, blood vessel endothelial cells, and the retinal pigmented epithelium in the eye and on epithelial surfaces throughout the body. The longer form, named Basigin-2 is expressed only by photoreceptor neurons. The two forms differ by one exon – exon 1A – that is found in Basigin-2 but not Basigin. Previous studies of Basigin gene expression in the neural retina suggested that the primary transcript contains exon 1A, which is spliced out in all cells but the photoreceptors, as trace amounts of exon 1A are found in the mRNA pool of Müller glial cells. Therefore, the purpose of this study was to analyze Basigin gene expression in various tissues of the mouse body to determine if the primary Basigin transcript does indeed contain exon 1A. Mice were sacrificed according to an accepted protocol and tissues were harvested for RNA isolation using the TRI reagent protocol. Quantitative RT-PCR was performed using primer sets specific for exon 1A and also for the mature Basigin transcript. The data indicate that exon 1A is present in the primary Basigin transcript, as it was detected in trace amounts in all tissues tested. This suggests that exon 1A is universally transcribed but then spliced out in all cells except for photoreceptor neurons. Future studies will be aimed at identifying the regulatory factors that regulate this splice specificity.

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Characterization of Basigin and Monocarboxylate transporter gene expression in the mouse olfactory system.

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Basigin, a member of the immunoglobulin superfamily, is known to associate with monocarboxylate transporters (MCTs) to form a complex thought to be necessary for photoreceptor function in the neural retina. Indeed, Basigin null mice do not express MCT1 or MCT4 at the photoreceptor or Müller cell plasma membrane and are blind from the time of eye opening. It has also been documented that Basigin null mice have olfactory deficits, in which they are anosmic and impervious to offensive odors such as acetic acid. Therefore, the purpose of this study was to examine the expression of Basigin and MCT-1, MCT-2, and MCT4 in the olfactory system of Basigin null mice and their control littermates in order to determine if a metabolic defect, like that in the retina, underlies the olfactory deficiencies of Basigin null animals. Immunohistochemical techniques were performed on the olfactory bulb (OB) of normal and Basigin null mice, as well as the main epithelium (MOE) of Olfactory Marker Protein (OMP)-GFP mice, using antibodies specific for Basigin, Embigin, MCT1, MCT2, and MCT4. The data indicate that MCT1, MCT2, and MCT4 expression is robust in the MOE. Conversely, MCT2 expression predominates in the OB, and is specifically associated with the glomeruli, the mitral cell layer, and the granule cell layer. The expression of MCT1 and MCT4 in the OB is limited to blood vessel endothelial cells. It is of interest to note that Basigin gene expression does not overlap that of MCT1 or MCT4 in the MOE, but does so in the OB, where it is found on blood vessel endothelial cells. A related protein, Embigin, is thought to interact with MCT2 in expressing cells, but was not detected in the OB of normal or Basigin null animals. The data suggest that a metabolic deficiency like that in the retina of Basigin null mice does not likely account for the acetic acid-specific anosmia in those animals.

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Human primary astrocytes express CD99: potential role in HIV brain infection.

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CD99 (MIC2) is an intracytoplasmic glycoprotein expressed in a wide variety of cells and has been shown to play a role in a variety of function ranging from cell differentiation and apoptosis. CD99 has also been observed to be important in lymphocyte adhesion and migration across the endothelium by activating alpha4beta1 and actin polymerization. Furthermore, induction of ICAM-1 by CD99 in human gingival fibroblasts has been shown to be important in the transmigration of leukocytes across the epithelial mucosa of the oral cavity thereby participating in pathogenesis of periodontal disease. HIV infection of the central nervous system is caused by the transmigration of virus-infected macrophages across the blood brain barrier (BBB) leading to the development of NeuroAIDS leading to deterioration of the CNS. This is facilitated by specific intercellular interactions with a variety of adhesion molecules expressed within the blood brain barrier which is comprised of endothelial cells, astrocytes, and pericytes. Indeed, previous studies have shown that endothelial cells express CD99 on their extracellular surface; however, to date, the presence of these molecules has not been identified among astrocytes. Further, the role of these molecules in the cellular physiology of these cells has not yet been elucidated. We have shown that human primary astrocytes robustly express CD99 as verified by Western blot and immunofluorescence assay. Currently their role in HIV infection of the CNS is being identified. Determining the role of these molecules in the semi-permeability of the blood brain barrier as well as synaptic regulation may provide further insight into the pathogenesis of NeuroAIDS the design of better therapies against HIV.

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Matrix metalloproteinase-14 cleaves the ectodomain of syndecan-2.

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Syndecans (SDCs) are a family of transmembrane heparan sulfate proteoglycans, consisting of four members (SDC1 to SDC4), and play critical roles in development, wound healing, inflammation, and tumor progression. Shedding of syndecan ectodomain found in many cultured cells is involved in biological events such as ligand binding and intracellular signaling. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases which are involved in degradation of extracellular matrix components during cancer invasion and metastasis. Recently, we identified that shedding of syndecan-2 (SDC2) is mediated by matrix metalloproteinase-7 (MMP-7) in colon cancer cells. To further analyze susceptibility of SDC2 to various MMPs, an extracellular domain (ECD) of rat SDC2 tagged with a crystallizable fragment of antibody (Fc) was overexpressed in HeLa cells. The Fc-tagged rat SDC2-ECD was cleaved the most efficiently by MMP-14, even better than by MMP-7. N-terminal amino acid sequencing revealed that MMP-14 cleaves rat SDC2 between Asn138 and Leu139. Fluorescence resonance energy transfer (FRET) assay using quenched fluorescent oligopeptide substrates containing cleavage sites of SDC2 showed that rat SDC2 oligopeptide is more efficiently cleaved by MMP-14 than human SDC2 oligopeptide. Taken together, our findings demonstrate that SDC2 is a novel substrate for MMP-14. Considering that MMP-14 is upregulated during invasion and metastasis, these results suggest that ectodomain shedding of SDC2 by MMP-14

may play a role in decreased SDC2-mediated cell-cell and cell-matrix adhesion and thus increased malignancy.

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The hemopexin but not the catalytic domain of Mmp3 is required for epithelial mammary invasion and branching morphogenesis.

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Sculpting of the mammary epithelial tree requires concerted and reciprocal signaling between the epithelium and its surrounding stroma, so that branch site initiation and timely invasion into the fat pad are ensured. Breakdown of normal branched architecture causes mammary gland dysfunction and may eventually create conditions to permit malignant transformation. Previous studies from our laboratory provided evidence that inappropriate expression of the stromal matrix metalloproteinase-3 (Mmp3) in mammary epithelia in engineered mice induces its morphological reshaping, unrestrained branching and ultimately malignant transformation. Although proteolytic activity appears to be the overriding regulator of Mmp3-induced disruption of mammary architecture, inhibition of MMP3 catalytic domain have yielded disappointing results in cancer trials. Here we explored extra-catalytic sites within MMP3 that contribute to impaired epithelial morphology. By expressing distinct Mmp3 mutants in mouse mammary epithelial cells, we uncovered a previously unrecognized role for the hemopexin domain of this enzyme in promoting cytoskeleton's reorganization, elongated morphology and scattering. We found that this domain is required also for Mmp3 to choreograph epithelial invasion and branching morphogenesis in murine mammary organoids cultured in three dimensional (3D) type I collagen gels. These findings uncover a novel mechanism by which Mmp3 regulates its function. Our data suggest also that conventional strategies to inhibit Mmp3 should be revisited, and targeting extra-catalytic sites of the protein may yield more effective inhibitors of its function. Ongoing experiments are focused on the identification of proteins that may interact with Mmp3 via hemopexin domain to allow mammary epithelial function and invasion during branching.

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A Novel Mechanism to Regulate MMP-2 Activity.

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Matrix metalloproteinase-2 (MMP-2) functions in diverse biological processes through the degradation of extracellular and non-extracellular matrix molecules. Because of its potential for tissue damage, there are several ways to regulate MMP-2 activity, including gene expression, compartmentalization, zymogen activation, and enzyme inactivation by extracellular inhibitors. Enzyme regulation through zymogen activation is important for the regulation of MMP-2 activity. In our previous studies, we showed that thrombin directly cleaved the propeptide of MMP-2 at specific sites for enzyme activation. We also demonstrated that heparan sulfate was required for thrombin-mediated activation of pro-MMP-2 by binding to thrombin, presumably through conformational changes at the active site of the enzyme. This suggests a regulatory mechanism for thrombin-mediated activation of pro-MMP-2. In this study, we found that MMP-2 formed a

reduction-sensitive homodimer in a controlled manner and that Ca²⁺ ion was essential for homodimerization of MMP-2. Homodimerization was not associated with protein kinase C-mediated phosphorylation of MMP-2. MMP-2 formed a homodimer through an intermolecular disulfide bond between Cys102 and the neighboring Cys102. Homodimerization of MMP-2 enhanced thrombin-mediated activation of pro-MMP-2. Moreover, the MMP-2 homodimer could cleave a small peptide substrate without removal of the propeptide. Taken together, our experimental data suggest a novel regulatory mechanism for pro-MMP-2 activation that is modulated through homodimerization of MMP-2.

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Biochemical purification and characterization of *Drosophila* MMPs.

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The vertebrate matrix metalloproteinase (MMP) family of extracellular proteases is very large with over 20 members that have apparent functional redundancy. Vertebrate MMPs can be classified according to their localization: some are secreted while others associate with the cell membrane. In *Drosophila melanogaster*, there are only two MMPs, DmMmp1 and DmMmp2. The fly MMPs do not have clear orthologs among the vertebrate MMPs, but DmMmp1 is known to be secreted into the media and DmMmp2 has a predicted GPI anchor. Thus both classes of MMPs are represented in the fly model. Because of this simplicity, *Drosophila* is an attractive system for the genetic analysis of MMP function. However, one limitation of the fly model is that few substrates have been identified biochemically or genetically. As a first step toward substrate identification, we are purifying full-length tagged enzymes from S2 cells and characterizing their activity. As expected, two isoforms of the secreted DmMmp1 exhibit activity by zymography. A third DmMmp1 isoform, which has a predicted GPI anchor, is enigmatic at this point. DmMmp2 remains associated with the cells, and we are working to demonstrate its activity in vitro. Our goal is to incubate crude extracts of insoluble *Drosophila* ECM with affinity-purified enzymes to identify substrates of *Drosophila* MMPs. We expect cleaved fragments will be released into solution, where they can be identified by mass spectrometry.

Cell-Cell Junctions I

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β -catenin is essential for tight junction maintenance under mechanical stress.

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Many tissues in our body experience and must tolerate mechanical stresses due to both internal and external forces. The skin, for example, is exposed to significant mechanical insults and is specially designed to have high mechanical resistance. Here we report a previously unrecognized role for β -catenin in providing stability to a tissue under stress. Loss of [beta]-catenin early in the development of the epidermis caused fully penetrant perinatal lethality. While broad regions of the epidermis appeared normal, sites of active morphogenesis and movement showed defects in tight junction localization and activated stress responses. These defects were not associated with detectable changes in Wnt signaling or adherens junctions in vivo. In addition, [beta]-catenin null cells formed adherens junctions and tight junctions normally in culture. Despite this, they had a specific defect in the maintenance of tight junction proteins when exposed to externally applied uniaxial stretch. In addition, [beta]-catenin null cells could not strengthen adherens junctions in response to cues from cortical microtubules, as wild-type

cells do. These data demonstrate that the full function of cell adhesion structures must take into account their role in response to mechanical stresses.

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Cell junctions, the actomyosin purse string and tension in dorsal closure.

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Cell sheet movements are an important aspect of embryonic development for organ and tissue formation. The functions of various junctional, cytoskeletal and motor proteins are coordinated to ensure that these movements occur at a correct time and place. Dorsal closure during *Drosophila* embryogenesis is an extensively used model to understand such movements. At the start of dorsal closure, contractile purse strings start to form as F-actin and myosin are accumulated at the leading edges of lateral epidermis. Here, we examine the roles of junctional proteins such as zyxin, α -actinin, cadherin and integrin. Zyxin and α -actinin localize to foci along the purse strings. Because zyxin localization coincides with the tension establishment along the purse string and it is responsive to the mechanical changes in cultured cells, we tested zyxin's response to mechanical changes in a multicellular environment using laser microsurgery. By cutting the tissue in different orientations, tension along the purse string can be abruptly increased or decreased. Contrary to the results in tissue culture cells, we found that zyxin foci disassemble when the tension along the purse string is released. In addition, the localization of zyxin and α -actinin into foci on the cell membranes indicates that the cell junctions have an important role in the formation of the purse string. We compare the functions of different cell junctional proteins, mainly cadherins and integrins, in making the purse string. Our results indicate that the cadherins, rather than the integrins, have primary role in making the purse string a supracellular structure. These results suggest that tension-sensitive actomyosin purse strings are made supracellular by cadherin-mediated cell-cell junctions. Grant support: GM33830 to DPK

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Spatial Regulation of Rho-A by GEF-H1 and p190RhoGAP in Response to ECM Stiffness.

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Breast epithelial cells sense and respond to the stiffness of their ECM environment using the small GTPase Rho, however the exact signaling mechanism has not been identified. Of the known Rho regulators, p190RhoGAP-B, and the GTP exchange factor, GEF-H1, are of particular interest to our lab because changes in both of these regulatory proteins were detected by microarray analysis of mouse epithelial cells cultured in low vs. high density collagen gels. In this study, we find that p190RhoGAP-B mediates down-regulation of RhoA activity and subsequent ductal morphogenesis in a compliant matrix, while GEF-H1 mediates RhoA activation in response to extracellular matrix stiffness. Under compliant matrix conditions both RhoA and p190RhoGAP-B co-localize with p120-catenin at sites of cell-cell contact. Consistent with these findings, we utilized a RhoA specific FRET biosensor and determined RhoA activity to be significantly decreased at cell-cell contact vs. cell-ECM adhesions. This suggests that Rho is held in an inactive pool at cell-cell contacts through a complex of p190B and p120-catenin, and is recruited to cell-ECM contacts within stiff matrices. Rho is activated in stiff matrices by GEF-H1, and we demonstrate the novel finding that microtubule stability is

diminished by a stiff 3D extracellular matrix, which leads to the activation of GEF-H1. Using acetylated-tubulin as a marker for microtubule stability, we observed a striking difference in acetylated-tubulin levels in cells that were cultured in stiff compared to compliant collagen gels: in compliant 3D matrix conditions, the acetylated microtubules appeared more linear and organized, while in stiff matrices acetylated microtubules were more punctate and disorganized. Additionally, loss of GEF-H1 decreases cell contraction of and invasion through 3D matrices. These data demonstrate that RhoA along with p120-catenin, p190B-RhoGAP-B, microtubules, and GEF-H1 function together in mechanosensing, which allows breast epithelial cells to detect and respond to changes in the stiffness of their tissue microenvironment.

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The desmosomal protein, desmoplakin, is a novel binding partner of the microtubule plus-end binding protein EB1.

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Desmoplakin (DP) is a desmosomal plaque protein that tethers intermediate filaments to sites of intercellular contact, and is essential for conferring mechanical integrity to tissues such as the epidermis and myocardium. DP and other desmosomal proteins are commonly mutated in arrhythmogenic right ventricular cardiomyopathy (ARVC), a cardiac disease in which arrhythmias can lead to sudden death. However, the pathogenicity of DP mutations is poorly understood. A yeast-two-hybrid screen using a construct of the DP N-terminus, which is a hot-spot for ARVC mutations, was conducted to identify binding partners of DP that contribute to disease development. Among the partners identified was the microtubule plus-end binding protein EB1 (end-binding 1). EB1 promotes border localization of the gap junction protein connexin 43 (Cx43), which is often aberrantly distributed in ARVC. Interference with EB1 binding and localization could thus be a potential mechanism by which mutation of DP leads to arrhythmogenesis. The DP-EB1 interaction was confirmed by co-immunoprecipitation of endogenous DP and EB1 from epithelial cell lysates. His-tagged EB1 constructs were used to demonstrate that the DP N-terminus binds full-length EB1 but not the EB1 head domain, suggesting that DP interacts with the EB1 C-terminus. *In situ* interaction of DP and EB1 was verified using a proximity ligation assay, in which DNA oligonucleotides produce a fluorescently detectable signal if two antigens of interest are in close proximity. To test if DP governs EB1 localization, we used structured illumination microscopy and confocal microscopy to compare the distribution of EB1 comets in control and DP-knockdown cells. Whereas control cells demonstrated a perpendicular alignment of EB1 comets with respect to cell-cell contacts, DP-deficient cells demonstrated a parallel alignment of EB1 comets and a loss of microtubule (MT) association with junctions. EB1 has been implicated in cortical capture of MTs, which confers stability to MT plus-ends. To test the importance of DP to MT stability, we treated WT and DP-knockdown cells with low concentrations of nocodazole known to preferentially target dynamic MT ends, and observed that MTs of DP-deficient cells were more susceptible to depolymerization. Importantly, cultured cardiac myocytes demonstrated a reduction in Cx43 border localization upon DP knockdown. Collectively, these results suggest potential mechanisms by which DP regulates MT organization and gap junction assembly, and by which mutations in DP contribute to ARVC development.

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Rac1 promotes maturation of cell-cell contacts by activating the E3 ubiquitin ligase Nedd4.

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The Rho-GTPase Rac1 promotes actin polymerization and membrane protrusions that mediate initial contact and subsequent maturation of cell-cell junctions. Here we report that Rac1 associates to the ubiquitin-protein ligase neural precursor cell expressed developmentally down-regulated 4 (Nedd4) and co-localizes with endogenous Nedd4 at epithelial cell-cell contacts. This interaction requires the hypervariable C-terminal domain of Rac1 and the WW domains of Nedd4. Reduction of Nedd4 expression by shRNA results in reduced transepithelial electrical resistance (TER) and concomitant changes in the distribution of adherens and tight junction markers. Conversely, expression of Nedd4 promotes TER, suggesting that Nedd4 cooperates with Rac1 in the induction of junctional maturation.

We found that activated Rac1 promotes Nedd4, but not Nedd4-2, -mediated ubiquitylation and degradation of the adapter protein dishevelled-1 (Dvl1), a negative regulator of cell-cell contact. Moreover, we observed endogenous Rac1 to co-localize with endogenous Dvl1 in intracellular puncta as well as on cell-cell contacts. Finally, we show that Dvl1 impairs epithelial cell-cell contacts by increasing the acetylation of microtubules (MTs). Bypassing the Rac1-Nedd4-Dvl1 pathway and promoting MT acetylation by inhibiting HDAC6 resulted in a loss of TER. Together, these data reveal a novel Rac1-dependent signalling pathway which, through Nedd4-mediated ubiquitylation of Dvl1, reduces the level of acetylated microtubules, which in turn promotes the maturation of epithelial cell-cell contacts.

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Epithelial Cell Contact Induces Zipcode-Mediated Contact Localized β -actin Monomer Synthesis.

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Epithelial cell-cell contact induces changes in the architecture of the actin cytoskeleton. These cytoarchitectural changes allow a cell to convert itself from a motile phenotype to an adhesive phenotype. Adherens junction complex assembly mediates the adhesive phenotype by generating a macromolecular adhesion complex that associates with the actin cytoskeleton. Previously it was demonstrated that the mRNA for β -actin contains a nucleotide sequence in its 3' UTR, called the zipcode, which is necessary for targeting and regulation of this transcript. The translation sequence is utilized by the zipcode binding protein, ZBP1. Consequentially deleting the zipcode sequence causes mislocalization of the mRNA transcript and its synthesized monomers inhibiting adherens junction assembly following myoblast cell contact. Here we report that the β -actin mRNA zipcode sequence is necessary for contact localized synthesis following epithelial cell contact. β -actin monomer synthesis was visualized using FIAsh & ReAsH to observe translation sites and calcium switching to induce epithelial cell contact. β -actin translation sites were observed at the base of filopods and later at the midline of the forming adherens junction. In fact quantifying the number of translation site at the cell contact there is a 7-fold decrease when the zipcode is deleted. Moreover under these conditions E-cadherin and F-actin failed to co-localize at cell contact sites. To quantify the extent of the adherens junction assembly defect we calculated the Pearson's correlation of E-cadherin and F-actin in the cytoplasm and cell contact sites. Dividing the Pearson's correlation calculation of the junction by the Pearson's correlation calculation of the cytoplasm yields the Asymmetry Coefficient of co-localization (AC). AC values greater than 1 represent adherens

junction events and AC values less than 1 represent adherens junction disassembly events. Zipcode-deletion causes the AC value to remain below 1 during the recovery stage of the calcium switch experiment. Taken together these data support a model where spatially and temporally regulated β -actin monomer synthesis drives adherens junction assembly following epithelial cell contact. Moreover this model may explain how ZBP1 functions as a potent metastasis inhibitor in cancer xenograph models.

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A novel role for Anillin in regulating cell-cell junctions in the intact epithelium.

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Anillin is a scaffolding protein that organizes and stabilizes the contractile ring by binding multiple cytokinesis regulators including F-actin, Myosin-2, Septins, the small GTPase RhoA, the RhoA activity regulator MgcRacGAP, and lipids. Anillin is required for successful cytokinesis in multiple biological organisms. Knock down of Anillin in isolated cells is associated with severe cytokinesis phenotypes such as multinucleation, oscillating cleavage furrows, improper midbody ring formation, and delocalization of RhoA, F-actin, and Myosin-2 from the contractile ring. Less is known about Anillin's role in intact epithelia where cells are polarized and make cell-cell junctions with their neighboring cells. In this situation, cells must maintain cell-cell junction integrity, which may become strained during cytokinesis, when the cell membrane experiences increased tension and shape changes as the contractile ring constricts. Here, using *Xenopus laevis* embryos as a model system, we identify a new role for Anillin in regulating junctional integrity and RhoA activation at cell-cell junctions in the intact epithelium. We find that a population of Anillin is localized at cell-cell junctions throughout the cell cycle. In Anillin knockdown embryos, both tight junctions and adherens junctions are disrupted, and F-actin at cell-cell junctions is disorganized. Additionally, we observe increased intercellular spaces, and increased permeability to small molecular weight fluorescent dextran in Anillin knockdown embryos. Because Anillin is reported to interact with RhoA, we tested the effect of knocking down Anillin on RhoA activity at cell-cell junctions. Intriguingly, Anillin knock down results in increased spontaneous flares of RhoA activity at cell-cell junctions in both dividing cells and non-dividing regions of the epithelium. These results reveal a novel role for Anillin in regulating junctional integrity through RhoA. We propose that Anillin is required to properly distribute cortical tension and RhoA activity in order to maintain cell-cell junctions.

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p115RhoGEF regulates epithelial architecture in breast epithelial cells.

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Maintenance of epithelial architecture is a critical feature for normal development and homeostasis. Defects in epithelial architecture can lead to pathological conditions such as cancer. The small GTPase Rho has been shown to play a critical role in the maintenance of epithelial architecture. However the regulation of Rho in this context has not been clearly understood. Rho is activated by guanine nucleotide exchange factors (GEFs). We have found that knockdown of p115 RhoGEF in MCF7 cells disrupted cell-cell junctions. When p115 RhoGEF was knocked down in MCF7 cells using siRNA, both adherens junctions and tight junctions showed a significantly disrupted morphology. In addition to disrupted junctions, the

knockdown cells also showed enhanced actin based lamella reminiscent of transformed cells. On the other hand, stable over expression of p115 RhoGEF in MCF7 cells significantly promoted both adherens junctions and tight junctions. p115 RhoGEF over-expressers also showed an enhanced cuboidal morphology as compared to GFP controls. We also tested the effect of manipulation of p115 RhoGEF expression in other cell types. The effect of p115 RhoGEF knockdown was more pronounced in MCF10A cells which are known to retain most of their epithelial characteristics. p115 RhoGEF knockdown in MCF10A cells disrupted junctions and significantly enhanced migration which was indicative of a more transformed phenotype. On the other hand over expression of p115 RhoGEF in MDA MB 231 cells which are known to be completely transformed showed a cuboidal morphology and significant inhibition of migration, thus indicating a reversal to epithelial morphology. These results indicate a novel role for p115 RhoGEF in the regulation of epithelial characteristics. To further test if the over expression phenotype was dependent on GEF activity, we made a GEF dead point mutant of p115 RhoGEF. Cells over expressing GEF dead p115 RhoGEF in MCF7 and MDA MB 231 cells did not display the over expression phenotype. This showed that the over expression phenotype was dependent on catalytic GEF activity. These results indicate a pivotal role for p115 RhoGEF in the regulation of epithelial morphology. Changes in the expression levels of p115 RhoGEF in epithelial cells could be an important contributing factor in cellular transformation.

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Zipcode-Mediated β -actin Monomer Synthesis is Required for Epithelial Cell Spreading, Adherens Junction Assembly, and Cell Adhesion.

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Epithelial cell contact triggers actin cytoskeleton remodeling permitting cells to switch from motile to adherent phenotypes. To elucidate molecular pathways driving epithelial cell adhesion following cell contact, we plated MDCK cells expressing GFP- β -actin with or without its zipcode sequence at low density and allowed them to contact each other. Adherens junction complexes were identified as sites with strong colocalization between E-cadherin immunofluorescence and GFP- β -actin. To quantify the extent of adherens junction assembly in these cultures, we calculated Pearson's correlations of colocalization for E-cadherin and GFP- β -actin. Calculating the ratio of the Pearson's correlation from the contact site and cytoplasm yields the Asymmetry coefficient of colocalization (AC). Interestingly, deleting the β -mRNA zipcode from the GFP- β -actin reporter significantly inhibits cell spreading, decreases the Asymmetry coefficient, and inhibits adherens junction assembly. Additionally, MDCK cells expressing zipcode-deleted GFP- β -actin mRNA are unable to assemble multi-cell clusters. Incredibly, overexpressing E-cadherin in the GFP- β -actin (zipcode-deleted) expressing MDCK cells rescues adherens junction assembly defect, increases the Asymmetry coefficient to wild-type levels, and repairs the clustering defects induced by zipcode-deletion. Together these data indicated spatially regulated β -actin monomer synthesis is required for epithelial cell spreading, adherens junction assembly, and cell adhesion.

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Identification of a phospho-domain in Alpha-Catenin that impacts epithelial sheet migration.

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Alpha catenin (α -cat) is the sole actin-binding component of the cadherin/catenin cell-cell adhesive complex, but how α -cat binds actin at cell junctions is under intense debate given

evidence that recombinant α -cat behaves as an allosteric protein that cannot bind F-actin and the cadherin complex simultaneously (Drees et al., 2005). However, it is unclear how post-translational modification of α -cat may regulate these interactions. Using a mass spectrometry-based phosphoproteomic approach, our lab has discovered a highly conserved phosphorylation domain consisting of five serine/threonine residues in α -cat that is situated just N-terminal to the F-actin-binding site of α -cat. These phosphorylations also lie within the C-terminal end of the mechano-sensitive, auto-inhibitory region of α -cat that restricts recruitment of vinculin, another actin-binding protein, to the cadherin/catenin complex (Yonemura, 2010, NCB). Using radioactive ^{32}P -orthophosphate, we confirm that these sites are the major targets of phosphorylation in cells. In vitro kinase assays performed on recombinant, purified GST-tagged α -cat demonstrate that these sites are targeted hierarchically by casein kinases (CK) 2 and 1, where phosphorylation at S641 by CK2 “primes” α -cat for processive phosphorylation by CK1 at S652, S655 and T658. Specifically, we show that a S641A phospho-mutant of α -cat is less heavily phosphorylated by CK1, whereas either a S641D phospho-mimic α -cat or WT α -cat that is pre-phosphorylated by CK2 is more heavily phosphorylated by CK1. Additionally, these CK1 sites are less accessible in the full-length α -cat protein compared with a C-terminal fragment (aa459-906). We hypothesize that combinatorial phosphorylation by CK2 and CK1 “activates” α -cat at junctions, resulting in a more open conformation that favors α -cat binding to vinculin and F-actin. Using a dog epithelial cell line (MDCK) that replaces endogenous α -cat with GFP-tagged forms that contain, lack or constitutively mimic phosphorylation, we find that phospho-mimic α -cat enhances epithelial wound closure by promoting the speed and persistence of cell migrations. Future studies will determine whether these enhanced activities are mediated through improved association with vinculin.

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Evaluation of Atrazine Permeation in Caco-2 Cells.

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Atrazine is one of the most commonly used herbicide in the United States, mainly in agricultural land. The use of atrazine as an herbicide is controversial. Some research studies have associated it with birth defects and menstrual problems when consumed by humans at concentrations below government standards, while other studies have reported that human exposure to atrazine does not pose a health risk. In addition, some animal model studies suggest that exposure to atrazine can cause catastrophic effects, such as feminizing male frogs. Based on these studies, it still remains unclear what the effects of atrazine are to human and animal health, and to our environment. The human intestinal cell line, Caco-2 cells, is an established model system for measuring the permeability of chemicals that can be ingested and adversely impact human health. We investigated the potential health risk of atrazine on humans by determining whether this herbicide could penetrate across the epithelial monolayer of the human gastrointestinal tract. We used Caco-2 cells to determine the transepithelial transport of atrazine from the apical or luminal side to the basolateral or serosal side. Our results from these investigations will be discussed.

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Structural and biochemical analysis of a novel, broadly specific claudin binder.

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Objective: Recent progress in understanding the biochemical structure of tight junctions has provided us with new insights for claudin (CL)-targeted drug development. CLs are a family of tetra-transmembrane proteins comprising over 24 members. CL binders enhance mucosal absorption of drugs, so CL targeting is a promising strategy for mucosal vaccination and cancer therapy. Previously, by using a library of mutated C-terminal fragments of *Clostridium perfringens* enterotoxin (C-CPE), we screened a CL binder, m19. Here, we performed structural and biochemical analyses of m19 and its mutants.

Methods: X-ray diffraction data on m19 were collected from a single crystal at 2.0 Å by using the BL44XU beamline at SPring-8, and processed with iMOSFLM and SCALA software. The structure of m19 was solved by using the BALBES system. The interactions of m19 and its mutants with CLs were investigated with ELISA and FACS analysis.

Results: C-CPE binds to CLs via interaction between the second extracellular loop domain of the CL and the 30 C-terminal amino acids of C-CPE. The structural backbone of m19 in the C-terminal domain is similar to that of C-CPE. An electrostatic surface map of m19 revealed that its C-terminal domain is more positively charged than that of C-CPE. Kimura et al. proposed an electrostatic interaction model for the binding of CPE and CLs (J. Biol. Chem., 285, 401, 2010) and showed that part of the second loop domain interacts with the CL-binding domain of C-CPE. The isoelectric point (pI) of CL-4 is higher than that of CL-1, -2, and -5, which interact less with CPE. Therefore, we hypothesized that if m19 binds to CL-1 via electric interaction, it would also bind to CL-2 and -5. m19 bound to CL-1, -2, -4, and -5-expressing cells; therefore, m19 is a broadly specific CL binder. Site-directed analyses showed that substitution of Ser at position 307 and Ser at position 313 with Arg and His, respectively, may be essential for the interaction with CL-1. We prepared double-substituted C-CPE mutants by changing the Ser (pI, 5.68) residue to His (pI, 7.59), Lys (pI, 9.8), or Arg (pI, 10.76). Increase in positive charge at position 307 and in negative charge at position 313, respectively, strengthened the interaction with CL-1. We also prepared double Ala (pI, 6.0)- or Asp (pI, 2.77)-substituted C-CPE mutants at positions 307 and 313 (S307A/S313A and S307D/S313D). The decrease in the charge attenuated the binding to CL-1.

Conclusion: Electrostatic interactions may be involved in the binding of m19 to CL-1. Therefore, modulation of the electrostatic surface may be a potent strategy for the development of CL binders.

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Regulation of Nectin-2 by Cadmium Chloride (CdCl₂).

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Nectin-2 is a major component of the adherens junctions (AJs) between Sertoli cells and germ cells in the testis. Recent studies have shown that male knockout mice of nectin-2 are sterile. Cadmium (Cd), an environmental toxicant, is known to be also an endocrine disruptor that affects spermatogenesis. In this study, we investigate whether cadmium chloride (CdCl₂) plays a

role in nectin-2 expression. CdCl₂ negatively regulates mRNA and protein levels of nectin-2 in mouse Sertoli cell line, TM4 cells. Luciferase reporter assays indicated that CdCl₂ reduces nectin-2 promoter activity within the region of nucleotides (nt) -246 and -211 (relative to the translation start site) where putative transcription factors (TFs) binding motifs are identified. However, site-directed mutational studies have shown that no specific motif is found to involve in CdCl₂-mediated nectin-2 gene repression. Hence, six consecutive cis-acting regions (each contains 6 nucleotides) between nt -246 and -211 are mutated respectively to identify the cis-acting region involved in the CdCl₂ effect. Results showed that the second 6-bp region (between nt -240 and -235) is involved in CdCl₂-mediated reduction of nectin-2 promoter activity. In addition, putative TFs binding to this region are identified. By EMSAs, we found that DNA (nt -240 to -235)-protein complexes are formed in a dose-dependent manner and CdCl₂ treatment could diminish the formation of the complexes. Antibody supershift assays have shown that TFs, E2F1, Sp1 and KLF4, are present in the complexes. We also found that CdCl₂ down-regulates the expression level of these TFs including E2F1, Sp1 and KLF4 in the nucleus. Apart from transcriptional regulation, cycloheximide assay indicated that CdCl₂ negatively modulates nectin-2 protein level via post-translational modification and we are now investigating the underlying mechanism. Taken together, CdCl₂-mediated down-regulation of nectin-2 is mediated through transcriptional modification by negatively affecting its basal gene transcription and post-translational modification. [This work was supported by Hong Kong Research Grants Council (HKU772009 and HKU773710) and CRCG Seed Funding for Basic Research.]

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MHC Class II Compartment in Human Autologous Macrophage-Lymphocyte Rosettes.

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The contact area between a T cell and antigen-presenting cell is known as "immunological synapse" (Grakoui et al, 1999) and the multiple interactions that occur leading to a "signal" for T cell activation. Cellular association between human blood monocyte-derived macrophages and lymphocytes T CD4+ from autologous cultures total leukocytes extracted from the blood, which bind selectively forming rosettes with central macrophage and lymphocytes adhered was described as the phenomenon of multiple immunological synapses on macrophage-lymphocyte rosette (MLR) (Cabral and Novak, 1992, 99). The processing and antigen presentation are involved in MLR phenomenon (Cabral and Novak, 1999, Novak and Cabral 2008, 09) and in this phenomenon the autologous antigens of senescent neutrophils are presented by endocytic way like phagocytosis of cells undergoing apoptosis naturally in the body. Along time of culture the cells that interact in the MLR phenomenon may present special features in their areas of cell-cell interaction, and in the surrounding cytoplasm such as mitochondrial translocation (Novak y Orquera, 2011). The spatial organization of MHC class II compartment (MIIC) changes in dendritic cells maturation with morphological characteristic such as multivesicular, multilaminar and tubular structures (Van Nispen tot Pannerden et al, 2010). Objectives: ultrastructural study to observe the organization of MIIC in the cells of the MLRs along culture time. Materials and methods: Healthy human blood samples, anticoagulated with heparin (n = 10) (donated by the Blood Bank, UNC, anonymity, data serology). Autologous cultures in TC199 medium (SIGMA, St. Louis, MO). Samples: 1, 2, 3, 20, 48, 96 and 144 h. MLR technique (Cabral y Novak, 1992, 99). Samples of MLRs underwent electron microscopy. MET: Zeiss LEO-906E. Results: MLR formation starts when monocytes transformed in macrophages and increased since 20 h. We observed multivesicular, multilaminar and tubular structures in spatial organization of MIIC along time culture, according to intermediated and late structures described. However in a lymphocyte that does not participate in MLR was observed at 2 h of culture a multilaminar structure like MIIC on plasma membrane. At this time neutrophils were observed whereas at 3h

a few of them appeared. In macrophages tubular structures occurs at 48 h. At 144 h multivesicular and multilaminar bodies coexists. Conclusion: This organization of MIIC which undergo major structural changes coincides with occurrence of macrophage transformation in cultures and his role as antigen-presenting cells in MLR.

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A549 and SW-13 Cell Biomarkers for 'Spice' Cannabinoid Drugs.

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'Spice' refers to designer drugs consisting of plant materials spiked with different synthetic mimetics of delta tetrahydrocannabinol, a psychoactive compound in marijuana. The toxicology and bioactivity of many synthetic cannabinoids are unknown including Spice brands: Barely Legal, Brain Storm and Voodoo.

It was hypothesized that the 'Spice' brands Barely Legal, Brain Storm and Voodoo Child contain different compounds, overlap in biological activities with delta THC and affect cell physiology and morphology. This study sought possible biomarkers for the effects of 'Spice'. Fluorescence microscopy detected cannabinoid receptor type 2 proteins but not type 1 receptors on A549 and SW-13 cells. The complement system regulatory proteins CD46 and CD59 but not CD55 were detected on A549 and SW-13 cells. Glycoconjugate profiles on these cells were characterized by lectin binding. Exposure to delta THC and Barely Legal Spice caused stronger Con A lectin binding to A549 lung cells than did exposure to Brainstorm Spice or control media. This study is the first description of the complement regulatory protein profile of adrenal gland cells and provided carbohydrate structure profiles of A549 and SW-13 cells as detected by lectin binding. The results will facilitate more detailed analysis of the toxicology and bioactivities of spice.

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Overexpression of proprotein convertase 1/3 induces an epithelial-mesenchymal transition-like phenotype in airway epithelial cells.

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The proprotein convertases are serine proteases responsible for the proteolytic maturation of many precursor proteins involved in upper airway remodeling during nasal polyposis. We have previously found that proprotein convertase PC1/3 is expressed in normal human nasal mucosa. However, whether PC1/3 is related to nasal polyp formation has not been investigated. To gain insight into the functional role of PC1/3 in the nasal polyp epithelium, we determined PC1/3 expression in nasal polyps and generated stable cell lines constitutively expressing PC1/3 using the airway epithelial cell line NCI-H292.

Nasal polyps exhibit increased PC1/3 expression compared to normal human nasal mucosa, as assessed by immunostaining, western blotting, and enzyme activity assays. PC1/3 was shown to be expressed in secretory neuroendocrine cell granules containing chromogranin A in normal nasal mucosa and it was also expressed in goblet and ciliated cells in nasal polyps. NCI-H292 stably transfected with intact PC1/3 cDNA displayed morphological changes, enhanced cell

proliferation and migration, down-regulation of E-cadherin and cytokeratin-18, and up-regulation of vimentin, matrix metalloproteinases, and $\alpha 5$ integrin.

Proteomic analysis showed that differentially produced proteins between PC1/3-overexpressing NCI-H292 cells and mock cells are implicated in carbohydrate metabolism, cell proliferation, cytoskeletal reorganization, DNA metabolism, and signal transduction. These proteins are directly or indirectly related to the epithelial-to-mesenchymal transition process. Interestingly, UCH-L1 expression, a neuroendocrine cell-specific protein of the airway epithelium, was significantly increased in PC1/3-overexpressing NCI-H292 cells, possibly suggesting that these cells can transdifferentiate into other epithelial cells.

Taken together, our data suggest that PC1/3 overexpression induces morphological and phenotypic epithelial-mesenchymal transition changes of airway epithelial cells and these changes may contribute to the pathogenesis of nasal polyps.

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Isolation and Characterization of Lacrimal Gland Progenitor Cells.

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The lacrimal gland (LG) is responsible for secretion of the aqueous layer of the tear film and is comprised of two main types of tissue, epithelium and mesenchyme. Degeneration of LG leads to a lack of corneal lubrication, irritation, pain, inflammation, and in severe/untreated cases, total blindness from extensive corneal damage. These conditions are known as dry eye syndrome (DES), and can result from a wide variety of causes such as aging, auto-immune diseases, or laser eye surgery. Although millions of people suffer from DES, it remains unrecognized and untreated until later stages when the LG may be severely degenerated. Developing new therapies to restore LG function would drastically improve the quality of life of people affected by DES and one such potential approach is the transplantation of progenitor cells. It has previously been reported that the LG has an innate and rather impressive regenerative capacity, suggesting that LG progenitor cells do in fact exist. We have begun to isolate and characterize potential progenitor cells in mice using two approaches: first, gene expression studies of stem cell transcription factors found only in the epithelium and second, fluorescence-activated cell sorting (FACS) using cell surface markers that have been used to isolate stem cells from similar glandular tissues. Because the epithelium progeny forms the ductal and acinar portion of the functional LG, our search has been focused on finding progenitor cells that are epithelial. We identify several stem cell transcription factors with expression restricted to the epithelial compartment of the LG. Moreover, the runt-related family of transcription factors (Runx1, 2, and 3) were found to be important regulators of LG development and regeneration. Through flow cytometry, we have isolated a putative population of epithelial progenitor cells expressing the stem cell factor receptor c-Kit and epithelial cell adhesion molecular (EpCAM). Our studies have shown the existence of one or more putative epithelial progenitor cells in the LG. The capacity of these progenitors to restore the function of damaged LG will be determined in future studies.

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Coordination of mitochondrial activity across the rat salivary glands epithelium imaged by intravital two-photon microscopy.

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In individual cells, levels of the reduced form of nicotinamide adenine dinucleotide (NADH) have been reported to oscillate periodically. These oscillations are linked to mitochondrial activity that is regulated through reactive oxygen species and are thought to correlate with the spatio-

temporal redox state of the cell. In order to investigate whether this phenomenon occurs in vivo, we imaged the salivary glands (SGs) of live rats using intravital microscopy, exploiting the fact that NADH emits upon two-photon excitation. Fast scan imaging allowed us to capture periodic oscillations of NADH levels under normal physiological conditions in vivo. Strikingly, we observed for the first time, that NADH oscillations are temporally and spatially synchronized across the SGs epithelium. To confirm that these oscillations were linked to mitochondrial activity, we used cationic dyes, such as TMRM and Rhodamine123, which are sensitive to mitochondrial membrane potential. Maximal mitochondrial activity was initially observed in specific areas of the epithelium and propagated throughout the tissue in a wave-like pattern. This phenomenon was not observed in confluent cell cultures, in explanted SGs, or in vivo, when the blood flow was reduced by ligation of the vessels. As expected, disruption of mitochondrial membrane potential led to the disruption of wave propagation. We hypothesized that synchronized oscillations might be related to basal secretory activity of the SGs that is mediated by several receptors. To test this hypothesis, we used a pharmacological approach. Inhibition or stimulation of muscarinic, alpha-adrenergic and purinergic receptors did not affect the propagation of the waves, whereas stimulation of beta-adrenergic receptors led to a sharp and uniform increase in NADH fluorescence across the tissue and the loss of the oscillations. Interestingly, both the oscillations and the propagation of the waves were restored 20 minutes after stimulation, consistent with the reported clearance of the agonist from the tissue. Based on our findings, we speculated that NADH oscillations and their synchronization might have implications in the coordination of tissue function under physiological conditions. Ongoing work is focusing on identifying the mode of signal propagation (e.g. through gap junctions or small diffusible molecule), on better understanding of its regulation, and the alteration of oscillations and signal propagation under pathological conditions.

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Ankyrin-B syndrome: Metabolic consequences of human genetic variations in the membrane adaptor ankyrin-B.

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The Ankyrin-B syndrome, caused by mutations in the membrane adaptor protein ankyrin-B, was initially identified and characterized in patients with cardiac arrhythmias and sudden cardiac death. In cardiomyocytes, ankyrin-B is required for stabilizing the Na/K ATPase, Na/Ca exchanger, and IP3 receptor proteins at specialized microdomains. In addition to the heart, ankyrin-B is selectively expressed in pancreatic beta-cells, where it is required for cholinergic modulation of insulin secretion. In isolated pancreatic islets, ankB haploinsufficiency results in reduced levels of IP3 receptor and impaired calcium signaling. These findings, along with the fact that one of the loss-of-function mutations in ankyrin-B associated with heart disease (R1788W) was also found in about 1% of Caucasians and Hispanics with type 2 diabetes, suggest that mutations in ankyrin-B affect multiple organ systems. Ankyrin-B mutations are commonly found in different ethnic patterns in 2-8% of human populations.

We have generated two knock in mouse strains to evaluate the physiological consequences of human ankyrin-B mutations and to obtain mechanistic insight into the role of ankyrin-B in disease pathogenesis. These transgenic animals bear either the L1622I variant, found in 6% of African Americans, or the R1788W mutation mentioned above, which is found in 0.2% of Caucasians. Consistent with previous findings, mutant mice show a cardiac phenotype characterized by reduced expression of ankyrin-B binding partners, arrhythmia, dilated cardiomyopathy, and lower heart rate. In addition, we found that ankyrin-B mutant mice display metabolic derangements at early ages, including abnormal oral glucose tolerance and insulin

secretion, and insulin insensitivity. Expression of mutant ankyrin-B causes reduction of ankyrin-B and IP3 receptor levels in pancreatic beta-cells, along with significant changes in the size of the pancreatic islets. Adding to the complexity of the syndrome, we also found that ankyrin-B is expressed in fat tissue, where the mutant animals show reduction in levels of ankyrin-B. Moreover, L1622I mutant mice exhibit increased accumulation of visceral and subscapular fat with bigger adipocytes, aging-associated obesity, and impaired hormone-stimulated lipolysis. Similarly, embryonic fibroblasts derived from mutant mice exhibit cell autonomous increased lipid accumulation following differentiation into adipocytes in culture.

In conclusion, Ankyrin-B syndrome is a recently recognized syndrome that affects the heart, pancreas and adipose tissue, and could potentially affect millions of people. Work underway focuses on additional metabolic and mechanistic characterizations, and additional analysis of human clinical data, all of which we expect will shed additional light into the pathophysiology of this condition.

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In vivo senescence in the Shwachman-Diamond syndrome pancreas.

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Loss of function mutations in the ancillary translation factor SBDS cause Shwachman-Diamond syndrome (SDS) resulting in severe exocrine pancreas pathology with acinar cell hypoplasia and fatty infiltration. SDS is one of a number of translation insufficiency disorders with shared features of poor growth, hematological anomalies and cancer predisposition but also possesses the distinguishing pancreatic phenotype. The translation burden of acinar cells is notable with their cued synthesis of over twenty digestive enzymes that are stored in zymogen granules. The targeted disruption of Sbd function in the murine pancreas results in markedly reduced organ size, perturbed ribosome profiles and loss of acinar cell markers and zymogen granules. We investigated how SDS-translation insufficiency results in this acinar cell failure.

In contrast to other organs affected in constitutive SDS mouse models, the severe hypoplasia of the pancreas was found not to be a result of apoptotic cell death. Ultrastructure analysis and an absence of immune infiltrate did not suggest prominent autophagy or necrosis, but cell morphology and acidic β -galactosidase activity indicated numerous senescent cells in the pancreas by 30 days of age. Transcript levels of genes commonly known to be involved in senescence pathways including osteonectin, fibronectin and genes of the innate immune response were elevated, but indicators of replicative and oxidative stress-induced senescence were not. Transcript analysis also revealed elevation of Tgfb1 and Cdkn2b (p15) consistent with cell cycle arrest. The downregulation of Myc was also noted and consistent with both cell cycle arrest and other models of acinar cell hypoplasia. A modest elevation of Trp53 (p53) transcript was also evident and was directly evaluated by breeding models to Trp53 deficient mouse strains resulting in resolution of gross pathology. However, patchy amylase immunostaining along with smaller cell size (a hallmark of translation deficiency), perturbed polysome profiles and apoptotic cells, identified by morphology and TUNEL staining, indicated that acinar cells remain dysfunctional. We conclude that the observed Tgf- β /Myc/p15 pathway prompts *in vivo* p53-mediated senescence and may provide tumor suppression capability for the SDS pancreas.

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Amylin Turnover and Toxicity in Pancreatic Cells Is Not Upregulated by Copper.*S. Singh¹, E. Lee¹, A. M. Jeremic¹; ¹Biological Sciences, The George Washington University, Washington, DC*

Amylin, a 37 amino acid peptide hormone, is co-secreted with insulin by the pancreatic beta cells and plays a role in regulating glucose homeostasis. Misfolding and subsequent intracellular accumulation and aggregation of human amylin is linked to mitochondrial dysfunction, oxidative stress and beta cell death, hallmarks of type 2 diabetes mellitus (T2DM). Similar to human amylin, increased serum concentrations of copper are found in diabetics. Although studies demonstrated that copper can bind to and upregulate amylin induced radical formation in cell free conditions, the extent to which this occurs in cells needs further clarification. Particularly, there is a lack of data on whether, following binding, copper can modulate amylin internalization and intracellular degradation, lack of which is associated with amylin aggregation and toxicity. Hence, the objective of this study was to determine the extent to which copper modulates amylin turnover and toxicity in pancreatic rat insulinoma beta cells (RIN m5F cells). To do so, we examined the effect of copper (1-20 μ M) on amylin internalization and intracellular trafficking using live confocal microscopy together with fluorescently labeled amylin and specific organelle markers. Colocalization analysis revealed that amylin internalized and trafficked to degradative compartments, autophagosomes and lysosomes, in beta cells. In contrast, amylin was largely absent from mitochondria and other organelles. Interestingly, addition of copper had no significant effect on intracellular amylin distribution or accumulation. Consistent with this finding, cell viability assays demonstrated a lack of stimulatory effect of copper on amylin toxicity. Taken together, these results suggest that binding of copper ions to amylin does not significantly contribute to amylin accumulation and toxicity in cells. In order to further elucidate the amylin-copper interaction, we tested the effect of amylin on copper turnover using a copper sensitive dye, Phen Green SK. Fluorescent spectroscopic analysis revealed that intracellular copper levels are increased, by ~20% relative to controls, in the presence of human amylin, indicating that copper can accumulate in cells as metalloprotein complex. Although it was previously shown that copper directly interacts with human amylin, our data does not support the model in which copper enhances its pro-oxidative and pro-apoptotic properties in cells. Further studies should clarify the biological significance of the stimulatory effect of amylin on intracellular copper accumulation and homeostasis.

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Nuclear receptor PPAR γ -regulated monoacylglycerol O-acyltransferase 1 (MGAT1) expression is responsible for the lipid accumulation in diet-induced hepatic steatosis.*Y. Lee^{1,2}, J-W. Kim^{1,2}; ¹Biochemistry and Molecular Biology, Yonsei University College of Medicine, Seoul, Korea, ²Brain Korea 21 Project for Medical Science, Seoul, Korea*

Recently, hepatic peroxisome proliferator-activated receptor (PPAR) γ has been implicated in hepatic lipid accumulation. We found that the C3H mouse strain does not express PPAR γ in the liver and, when subject to a high-fat diet, is resistant to hepatic steatosis, compared with C57BL/6 (B6) mice. Adenoviral PPAR γ 2 injection into B6 and C3H mice caused hepatic steatosis, and microarray analysis demonstrated that hepatic PPAR γ 2 expression is associated with genes involved in fatty acid transport and the triglyceride synthesis pathway. In particular, hepatic PPAR γ 2 expression significantly increased the expression of monoacylglycerol O-acyltransferase 1 (MGAT1). Promoter analysis by luciferase assay and electrophoretic mobility shift assay as well as chromatin immunoprecipitation assay revealed that PPAR γ 2 directly

regulates the MGAT1 promoter activity. The MGAT1 overexpression in cultured hepatocytes enhanced triglyceride synthesis without an increase of PPAR γ expression. Importantly, knockdown of MGAT1 in the liver significantly reduced hepatic steatosis in 12-wk-old high-fat-fed mice as well as ob/ob mice, accompanied by weight loss and improved glucose tolerance. These results suggest that the MGAT1 pathway induced by hepatic PPAR γ is critically important in the development of hepatic steatosis during diet-induced obesity.

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Gender Differences Following Attenuation of High Cholesterol-Induced Hepatotoxicity by Rutin in Rats.

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Background: Hyperlipidaemia has been implicated in atherosclerosis, a leading cause of death worldwide and accounting for almost 17 million deaths annually. Experimentally, high-cholesterol-diet (HCD) supplementation to rats impairs lipid metabolism resulting increase in blood and tissue lipid profile. Earlier gender studies established that females are more protected from hypercholesterolemia-related diseases such as CVD until menopause. Furthermore, meager information expressed the different gender response following HCD and the possible potential effect of antioxidant vitamins like rutin (RT) and ascorbic acid (AA) supplementation on experimentally-induced hypercholesterolemia in rats.

Objective: The present study was designed to investigate the potential beneficial effects of dietary RT and AA on HCD induced hepatotoxicity in male and female Wistar rats.

Methods: Thirty six of each sex of Wistar albino rats was used in this study. They were subdivided into six groups; control, RT, AA, HCD, RT+HCD and AA+HCD. Animals received freshly prepared experimental diets for 6 consecutive weeks. In hepatic cells nucleic acids, total protein, thiobarbituric acid reactive substances (TBARS), Glutathione (GSH), total cholesterol (TC) and triglycerides (TG) levels were estimated. Histopathological changes following HCD with or without both antioxidants were investigated.

Results: In hepatic cells GSH levels significantly decreased while TC, TG and TBARS concentrations were increased in HCD fed rats regardless of sex. The supplementation of RT and AA with HCD brought back these changes to normal in both gender groups. However, the correction of changes in all parameters showed more significant in female than male rats.

Conclusion: Present data suggest the gene and/or metabolic regulations by dietary cholesterol could interact with that of sex hormones and explains why males are more sensitive to dietary cholesterol. And also highlights the importance of dose fixing of nutritional supplements among males and females.

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BMP4 regulates podocyte injury in the diabetic nephropathy.

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It has been proposed that podocyte injury provides the important role for diabetic nephropathy (DN), however its pathological mechanism is not clear. We have shown that bone morphogenetic protein 4 (BMP4) signaling leads glomerular changes, which are characteristic of DN.

In order to analyze the molecular mechanism of podocyte injury, the effect of BMP4 was investigated in in vivo and in vitro cultured podocyte. We studied the BMP4 expression in streptozotocin (STZ)-induced diabetic mice. In order to examine its role in vivo, the induced-

Bmp4 transgenic mice (Bmp4 tgm) were generated by the tamoxifen-regulated Cre-loxP system. Finally diabetic Bmp4 heterozygous knockout mice (Bmp4+/-) were analyzed to investigate suppressant effect of Bmp4 in vivo.

Diabetic STZ mice represented the significant expression of Bmp4, which degrees were associated with the levels of glomerular matrix hyperplasia in STZ mice ($r = 0.927$, $p < 0.01$). Bmp4 tgm were indicated significant mesangial matrix expansion and thickening of glomerular basement membrane, which are characteristic pathological findings of DN. Albuminuria was dramatically increased in Bmp4 tgm compared with non-inducible mice. The Bmp4 tgm decreased both the expression of nephrin and the count of WT1 positive cells, both of them are specific markers of podocyte. The glomerular matrix hyperplasia progressed in accordance with the decreased number of WT1 positive cells. The number of podocyte also decreased inversely with an increase of the albuminuria. We investigated whether the reduction of Bmp4 expression improved the diabetic glomerular changes by using Bmp4+/- mice. The diabetic Bmp4+/- mice showed to improve the nephrin expression compared with the decreased nephrin expression in diabetic wild mice. Proteinuria also decreased in diabetic Bmp4+/- mice compared with diabetic wild mice. Finally we confirmed increased expression of cleaved caspase 3 in both STZ mice and Bmp4 tgm. Cultured podocyte showed the decreased expression of Nephrin by Bmp4 treatment in a dose dependent manner in vitro. Bmp4 treatment (50ng/ml) activated p38 expression and increased cleaved caspase 3 in cultured podocyte.

These data suggest that BMP4 plays an essential role for development of podocyte injury in DN.

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AKT and AMPK activation after high-fat and high-glucose in vitro treatment on prostate epithelial cells.

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Obesity is an increasing health problem worldwide. It has been proposed that prostate cancer development and prognosis is adversely affected by the comorbidities of obesity and diabetes mellitus, which are conditions associated with elevated levels of circulating fatty acids, hyperglycaemia and hyperinsulinaemia. AMPK is a cell energy-sensor kinase activated by metabolic stressors that reduces ATP-consuming functions and increases ATP-producing to restore cell energy homeostasis. Thus, AMPK is altered in obesity and cancer and currently is under investigation as a tool for treatment of these disorders. It was investigated the effects of exposure of nonmalignant human prostate epithelial cells to elevated levels of fatty acids and glucose on their proliferation, survival, AKT and AMPK activation. PNT1A cells were cultivated in RPMI medium containing palmitate (100 or 200 μ M) and/or glucose (450 mg/dl) during 24 or 48 hrs. MTS assay evaluated cell proliferation, TUNEL test measured dead cells and western blotting for phosphorylated forms of AMPK and AKT were preformed after cell treatments. After 24hrs of glucose and fatty acid treatment cell proliferation did not change but there was an increase in apoptosis. Increased cell proliferation was detected after 48hrs of treatment in PNT1A cells. Initially, in 24hrs of both high-fat and high-glucose exposure, there is an increase in AMPK and AKT activation. AMPK activated blocks protein synthesis and thus, cell proliferation analyzed by MTS is not increased in this period. Following 48hrs of treatment, cells became adapted to the high-energy environment and decrease AMPK activation, but maintained activated AKT levels. In this regard, inactivation of AMPK and increased AKT after 48hrs stimulates cell proliferation which is increased in this period. In conclusion, this research shows that high concentrations of fatty acids and also glucose stimulate proliferation of PNT1A cells by decreasing the activation of AMPK and increased activation of AKT. These data open

new perspectives for the elucidation of the molecular elements responsible for the positive association between obesity and prostate cancer.

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Biomarkers of oxidative stress in rat male reproductive organs under experimental diabetes and interference of melatonin treatment.

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Hyperglycemia and oxidative stress are major factors which impair organ physiology in diabetes. Reproductive dysfunction is a well recognized consequence of diabetes, but the oxidative stress involvement in male reproductive damage in this disease is poorly understood. The present study investigated the antioxidant system biomarkers response in rat prostate, testis and epididymis during early and late phases of streptozotocin (STZ)-induced diabetes. It also assessed the influence of treatment with low melatonin (MLT) doses on oxidative stress biomarkers and its interference in experimental diabetes. MLT was administered to male Wistar rats, from 5th week old to the end of experiment, in the drinking water (2.5µg/Kg b.w daily) and diabetes was induced at 13th week old by STZ (4.5mg/100g b.w., i.p.). The animals were killed in the 14th (short-term diabetes) and 21st (long-term diabetes) week old. The activities of oxidative stress biomarkers - catalase (CAT), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) - were quantified in blood, ventral prostate, testis and epididymal extracts using biochemical assays. The changes in antioxidant biomarkers activity were more pronounced in ventral prostate in comparison with other organs. GST activity in this gland increased in both short and long-term diabetes, but the early response involves increase in GPx and the late in CAT activities. The epididymis responded to short-term diabetes with increase in CAT activity, but no changes were detected at longer time. No alterations were observed in antioxidant enzymes activity of testis at both periods of diabetes whereas in the plasma it was observed an increase in GPx and decrease in GST activities only after two-month-diabetes. MLT treatment did not affect the activities of antioxidant enzymes in studied tissues except for an enhancement of GPx activity in the epididymis of control rats at short-term and for an inhibitory effect in CAT testis at long-term diabetes. The present study showed that testis are less susceptible to diabetes effects and it is probably related to the protection provided by the Sertoli cells in the seminiferous tubules. The GST proved to be a good marker of compensatory antioxidant defense in prostate corroborating our previous data on medium-term diabetes. Furthermore, MLT, even at low dosages, was able to normalize the enzymatic disorders in prostate and epididymis, which shows its effective antioxidant role.

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Expression of adrenoceptors on peripheral blood mononuclear cells in central obesity.

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Background and Aim: Sympathetic nervous system overactivity and a state of chronic immune activation are characteristics of central-obesity-related comorbidities, such as cardiovascular

diseases. Catecholamines (CA), adrenaline (AD) and noradrenaline (NA), regulate both metabolism and innate immune response through adrenoceptors (AR). Since immune cells and adipocytes are CA-producing cells we investigated the association between central obesity (CO), AR expression in peripheral blood mononuclear cells (PBMCs), CA and innate inflammatory markers in blood donors (BD).

Methods: We studied 63 BD (35 men and 28 women) considering waist circumference, plasmatic CA, blood monocyte subpopulations, being the latter investigated by flow cytometry. AR expression in PBMCs was evaluated by quantitative real-time PCR. Given that β 2-AR are the most expressed AR in these cells, all the population was studied for these receptors. We have also looked for α 1A, α 1B, α 1D, α 2A, α 2B, α 2C, β 1, and β 3 –AR mRNA expression in a subgroup of subjects with and without CO, which was defined by the International Diabetes Federation criteria.

Results: 89% of women and 60% of men were centrally obese. Centrally obese individuals showed a more proinflammatory monocyte pattern of the CD16+ cells: lower expression of CD14 ($p=0.025$) and lower cellular complexity ($p=0.014$). AD correlated positively with the number of CD16+ pro-inflammatory cells ($p=0.309$, $p=0.044$). CO was associated with lower β 2-AR expression in PBMCs ($p=0.002$). There were no significant differences between groups considering α 1A, α 1B, α 1D, α 2A, α 2C and β 3 –AR mRNA levels. α 2B and β 1-AR were not detected on PBMCs.

Conclusion: In our population, CO was related with a higher activation of innate immune response and with a lower β 2-adrenoceptor expression in PBMCs, which might be associated with a higher cardiovascular risk. We did not find differences in the expression of the other AR studied in PBMC, and α 2B and β 1-AR subtypes were undetected in these cells. The positive correlations between CA and pro-inflammatory monocytes highlight the role of catecholaminergic system in obesity and cardiovascular risk assessment.

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Modulation of adipocyte differentiation by omega-3 polyunsaturated fatty acids involves proteasome-dependent degradation of fatty acid synthase and other adipogenic proteins.

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We have evaluated the effects of three different omega-3 polyunsaturated fatty acids (PUFAs) - docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) on fat accumulation and expression of adipogenic and inflammatory markers using both 3T3-L1 pre-adipocytes and differentiated 3T3-L1 adipocytes. Our results indicate that omega-3 PUFAs have beneficial metabolic effects on adipose cells. They decrease expression of adipogenic transcription factors (PPAR γ and SREBP1) and fatty acid synthase through a proteasome mediated mechanism. Moreover, adipocytes do not undergo cell death with prolonged proteasome inhibition, which induces adipophilin, while decreasing perilipin expression. Omega-3 PUFAs induce adipophilin expression, while decreasing expression of leptin and GLUT4 at the mRNA level. Moreover, all three ω -3 PUFAs appear to induce expression of the proinflammatory TNF α cytokine, while at the same time decreasing NF κ B levels. While the effects of all three PUFAs are similar, they appear to differ in their relative potency. Therefore, further research is needed to elucidate their effects, in particular how omega-3 PUFAs trigger proteasome-dependent degradation of several adipogenic factors.

Lipids and Membrane Microdomains I

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A Role for Sphingomyelin-Rich Lipid Domains in the Accumulation of Phosphatidylinositol-4,5-Bisphosphate to the Cleavage Furrow during Cytokinesis.

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Cytokinesis is a crucial step in the creation of two daughter cells by the formation and ingression of the cleavage furrow. Here, we show that sphingomyelin (SM), one of the major sphingolipids in mammalian cells, is required for the localization of phosphatidylinositol-4,5-bisphosphate (PIP₂) to the cleavage furrow during cytokinesis. Real-time observation with a labeled SM-specific protein, lysenin, revealed that SM is concentrated in the outer leaflet of the furrow at the time of cytokinesis. Superresolution fluorescence microscopy analysis indicates a transbilayer colocalization between the SM-rich domains in the outer leaflet and PIP₂-rich domains in the inner leaflet of the plasma membrane. The depletion of SM disperses PIP₂ and inhibits the recruitment of the small GTPase RhoA to the cleavage furrow, leading to abnormal cytokinesis. These results suggest that the formation of SM-rich domains is required for the accumulation of PIP₂ to the cleavage furrow, which is a prerequisite for the proper translocation of RhoA and the progression of cytokinesis.

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Dissecting the role of PI4P in PI(4,5)P₂ synthesis at the plasma membrane.

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PI(4,5)P₂ is lipid with pivotal roles in plasma membrane (PM) organization and function. Through its interaction with a multitude of effector proteins, it organizes the cortical actin cytoskeleton and adhesion complexes, nucleates sites of clathrin-mediated endocytosis, maintains the activities of ion channels and transporters, facilitates regulated exocytosis and acts as a precursor for the ubiquitous PLC and PI3K signaling pathways. We recently showed that PI4P, the major intermediate lipid in PI(4,5)P₂ synthesis, is present in the PM where it is paradoxically mostly redundant for PI(4,5)P₂ synthesis. Instead, PI4P provides a metabolically distinct pool of polyanionic lipid that together with PI(4,5)P₂ regulates localization or activity of several effector proteins specifically at the PM (PMID 22722250). However, this raises the question as to how cells synthesize and maintain their functionally crucial PI(4,5)P₂ pools independently of PM PI4P. To address this question, we have used a novel PI4P biosensor derived from the Rab1 guanine nucleotide exchange factor SidM from *Legionella pneumophila*. This biosensor recognizes both the PM and Golgi PI4P pools in mammalian cells, which is a substantial improvement on previous PI4P biosensors. Chemically-inducible recruitment of PI4P phosphatase activity to either the Golgi or PM removes this sensor exclusively from the targeted organelle. Recruitment of a PI(4,5)P₂ 5-phosphatase to the Golgi is without effect, confirming the low abundance of this lipid in this organelle - whereas targeting the same enzyme to the PM

results in further recruitment of PI4P biosensor, owing to the conversion of PI(4,5)P₂ into PI4P. Treatment of cells with compounds that inhibit PI4KA activity show that the basal PI4P pool is rapidly (< 10 min) depleted, but that the pool provided by 5-phosphatase degraded PI(4,5)P₂ is more resistant. These data confirm the distinct metabolic profiles of the steady-state PI4P and PI(4,5)P₂ pools, and support a model whereby the two stages of PI(4,5)P₂ synthesis (namely 4- and 5-phosphorylation) are tightly coupled and do not produce substantial levels of PI4P intermediate.

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PI4P synthesis at the plasma membrane by PI 4-kinase III α and its impact on PI(4,5)P₂ dynamics and plasma membrane identity.

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Phosphoinositides are minor phospholipids but critical regulatory components of cellular membranes. PI4P, the most abundant mono-phosphorylated phosphoinositide, is mainly localized at the Golgi complex, in a subset of endosomes and in the plasma membrane. Regulation of this lipid, particularly at the plasma membrane, is critically important as it serves as a metabolic precursor of PI(4,5)P₂ and thus of several other signaling molecules derived from this phosphoinositide. Additionally, PI4P has signaling functions of its own. However, mechanisms that control this PI4P pool in cells of higher eukaryotes remain elusive because the enzyme thought to synthesize this PI4P pool, PI4KIII α , was reported to be localized in the ER. Here we show the presence of PI4KIII α at the plasma membrane and demonstrate that its targeting to this membrane is mediated by an evolutionarily conserved protein complex containing TTC7 and EFR3/rolling blackout (see accompanying poster by Baskin et al) (see also Baird et al. JCB 183:1061-74, 2008). Analysis of PI4KIII α KO cells derived from conditional PI4KIII α KO mice that we have generated revealed a profound reduction of total cellular PI4P (with a dramatic reduction of the PI4P signal at the plasma membrane) but surprisingly only a modest reduction of total cellular PI(4,5)P₂, due to robust upregulation of PI4P 5-kinases. In these cells, a major fraction of both PI4P 5-kinases and PI(4,5)P₂, which are typically restricted to the plasma membrane, were localized on intracellular membranes, along with several normally plasma membrane-resident proteins. Collectively, these results provide insight into the mechanisms leading to the generation of PI4P in the plasma membrane by PI4KIII α and reveal a major function of PI4KIII α in the definition of plasma membrane identity.

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Recruitment of PI 4-kinase III α to the plasma membrane is mediated by TTC7 and EFR3/Rolling blackout, a palmitoylated peripheral membrane protein.

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Phosphatidylinositol 4-phosphate (PI4P), the most abundant monophosphorylated phosphoinositide, is present in multiple cellular pools located on the Golgi apparatus, endosomes, and the plasma membrane. The plasma membrane pool of PI4P serves as a metabolic precursor to PI(4,5)P₂ and its downstream metabolites PI(3,4,5)P₃, IP₃, and diacylglycerol. Despite the importance of this PI4P pool in membrane biology, many questions remain about the control of its biosynthesis. Of the four mammalian PI 4-kinase isoforms, Type

III α (PI4KIII α) is thought to produce the bulk of this pool, in spite of the proposed localization of the enzyme in the ER, a localization that has been controversial. Here we show that EFR3/Rolling blackout and TTC7, homologs of yeast Efr3 and Ypp1 respectively, can recruit PI4KIII α to the plasma membrane in a complex that bears some similarity to but also differences from yeast PIK patches containing the PI 4-kinase Stt4. We also show that EFR3 is a peripheral membrane protein, in contrast to studies of its *Drosophila* homolog, rolling blackout, which was proposed to function as an integral membrane lipase. Moreover, we show that membrane targeting of mammalian EFR3 is mediated by palmitoylation of an N-terminal Cys-rich motif that is conserved in higher eukaryotes but absent from yeast. Collectively, our studies, combined with an analysis of PI4KIII α KO cells (see accompanying poster by Nakatsu et al.), provide insight into the mechanism of PI4P generation at the plasma membrane by PI4KIII α and more generally propose a role for PI4KIII α in the determination of plasma membrane identity. Work to probe the precise functions of EFR3 and TTC7 in PI4P synthesis and to identify additional members of the mammalian PI4KIII α /EFR3/TTC7 complex are ongoing.

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Phosphorylation of ACAP4 BAR domain regulates membrane tubulation.

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ACAP4 is an ARF6 GTPase-activating protein essential for EGF-elicited cell migration. Many ARF GAPs contain a BAR domain, a structure element to sense and elicit membrane curvature. Here, we show that ACAP4 contains a functional BAR domain and phosphorylation of BAR domain at Tyr34 is critical for EGF-elicited membrane remodeling. Domain structure analysis established that N-terminal region of ACAP4, named the BAR domain, regulates membrane curvature. EGF stimulation phosphorylates ACAP4 at Tyr-34 which subsequently promotes ACAP4 homodimer curvature. Phospho-mimicking mutant ACAP4 promotes its lipid binding activity and tubulation in vitro and facilitates the recruitment of ARF6 to the membrane ruffles of the leading edge during EGF stimulation. Persistent expression of non-phosphorylatable ACAP4 attenuates EGF-elicited cell migration. Thus, our study suggests a novel mechanism by which EGF-elicited phosphorylation of BAR domain controls ACAP4 molecular plasticity and plasma membrane dynamics during cell migration.

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Diverse membrane-deforming activities of F-BAR proteins.

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Subcellular membrane compartments are the hallmark feature of eukaryotic cells, but we do not understand how these compartments are sculpted into characteristic shapes that undergo dynamic remodeling and turnover. BAR domain proteins are critical for deforming subcellular membranes in the endocytic pathway, by forming a crescent-shaped dimer with varying degrees of curvature that binds to and bends the lipid bilayer. Diverse members of this protein family, including the shallow-curvature F-BAR subfamily, have adapted the BAR domain structure to specialized cellular roles, but the rules governing how these variants function to create different dynamic membrane geometries remain unknown. We have found that three neuronal *Drosophila* F-BAR proteins (Cip4, Syndapin, and Nwk) each generate different membrane tubules in cells and on purified giant unilamellar vesicles (GUVs). Remarkably, Nwk can generate both positive and negative membrane curvature, while Cip4 and Synd generate only positive curvature. We are testing the hypothesis that differential F-BAR domain assembly into

higher order structures, mediated by tip-to-tip or lateral interactions, determines the direction of membrane deformation. We have used molecular modeling together with electron microscopy and single particle analysis to determine the structural differences between diverse F-BAR domains, together with kinetic analysis of F-BAR assembly on GUVs to understand the self-assembly properties of these different proteins. Our results indicate that even related F-BAR domain proteins have unique membrane deforming properties that are specifically tailored to their cellular roles.

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Membrane-sculpting BAR and F-BAR domains generate stable lipid microdomains.

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Bin-Amphiphysin-Rvs (BAR) domain proteins are central regulators of cellular processes involving plasma membrane dynamics. BAR domains bind phosphoinositide-rich membranes with high affinity. Depending on the geometry of the lipid-binding interface they generate either plasma membrane protrusions or invaginations to promote cell migration or endocytosis, respectively, or stabilize planar membrane sheets. Here, we report that BAR domains also generate very stable lipid microdomains by inhibiting lateral diffusion of lipids in membranes by at least two orders of magnitude. This is a general feature of BAR domains because all budding yeast endocytic BAR and F-BAR domains, and the eisosomal BAR protein Lsp1 induced phosphoinositide-clustering and inhibited lipid diffusion, despite differences in lipid-specificities and interactions with the acyl-chain region of lipid bilayer. Furthermore, the BAR domain of Pinkbar, which stabilizes planar membrane sheets, efficiently inhibited lateral diffusion of phosphoinositides. Fluorescence-recovery-after photobleaching (FRAP) analyses revealed that Lsp1 displays comparable low diffusion rates in vitro and in vivo, suggesting that BAR domain proteins form also stable scaffolds and generate lipid microdomains in cells. Collectively, these results uncover an unexpected and conserved role of BAR superfamily proteins in regulating the dynamics of lipid molecules within membranes. Stable microdomains induced by BAR domain scaffolds and specific lipids can generate phase boundaries and diffusion barriers, which may have profound importance in diverse cellular processes including endocytosis.

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Membrane scalloping by the F-BAR protein Nervous Wreck.

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Proteins of the crescent-shaped BAR domain superfamily are important mediators of membrane deformation and act in a variety of cellular contexts to create membrane buds and tubules of defined geometry and lipid composition. Membrane binding by different BAR proteins has been shown to generate invaginating tubules, protrusive tubules, or flat regions of membrane. Here we have identified a new mode of membrane deformation, consisting of periodic scallops, mediated by the F-BAR family protein Nervous Wreck (Nwk). In a cell-based assay, both *Drosophila* Nwk and its mammalian homolog (FCHSD2) induce extensive actin cytoskeleton-dependent cellular protrusions, in contrast to the invaginating tubules formed by the related F-BAR proteins Cip4 and Syndapin. Using cryo-electron microscopy of purified protein on liposomes and thin-section electron microscopy of Nwk-expressing cells, we find that these protrusions are not smooth tubules like those generated by other F-BAR proteins, but instead

are composed of undulating membrane scallops, which at the micron scale can deform into structures of either net negative or net positive curvature. To further understand the activity of Nwk we performed structure-function analyses to identify key features of the protein critical to its membrane deforming function, both *in vitro* and *in vivo* at the *Drosophila* neuromuscular junction (NMJ). We show that a novel structural determinant located at the tips of the F-BAR dimer as well as the conventional convex F-BAR membrane-binding surface are required for Nwk activity *in vivo* and lipid binding *in vitro*. Furthermore we show that the activity of the F-BAR domain is intra-molecularly inhibited by C-terminal sequences *in vivo*. Our data uncover a novel membrane deforming activity for a BAR family protein, and suggest a mechanism by which highly ordered local alterations in membrane curvature can induce unexpected global changes in membrane shape.

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A novel mutation causing retinopathy and widespread abnormal membrane structures is linked to a small subunit of the serine palmitoyltransferase.

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Sphingolipids are crucial components of cellular membranes. Disruptions in sphingolipid metabolism has been implicated in both retinopathy and neurodegenerative diseases, suggesting a critical role of sphingolipid homeostasis in the health of the retina and the brain. We have identified a novel mouse mutant named *Stardust* (*sd*), which exhibits shiny flecks by indirect ophthalmoscopy, and photoreceptor degeneration, as well as deposition of ubiquitylated proteins and presence of aberrant membrane structures in both the retina and the brain. A missense mutation was identified in one of the small subunits of serine palmitoyltransferase (SPT), which is the first enzyme of the *de novo* biosynthetic pathway of ceramide, a molecule that serves as the backbone of all complex sphingolipids. Our preliminary results suggest that the *sd* mutation may cause hyperactivation of SPT, resulting in elevation of particular types of ceramide species. This mouse mutant thus provides a unique model to study how the regulation of sphingolipid homeostasis affects the health of the eye and the brain.

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In the erythrocyte membrane, cholesterol and membrane:cytoskeleton anchorage complexes oppositely regulate micrometric segregation of fluorescent lipid analogs.

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Fluorescent (BODIPY, *) analogs of glucosylceramide (GlcCer*), sphingomyelin (SM*) and phosphatidylcholine (PC*), inserted at tracer levels into the outer plasma membrane leaflet of living cells, spontaneously gather into structurally distinct (sub)micrometric domains, visible by confocal microscopy. Specific disappearance of SM* domains upon SM depletion suggests a link with endogenous lipids. We here address the mechanisms governing micrometric lipid* domains at 37°C on erythrocytes attached on poly-L-lysine, as a flat featureless living cell model, with high cholesterol content and strong membrane:cytoskeleton anchorage via non-redundant 4.1R and ankyrin complexes. We found that the three lipids* formed well-defined (sub)micrometric domains, organized into immobile assemblies of exchangeable constituents as shown by fluorescence recovery after photobleaching. Stretching by increasing poly-L-lysine

concentration and time of erythrocyte adhesion suppressed all domains, indicating repression by membrane tension. Minor cholesterol depletion preserved GlcCer* but suppressed SM* and PC* domains. Uncoupling of membrane:cytoskeleton anchorage at 4.1R complexes by PKC activation increased the abundance of GlcCer* and SM* but not PC* domains; uncoupling at ankyrin complexes in spherocytotic patients increased the abundance of SM* and PC* but not GlcCer* domains. Thus, clustering into micrometric domains exhibited differential dependence on the two anchorage complexes. This was confirmed by confocal microscopy: distinct submicrometric protein patches, respectively enriched in 4.1R (glycophorin C) and ankyrin complexes (CD47), differentially decorated the three types of lipid* domains. Taken together, these data suggest that the high cholesterol content of erythrocytes and the two complementary membrane:cytoskeleton anchorage complexes oppositely modulate clustering of fluorescent polar lipids into distinct types of (sub)micrometric domains. Lipid segregation into micrometric domains may be relevant for erythrocyte deformability, critical for squeezing into the narrow pores of spleen sinusoids >10.000 times in the cell lifespan.

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Influence of membrane cholesterol content on the activity of the sodium taurocholate cotransporting polypeptide.

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Introduction:

Previous studies have shown that canalicular liver plasma membranes contain microdomains defined by their resistance to solubilisation by detergents at cold temperature (Hepatology 49: 1673, 2009). These detergent resistant microdomains (DRMs) are enriched in cholesterol and ABC transporters involved in bile secretion. Cholesterol has been shown to regulate the activity of various membrane enzymes and variation of cholesterol concentration correlates with changes in membrane transport activity. In the present study we tested whether basolateral liver plasma membranes (bLPMs) also contain DRMS and we investigated the effect of membrane cholesterol content on the transport activity of the key organic anion transporter involved in bile formation: sodium taurocholate cotransporting polypeptide (Ntcp).

Methods:

bLPMs were extracted with either 1% Triton X-100 or 1% Lubrol-WX for 30 min on ice and subsequently submitted to flotation on sucrose step gradients. Gradient fractions were then used for Western blotting and lipid composition analysis. Ntcp was expressed with the baculovirus system in Sf9 insect cells. Infected cells were loaded with cholesterol by incubation with 1mM cholesterol@RAMEB complex for 30 min at 37°C. After cholesterol loading, radiolabeled taurocholate uptake assay were performed with cells or isolated Sf9 cell membrane vesicles and transport activity was compared with control condition (no cholesterol loading).

Results:

Extraction with both detergents generated DRMs containing the raft marker proteins flotillin-1 and flotillin-2. These conventional markers for microdomains remained mainly in the soluble phase indicating that they are only partially associated with microdomains in rat bLPM. Ntcp was mostly soluble in Triton X-100 whereas after extraction with Lubrol-WX Ntcp was mainly found in the DRMs. Taurocholate uptake assays performed in cells show a high transport activity in absence of cholesterol, in cholesterol loaded cells the transport activity is dramatically reduced. Taurocholate uptake assays performed in vesicles show lower transport activity in absence of cholesterol compared to cell experiments however the inhibition of transport activity in presence of cholesterol is still observed.

Conclusions:

We were able to show that bLPMS contain two different types of microdomains: Triton X-100 and Lubrol-WX microdomains. We have demonstrated that Ntcp transport activity is dramatically reduced in presence of cholesterol hence suggesting that localisation of Ntcp inside or outside of cholesterol rich microdomains might have a major impact on the regulation of transport activity.

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Cholesterol transbilayer distribution in mammalian cells.

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Plasma membrane (PM) bilayer asymmetry is a ubiquitous feature of eukaryotic cells involved in maintaining cellular homeostasis. Both proteins and lipids are transversely asymmetric in the PM. Sterols are a major constituent in eukaryotic PM representing about 30% of total PM lipid. In model membranes, cholesterol transbilayer movement is rapid and spontaneous. This is in contrast to phospholipids, which require energy dependent flip-flop transporters. Curiously, recent studies with fluorescent cholesterol analogues suggest that sterols are enriched in the inner leaflet of the PM. It is however not known whether native cholesterol shares this transverse asymmetry. In addition, cholesterol is heterogeneously distributed laterally in the plane of the PM; it is enriched in various dynamic assemblies of lipids and proteins, known as lipid rafts. Lipid raft formation also requires sphingomyelin, a phospholipid almost exclusively resided in the outer leaflet of the PM. If cholesterol is enriched in the inner leaflet of the PM, similar to its fluorescent analogues, the lipid raft concept would need to be substantially updated.

The aim of this study is to analyze mammalian PM cholesterol transbilayer distribution in order to investigate mechanisms and functions of this asymmetry. For this, we developed a protocol that can analyze cholesterol in a leaflet-specific manner. Specifically, we show that, at 37°C, trace cholesterol in large unilamellar vesicles (LUV) can be 100% extracted by methyl- β -cyclodextrin (MCD), consistent with rapid cholesterol flip-flop between bilayers. However, at 0°C, MCD can only extract 50% of the cholesterol from LUVs. The process of cholesterol extraction by MCD itself is not temperature-sensitive and LUV remains intact. This suggests cholesterol flip-flop between leaflets can be effectively stopped or drastically reduced at 0°C, which allows cholesterol extraction exclusively from the outer leaflet of LUVs.

We then devised a donor/acceptor exchange protocol using LUVs containing 20% cholesterol. Low concentration of β -cyclodextrin was used as shuttle to allow cholesterol exchange between donor LUV, labelled with ³H-cholesterol, and excess acceptor LUV. We found that ³H-cholesterol in the donor LUVs can be 100% transferred to acceptor LUV membranes at 37°C. However, at 0°C only 50% donor cholesterol appears in acceptor LUV. We conclude that we have established a method to analyze leaflet-specific distribution of cholesterol. We plan to apply this method to PM vesicles, red blood cells, and eventually the PM of live nucleated cells.

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Novel function of sphingolipid-enriched compartments ("sphingosomes") in vesicular transport, exosome formation, and ciliogenesis.

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Until recently, the sphingolipid ceramide has been regarded as a metabolic precursor or a cell signaling factor inducing growth arrest and apoptosis. Studies in our laboratory have shown for the first time that ceramide-enriched compartments termed "sphingosomes" constitute lipid platforms for the assembly of protein complexes and scaffolds in cell polarity, exosome

formation/secretion, and ciliogenesis. The unique biophysical properties of ceramide based on the chain length and saturation of the fatty acid linked to sphingosine leads to the formation of ceramide microdomains in the cell membrane and the endosome. Accordingly, we have found that specific ceramides, in particular C16, C18 and C24:1 ceramide are enriched in cell membrane and endosome-derived compartments. These compartments include a novel “apical ceramide-enriched compartment” (ACEC) at the base of the primary cilium and ceramide-enriched lipid vesicles generated in multivesicular endosomes (MVEs) and secreted to the medium as exosomes. Our studies suggest that these sphingolipid-enriched intra- and extra/intercellular compartments termed “sphingosomes” are critically involved in the etiology of Alzheimer’s disease and ciliopathies caused by aberrant ciliogenesis. Central to sphingosomes is the association of ceramide with atypical PKC (i.e. PKCzeta) and its interaction with proteins regulating vesicle transport (i.e. Rab11a) and cell polarity (i.e. Cdc42) in a “sphingolipid-induced protein scaffold” (SLIPS). Surprisingly, these sphingosome-associated SLIPS appear to be key regulators for microtubule nucleation and acetylation important for cell polarity and primary cilium formation. In summary, our studies reveal a previously unknown function of ceramide in the spatially organized regulation of vesicular transport and the cytoskeleton by sphingosomes. This work is supported by the NSF (1121579) and NIH (R01AG034389).

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Correlative light and electron microscopy (CLEM) with sub-micrometer alignment precision for cryo specimens: revisiting caveolae.

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Electron cryo tomography of vitrified cells offers the opportunity to analyze molecular structures in three-dimensions in their native cellular context. However, precisely locating a specific event of interest inside a frozen-hydrated cell has remained extremely challenging. The work presented here shows that by combining correlative light and electron microscopy (CLEM) approaches (1, 2); it is possible to achieve a high correlation precision between LM and EM of intact cells at cryogenic temperatures.

The positions of fluorescently tagged proteins are first recorded in cells grown on dedicated support grids using fluorescent live cell imaging. Following plunge-freezing of cells, we re-examine the specimen by cryo fluorescence microscopy using a dedicated cryo stage (3). The positions of electron dense Fluospheres on the support film are then used as markers to establish a common coordinate system of sub-micrometer precision between medium magnification EM grid maps and externally acquired fluorescent maps, thus giving each carbon hole a unique ‘address’. Next, electron cryo-microscopy/tomographic data sets are acquired on desired positions based on the localization information for the fluorescently tagged proteins.

We exemplify the use of this correlative pipeline for the localization of caveolar protein coats. This allows us to analyse the molecular organisation of fluorescently tagged cellular vesicles at high resolution and to define the ultrastructure of their surrounding native context.

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Cavins and Caveolae.

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Caveolae constitute up to 20% of the surface area of continuous microvascular endothelia in many mammalian tissues, and are thought to mediate transcytosis across endothelial cells. How morphologically uniform caveolae function in endothelia that have different permeability properties is not clear. The cavin proteins are important components of caveolae, and are expressed at varied amounts in different tissues. We have investigated this in detail using biochemical, electron microscopy and fluorescence microscopy methods. Our data provide direct evidence for structural and functional heterogeneity between caveolae in different capillary beds.

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Intra membrane surface flow in response to protein induced spontaneous curvature.

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Lipid bilayers are unique in their biophysical properties. They are fluid in-plane and solid in bending. While traditional models use the Helfrich approach to model the elastic deformations of the bilayer membrane, these approaches only provide insight into the equilibrium configurations of the elastic deformations. But many membrane deformations are dynamic—viscoelastic—and can occur in response to protein binding. We have developed a model that accounts for the viscoelastic behaviour of the membrane and captures the flow of lipids on the surface of the membrane in response to deformations and/or surface pressure gradients.

An important process driving membrane deformations is the binding of proteins to the membrane surface. We use the viscoelastic model of the bilayer membrane to capture the dynamics of membrane shape change in response to protein binding. The binding of proteins to the membrane is modelled as a kinetic process, and the spontaneous curvature of the membrane is assumed to be proportional to the surface density of the proteins in that region. Numerical simulations show that binding of proteins changes the shape of the membrane and induces lipid flow at the boundaries resulting from the increase in surface area of the membrane. Additionally, the surface pressure field evolves from a uniform distribution to an inhomogeneous distribution. Regions with higher protein concentration also have a lower surface pressure. Together, these results capture the dynamics of lipid flow and surface evolution in response to protein binding.

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Low PIP₂ molar fractions induce nanometer size clustering in giant unilamellar vesicles containing POPC.

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One of the leading causes of death in the world is cancer. This has propagated expanding areas of research in pathways involved in cancer cell signaling. Phosphatidylinositol (4,5) bisphosphate (PIP₂) is a key lipid signaling molecule found on the inner leaflet of the cell membrane. Polyphosphoinositides signaling play an important role in regulation of many cell processes such as cell survival, proliferation, migration, and apoptosis. Phosphoinositides regulate signal transduction by providing binding or docking sites for intracellular proteins. In order to perform these functions, it has been suggested that PIP₂ must be able to form localized clusters within the cell membrane.

In this study, single point fluorescence correlation spectroscopy and brightness analysis of various BODIPY-labeled PIP₂ was utilized to detect the presence of clusters in the membrane of giant unilamellar vesicles (GUVs) consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). The number of freely diffusing fluorescent BODIPY molecules in the membrane was calculated and we found that in GUVs containing various amounts of labeled PIP₂ had significantly lower number of fluorescent molecules than in GUVs made with the control BODIPY labeled hexadecyl phosphatidylcholine (BODIPY-HPC). Also, an increase in brightness of the labeled PIP₂ particles with increasing labeled PIP₂ molar fraction was noted. These results show the existence of PIP₂ enriched clusters in the liquid disordered phase of GUVs that are smaller than the resolution limit of the fluorescent microscope. Using LAURDAN generalized polarization function, we demonstrated that the formation of these clusters is not due to the presence of the BODIPY moiety on the lipids. In addition, we report for the first time the presence of a previously uncharacterized hypsochromic shift of the fluorescence for the BODIPY labeled lipids that we attributed to BODIPY dimers. Finally, by adding cholesterol to the GUVs, we showed that the BODIPY dimers tend to favor the liquid ordered phase.

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The signaling lipid phosphatidylinositol 3,5 bis phosphate serves as a landmark for the TORC1 pathway.

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The Target Of Rapamycin (TOR) is a Ser/Thr protein kinase that functions in multiple pathways and forms two distinct complexes, TORC1 and TORC2. TORC1 is a central regulator of cell growth and metabolism. In mammals, in response to amino acids, TORC1 moves to the lysosome membrane where it is activated. However, it has not been understood how mTORC1 or its activators are targeted to the lysosome membrane, and the significance of this localization is not yet known. Here we show that in the yeast *Saccharomyces cerevisiae* the signaling lipid phosphatidylinositol 3,5 bis phosphate (PtdIns(3,5)P₂), plays a crucial role in the recruitment of TORC1 to the endosomal membrane. We show that Kog1, a yeast homologue of Raptor and a major regulator of TORC1, binds directly to PtdIns(3,5)P₂. Moreover, PtdIns(3,5)P₂ is required for the proper localization of TORC1 to the yeast vacuole/endosomes. In addition, yeast

mutants with low levels of PtdIns(3,5)₂ display reduced TORC1 function. Furthermore, we found that a major substrate of TORC1 localizes on the vacuole membrane via binding PtdIns(3,5)P₂. Together, these studies reveal that PtdIns(3,5)P₂ is required for TORC1 function via its role in recruitment of TORC1 to the vacuole membrane, and establish a novel link between the PtdIns(3,5)P₂ and TORC1 signaling pathways. In addition, the identification of Kog1 along with other novel unexpected candidates predicts that functions of PtdIns(3,5)P₂ extend beyond pathways currently known to be regulated by this lipid.

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A role for the PLD1 pathway in autophagosomal membrane dynamics and autophagy modulation.

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Macroautophagy (simply referred to as autophagy) is an essential homeostatic process that entails the biogenesis of autophagosomes (APs), which sequester cytoplasmic substrates and undergo a maturation process before ultimately fusing with lysosomes, where the degradation of sequestered material occurs. While the autophagy process involves major alterations in membrane architecture, the lipid composition of the AP and changes underlying autophagosomal membrane dynamics are largely undetermined. We have previously reported that phospholipase D1 (PLD1), an enzyme producing bioactive lipid phosphatidic acid (PA), acts downstream of class III PI 3-kinase Vps34 during starvation-induced autophagy and positively regulates autophagy at both the biogenesis and maturation steps. However, the precise molecular mechanisms underlying the autophagic actions of PA and the specific role of this lipid in regulating mTOR in the context of autophagy are unknown. Here we tested the role of PLD1 in the acute amino acid activation of mTORC1 signaling following nutrient deprivation using a combination of pharmacological and genetic tools. Additionally, to better understand the role of PA in AP maturation, we performed cell-free heterotypic fusion assays using organelles purified from wild-type and PLD1-deficient mice. We found a decrease in fusion efficacy between PLD1-deficient APs and purified endosomes and lysosomes. A lipidomic analysis of these same organelles revealed a variety of lipid changes, which may contribute to the fusion defect phenotypes observed in the absence of PLD1. Overall, our studies clarify the lipid-based mechanisms controlling AP membrane dynamics and provide important clues on how PA intersects with the metabolism of other lipid families in the context of nutrient deprivation.

Establishing and Maintaining Organelle Structure I

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Actin distribution in HepG2 cell-spheroids: A fluorescence microscopic study/

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HepG2 cells, human hepatocellular carcinoma cell line, are very popular for the studies of normal liver cells since they demonstrate numerous functions of normal human hepatocytes. Studies are continuing to produce a cell type which more resembles normal liver cells in 3D

environmental conditions. HepG2 cells tend to form spheroids in cultures instead of spreading over the surfaces. The current study aimed to investigate actin distribution in these spheroids. HepG2 cells (ATCC), were seeded on glass coverslips. They were grown in Minimum Essential Medium (ATCC) which contain 10% fetal serum bovine. Cells were labeled with fluorescent phallotoxins (Invitrogen). Labeling of the cells at the beginning of the formation of spheroids were compared with the labeling intensity of the cells which are seven days old after the formation of spheroid. Axioscope fluorescence microscope and Eclipse 90i Nikon confocal microscope were used for comparison of the location of actin. Antibodies which are specific for Golgi, mitochondria and endoplasmic reticulum were also used to investigate the changes of these organelle distribution during spheroid formation.

Results demonstrated that actin especially located on the cell membrane, between the adjacent cells in spheroids. Organells were also increasing during aggregate formation. There were no significant difference between the labeling density of the cells at the center of the spheroids and the cells which were located peripherally.

It has been concluded that spheroid formation stimulates adhesion between cells. Actin accumulation on cell plasma membrane is probably a sign of cell junction formation between the cells. More studies need to determine if the location of actin can also be a sign of newly formed bile ductuli between the cells as shown in different 3D culture models.

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SAS-6 coiled coil structure and interaction with SAS-5 suggests a mutual regulation in centriole assembly.

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The centriole is a conserved microtubule-based organelle essential for both centrosome formation and cilium biogenesis. Five centriolar proteins have been identified in *Caenorhabditis elegans* and their homologues in other species have also been reported. Two of them, SAS-5 and SAS-6, physically interact with each other and are codependent for their targeting to procentrioles. However, it remains unclear how these two proteins interact at the molecular level and why the *C. elegans* centriole has a unique central tube that is absent in non-nematode centrioles. Here, we demonstrate that the SAS-5 C-terminal domain (CTD) specifically binds to the central region of the SAS-6 coiled coil. To further investigate their interaction, we have solved the crystal structure of the SAS-6 coiled-coil domain (CCD, residues 248-410) and established that the association of the SAS-6 CCD and the SAS-5 CTD is mediated by synergistic hydrophobic and electrostatic interactions. The crystal structure also shows a periodic charge pattern along the SAS-6 CCD which, in the absence of SAS-5, gives rise to an anti-parallel tetramer of its CCD. Electron microscopy studies of the SAS-5/SAS-6 complex suggest that the central tube of *C. elegans* centrioles is formed by SAS-5 circularly arranged on SAS-6; SAS-5 alone forms aggregates. Overall our findings establish the molecular basis of the specific interaction between SAS-5 and SAS-6, and suggest that both proteins individually adopt a self-associated conformation that is disrupted upon the formation of the hetero-complex to facilitate the correct assembly of the nine-fold symmetric centriole.

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Mechanisms of Tubular Recycling Endosome Biogenesis.

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Endocytic recycling is a critical transport step that regulates the return of internalized receptors and membrane back to the cell surface. Over the last decade, our laboratory has focused on the function of the C-terminal Eps15 homology domain-containing (EHD) protein, EHD1. We and others have demonstrated that EHD1 is an ATPase that localizes to tubular recycling endosomes and regulates transport from the endocytic recycling compartment to the plasma membrane. However, the composition and function of these tubular endosomes to which EHD1 localizes has not been studied extensively, and their mode of biogenesis remains unknown. We have identified the molecular and atomic basis for the interaction between the EHD1 EH-domain and proteins containing the tripeptide asparagine-proline-phenylalanine (NPF), and we have identified a number of new EHD1 interaction partners that regulate endocytic events. One such protein is known as MICAL-L1, which associates with membranes through a C-terminal coiled-coil and recruits both EHD1 and Rab8a to the tubular membranes. To gain insight into the mode by which MICAL-L1 associates with membranes, we purified the coiled-coil and found that it is only one of a handful of proteins that selectively interacts with phosphatidic acid (PA). We have also discovered a novel interaction between a proline-rich domain of MICAL-L1 and the SH3 domain of the BAR-domain-containing protein, Syndapin2. Similar to MICAL-L1, Syndapin2 too showed a selective binding to PA. To determine whether PA comprises recycling tubule membrane in cells, we used a GFP-tagged version of the well-characterized yeast PA-binding protein, Spo20p. Indeed, PA could be identified as part of MICAL-L1-decorated tubules. Moreover, phospholipase D inhibitors that decrease PA synthesis severely impair the recycling of transferrin to the plasma membrane. Our data are consistent with a novel model by which PA recruits MICAL-L1 and Syndapin2 to membranes and facilitates the nucleation and biogenesis of recycling tubules that are required for optimal endocytic recycling.

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Identifying the members of the VPS16B and VPS33B complex in mammalian cells.

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Platelets are small blood cells that initiate and coordinate blood clotting at wound sites, where they adhere, aggregate and secrete an assortment of molecules in the process of blood clotting. Platelets contain specialized granules that secrete their contents upon platelet activation and are required for platelet function. The two distinct types of platelet granules (alpha-granules and dense-granules), originate by non-redundant developmental pathways. We have shown that vacuolar protein sorting protein 33B (VPS33B), a member of the Sec1/Munc18 protein family predicted to interact with SNAP (Soluble NSF Attachment Protein) receptor (SNARE) proteins to facilitate docking and fusion of intracellular vesicles, is required for alpha-granule biogenesis. Recently we have identified another protein, VPS16B, which interacts with VPS33B. Our laboratory has also shown that VPS16B is required for platelet alpha-granule biogenesis. Using blue native gel analysis, we have determined that the VPS33B-VPS16B subunits exist as part of larger complexes (480 kDa and 720 kDa). My research entailed identifying additional components of the VPS33B-VPS16B complex and characterizing the role of these interactions in platelet alpha-granule formation. The identification of potential interacting proteins was done

through yeast hybrid assays combined with affinity purification and mass-spectrometry. Results from a Yeast-3-Hybrid assay using VPS16B as bait and VPS33B as the bridge indicated several interesting candidates that function within the Golgi-endosome trafficking pathway, such as COG1/5 (Conserved Oligomeric Golgi complex protein) and VPS52 (part of Golgi associated retrograde protein (GARP) complex). Further experiments are currently underway to confirm these interactions. Preliminary co-immunoprecipitation results indicate that VPS52 interacts with VPS16B in the 720kDa complex. Interestingly, blue native gel analysis of purified VPS33B-VPS16B complexes showed that the two proteins are capable of forming multimeric complexes by themselves (240 kDa and 480kDa). Further studies are currently underway to investigate the structure and function of these large complexes in alpha-granule biogenesis.

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Vacuole Size Scaling in Budding Yeast is Maintained by a Balance between Synthesis and Inheritance.

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Organelle size, including that of lysosomes, impacts function and is therefore regulated by the cell. However, relatively little is known about how lysosome size is kept in balance with the overall cell size. To address this question, we have developed a computational image analysis method to quantitatively measure the size of budding yeast vacuoles/lysosomes. Wild-type yeast show characteristic scaling trends when comparing vacuole and cell size. These trends are altered in membrane trafficking and inheritance mutants, showing that these are key pathways in determining vacuole size. The mutant *vac8Δ* is defective in normal inheritance of the vacuole from the mother to the bud during the cell cycle. Interestingly, even though daughters begin with smaller vacuoles, the population as a whole shows increased vacuole size scaling. Timecourse analysis reveals that vacuole is continuously generated in *vac8Δ* at rates comparable to wild-type despite the defect in delivery to the bud. Repeated cell cycles leads to accumulation of the generated vacuole in the mother and an age-dependence of vacuole-to-cell size scaling. These results are consistent with a model where wild-type organelle size scaling arises passively from consistent cell and vacuole growth patterns rather than through active regulatory mechanisms. Pulse-chase fluorescence labeling will be applied to determine the contributions of de novo synthesis and inheritance to determining vacuole size.

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***Saccharomyces cerevisiae* Env7 is a palmitoylated serine/threonine kinase 16 (STK16)-related protein kinase and negatively regulates organelle fusion at the lysosomal vacuole.**

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Membrane fusion depends on conserved components and is responsible for organelle biogenesis and vesicular trafficking. Yeast vacuoles are dynamic structures analogous to mammalian lysosomes and serve as a model for membrane fusion and fission. Here we report that Env7 is a novel yeast palmitoylated protein kinase ortholog that negatively regulates vacuolar membrane fusion. Microscopic and biochemical studies confirm localization of tagged Env7 at vacuolar membrane via palmitoylation of N-terminal cysteine residues. Using custom prepared polyclonal anti-Env7 antibodies, we also show that wild-type Env7 is endogenously expressed at detectable levels and localized to vacuolar membrane. *in vitro* kinase assays of Env7 expressed in yeast as well as *E. coli* establish Env7 as a protein-kinase capable of autophosphorylation and phosphorylation of exogenous substrates. Site-directed mutagenesis

of Env7 'APE' motif Glu269 residue to Alanine (E269A) results in an unstable kinase-dead allele; Env7-E269A is stabilized and redistributed to detergent resistant fraction by interruption of proteasome system *in vivo*. Palmitoylation-deficient Cys13-15Ser mutant Env7 is cytoplasmic and lacks kinase activity suggesting that membrane association is necessary for Env7 kinase activity. Microscopic studies establish that *env7Δ* is defective in maintaining fragmented vacuoles during hyperosmotic response and in buds. *ENV7* function is not redundant with similar role of vacuolar membrane kinase Yck3 as the two do not share a common substrate, and *ENV7* is not a suppressor of *yck3Δ*. Bayesian phylogenetic analyses strongly support *ENV7* as an ortholog of human STK16, a Golgi protein kinase with undefined function. We propose that Env7 function in fusion/fission dynamics may be conserved within the endomembrane system and regulated by interplay of post-translational modifications.

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Lysosome tubules represent a more motile lysosome population and require the Arl8b and Rab7 GTPases for tubulation.

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Lysosomes are essential organelles involved in many cellular functions including the breakdown of endocytic and biosynthetic cargo, pathogen killing, autophagy and even membrane repair. In most cells, lysosomes typically appear as small, punctate structures under the fluorescence microscope. By contrast, LPS-activated macrophages possess strikingly tubular lysosomes (TLs). TLs are suggested to play a role in phagosome maturation and retention of fluid-phase endocytic uptake in activated macrophages. In addition, LPS-activated dendritic cells form tubular MHC-II compartments, which are lysosome-related organelles and that may be involved in antigen presentation. However, remarkably little else is known about how TLs form and function. Therefore, we have initiated pioneering studies on the molecular requirements for lysosome tubulation in macrophage cell lines. Our work confirmed the requirement for microtubules as a template necessary for tubulation, along with both dynein and kinesin microtubule-dependent motors. In addition, we identified the first molecular components needed for lysosome tubulation; TL biogenesis required the concerted action of the Arl8b GTPase, along with its effector SKIP, a kinesin-adaptor protein, and the Rab7 GTPase, along with its effectors RILP and FYCO1, which are adaptor proteins for dynein and kinesin, respectively. Most importantly, we observed that TLs are highly dynamic structures that are almost always moving, whereas punctate lysosomes are conspicuously more static. We postulate that the more dynamic nature of TLs will lead to higher rates of endocytic content acquisition and lysosomal content exchange, which may have consequences for antigen delivery, sharing and processing within the lysosomal population.

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Rab11FIP1 interacts with the BLOC-1 complex to retrieve melanogenic proteins from the recycling pathway and a dominant negative mutation in *RAB11FIP1* causes Hermansky-Pudlak Syndrome Type 10 (HPS-10).

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Hermansky-Pudlak Syndrome (HPS) is a genetically heterogeneous disorder of lysosome-related organelle (LRO) biogenesis and is characterized by oculocutaneous albinism and a bleeding diathesis. There are currently 9 known genes that cause HPS; all of whose protein

products function in the biogenesis of LROs. The Biogenesis of Lysosome related Organelle Complex 1 (BLOC-1) contains 8 subunits but relatively little is known about the intracellular function of the complex, although a role in endosomal protein sorting has been suggested. Using his-tagged BLOC-1 subunits expressed in HEK293 cells and mass spectroscopy, we discovered that Rab11FIP1 is a novel interacting protein of the BLOC-1 complex. *RAB11FIP1* encodes a Rab11a interacting protein that homo-dimerizes to interact with Rab11a. A yeast-2-hybrid assay showed that the Dysbindin subunit of BLOC-1 directly interacts with Rab11FIP1; this was confirmed by co-immunoprecipitation and confocal immunofluorescence microscopy in melanocytes. Here we report a girl who had previously been screened for mutations in HPS-1 through HPS-6 and all the genes encoding the BLOC-1 complex. No mutations were found, although the patient had typical signs and symptoms of HPS and a cellular phenotype mimicking that of BLOC-1, i.e., increased plasma membrane cycling and endosomal accumulation of a melanogenic protein, TYRP1. Whole exome sequencing revealed a *de novo* heterozygous frameshift mutation in *RAB11FIP1*. The short protein fragment from this allele was expressed and interacted with the full-length protein, resulting in a dominant negative effect. Known cargos of the BLOC-1 complex in melanocytes are TYRP1 and ATP7A. How these cargos traffic to LROs was unknown, but we discovered that GFP-TYRP1 traffics to the plasma membrane, is endocytosed and only then directed to LROs. We demonstrated that TYRP1 interacts with the AP-1, AP-2 and AP-3 complexes, allowing this trafficking to occur. ATP7A, however, appears to traffic directly to endocytic vesicles, where Rab11FIP1 and the BLOC-1 complex are required for retrieval to LROs. Taken together, these data suggest a function of the BLOC-1 complex in retarding protein recycling by forming a physical brake between early endosomes (through the BLOC-1 interactor, Syntaxin-13) and recycling endosomes (through the BLOC-1 interactor, Rab11FIP1). This would allow more time for proteins to be retrieved from the endosomal compartment (by the AP-3 complex) and directed to LROs.

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Establishing *Drosophila* S2 cells as a system for genetic studies of exosome/microvesicle biogenesis.

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Animal cells secrete small, membrane-bound vesicles that have the same topology as the cell. These vesicles, termed exosomes/microvesicles (EMVs), mediate cellular signaling events with their capability to shuttle proteins, lipids, and RNAs among cells. EMVs are implicated in a wide variety of both normal biological processes and pathological conditions. However, despite their biomedical importance, little is known about the factors involved in EMV biogenesis. *Drosophila* S2 cells have emerged as an important experimental system for understanding basic cell biological processes, and here we report their use as a system to study EMV biogenesis. Using S2 cells that express two EMV cargo proteins, CD63-GFP and AcylTyA-GFP, we screened a variety of candidate genes for their roles in EMV biogenesis. Our results indicate that CD63-GFP budding is inhibited by the actin network, is independent of the endocytosis process, is ESCRT-independent, and is impaired by the depletion of Rab1, Rab6, and Fwd (a *Drosophila* PI4K) proteins. Similar results were also seen for the budding of AcylTyA-GFP. Taken together, these observations establish the usefulness of *Drosophila* S2 cells as a system for studying EMV biogenesis and provide novel insights on the EMV biogenesis pathway.

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Type II phosphatidylinositol 4-kinase regulates trafficking of secretory granule proteins in *Drosophila*.

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Type II phosphatidylinositol 4-kinase (PI4KII) is responsible for production of the lipid phosphatidylinositol 4-phosphate (PI4P), a crucial resident of Golgi membranes that regulates membrane trafficking events such as secretion. PI4KII function is required for normal development of the *Drosophila* larval salivary gland. In PI4KII mutants, mucin-containing glue granules were considerably smaller than in wild type. Furthermore, PI4KII formed dynamic tubular networks along microtubules. Portions of these tubules colocalized with late endosomes and lysosomes marked with YFP-Rab7 and GFP-LAMP, suggesting these PI4KII positive tubules were dynamic endosomal structures. In PI4KII mutants, these PI4KII positive tubular structures were absent and enlarged lysotracker positive endosomes were observed instead. These enlarged acidic late endosomes/lysosomes contained mucin cargo proteins as well as the glue granule associated SNARE SNAP24. Our data thus far suggested that PI4KII function is required for the proper trafficking of granule proteins, where mistrafficked granule proteins accumulated in the late endosome. To further examine the role of PI4KII in granule biogenesis, we employed a reverse genetic screen to identify potential genetic interactions using publicly available transgenic RNAi lines. A selection of candidate genes including Rabs, SNAREs, synaptotagmins, and BAR domain containing proteins were screened. Interestingly, RNAi directed against early endosome associated Rab5 resulted in a more severe phenotype, where the salivary glands were immature and granule protein expression was scarce. Although PI4KII partially colocalized with Rab7, Rab7 RNAi showed no changes in granule size. In contrast, early endosome associated SNARE Syntaxin13 (Syx13) RNAi generated a similar phenotype to PI4KII mutants. Our screen results suggest that PI4KII activity may affect how proteins are trafficked from the early endosome, leading to potential defects in vesicular homotypic fusion facilitated by the Syx13 SNARE complex.

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Dual role of the Arf1 exchange factor GBF1 in lipid and vesicular trafficking.

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Lipid droplets are dynamic organelles that interface with membrane trafficking pathways, and are involved in lipid homeostasis and signaling (Farese and Walther, 2012). The Arf1 exchange factor GBF1, its substrate Arf1 and effector COPI regulate trafficking both in the early secretory pathway, and in trafficking of a subset of LD resident proteins from the ER to the LD surface. In addition to their well-characterized localization to ER-Golgi intermediate compartment (ERGIC) and Golgi membranes, GBF1, Arf1 and COPI localize as well to the region surrounding LDs by fluorescence microscopy. We determined by thin section electron microscopy and tomography that bilayer ERGIC membranes are in close proximity to the LD surface (Soni et al., 2009). These results raise two interesting questions. First, do GBF1, Arf1 and COPI bind directly to the phospholipid monolayer of LDs, or are they only associated with bilayer ERGIC membranes in close proximity? Second, what localization determinants direct GBF1 either to LDs or to the

Golgi? We are exploring these questions using in vivo and in vitro approaches. We have identified a domain of GBF1 that binds directly to both liposomes and phospholipid monolayer droplets in vitro, and which localizes on its own to LDs in cells. This domain, called HDS1, is located downstream of the catalytic Sec7 domain. The N-terminal region of GBF1 upstream of the Sec7 domain is required for Golgi localization of GBF1. The Sec7 domain itself appears to be a major determinant for bilayer membrane localization, since the tandem Sec7-HDS1 does not localize to LDs, but rather to perinuclear membranes in cells.

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Hormone-sensitive lipase Ser660 phosphorylation is responsible for shrinking of lipid droplets in adipocytes.

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Lipid droplet (LD) is the most characteristic organelle of adipocytes and is recognized as highly dynamic organelle that plays a crucial role in regulating energy homeostasis in living organisms. When adipocytes are stimulated with catecholamines, triglyceride stored in LDs is hydrolyzed (lipolysis) and released as free fatty acid and glycerol from the cells. To date, numerous studies on the complicated molecular processes involved in lipolysis have revealed that hormone-sensitive lipase (HSL), which possesses multiple phosphorylation sites, is the major rate-limiting lipase in the catecholamine-stimulated lipolysis. However, there are only few studies on the morphological changes of LDs in adipocytes during catecholamine-stimulated lipolysis. Thus, in the present study, we clarified the relationship between various phosphorylated forms of HSL and morphological changes such as shrinking of LDs.

The study used adipocytes differentiated from primary preadipocytes or 3T3-L1 cells in vitro. At first, we performed time-lapse observation of adipocytes stimulated with norepinephrine (NE) using a DIC microscope to investigate morphological changes of LDs. In 35% adipocytes, large LDs ($\approx 10 \mu\text{m}$) shrunk from immediately to several hours after NE stimulation, and a part of them disappeared. We next performed immunofluorescence staining of HSL phosphorylated at either serine residues 563 (p-Ser563), 565 (p-Ser565), or 660 (p-Ser660). Without NE stimulation, p-Ser563 and p-Ser660 were hardly detected, while p-Ser565 was distributed throughout the cytoplasm. At 15 min after NE stimulation, the three phosphorylated forms were localized on the surface of LDs. At 6 h after the stimulation, p-Ser563 and p-Ser565 were hardly detected and dissociated from the LDs surface, respectively. On the other hand, marked localization of p-Ser660 on the surface of LDs was maintained even at 6 h after the stimulation, corresponding to the time course of LDs shrinking. In summary, these results suggest that the phosphorylation of HSL at serine residue 660 as well as translocation/localization of HSL on the LD surface is responsible for shrinking of LDs in adipocytes during catecholamine-stimulated lipolysis.

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Ldb16 associates with Fld1/seipin to control lipid droplet size in budding yeast.C-W. Wang¹, C-W. Wang¹; ¹Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan

The rare human disease congenital generalized lipodystrophy (CGL) is linked to mutations in BSCL2/seipin. While the molecular function of seipin is unknown, knocking-out the yeast seipin homolog FLD1 results in the accumulation of supersized lipid droplets (LDs) and altered lipid homeostasis. Here, we provide evidence for a protein termed Ldb16 that works in concert with Fld1 in yeast. Ldb16 null mutant accumulated supersized LDs and showed aberrant lipid profiling similar to that found in *fld1Δ*. Like Fld1, Ldb16 is a transmembrane protein localized to the endoplasmic reticulum (ER), and most importantly these membrane proteins assembled on the ER to mark a unique subdomain that makes contacts with LDs, a region that is believed to link neutral lipid synthesis and LD assembly. We found that Ldb16 interacts with Fld1 through its hydrophobic segments and the interaction is crucial for the control of LD morphology. Moreover, we revealed that Ldb16 is a natural substrate for ER-associated degradation (ERAD) and is stabilized when Fld1 is present. Together, our findings suggest for the first time that Fld1/seipin might regulate LD homeostasis through assembling together with other ER transmembrane protein like Ldb16 to scaffold the channel opening complex at the ER/LD contact site for the LD size control.

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Enhancer Mutagenesis Screen Yields Genetic Interactions between the BBSome and Transition Zone Complexes.S. Henke¹, S. Masyukova¹, C. Williams², D. Landis¹, J. Pieczynski³, B. Yoder³; ¹Genetics and Genomics, University of Alabama at Birmingham, Birmingham, AL, ²University of Michigan, Ann Arbor, MI, ³CMBD, University of Alabama at Birmingham, Birmingham, AL

The cilium is a specialized signaling organelle essential for vertebrate development. Mutations disrupting its function result in a group of human diseases, termed *ciliopathies*. The cilium's signaling capabilities rely on maintaining a unique protein composition distinct from both the cell cytosol and membrane. To achieve this distinction, a diffusion barrier is believed to be established at the cilium's base. This subcellular region is called the transition zone (TZ) and is thought to help establish the cilia as a signaling compartment. Further evidence of this is found in ciliopathy mutations such as those observed in both Meckel-Gruber Syndrome (MKS) and Nephronophthisis (NPHP) which affect TZ proteins; emphasizing the importance of the sub-domain in which they localize. While TZ dysfunction is associated with disease its exact function remains elusive. *Nphp-4* is a well characterized TZ protein associated with NPHP; and unlike many ciliary proteins, the loss of this protein does not affect the ability for the ciliated sensory neurons of *C. elegans* to dye-fill. Here we utilize *C. elegans* in a mutagenesis screen to generate mutations that cause a dye-filling defective phenotype in a manner dependent on a background mutation in *nphp-4*. We aimed to identify novel TZ interactors to better understand the function of this sub-domain. The results uncovered exciting genetic interactions between *nphp-4* and proteins associated with both MKS and the ciliopathy Bardet-Biedl Syndrome (BBS). Thus, identification of genes from this screen has provided potential loci for human ciliopathies and new proteins that function with NPHP and MKS to establish the TZ.

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Regulated translocation of a cell surface membrane protein into the ciliary/flagellar membrane in *Chlamydomonas* relies on microtubule-dependent concentration at the flagellar base and is independent of intraflagellar transport.

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Key developmental pathways in multicellular animals depend on the cellular compartment created by the primary cilium/flagellum, yet we know little about the mechanisms that specify the membrane protein composition of this important sensory organelle. Intraflagellar transport (IFT), which carries soluble, axoneme-associated proteins to and from the cilium/flagellum, is implicated in regulating ciliary membrane protein composition, but whether cytoplasmic microtubules and anterograde IFT are essential for movement of membrane proteins from cell body plasma membrane into the flagellar membrane is unknown. Here, in immunolocalization/cell fractionation studies with an epitope-tagged form of the *Chlamydomonas* membrane protein SAG1, we report that a SAG1-derived polypeptide is homogeneously distributed on the cell body plasma membrane of resting gametes, and de-enriched in the flagellar membrane. Upon signaling triggered during fertilization, the cell body SAG1 polypeptide undergoes a striking and rapid (~ 5-10 min) redistribution to become concentrated in the cell membrane at the base of the flagella, followed by rapid translocation into the flagellar membrane. In a mechanism regulated by cell-cell interactions, the SAG1 polypeptide is shed into the medium in a novel flagellar membrane fraction that is de-enriched in known flagellar proteins, including the major membrane protein, FMG, and IFT complex proteins. Cytoplasmic microtubules facilitate SAG1 polypeptide translocation to the flagella and are required for SAG1 enrichment at the base of the flagella. Use of a conditional IFT kinesin 2 mutant indicates that both SAG1 polypeptide enrichment at the flagellar base and translocation into the flagellar membrane are independent of kinesin 2. Our work demonstrates that trafficking of a membrane protein between the cell body and the flagellum can be one-way; and that, unlike transport of most axoneme-associated proteins, translocation of an integral membrane protein into the flagellar membrane is independent of anterograde IFT.

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Comparative proteomics of half-length with full-length flagella reveals tip proteins required for flagellar assembly in *Chlamydomonas* and ciliogenesis in vertebrate cells.

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Ciliary tips are important hubs where IFT is re-structured from anterograde to retrograde trains, cargo is released for cilia assembly, motor exchange occurs and turnover products are picked up for return to the cell body. Also at the tip, structures of unknown function are inserted into the ends of A-tubules of the outer doublets, each with a filament linked to the ciliary membrane. To identify proteins that constitute these ciliary structures as well as proteins involved in IFT turnover at the tip we carried out quantitative, comparative proteomic analysis (iTRAQ) of half-length with full-length flagella in *Chlamydomonas*, based on the hypothesis that proteins localized at the flagellar tip will be enriched ca. two-fold in half-length flagella. Such a comparative study is possible only in *Chlamydomonas*, as isolation of intact half-length and full-length flagella can be easily done. The proof of principle for this approach is that CrEB1, a known flagellar tip protein in *Chlamydomonas*, is enriched two fold in half-length flagella. As proteins involved in flagellar assembly could be enriched in the flagellum during growth, we also found several known proteins like IFT polypeptides and motors with increased abundance in

short flagella. Localization studies on eleven candidate proteins that have close human homologues using mammalian antibodies were done in RPE1 cells in tissue culture. Five of these candidate proteins were localized to basal bodies and one, CEP104, localized to the ciliary tip and daughter centrioles. In actively growing RPE1 cells, CEP104 localizes at one end of each centriole throughout the cell cycle. Upon induction of ciliation by serum starvation, CEP104 from the mother centriole moves to the tip of the cilium. It remains at the ciliary tip during ciliary growth and resorption. The daughter centriole retains a cap of CEP104 during ciliary growth and centrosomal separation. Depletion of this protein using CEP104-siRNAs inhibits ciliogenesis in RPE1 cells. The *Chlamydomonas* homologue of CEP104, FAP256 also localizes to flagellar tips and an insertional mutant of FAP256, (Roc22) is defective in flagellar growth and regeneration, indicating its conserved role in flagellar assembly. Together, these results indicate that conserved tip proteins important for ciliogenesis in vertebrate cells can be identified through this proteomic approach in *Chlamydomonas*. Support-(NIH-GM14642)/JR

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Eyespot Assembly in *Chlamydomonas reinhardtii* After Cell Division.

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Chlamydomonas reinhardtii is a biflagellate unicellular green alga that uses an organelle known as the eyespot to sense light and direct phototaxis. The eyespot is a complex structure that requires coordinated contributions from the plasma membrane and chloroplast. The eyespot disassembles and forms *de novo* after each cell division at the equator of the cell, near the tip of the plus end of the daughter four-membered (D4) rootlet. The D4 rootlet is one of two highly acetylated rootlets that emanate from the region around each basal body at the anterior of the cell. Fluorescence microscopy with antibodies targeted against acetylated tubulin, photoreceptors ChR1 and ChR2, chloroplast envelope protein EYE2, and pigment granule protein EYE3, suggests that ChR photoreceptors are directed by the D4 rootlet to the equator of the cell where they interact with the other eyespot proteins to assemble an eyespot. The order of assembly of eyespot proteins is unknown, and is a focus of our current research. In dividing wild-type cells, ChR1/ChR2 and EYE2 patches are not observed at the position of eyespot formation until the D4 rootlet has extended to the equator of the cell. The patches of ChR1/ChR2 and EYE2 remain at the equator and enlarge as the D4 rootlet extends to the posterior of the cell and recedes back to the position of eyespot formation at the equator. In dividing *mlt1* (multi-eyed) mutant cells, an EYE2 patch positions properly in the absence of ChR1/ChR2, which accumulate at the anterior of the cell. These data suggest that ChR1/ChR2 do not regulate eyespot placement. Efforts are currently focused on: 1) isolating a double mutant defective in ChR1 and ChR2 (*cop3 cop4*), and, 2) identifying proteins that interact with EYE2. These approaches will help to more precisely define the role of these proteins in eyespot assembly and placement. Also under way is a screen designed to identify other potential regulators of eyespot assembly and/or placement.

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Mitochondrial fission and fusion in *Dictyostelium discoideum*: a search for proteins involved in membrane dynamics.

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Mitochondrial morphology is maintained by two distinct membrane events -fission and fusion. Altering these conserved processes can disrupt mitochondrial morphology and distribution,

thereby disrupting the organelle's functionality and impeding cellular function. In higher eukaryotes, these processes are mediated by a family of dynamin-related proteins (DRP's). In the lower eukaryotes, for instance *Dictyostelium discoideum*, mitochondrial fission and fusion have been implicated but not yet established. To understand the overall mechanism of these dynamics across organisms, we developed an assay to identify fission and fusion events in *Dictyostelium* and to assess the involvement of the mitochondrial proteins, MidA, CluA, and two DRPs, DymA and DymB. Using laser scanning confocal microscopy to observe real time movement of fluorescently labeled *D. discoideum* mitochondria, we show, for the first time, that lower eukaryotes mediate mitochondrial fission and fusion. In *D. discoideum*, these processes are balanced, occurring approximately 1 event/minute. Quantification of the rates of fission and fusion in *midA*-, *cluA*-, *dymA*-, or *dymB*- strains established that MidA appears to play an indirect role in the regulation of fission and fusion, while the DRP's are not essential for these processes. Rates of fission and fusion were significantly reduced in *cluA*- cells, indicating that CluA is necessary for maintaining both fission and fusion. We have successfully demonstrated that *D. discoideum* mitochondria undergo the dynamic processes of fission and fusion. The classical mediators of membrane dynamics, the DRP's, are not necessary for these dynamics, whereas CluA is necessary for both processes. This work contributes to our overall understanding of mitochondrial dynamics and ultimately will provide additional insight into mitochondrial disease.

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The formin INF2 mediates a novel actin-dependent step in mitochondrial fission.

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Mitochondrial fission is a fundamental process in cellular physiology, playing roles in cellular and mitochondrial health, apoptosis, and the proper function of many cell types such as neurons and cardiac muscle. Current understanding is that the dynamin-related GTPase Drp1 mediates fission, but the exact mechanism of mitochondrial division is unclear. In particular, the mechanism by which Drp1 is brought to the constriction site is unknown in mammals. A recent study showed that fission occurred at sites where mitochondria contacted endoplasmic reticulum (ER) and that, even in mammalian cells blocked for fission by a dominant-negative Drp1 construct, mitochondria still partially constricted at ER contact sites. Our results show that the ER-bound isoform of the formin INF2 (INF2-CAAX) is required for efficient mitochondrial fission in mammalian cells. Suppression of INF2-CAAX by siRNA results in a 2-fold increase in mitochondrial length, whereas expression of a dominant active INF2-CAAX construct (INF2-DA) results in a 2-fold decrease. Live-cell confocal microscopy shows that INF2-DA causes tight ER-mitochondria association, a decrease in mitochondrial motility, and an increase in fission events. INF2 does not interact with Drp1 but functions up-stream of Drp1, since the number of mitochondrially-associated Drp1 puncta correlate with INF2 expression levels and since dominant-negative Drp1 blocks INF2-DA's ability to decrease mitochondrial length. INF2's effects on mitochondria depend on its actin polymerization ability. The actin sequestering drug Latrunculin B or an actin polymerization-deficient INF2 mutant block the effects of INF2-DA on mitochondria size. Careful examination shows that actin filaments accumulate at mitochondrial constriction sites, between mitochondria and INF2-enriched ER membranes. These results lead to a model whereby INF2-induced actin filaments mediate initial constriction of the mitochondrial outer membrane. This initial constriction stimulates Drp1 accumulation at the constriction site, leading to a secondary, Drp1-dependent constriction and fission. Thus, we link for the first time actin polymerization with the mitochondrial fission process. This process has interesting

parallels to and differences with endocytic vesicle fission, which is also mediated by actin polymerization and a dynamin GTPase.

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Determining the role of CluA in mitochondrial distribution in *Dictyostelium discoideum*.

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Mitochondrial distribution in the social amoeba *Dictyostelium discoideum* is dependent upon CluA. Lack of CluA, or its homologs, produces mitochondrial clustering in various organisms. In budding yeast, mitochondrial fission defects result in highly interconnected mitochondria that cluster to one side of the cell. Therefore to clarify the function of CluA, we determined the rates of fission and fusion in *cluA*- cells. Unexpectedly, we observed that CluA is necessary for both fission and fusion with rates in *cluA*- cells about half that of a wild-type cell.

Studies of flies and plants suggest that CluA regulates mitochondrial movement along the cytoskeleton. In order to explore how CluA regulates both fission and fusion, we want to determine if the cytoskeleton is necessary to establish mitochondrial clusters. It is possible that in the absence of CluA the mitochondria are transported by the cytoskeleton improperly to a central location resulting in the clusters. Alternatively, electron microscopy has shown that the mitochondria in *cluA*- cells are interconnected; perhaps the cytoskeleton is necessary to complete the fission and fusion events in these cells. It has been theorized that movement along the cytoskeleton may complete fission or fusion of the organelles.

We will analyze mitochondrial morphology in *cluA*- cells treated with cholchicine and latrunculin-A. Using time-lapse confocal microscopy, we will also determine the dynamics, distance, and direction of mitochondrial movement in wild-type and *cluA*- cells, to see whether mitochondria move randomly or directionally, such as along cytoskeletal tracks in these cells. We expect that, if the cytoskeleton is necessary to establish this clustered phenotype, disruption of the cytoskeleton will allow the mitochondria to scatter throughout the cell. If the cytoskeleton is necessary to complete fission or fusion, we expect the clustered mitochondria to remain unchanged. The results from these experiments will provide insight into how CluA determines mitochondrial distribution either through a cytoskeletal dependent mechanism or by functioning as a novel regulator of both fission and fusion.

Nuclear Bodies and Dynamics

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Temporal alteration of nuclear structure and stiffness accompany gene expression changes from force and cytokine treatment.

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Genome regulation requires delicately balanced temporal and spatial control for proper cellular homeostasis and adaptation. Although DNA is a linear genetic code, recent studies have illuminated that genome organization within the nucleus aids in regulation, and there is evidence that gene translocation within the nucleus leads to differential expression. The correlation of gene expression with gene position and movement in the mechanically stiff nucleus suggests there may be mechano-coupling impacting gene expression. We examine the effects of

extracellular mechanical and biochemical stimulation on nuclear organization. In addition to the cellular chemical signaling cascades that activate nuclear transcription factors leading to gene expression, we examine time-dependent reorganization of nuclear structures, altered chromatin condensation, gene movement and altered gene expression. We consider the response of endothelial cells to extracellular shear stress; we observe a dose-dependent response of the nuclear interior, with transition states at 30 minutes after the application of stress. We also consider chemical stimulation with VEGF on endothelial cells. These chemical treatments similarly result in an effective intranuclear softening. This effective softening correlates with chromatin decondensation and nuclear reorganization. This decondensation is coupled with increased motor protein-driven cytoskeletal stress on the nucleus. We suggest that these cytoskeletal stresses increase nucleoplasmic agitation to enhance DNA and protein diffusion and, thus, collision frequency to drive the favorable binding events necessary for transcriptional activation. We observe a time-dependent response with significant attenuation in the VEGF response after 2.5 hours. Endothelial cells exposed to shear following VEGF stimulation showed aspects of both stimulated responses, with an effective intranuclear softening that paralleled VEGF stimulation and the transition in the time-dependent response at 30 minutes from shear. Understanding genome organization within the nucleus and the mechanisms underlying stimulated cellular transition states are vital to elucidating genome function. Our results indicate that the large changes in gene expression associated with mechanical and VEGF stimulation of endothelial cells correspond to an effective nuclear softening and changes in global chromatin condensation, rather than a purely localized and gene-specific effect. Thus, our work fortifies the dynamic view of the genome, suggesting stimulated gene repositioning and implying a role for externally imposed nuclear stresses in stimulated changes in gene expression.

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A novel role for Protein Kinase C in nuclear size regulation.

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Cell and nuclear size are tightly regulated amongst species, cell types, developmental stages and disease states. During *Xenopus laevis* early embryonic development the single fertilized cell (~1mm diameter) divides into several thousand 50µm diameter and smaller cells with concomitant reductions in nuclear size. From the cleavage stage embryo through gastrulation, there is an approximate 8-fold reduction in nuclear envelope surface area. These drastic nuclear size changes occur without changes in DNA content per cell, suggesting nuclear size is regulated by yet unknown cytoplasmic factors. In order to discover developmentally regulated proteins that control nuclear size during *X.laevis* embryogenesis, we developed an assay whereby large nuclei assembled in egg extract shrink in the presence of late embryo extract. We hypothesize that phosphorylation of lamins, an integral structural component of the nuclear envelope, may be responsible for nuclear size changes. We tested whether specific kinases were necessary for nuclear shrinking in this assay. Selective inhibition of protein kinase C (PKC) with chelerythrine, but not cyclin-dependent kinase with olomoucine, blocked shrinking. Interestingly, during time course immunofluorescence experiments it was observed that nuclei resuspended in buffer shrink and re-expand while nuclei in late embryo extract only shrink, suggesting that late embryo extract inhibits nuclear re-expansion. This inhibition of re-expansion is PKC-dependent as addition of chelerythrine promotes re-expansion. Furthermore, by depleting calcium and diacylglycerol, the most likely subfamily of PKC isozymes responsible for this activity has been identified. Future depletion or selective PKC isozyme inhibitor experiments will be performed to pinpoint a single isozyme that contributes to reducing nuclear size during developmental progression. We will use these data to alter nuclear size in a minimally invasive fashion in order to characterize the functional impacts of nuclear size modifications.

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Atomic force microscopy of the nucleus and nucleolus.

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Although nuclear structure, function and dynamics are well known by using different molecular and cellular approaches, few *in situ* studies have been done using atomic force microscopy. Here we present recent advances in this topic. We prepare samples of different sources as for transmission electron microscopy. Semithin sections are observed under atomic force microscopy. We have been able to recognize different substructures as chromatin, nucleolar dense fibrillar component and heterogeneous fibrillar centers. Also, Cajal bodies are seen under these conditions in some cells as tomato (*Lycopersicon esculentum*). In HeLa cells, interchromatin granule clusters were observed. Nanometric profiles of some nuclear territories as the nucleoplasm reveals zones similar to IGC. Nuclear structure of parasites as *Giardia lamblia*, *Entamoeba histolytica* and *Trypanosoma cruzi* are also shown. Discontinuities along nuclear envelope also indicates nuclear pores complexes (PAPIIT IN227810, CONACyT 180835, PAPIIME PE211412).

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New methods for quantitative immunolocalization reveal that stress and gold nanoparticles alter the functional organization of the nucleolus.

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Nucleoli, the sites of ribosome biogenesis, are conspicuous compartments inside the nucleus that are composed of several thousand different proteins. Nucleoli are crucial for the proper execution of diverse cellular processes, many of which are directly relevant to human health. As such, nucleoli associate with key molecules that regulate cell cycle progression, apoptosis, the stress response and tumorigenesis. The molecular make up of nucleoli is highly dynamic and thus supports the rapid response to changes in cell physiology. This dynamic nature of nucleoli presents an obstacle to quantitative analyses of nucleolar proteins, and it demands powerful tools that measure the changes in composition and activities of the nucleolus under a wide variety of conditions. Although proteomics was indispensable to define the complexity of the nucleolar proteome, it is not efficient to examine those nucleolar processes that are highly dynamic or require large sample sizes. To achieve this goal, we developed novel quantitative immunofluorescence approaches that were combined with computer-based image analysis. These methods were used successfully to measure the association of fluorescent molecules with nucleoli. Our protocols provide robust, fast and reliable tools to analyze the functional organization of the nucleolus in a timely fashion. Importantly, these protocols are compatible with automated high throughput screening platforms, which permit simultaneous analyses of many of the biological processes that are located in nucleoli.

Here, we applied our protocols to define the effects of two different types of interventions on the functional organization of nucleoli. First, we selected heat shock as an environmental stress that is frequently experienced by living organisms. Second, we investigated the impact of gold nanoparticles, which have emerged as powerful tools for drug delivery, cellular imaging, biomedical diagnostics and therapeutics. Our results demonstrate that heat stress and gold nanoparticles alter the organization and function of the nucleolus. Furthermore, we show that the shape of gold nanoparticles is critical for their effect on the nucleolus.

Taken together, our research provides a novel platform to study the biology of the nucleolus. This platform combines quantitative immunofluorescence, computer-based image analysis and nanotechnology.

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Cohesin – A protein clip to fix the folds of the genome.

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The cohesin complex is fundamental for all eukaryotes to ensure the stable transmission of the genome over numerous generations. To achieve this, cohesin tethers the replicates of the genome together to ensure proper identification and distribution of the copies during mitotic and meiotic cell divisions.

Starting with the genome-wide identification of cohesin-binding we realized that cohesin has more functions such as chromatin insulation (in cooperation with CTCF) [1] and transcriptional control and is important for chromosomal long-range interactions[2].

The chromatin fibre is organized in domains termed “fractal globules”[3] or “topological domains”[4] and CTCF is often found at the boundaries of these domains.

We aimed to investigate which role cohesin plays for the general organization of the chromatin fibre, especially whether cohesin is necessary to organize chromatin domains. To study this we have destroyed functional cohesin by protease cleavage which releases the cohesin complex from chromatin but not CTCF.

Using chromatin conformation capturing methods (4C and Hi-C)and 3D-FISH we have analyzed the impact of releasing cohesin from chromatin on chromatin structure genome-wide and in detail for the H19/IGF2 locus. By ChIP-sequencing and RNA-sequencing we will correlate these structural changes with altered cohesin binding and changes in gene activity. We will present the results of this study and discuss impact of our observations for the understanding of genome organization and cohesin function.

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Nucleography: Surveying the nuclear landscape.*K. V. Laster¹, S. Kosak¹; ¹Cell and Molecular Biology, Northwestern University, Chicago, IL*

In the mammalian nucleus, chromosomes are non-randomly organized in a cell-type specific manner. Using a murine hematopoietic progenitor cell model, our lab provided evidence that this cell-type specific chromosome organization is intimately correlated with gene expression. Specifically, we found that co regulated gene sets facilitate the establishment of emergent order in the nuclear architecture, suggesting that the differentiating nucleus functions as a self-organizing system. We have now extended our initial findings to human embryonic stem cells (hESCs), testing whether a similar phenomenon may underpin the loss of pluripotency during neuronal differentiation. Implementing a tendency based, bi-clustering algorithm on a multi-timepoint gene expression dataset, we observe that genomic order, as a function of entropy, is lost and regained over differentiation into committed neuronal precursor cells. This dynamic change in entropy is concomitant with phenotypic changes during the differentiation time course. We are investigating whether this modulation of gene expression at key timepoints is manifested in changes in global chromatin architecture utilizing a novel visualization technique. Using probe material generated from a micro-dissected nucleus, we expect to see increased variability in proximity of chromatin clusters at the entropic minima relative to the maxima. Additionally, we are examining the differential localization of specific genes involved in neuralization. These findings confirm that cellular differentiation is a highly dynamic process reflected at the level of genomic organization.

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Assistance of the SMN-complex component Unrip in snRNA 5' cap hypermethylation.*B. Boysen¹, O. Gruss¹; ¹DKFZ-ZMBH-Alliance, Heidelberg, Germany*

The survival of motor neurons (SMN) complex contributes to the biogenesis of small nuclear ribonucleoprotein particles (snRNPs). It binds snRNA and sm proteins in the cytoplasm and assembles the sm proteins in a ring-like structure around the snRNA. Afterwards it accompanies the snRNP on its import into the nucleus. Then the complex dissociates from the snRNP in the Cajal bodies. The SMN complex consists of nine different components, SMN, Gemin2-8 and Unrip. In contrast to other complex components Unrip dissociates at a certain step from the complex. It has been shown that Unrip leaves the SMN complex prior to accumulation of the SMN-snRNP-complex in nuclear Cajal bodies. However, whether Unrip dissociates prior or after nuclear import of the SMN-snRNP-complex is not yet fully clarified. Interestingly, loss of Unrip by RNAi causes an increase of nuclear as well as generation of cytoplasmic SMN foci. Here we show that Unrip is still part of the Snurportin-SMN import-complex and present our results about Unrip association with the complex. Furthermore fluorescence analysis upon Unrip knockdown reveals that the cytoplasmic foci depict an accumulation state of the SMN-snRNP-complex prior to 5' cap hypermethylation of the snRNA. Pulse-chase analysis indicates that loss of Unrip impairs 5' cap hypermethylation kinetics. Taken together our results show a contribution of Unrip in the process of snRNA 5' cap hypermethylation.

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Cell-Cycle-Dependent Structural Transitions in the Human CENP-A Nucleosome In Vivo.*M. Bui¹, Y. Dalal¹; ¹National Cancer Institute, National Institutes of Health, Bethesda, MD*

In eukaryotes, DNA is packaged into chromatin by canonical histone proteins. The specialized histone H3 variant CENP-A provides an epigenetic and structural basis for chromosome segregation by replacing H3 at centromeres. Unlike exclusively octameric canonical H3

nucleosomes, CENP-A nucleosomes have been shown to exist as octamers, hexamers, and tetramers. An intriguing possibility reconciling these observations is that CENP-A nucleosomes cycle between octamers and tetramers in vivo. We tested this hypothesis by tracking CENP-A nucleosomal components, structure, chromatin folding, and covalent modifications across the human cell cycle. We report that CENP-A nucleosomes alter from tetramers to octamers before replication and revert to tetramers after replication. These structural transitions are accompanied by reversible chaperone binding, chromatin fiber folding changes, and previously undescribed modifications within the histone fold domains of CENP-A and H4. Our results reveal a cyclical nature to CENP-A nucleosome structure and have implications for the maintenance of epigenetic memory after centromere replication.

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Nuclear Actin Filaments Inhibit Transcription.

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Why are there no actin filaments in the nucleus? Although the concentration of actin in the nucleus is above the critical concentration for polymerization, actin filaments are rarely detected in the normal interphase nucleus. Nevertheless, it has been established that nuclear actin has several important functions, including transcription by all three RNA polymerases, chromatin remodeling, and cell fate decisions. How actin performs these functions in the absence of filaments remains a mystery. Nuclear actin is thought to exist as monomers or in highly dynamic and short polymers, and the dynamic state of nuclear actin has been shown to be a crucial regulator of its nuclear functions. To more closely investigate the effects of nuclear actin dynamics on transcription, we have used several models to induce the formation of stable nuclear actin filaments and determined the effects of nuclear actin filaments on transcription. Targeting α -catenin to the nucleus, or expressing V163M α -actin, S14C β -actin or a sub-domain of supervillin results in the formation of nuclear actin filaments. Importantly, the presence of nuclear actin filaments strongly correlates with the accumulation of RNA polymerase II into large granules, a decrease in transcription, and alterations in chromatin structure. We are currently performing additional experiments to determine how G-actin impacts RNA polymerase II targeting in the nucleus. Our results provide insights into the function/polymerization relationship of nuclear actin and why, despite the propensity for actin to polymerize into filaments, it avoids doing so in the nucleus.

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Molecular dynamics of the survival of motoneuron (SMN) protein in the nucleus – mobilization of SMN in Cajal bodies.

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The loss of survival of motoneuron (SMN) protein leads to Spinal Muscular Atrophy, a neurodegenerative disease affecting motoneurons. In the nucleus, SMN is associated with two types of nuclear bodies denoted as gems and Cajal Bodies (CBs). The 23 kDa isoform of Fibroblast growth factor-2 (FGF-2²³) is a nuclear protein which binds to SMN and destabilizes the SMN-Gemin2 complex resulting in a decreased number of nuclear gems (Claus et al., 2003; Claus et al., 2004; Bruns et al., 2009). In the present study, we analyzed the effect of this FGF-2

isoform on CBs. We have shown that FGF-2²³ depletes SMN from CBs without affecting their general structure. FRAP analysis of SMN-EGFP in CBs demonstrated that the majority of SMN in CBs remained mobile and allowed quantification of fast, slow and immobile nuclear SMN populations. The potential for SMN release was confirmed by in vivo photoconversion of SMN-Dendra2, indicating that CBs concentrate immobile SMN that could have a specialized function in CBs. This is mechanistically regulated by a competition of coilin with FGF-2²³ for binding to SMN. These results demonstrate that FGF-2²³ promotes release of SMN from its immobile pool in the CB and further defines the function of this nuclear growth factor. Furthermore, FGF-2²³ caused snRNP accumulation in CBs. We propose a model in which Cajal bodies store immobile SMN that can be mobilized by its nuclear interaction partner FGF-2²³, leading to U4 snRNP accumulation in CBs, indicating a role for immobile SMN in tri-snRNP assembly.

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Nucleocytoplasmic Transport

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Importin-β and Ran regulate the passive permeability barrier in the nuclear pore complex.

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The nuclear pore complex (NPC) is a large, multi-protein assembly that mediates the selective transport of molecules between the cytoplasm and nucleus in eukaryotic cells. While the proteins involved in the transport pathway have largely been identified, the physical mechanism by which this complex can support both efficient and selective molecular transport remains unclear. Using fluorescence microscopy and super-resolution imaging techniques, we have examined how nuclear transport receptors influence the permeability properties of the NPC for both active and passive transport processes. We find that importin-β binding to the nucleoporin Nup153 significantly slows passive transport through the NPC; however, Ran in its GTP-bound form reverses this effect. FRAP studies reveal that RanGTP weakens the binding interaction of importin-β to the NPC. Furthermore, STORM imaging of individual importin-β localizations inside the NPC show that RanGTP dramatically changes the importin-β distribution within the channel. These results suggest that importin-β in conjunction with Nup153 is an integral component of the NPC permeability barrier which is regulated by RanGTP.

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Defining the mechanisms regulating selective mRNA export during heat shock-induced stress.

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Eukaryotic cells respond to major environmental stresses through dramatic alterations in gene expression. An essential process during these events is the export of messenger RNA (mRNA) from the nucleus to the cytoplasm. As mRNA is synthesized, the transcript is packaged with proteins to form export competent messenger ribonucleoprotein particles (mRNPs). The protein composition of these mRNPs is highly dynamic, characterized by the association and dissociation of specific proteins that mediate efficient mRNA translocation through nuclear pore complexes. In the budding yeast *Saccharomyces cerevisiae* during the heat shock stress response, others have shown that the transcription and export of specific heat shock-induced mRNAs are upregulated. In contrast, non-heat shock-induced mRNAs are retained in the nucleus. This allows the translation machinery to be geared for the synthesis of heat shock proteins. However, the precise molecular determinants that control this selective mRNA export remain unresolved.

Previously, our laboratory demonstrated that the mRNA-binding proteins Nab2 and Yra1 colocalize to Mlp1-dependent intranuclear foci following exposure to heat shock stress conditions (Carmody et al. *Mol Cell Biol.* 2010 30(21):5168-79). To gain a better understanding of how mRNA export factors such as Nab2 are affected during heat shock stress, we employed a previously characterized nab2 mutant (*nab2-C437S*) that exhibits significantly reduced RNA-binding affinity and altered mRNP assembly (Tran et al. *Mol Cell.* 2007 28(5):850-9; Brockmann et al. *Structure.* 2012 20(6):1007-18). Surprisingly, using *in situ* hybridization analysis, we observed that poly(A) mRNAs were not retained in the nucleus following heat shock in the *nab2-C437S* mutant. This suggested a defect in the regulation of mRNA export. Moreover, colocalization of Nab2 and Yra1 to intranuclear foci upon heat shock stress was disrupted in the *nab2-C437S* mutant. We are currently analyzing the localization of non-heat shock and heat shock mRNAs in this mutant using single molecule RNA FISH. Recent evidence indicates that post-translational modifications of RNA binding proteins are important for the mRNA export mechanism. Our laboratory has previously shown that during heat shock stress Nab2 is phosphorylated by the mitogen-activated protein (MAP) kinase Sit2/Mpk1 (Carmody et al. *Mol Cell Biol.* 2010 30(21):5168-79). In addition, others have recently revealed that Yra1 is ubiquitinated by the E3 ligase Tom1 (Iglesias et al. *Genes Dev.* 2010 24(17):1927-38). We speculate that a potential role of these post-translational modifications may be to regulate selective mRNA export during stress responses. Our ongoing studies are focused on further characterizing the heat shock response in the *nab2-C437S* mutant and addressing this hypothesis. Together, these strategies aim to reveal the mechanism for selective mRNA export during the heat shock stress response.

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Traveling through the pore: dynamics and conformation of phenylalanine-glycine repeat domains inside the nuclear pore complex.

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All kind of proteins and RNA transport across the nuclear envelope in eukaryotes is selectively regulated by nuclear pore complexes (NPCs). While the research field of the NPC is now entering its fifth decade, selective gating mechanism across the pore is still ambiguous. This

obscurity mainly stems from lack of accurate information on dynamics and conformations of the natively unfolded phenylalanine-glycine (FG) repeat domains that constitute about 30% of the of the NPC structure and are believed to be the main component of selective gating.

Due to their natively unfolded structure and extremely delicate nature, however, their functional state cannot be captured in vivo or in vitro. Indeed, FG-repeats are confined to the NPC central channel, and the compact architecture of the channel makes the investigation even more challenging. Thus, conformational behavior of the FG-repeats during transport has been the subject of speculation for the past 13 years, and still there is not a clear consensus that how these repeat domains form the selective barrier.

Nevertheless, there are extensive, and sometimes contradictory, data in the literature on structural and functional aspects of the FG-repeats. Currently, the time is truly ripe to gather this large pool of data and make a computational model to reveal dynamics and conformation of these repeat domains with high spatiotemporal resolutions.

Based on the available empirical data and following polymer physics principles, last year we published a computational 2D model of the whole NPC structure, which was validated against experimental results. In this study, we have focused on the mysterious central channel of the NPC to exclusively shed light on dynamics and conformation of the FG-repeat domains confined in the channel. To be able to mimic the real geometry of the pore, we have extended the model to the 3D version that capable of capturing true geometrical features of the channel. Our model suggests that FG-repeats form a layer of hydrophobic 'lubricant' on the channel wall and leave a central tube in the channel, but the formation of the channel-filling hydrogel is not feasible even at extreme pHs. Then we examined the same conditions for free, not confined, FG-repeats domains and found that they can form a hydrogel, both at the extreme and normal pHs. This indicates the essential role of the confined geometry of the NPC that has been neglected so far. Thermodynamic analysis reveals that for the hydrogel to form, 'enthalpic gain' must overcome 'entropic loss' of oppositely grafted FG-repeats in the channel.

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The Nup-Imp β affinity gradient of the nuclear pore complex is highly optimized to maximize import rate.

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Background: The nuclear pore complex (NPC) regulates transport between cytoplasmic and nuclear compartments of the cell. The mechanism of such transport is an active topic of debate and has lead to the suggestion of several theoretical models of transport. Among these is the affinity gradient model, which suggests that transport is based on the experimentally observed, steeply increasing affinity gradient across the pore. Although this model alone cannot account for transport, the observed affinity gradient likely plays a role.

Objective: Explore the effect of gradients in affinity between nucleoporins (Nups) and Importin- β in the NPC on import rate and to determine the distribution of transport rates the range of experimentally observed affinities can support.

Methods: A 3D agent-based model (ABM) of the nuclear pore complex was constructed comprising static agents representing the NPC scaffold, nuclear basket, cytoplasmic filaments and nuclear envelope. Mobile agents were restricted to the cytoplasmic filament region, central channel and nuclear basket. Mobile agents consisted of Imp β -binding (FG agents) and non-Imp β -binding (non-FG agents); the quantity of the FG-agents being selected based on the number of FG-containing Nups within the NPC and the non-FG agents selected to represent the

volume of the channel occupied by Nups. Mobile agents representing Imp β were present in the cytoplasmic region at a concentration of 3 μ M along with agents representing RanGTP in the nucleus at a concentration of 1 μ M. Imp β agents were capable of translocating through the channel via a combination of diffusive events and binding/unbinding events with FG agents. The channel was partitioned into three regions consisting of cytoplasmic, central channel and nuclear basket. FG-Imp β affinity was varied over a range of $K_D=0.1$ nM to $K_D=10$ μ M for all three regions and Imp β transit rates were measured and averaged over 100 simulations per data point.

Results: Peak transport rate of Imp β across the channel was observed for an affinity gradient of 1000nM, 200nM and 10nM in the cytoplasm, central channel and nucleus, respectively. The effect of cytoplasmic Nup affinity on transport rate peaked at $K_D=1000$ nM with no appreciable increase in transport rate at higher values of K_D . Similarly, the effect of nuclear basket Nups on transport rate peaked at $K_D=10$ nM with no noticeable increase at lower K_D . Adjusting central channel Nup affinity resulted in a biphasic behavior in transport rates with an observed maximum transport rate at $K_D=200$ nM. Additionally, the transport rate for the optimal affinity gradient was approximately 23% greater than that of the most optimal channel lacking an affinity gradient.

Conclusions: The presence of an affinity gradient can increase Imp β transport rates across the nuclear pore complex. Furthermore, our model and simulations predict that the transport rate reaches a maximum at optimum affinities for each nuclear pore region. Interestingly, these optimal values are in very near agreement to experimentally measured dissociation constants.

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Exploring the Potential Impact of the O-GlcNAcylation of Nucleoporins on Nucleocytoplasmic Transport.

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Introduction: O-GlcNAcylation, or the addition of *N*-acetylglucosamine (GlcNAc) to serine or threonine amino-acid residues in the nucleus and the cytosol, was discovered only several decades ago and has since garnered significant attention as a novel form of dynamic post-translational modification. While notable progress has been made in uncovering the role that O-GlcNAcylation may play in cellular processes such as transcription, translation, and signal transduction, very little is known about its potential role in nucleocytoplasmic transport (NCT).

The objective of this work is to study how the experimentally-validated O-GlcNAcylation of residues proximal to functionally-important phenylalanine-glycine (FG)-repeat domains on nucleoporin proteins (Nups) affects their interaction with hydrophobic patches on karyopherin proteins during NCT.

Methods: We have used the CHARMM molecular dynamics modeling package, along with CHARMM27 protein and CHARMM36 carbohydrate force fields, to generate multiple distinct O-GlcNAcylated peptides (glycopeptides) spanning 10-amino-acid sequences within the FG-repeat-rich portions of human Nup153 and Nup214. We then used VMD to solvate and ionize each glycopeptide in 139mM KCl, and NAMD to minimize and equilibrate them under periodic boundary conditions and an NPT ensemble. A crystal structure of human importin β 1 bound to the IBB domain of importin α 2 (hImp β 1/Imp α 2-IBB) was also minimized and equilibrated separately in identical conditions. Using VMD, we constructed systems involving each equilibrated peptide – either with or without the O-GlcNAc modification – separately replicated

around the equilibrated hImp β 1/Imp α 2-IBB for temporal simulation of interaction and binding dynamics.

Results and Conclusion: Preliminary data suggest that glycosylated and unglycosylated peptides bind to the hImp β 1/Imp α 2-IBB surface regions that are consistent with or in close proximity to some of the regions previously reported in the literature. The free energy change upon binding (FEB) that is attributed to van der Waals interactions between FG-repeat residues and hImp β 1/Imp α 2-IBB is on the order of 17.4 ± 2.3 and 16.6 ± 2.2 kcal/mol for glycopeptides and peptides, respectively. In addition, where the GlcNAc is in the vicinity of the binding event, it contributes an additional 9.8 ± 1.3 kcal/mol to van der Waals FEB. These observations may suggest that having O-GlcNAcylation near the FG-repeat domains of Nups contributes energetically to their association with Imp β 1 as well as possibly other karyopherins.

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The impact of nuclear size on *Xenopus* early development.

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Answering a fundamental question in cell biology like how nuclear size is regulated could shed light on understanding diseases associated with altered nuclear size, such as cancer. It has been previously shown that nuclear size in *Xenopus* can be either increased or decreased by altering the amounts of importin α , Ntf2, and the nuclear import cargo lamin B. During early frog embryogenesis nuclear and cell size decrease with the nuclear to cytoplasmic volume ratio reaching a maximum at the midblastula transition (MBT). MBT is mainly characterized by embryonic transcription upregulation, longer cell cycles and cell division asynchrony. We hypothesize that MBT timing is determined by the nuclear to cytoplasmic volume ratio, which we can experimentally manipulate by altering nuclear size.

Our approach is to alter nuclear size in early *Xenopus* embryos by mRNA microinjection of one blastomere in a two-cell embryo to ectopically express nuclear scaling factors, enabling us to investigate the correlation between nuclear size and developmental progression. We monitor the molecular and cellular hallmarks of MBT in the two halves of the embryo by whole mount in situ hybridization and fluorescent time lapse microscopy. According to our preliminary data, increasing nuclear size in early embryos leads to a premature MBT indicated by earlier zygotic transcription, earlier onset of cell division asynchrony, and longer cell cycles in the injected half of the embryos. Conversely, decreasing nuclear size leads to a delayed MBT detected by later zygotic transcription, later onset of cell division asynchrony, and more cell divisions and smaller cells in the injected half of the embryo compared to the uninjected half.

Further we want to address the follow-up question: does altering nuclear size in early embryos affect development and differentiation in later stage embryos during gastrulation and organogenesis? Also it would be interesting to investigate the long-term effects of altering nuclear size in swimming tadpoles and beyond. This knowledge will provide insight into the link between nuclear size and normal embryonic development, cell function, physiology and morphology.

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Constructing Finite Element Models for Exportin Cse1p and Xpot.

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Studying conformational dynamics of proteins is beneficial to understand their biological functions. Here, we utilized Finite Element Analysis (FEA), a mature engineering technique, to investigate the conformations of Cse1p and Xpot, two of the known transport factors, which play crucial roles in Nucleocytoplasmic Transport (NCT). Cse1p and Xpot are exportins, which

shuttle specific cargos from the nucleus to the cytoplasm by passing through the Nuclear Pore Complex (NPC). During the process, they are capable of adapting at least two different conformations to execute association and dissociation of cargos. Therefore, the flexibility of transport factors has been considered one of the most important characteristics of the NCT. A spring-loading hypothesis was proposed by another research group, which suggested that the Cse1p has a distorted conformation which may act like a loading spring after accommodating cargos in the nucleus. After passing through the NPC, conformational changes might be produced due to the strain stored in the complex structure, which allows the cargos to be released in the cytoplasm. In order to test this hypothesis, we first calculated the solvent-excluded surface (SES) of Cse1p's conformation in the nuclear/bound state. Second, we created an enclosed volume based on the SES, and then discretized the volume into a FEA model. Finally, a free-vibration analysis was carried out to obtain the nature vibrational modes of Cse1p that were found to be relevant to the mechanism of binding cargos. More precisely, a dominant mode was identified to have the most potential to form the unbound conformation in the cytoplasm. Furthermore, a distribution of strain/stress on Cse1p's FEA model was established, and the results indicated that Cse1p's distorted conformation in the nucleus did have stored strain/stress to facilitate the process of releasing cargos in the cytoplasm. In other words, we were able to quantitatively point out where the most flexible regions of Cse1p's complex structure were. The same procedure was applied on Xpot as well, and the results also revealed a dominant mode relevant to binding mechanism and a strain/stress distribution mapped to highly flexible regions of Xpot's bound conformation. In conclusion,, FEA can be a useful tool for exploring conformational dynamics of proteins and beneficial to understand their biological functions.

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Characterization of NLS2 from Influenza A virus nucleoprotein.

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Influenza A virus nucleoprotein (NP) is an abundant essential protein, participating in genome organization, nuclear trafficking, and RNA transcription and replication. Two nuclear localization sequences (NLSs) on NP are known to mediate the nuclear import of the influenza genome: NLS1 at the N-terminus of NP, and NLS2 spanning residues 198-216. Sequence alignment shows both NLSs on NP are highly conserved between different strains of influenza A virus, suggesting that these NLSs are ideal candidate for novel antiviral approaches. The functional role of NLS1 has been very well characterized in previous studies. Using chimera proteins fused to NLS2, we are characterizing the contribution of NLS2 to nuclear import and defining the amino acids that are important for NLS2's function. NLS2 has been previously classified as a classical bipartite NLS, containing two clusters of basic amino acids separate by a linker of 13 amino acids. Through sequence alignment, we found that there is one amino acid different in the second cluster of basic amino acid at position 17 on NLS2. Interestingly, with only this one basic amino acid difference (K:lysine to R:arginine) the NLS2 containing a K at position 17 (NLS2K) contributes stronger to the nuclear import of the chimera protein than the NLS2 containing an R at position 17 (NLS2R). To understand the nuclear import function differences between NLS2K and NLS2R, site-directed mutagenesis was performed to the basic clusters of each NLS2. Mutations in either cluster of NLS2K did not affect the nuclear accumulation of the chimera protein containing NLS2K, which corroborate that NLS2K is a classical bipartite NLS. In contract, mutagenesis of the first basic cluster of NLS2R yielded a significant decrease in the nuclear accumulation of a chimera protein containing NLS2R, and the same result was obtained when both amino acid clusters of NLS2R were mutated. This result indicates that NLS2R behaves as an unconventional monopartite NLS. We are now performing deletion mutants of the NLS2R-chimera protein to define this novel NLS. Together, these strategies aim to reveal

functional amino acids on NLS2 of influenza A virus NP that mediate the nuclear import of the viral genome.

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Elevated level of Human RPA Interacting Protein α (hRIP α) in tumor cells is involved in cell proliferation through regulating RPA transport.

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Replication protein A (RPA) is a eukaryotic single-stranded DNA binding protein that is essential for DNA replication, repair, and recombination, and human RPA interacting protein α (hRIP α) is the nuclear transporter of RPA. Here, we report the regulatory role of hRIP α protein in cell proliferation. hRIP α protein expression is often increased in human cervical tumor cells and hRIP α knockdown by siRNA inhibited cellular proliferation through deregulation of the cell cycle. In addition, overexpression of hRIP α resulted in increased clonogenicity. These results indicate that hRIP α is involved in cell proliferation through regulation of RPA transport.

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Control of gene expression through regulation of DEAD-box helicases.

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The mRNA life cycle is a fundamental cellular process comprised of a series of dependent steps from transcription to mRNA decay. During each step, the cohort of mRNA-associated proteins and the structure of the mRNA itself are constantly changing. The DEAD-box protein family is a major component of the machinery that facilitates these changes, "remodeling" mRNPs through the ability to both remove proteins bound to RNA and unwind structured mRNA. Importantly, appropriate control of DEAD-box protein activity and how DEAD-box proteins remodel RNP complexes are not fully defined. Only a small number of factors that affect DEAD-box protein activity have been identified. Among these is Gle1, which stimulates the activity of the DEAD-box protein Dbp5 in mRNA export. Our prior studies have revealed new functions of Gle1 in protein translation, acting both in initiation and termination. In termination, Gle1 likely activates Dbp5 as in export. In initiation, we found that Gle1 inhibits the activity of a different DEAD-box protein, Ded1. Because Gle1 is a multifunctional DEAD-box protein regulator (acting in nuclear export, translation initiation and translation termination), we suggest it is a key factor in regulating mRNP remodeling during these steps. Our current goals are to determine the mechanism and biological consequences of Gle1 regulation of Ded1 in translation initiation. This study will further our long-term goal of understanding how DEAD-box proteins and their regulatory factors direct the fate of mRNAs. In addition, the human homolog of Ded1, DDX3, has been implicated in several disease pathologies, including cancer development and HIV infection, and better understanding its cellular functions will be crucial in unraveling its roles in disease.

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Btf has functions unique from TRAP150 in regulating the subcellular distribution of mRNAs.

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Transcription of protein-coding genes is coordinated with pre-mRNA processing as well as mRNP assembly and nuclear export in mammalian cells. Btf (BCLAF1) and TRAP150 are serine-arginine-rich (SR) proteins that share 39% sequence identity and 66% sequence similarity, but the extent of their functional overlap is not clear. Btf and TRAP150 were previously reported to associate with synthetic affinity-purified in vitro spliced mRNPs, and also as a part of the spliceosome complex, suggesting they are involved in pre-mRNA processing. We used a combined immunofluorescence/RNA-FISH approach to show that both Btf and TRAP150 localize at a constitutively active beta-tropomyosin (BTM) reporter minigene locus in HeLa cells. Both proteins also localize at a U2OS 2-6-3 reporter gene locus in a RNA polymerase II transcription-dependent manner. While Btf and TRAP150 showed some overlap with reporter RNA and other pre-mRNA processing factors at transcription loci, they showed the most extensive overlap with the exon junction complex (EJC) protein Magoh. Since EJC components have roles in nuclear export, we examined nuclear/cytoplasmic mRNA distribution by qRT-PCR. Depletion of Btf or TRAP150 did not alter splicing of BTM transcripts or the recruitment of other RNA processing factors to transcription sites. Specifically, Btf depletion (but not TRAP150 depletion) caused an increase of beta-tropomyosin minigene reporter transcripts in the cytoplasm. In addition, RNA-FISH using fluorescently tagged oligo-dT probes showed increased abundance of endogenous polyadenylated RNA in the cytoplasm of HeLa cells specifically following Btf depletion but not TRAP150 depletion. Since immunoblots revealed that Btf depletion resulted in increased expression of TRAP150, we prevented compensatory TRAP150 expression upon Btf depletion by adding limiting amounts of TRAP150 siRNA duplex to the siRNA mixture. Our experiments demonstrate that it is Btf depletion, but not compensatory TRAP150 upregulation, that leads to a global change in subcellular distribution of mRNA. We are currently mining our exon microarray data generated from Btf-depleted cells to identify mis-spliced endogenous mRNAs and to determine if incorrectly spliced transcripts reach the cytoplasm. In summary, our data suggests that Btf and TRAP150 have both redundant and distinct functions in human cells. We reveal that modulating the expression of a single protein, Btf, has global impact upon the nuclear/cytoplasmic distribution of mRNAs.

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Nucleolar localization signals interact electrostatically with nucleolar components.

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The majority of nucleolar proteins are dynamically exchanged between the nucleolus and the surrounding nucleoplasm. How proteins are accumulated (sometimes in relatively high concentration) within such non-membrane-bound substructures remains unclear. One way of protein retention in the nucleolus is associated with the presence of specific short amino acid sequences – nucleolar localization signals (NoLSs). At present, the mechanism of their action is unknown. The analysis of published data showed that all described NoLSs are enriched with positively charged amino acids (~50% of the total amino acid content). One can suggest that the mechanism of interaction with the nucleolar components is electrostatic. To ascertain this

assumption, we used chimeric proteins containing EGFP and short tags containing different amounts (from 0 to 19) of positively charged amino acids – lysines or arginines (such tags were referred to as imitative NoLSs). We found that there was dependence between the charge of the imitative NoLSs and the level of EGFP accumulation inside nucleoli. The level of EGFP accumulation was inversely correlated with the speed of EGFP exchange between the nucleolus and the nucleoplasm. We found that the level of accumulation is higher in case of imitative NoLSs in which positively charged amino acids were not separated by uncharged amino acids. Also, we developed a method for the mapping of the NoLS boundaries. We mapped in detail the signals and their structure in two proteins. The presence of positively charged amino acids in close vicinity of the signal leads to the "blurring" the NoLS boundaries. In fact, in this case, the boundaries can be mapped only approximately. The results of this study confirm that NoLSs interact electrostatically with the nucleolar components.

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Structural basis for cell-cycle dependent nuclear import mediated by Kap121p in *Saccharomyces cerevisiae*.

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In all eukaryotes, precise regulation of the bidirectional transport of macromolecules between the nucleus and the cytoplasm is an essential aspect of many cellular processes, including gene expression, signal transduction, cell cycle progression, and differentiation. Nuclear transport occurs through nuclear pore complexes (NPCs) that form aqueous channels across the nuclear envelope, and altering the structure of NPCs is an emerging mechanism to control nuclear transport. The yeast nucleoporin Nup53p is a target of phosphorylation events in the final stages of mitosis. The phosphorylation of Nup53p alters the interactions of Nup53p with its neighbors during mitosis. This M phase specific molecular rearrangements in the NPCs allow Nup53p to bind the karyopherin Kap121p (also known as Pse1p) specifically during mitosis, slowing its movement through the NPCs and inducing cargo release. The M phase specific regulation of Kap121p-mediated nuclear import is required for normal progression through mitosis. Here we report crystal structures of Kap121p in isolation and also in complex with either its import cargos or Nup53p or RanGTP, which establish the structural basis for recognition of a novel nuclear localization signal (NLS), cargo-Nup53p competition and cargo-displacement by RanGTP. Kap121p has a superhelical structure composed of 24 HEAT repeats. Kap121p is almost exclusively α -helical except for surface loops connecting the inner and outer helices of HEAT repeats and a large insert in HEAT15 that is folded into a small protruding domain involved in Ran-binding. The structures of the Kap121p-cargo complexes define a novel NLS that has a consensus sequence of **KV/IxKNxK/H**. The NLSs bind in the central portion of Kap121p on the inner concave surface of HEAT repeats 8 to 12 through multiple hydrogen bonds, electrostatic interactions and van der Waals contacts. The NLS- and Nup53p-binding sites are exactly the same, suggesting that Nup53p displaces import cargos by direct competition. The binding of cargos or Nup53p to Kap121p is not associated with conformational changes of Kap121p. By contrast, the binding of RanGTP drastically opens up the N-terminal arch of the Kap121p superhelix. Comparison of the NLS- and RanGTP-complexes revealed that RanGTP-mediated dissociation of import cargos involves both a global conformational change that locks the Kap121p superhelix into a cargo-incompatible conformation and a direct displacement by RanGTP.

Endocytic Trafficking I

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Divergent modes for cargo-mediated control of clathrin-coated pit dynamics by signaling receptors.

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Clathrin-mediated endocytosis has long been viewed as being driven by core endocytic proteins, with the internalized proteins being passive cargo. Contrary to this, an emerging view suggests that signaling receptor cargo may actively control their own fate by regulating the dynamics of clathrin-coated pits (CCPs) that mediate their internalization. Despite its physiological implications, very little is known about such "cargo-mediated regulation" of CCPs by signaling receptors. Here, using multi-color TIR-FM imaging in live cells, we show that the mu-opioid receptor, a physiologically relevant G protein-coupled signaling receptor, delays the dynamics of a subset of CCPs in which it is localized. This delay is mediated by the interactions of two critical leucines on the receptor cytoplasmic tail, which regulate key components of CCP scission. These results identify a novel means for selectively controlling the endocytosis of distinct cargo that share common trafficking components, and indicate that CCP regulation by signaling receptors can operate via divergent modes.

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Exploring the Role of Protein-Protein Crowding in Clathrin Mediated Endocytosis.

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Two major mechanisms of membrane bending during clathrin-mediated endocytosis have been investigated: bending by curved protein scaffolds such as the clathrin coat, and bending by insertion of wedge-like amphipathic helices into the membrane by adaptor proteins such as epsin1. Recently we have reported a third general mechanism – membrane bending by protein-protein crowding, where collisions between proteins bound densely to membrane surfaces drive bending (NCB 2012). Endocytic adaptor proteins frequently consist of a folded N-terminal membrane binding domain, and an unfolded C-terminal domain that binds clathrin and other proteins. Due to their lack of structure, unfolded protein domains have much larger hydrodynamic radii than folded protein domains of equal molecular weight. Therefore, based on the finding that molecular crowding can drive bending, we hypothesized that the unfolded portions of adaptor proteins might play an important role in curving membranes. In this study, we show that the unstructured epsin C-terminus can bend model membranes using 3-4 times fewer molecules than the structured epsin N-terminal homology (ENTH) domain, which has traditionally been thought to drive bending. These findings suggest that concentrating the unfolded domains of adaptor proteins at endocytic sites may have a previously unappreciated role in promoting membrane bending. Very recently, Dannhauser et al. (NCB 2012) have shown that clathrin can bend membranes in the absence of the ENTH domain, when recruited to the membrane surface by the epsin C-terminus. This result, in combination with our finding that the epsin C-terminus can drive bending by itself, points to two possible roles for clathrin in membrane bending: (i) clathrin could bend membranes directly through scaffolding, or (ii) it could bend membranes by locally concentrating adaptors and producing steric pressure between them. To investigate these possibilities, we are using epsin C-terminus to recruit

clathrin to the surface of giant unilamellar vesicles. We find that the presence of clathrin reduces the amount of epsin C-terminus needed to bend the membrane by a factor of approximately 2 and that the concentration of epsin on the membrane surface is locally increased by clathrin coats. The aim of our ongoing experiments is to reveal how clathrin and adaptor proteins work together to curve membrane surfaces.

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Live cell imaging of membrane topology during clathrin mediated endocytosis reveals regions of high curvature adjacent to growing pits.

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Induction of membrane curvature is a key feature in the classical model of vesicle formation during clathrin mediated endocytosis. Details of clathrin coated pit geometries and composition have been shown using electron microscopy, superresolution imaging and a suite of biochemical approaches. However, dynamic changes in membrane topology associated with clathrin assembly, pit formation and dynamin-mediated scission, remain poorly characterized. Furthermore, clathrin coated pits and invaginations are smaller (50-120 nm) than the resolving power of traditional fluorescence microscopes (approx. 250 nm). Improved techniques are required to monitor these sub-diffraction-limited objects in living cells. Polarized total internal reflection fluorescence (pol-TIRF) microscopy permits visualization of sub-resolution membrane curvature in cells labeled with lipophilic fluorophores that are oriented within the plasma membrane. We describe an approach to preferentially excite carbocyanine fluorophores (e.g. DiI) whose dipoles are oriented perpendicular or parallel to the glass substrate. A polarized laser beam is focused at orthogonal azimuthal positions in the back focal plane of a high numerical aperture lens to distinguish membrane curvature based on ratio imaging of the two excitation polarizations. Visualization of membrane topology with DiI in skin melanoma cells expressing endogenously-tagged GFP-dynamin permitted high spatiotemporal resolution imaging of clathrin mediated endocytosis. Unexpectedly, regions of high curvature were often observed adjacent to clathrin pits. The topology observed here is consistent with a role for membrane protrusions in the completion of endocytosis.

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Time-resolved electron tomography reveals how the plasma membrane is reshaped during endocytosis.

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Endocytosis, like many dynamic cellular processes, requires precise temporal and spatial orchestration of a complex protein machinery to mediate vesicle budding. To understand how the yeast plasma membrane is reshaped by the components of this machinery, we applied our recently developed correlated fluorescence microscopy and electron tomography procedure. This approach allows to directly correlate specific time windows, which are defined by the presence or absence of key protein pairs, with ultrastructure. Our analysis spans the time from recruitment of the early endocytic module, including Ede1, to the disassembly of the actin network, represented by Abp1. We systematically located 211 endocytic intermediates, assigned each of them to one of nine defined time windows during endocytosis, and reconstructed their ultrastructure in 3D. These reconstructions revealed flat membranes, invaginations of various depth and vesicles at endocytic sites. We then extracted parameters describing these membrane ultrastructures and performed a quantitative analysis of shape changes. The resulting virtual ultrastructural movie describes the protein-mediated membrane

shape changes during endocytosis. We find that clathrin is recruited to flat membranes and does not initiate curvature; membrane invagination only begins upon actin network assembly. Subsequently, approximately 3 s after initial bending and when invaginations are approximately 50 nm deep, amphiphysin binding to parallel membrane segments promotes elongation of the invagination into a tubule, which constricts into a neck at 1/3 of its depth. Scission occurs on average 9 s after initial bending, releasing non-spherical vesicles with 6'400 nm² mean surface area. These vesicles often retain the teardrop-shape of necked invaginations. In summary, our large, quantitative dataset reveals the sequence of changes in membrane shape occurring during endocytosis, and places them in the context of recruitment and disassembly of endocytic protein modules.

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Phosphorylation of clathrin light chain b regulates GPCR trafficking.

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Transmembrane receptors are involved in a myriad of physiological functions and disease can result if they are defective. G protein-coupled receptors (GPCRs) can signal at the cell surface as well as throughout the endocytic pathway and consequently the signal output may differ depending on cellular location. Regulation of the core endocytic machinery facilitates differential uptake of individual cargoes and ensures their appropriate trafficking. Clathrin-mediated endocytosis is a major entry portal into cells. Clathrin forms a lattice around invaginating pits that have captured cargo. Upon vesicle formation, regulated uncoating of components ensures efficient delivery to early endosomes.

We have investigated how clathrin regulates GPCR trafficking. In solution clathrin exists as triskelia composed of three molecules of clathrin heavy chain and associated clathrin light chains (CLC). We show that knockdown of both CLCs inhibits the endocytosis of GPCRs. Strikingly, phosphorylation of CLCb by G-protein-coupled receptor kinase 2 (GRK2) is required for efficient internalization of a subset of GPCRs. Overexpression of CLCb phospho-mutant inhibits uptake of GPCRs, whose internalization is GRK2-dependent. These results identify a new role for CLCb phosphorylation in the trafficking of a subset GPCRs.

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Girdin regulates endocytosis as a GAP of dynamin2.

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Clathrin mediated endocytosis (CME) is the main pathway of endocytosis and large GTPase dynamin is a central player in this process. However, the regulation of dynamin GTPase activity is not completely understood. Here, we identified actin-binding protein Girdin interacts with dynamin2 through its NT domain, especially N2 domain. And dynamin2 GTPase and GED domain contain Girdin-binding site. In addition, Girdin-NT domain binds with dynamin2 GTP-form and increases dynamin2 GTPase activity in a dose and time dependent manner. Girdin-NT has no effect on dynamin2 self-assembly. All of these results strongly indicate Girdin as the GAP of dynamin2. In consist with Girdin's GAP function, knockdown of Girdin inhibits transferrin endocytosis, overexpression dynamin interacting domain (Girdin-NT or N2) also inhibits transferrin endocytosis, which indicates Girdin-dynamin interaction is essential for endocytosis. In conclusion, this study shows Girdin functions as a GAP for dynamin2 to regulate endocytosis.

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Novel functional roles of non-muscle myosin II in clathrin-mediated endocytosis and synaptic transmission.

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Clathrin-mediated endocytosis (CME) aids in nutrient uptake, receptor mediated signaling, and reabsorption in most cell types. It also plays a major role in synaptic vesicle recycling and therefore is important for sustaining normal synaptic transmission. From electrophysiology experiments on cultured hippocampal neurons isolated from myosin IIB (MIIB) knockout (KO) embryos we found that synaptic transmission was severely impaired compared to control neurons from wild type (Wt) embryos. FM1-43 dye uptake during K⁺ depolarization showed abnormal accumulations in vacuolar structures in the KO neurons that also stained with an early endosomal antigen (EEA1) antibody, which lead us to classify them as recycling endosomes. This data and additional electrophysiology and EM experiments on the hippocampal neurons suggested that the impairment is at least partially due to a presynaptic defect in the synaptic vesicle recycling pathway that involves endocytosis. To obtain mechanistic insights into the role of MIIB we studied embryonic fibroblasts isolated from Wt and MIIB KO mice. We found CME was defective in MIIB KO fibroblasts especially during transferrin-mediated retrieval. Acute inhibition of MII using blebbistatin or knock down of MIIA or MIIB isoforms resulted in decreased uptake of fluorescent transferrin by the fibroblasts. TIRF microscopy of KO cells expressing YFP-clathrin light chain or GFP-dynamin revealed decreased coated pit dynamics. Exogenous expression of MIIB rescued the coated pit dynamics in the KO fibroblasts. Electron microscopy showed abnormally shaped coated pits in the KO fibroblasts. EM studies using HRP transferrin showed increased number of shallow and invaginated pits in KO and blebbistatin treated fibroblasts. In Wt cells, immuno EM detected MIIB in association with a network of actin filaments connected to coated pits. Taken together, our findings indicate MII is an important molecular player in CME and is involved in both development and maintenance of synaptic transmission.

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Synaptojanin 1 plays a role in endocytic trafficking in zebrafish cone photoreceptors.

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Phosphoinositides (PIs) play key roles in regulating membrane trafficking and cells contain multiple PI kinases and phosphatases to regulate the location and levels of PI species. Synaptojanin 1 (SynJ1) is a polyphosphoinositide phosphatase that is involved in recycling of clathrin coated vesicles at synapses in neurons. We have previously identified a zebrafish mutant, no optokinetic response c (*nrc*), which lacks SynJ1. In zebrafish, this mutation results in vision defects and morphological and functional abnormalities at cone photoreceptor synapses. In addition to this expected role of SynJ1 at the photoreceptor synapse, we found that SynJ1 localizes to cone photoreceptor inner segments (IS) and that the loss of SynJ1 results in the accumulation of the synaptic vesicle protein VAMP2/synaptobrevin in the IS.

In this study, we characterized the IS phenotype of *nrc* cone photoreceptors. We found that the ribbon synapse protein ribeye also accumulates in the *nrc* cone IS, and colocalizes with mislocalized VAMP2/synaptobrevin. Using electron microscopy, we observed that the *nrc* cone IS contains large vesicular structures of unknown origin. These structures lack a clathrin coat and are much larger than the vesicles that accumulate at the synapses of neurons lacking SynJ1. In order to determine the origin of these vesicles, we created fluorescent protein markers for various subcellular organelles including the Golgi apparatus, the endoplasmic reticulum and multiple endocytic compartments. These markers were expressed in cones and zebrafish larvae were analyzed at 5 days post fertilization by confocal microscopy. We found that *nrc* photoreceptors contain disrupted Golgi and enlarged Rab5 positive endosomal compartments. Interestingly, the Golgi disruption is not caused by abnormal Golgi development. These results demonstrate a novel role for SynJ1 at the Golgi and in the regulation of early endosomal dynamics in zebrafish cone photoreceptors.

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One track, two destinations: Elucidating how the endosomal Retromer complex mediates distinct trafficking itineraries.

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To maintain the unique identities, compositions, and activities of organelles, divergent membrane trafficking routes for proteins and lipids must exist. The Retromer complex is known to mediate trafficking of cargo from endosomes to the trans-Golgi network (TGN), in a process known as retrograde transport. Interestingly, it was recently discovered that Retromer can also mediate the recycling of the β 2-adrenergic receptor (β 2-AR) from endosomes to the plasma membrane (PM). Using the β 2-AR as a model cargo for the recycling pathway and the Wnt transport receptor Wntless (Wls) as a model cargo for Retromer-dependent retrograde transport, it was determined that these cargos converge at a shared vesicular compartment in their trafficking itineraries when expressed in the same cell. Additionally, it was confirmed that the β 2-AR and Wls have distinct trafficking itineraries, as surface labeled β 2-AR traffics between the PM and intracellular vesicles, while surface labeled Wls traffics between the PM, intracellular vesicles, and the TGN. In the future, we plan to use this system to further elucidate how the Retromer complex is able to physically and biochemically segregate cargos between the recycling and retrograde transport pathways.

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Dopamine receptor D3 regulates endocytic sorting by a Prazosin-sensitive interaction with the coatomer COPI.

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Macromolecules enter cells by endocytosis and are sorted to different cellular destinations in early/sorting endosomes. The mechanism and regulation of sorting are poorly understood, although transitions between vesicular and tubular endosomes are important. We found that the antihypertensive drug Prazosin inhibits endocytic sorting by an off-target perturbation of the G protein-coupled receptor dopamine receptor D3 (DRD3). Prazosin is also a potent cytokinesis

inhibitor, likely as a consequence of its effects on endosomes. Prazosin stabilizes a normally transient interaction between DRD3 and the coatomer COPI, a complex involved in membrane transport, and shifts endosomal morphology entirely to tubules, disrupting cargo sorting. RNAi depletion of DRD3 alone also inhibits endocytic sorting, indicating a noncanonical role for a G protein-coupled receptor. Prazosin induces rigid sorting and recycling endosomal tubules and locks endocytic cargos in these tubules, which is different from Brefeldin A, another small molecule commonly used to disrupt membrane trafficking. Prazosin is therefore a powerful tool for rapid and reversible perturbation of endocytic dynamics.

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Ubiquitination and a Ser/Thr motif are required for internalization of an epsin-specific cargo.

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The epsins are a conserved family of endocytic adaptors involved in Notch and RhoGTPase signaling. Although epsins participate in the endocytosis of several ubiquitinated transmembrane proteins, no epsin-specific cargo has been unequivocally identified to date.

Using a combination of genetics, biochemistry and cell biology, we determined that the endocytosis of the yeast Na⁺/H⁺ antiporter ENa1 (Exitus Natru 1) exclusively depends on the presence of the epsin adaptor. In addition, our results indicate that epsin-mediated internalization requires ENa1 ubiquitination and the simultaneous presence of a Ser/Thr (ST) motif in its intracellular tail. Importantly, the ENa1 ST motif is required for epsin function but not for the transporter ubiquitination.

Specifically, we report that the internalization of GFP-ENa1 is abolished in double epsin knock-outs, but can be rescued by introducing either of the two yeast epsins, Ent1 or Ent2. Further, we established that the UIMs (Ubiquitin Interacting Motifs) of epsin are necessary for ENa1 internalization. Additionally, we have mapped crucial determinants present at the C-terminal intracellular tail of ENa1 that are required for its epsin-dependent internalization. First, we identified a requirement for Lys¹⁰⁹⁰ that can be bypassed by fusion of an in-frame ubiquitin in the C-terminal ENa1 tail. These observations suggest that ENa1 ubiquitination mediates UIM-mediated epsin recognition. Interestingly, although ubiquitination of the yeast GPCR, Ste3, is also required for its internalization, we verified that the endocytosis of this cargo is independent of epsin.

Therefore, we hypothesized the existence of other determinants responsible for conferring epsin specificity. Indeed, we determined the presence of an ST motif (S¹⁰⁷⁶TST¹⁰⁷⁹) essential for ENa1 internalization. Extensive mutational analysis suggested that this ST motif might be targeted for phosphorylation. However, this post-translational modification was not important for ubiquitination, but instead led to enhanced epsin binding as detected by in vitro binding assay. Indeed, both ubiquitination and integrity of the ST motif are simultaneously required for epsin-mediated internalization of ENa1.

In summary, we have identified the first epsin-specific cargo and are in the process of defining an epsin-specific recognition sequence. In addition, these findings set up the basis for more comprehensive studies of epsin function. Further, our results suggest the existence of sequence motifs in ubiquitinated cargo that contributes to the specificity for ubiquitin-binding endocytic adaptors.

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MICAL-L1 regulates the intracellular transport of c-Src.

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Increased activity of the non-receptor tyrosine kinase c-Src (Src) is found in numerous cancers including those of the breast, lung and colon. The intracellular trafficking of Src is intimately associated with its activation and function and the regulation of Src localization within the endocytic pathways is crucially important. Inactive Src localizes to the perinuclear endosomal recycling compartment (ERC). Upon activation, Src translocates from the ERC to the plasma membrane (PM). We have found that Src co-localizes with the endosomal regulatory protein Molecule Interacting with Cas Ligand Like-1 (MICAL-L1). Using immunofluorescence analysis, we demonstrate that MICAL-L1-depletion in HeLa cells leads to Src retention in the ERC. The Src-ERC accumulation is specially pronounced upon serum stimulation, following which Src normally is transported to the PM. By immunoblot analysis, there is a significant reduction in active Src in MICAL-L1-depleted cells. In addition, we find that loss of MICAL-L1 in human fibroblasts leads to accumulation of active Src in intracellular vesicles. Previous studies have shown that intracellular sequestration of Src in fibroblasts stabilizes focal adhesions, leading to defects in cell spreading and migration. In agreement with this, the number and size of focal adhesions in MICAL-L1-depleted fibroblasts is increased. A large number of cells display a singular broad lamella, suggestive of defects in cell spreading. In total, we provide novel data supporting a model in which MICAL-L1 is a crucial endocytic protein regulating the transport of Src, an intracellular kinase that is overactive in numerous cancers.

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Reciprocal regulation of fibroblast growth factor receptor signalling and trafficking mediated through Src and Eps8.

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Fibroblast growth factor receptors (FGFRs) regulate fundamental cellular processes, including proliferation, differentiation and angiogenesis and have emerged as growth factor receptors central to oncogenesis. We developed a live-cell assay system for studying FGFR endocytosis and trafficking by employing both confocal and total internal reflection fluorescence (TIRF) microscopy in cells expressing a previously characterised GFP-tagged FGFR2 construct. Our analyses have demonstrated that endocytosis of activated FGFR occurs through a dynamin dependent pathway which we have identified as clathrin-mediated endocytosis. While AP2 siRNA and Dynasore treatment completely inhibited FGFR endocytosis, caveolin1 siRNA had no effect. Additionally, following stimulation significant colocalization of FGFR and clathrin is observed, while no colocalization with caveolin1 was detected. Interestingly, FGF treatment also significantly increased the number of CCPs as well as the number of clathrin-mediated endocytic events. However, as FGF treatment did not increase transferrin uptake we hypothesize that these may represent a cargo specific population of clathrin-coated pits.

Recent proteomic studies have identified the multifunctional scaffolding protein Eps8 as a prominent target of Src kinase activity with potential roles in the regulation of the signalling and trafficking of FGFR. Our analysis shows that treatment of cells with the Src family inhibitor Dasatinib or depletion of Eps8, prevents the FGF induced increase in plasma membrane clathrin and reduces the internalization of FGFR. Following stimulation, FGFR passes through an Eps8 positive peripheral compartment en route to the peri-nuclear recycling compartment (PNRC). Both Src and Eps8 are required for receptor to exit from this EEA1 positive peripheral compartment into the Rab11 positive PNRC. Eps8 depletion also inhibits the early phases of

ERK activation in response to FGFR activation, placing this signalling event early in the trafficking pathway of the receptor. Thus, these results have identified the endocytic pathway for endocytosis of FGFR2 and described Eps8 and Src as a key mediator of the early phases of activated FGFR trafficking and signalling.

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Examining the Role of the ArfGAP Asap1 in Coupling Endocytosis and Actomyosin Downregulation during *Drosophila* Early Embryogenesis.

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Plasma membrane structure is controlled by membrane trafficking and cytoskeletal activity. Arf small G proteins are major regulators of such events, and are activated by Arf GEFs (Guanyl Nucleotide Exchange Factors) and inactivated by ArfGAPs (GTPase Activating Proteins). Here we show the requirement of the ArfGAP Asap1 in the proper development of the *Drosophila* early embryo. Asap1 RNAi led to abnormal expansion of actomyosin-coated membranes at the tips of invaginating plasma membranes of cellularizing embryos. This actomyosin expansion was accompanied by the pinching and expulsion of nuclei from the embryo periphery. To determine the initial time point of actomyosin membrane expansion, we monitored actomyosin dynamics live and discovered the expansions arise during syncytial stages, prior to cellularization. Consistent with a role in membrane trafficking and endocytosis, Asap1 RNAi also inhibited the formation of Amphiphysin (Amph) positive tubules during early cellularization. Strikingly, RNAi of the ArfGEF Steppke (Step) or Arf1 also results in abnormal actomyosin network expansion and the inhibition of Amph positive tubules. This prompted us to pursue whether Asap1 might function with Step and Arf1. Co-expressed Asap1::GFP and mCherry::Step fusion proteins colocalized throughout the cortex of the forming epithelium and both were enriched at the tips of invaginating plasma membranes. Interestingly, co-expression of these proteins also led to an increase in the number of embryos with strong cortical mCherry::Step compared to the expression of mCherry::Step alone. We hypothesize that Asap1 may stabilize Step at the plasma membrane via Arf GTPase cycling and we are testing whether Step also affects cortical Asap1 levels. However, overall Asap1::GFP localization was unaffected with Step RNAi, suggesting other mechanisms may be involved in the proper placement of Asap1. To determine whether Asap1 localization is dependent on its activity towards Arfs, we generated a GFP::Asap1 fusion protein that lacks its GAP domain. Intriguingly, this deletion led to abnormal localization to Amph positive tubules, suggesting Arf binding may be somehow coupled to tubule regulation. Together, these data outline a regulatory system important for coupling endocytosis and actomyosin downregulation in the early *Drosophila* embryo.

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Dynein provides the mechanical force for membrane tubulation in clathrin-independent endocytosis.

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The mechanisms responsible for inducing membrane curvature in the early stages of raft-dependent, clathrin-independent endocytic pathways remain poorly defined. Recent studies have identified a role for certain cargo molecules of these pathways, such as the glycolipid binding toxins Shiga toxin and cholera toxin, in mechanically deforming the plasma membrane

to produce tubular endocytic intermediates. However, it is currently unclear if cellular components also contribute to the process of membrane tubulation in this pathway. In the current study, we tested for a potential role of microtubules and microtubule motors in this process. As a model system, we visualized tubule formation in COS-7 cells labeled with fluorescent cholera toxin B-subunit (CTxB) under conditions previously shown to be permissive for tubule growth but that inhibit tubule scission. Pretreatment of cells with nocodazole prior to the addition of CTxB blocked tubule formation, indicating an intact microtubule network is required. In contrast, the number and length of tubules were unaltered when microtubules were stabilized with taxol, ruling out a role for microtubule dynamics in the tubulation process. Time lapse imaging revealed the tubules underwent complex motions including branching events and bi-directional movements, suggesting microtubule motors may also be important for tubule extension. Consistent with this, disruption of dynactin by expression of dynamitin or CC1, or direct inhibition of the ATPase activity of dynein using the small molecule inhibitor ciliobrevin A inhibited tubule formation. These findings suggest the microtubule motor dynein provides the primary mechanical force for membrane tubulation, thus defining a novel role for microtubule-based motility in early stages of clathrin-independent endocytosis.

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Endosomal Network Analysis identifies a role for the metabolic enzyme ATIC and the putative tyrosine phosphatase PTPLAD1 on insulin receptor (IR) regulation.

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Following insulin binding at the cell surface of target cells, there is within seconds internalization of the activated complexes into the endosomal apparatus. At this intracellular locus, the IR is deactivated by the concerted action of several mechanisms including insulin dissociation at the intraluminal acidic pH followed by degradation of free insulin and rapid tyrosine dephosphorylation of IR regulatory phosphotyrosine sites. A proteome of a Golgi/endosomes (G/E) fraction prepared from the mouse liver was previously described by us. Bioinformatics analyses using Mouse Genome Informatics (MGI) facilities now show that they enriched for specific groups of proteins and depleted for others. Proteins identified in the G/E fraction can be grouped and linked according to their functional and molecular associations in the cell whereby they form tight and interconnected clusters. Altogether, these results can be now summarized as a network of functional links between proteins known to be involved in specific cellular functions and mechanisms. Among the yet to be characterized proteins associated with the internalized IR, the higher probability Mascot score was attributed to a metabolic enzyme, ATIC, a homodimeric enzyme implicated in the two final steps of the de novo purine biosynthesis pathway. We confirmed the association in hepatic endosomes and demonstrated that IR/ATIC binding can be dynamically reconstituted in vitro by using IR-loaded endosomes and that ATIC increases IR autophosphorylation activity. The association was not observed when the IR was replaced with the internalized epidermal growth factor receptor (EGFR). We also verified the IR/ATIC association in HEK293 cells partially depleted with ATIC and have confirmed the presence of IR/ATIC complexes and decreased IR tyrosine phosphorylation. The transmembrane protein PTPLAD1 was also detected in IR complexes. The deletion of PTPLAD1 in HEK293 cells induced IR hyperphosphorylation. The results indicates the presence of a mechanism whereby ATIC counteracts the IR tyrosine dephosphorylating activity of PTPLAD1 in endosomes.

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Cell surface GAPDH recruits Apo Tf to facilitate iron export in iron overloaded cells.

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Iron is an essential element for vital metabolic processes but is toxic in excess. Nature has evolved a delicately balanced system for its storage and recycling where the reticuloendothelial system plays an important role in maintaining this homeostasis. Though the mechanism of iron import into cells involving carrier proteins like transferrin and transferrin receptors is well characterized, iron export from cells still remains poorly understood. Till date ferroportin is the only known mammalian iron exporter and is highly expressed in duodenal enterocytes and macrophages. In the current study we report that cells (J774, differentiated THP1, primary splenic & peritoneal macrophages and hepatocytes) express the glycolytic enzyme glyceraldehyde -3-phosphate dehydrogenase (GAPDH) on their surface upon iron overload which results in increased Apo transferrin (Apo Tf) binding to their surface. Apo Tf is one of the best known biological chelators of iron and we hypothesize that cell surface GAPDH and Apo Tf assist cells to remove excess intracellular iron. GAPDH and Apo Tf interaction was confirmed by solid phase assays, co-immunoprecipitation, confocal microscopy colocalization and Förster resonance energy transfer (FRET). The equilibrium dissociation constant of GAPDH-Apo Tf interaction was found to be 5.3 nM by biacore analysis. The results were further confirmed in GAPDH knockdown THP1 cells which failed to present GAPDH to their surface upon iron overload and abolished the Apo Tf binding. Iron overload presented in form of erythrophagocytosis (in J774, differentiated THP1 and primary splenic macrophages) also resulted in surface presentation of GAPDH and increased Apo Tf binding. We further observed that this surface GAPDH interacts with ferroportin as confirmed by co-immunoprecipitation, confocal microscopy colocalization and FRET. By loading cells with radioactive iron we confirmed that addition of Apo Tf to cells facilitated the exit of iron. Our results demonstrate that upon iron overload cell surface GAPDH functions as high affinity receptor for Apo Tf increasing its local concentration in close proximity to cells so that iron released by ferroportin is quickly chelated by it to avoid any toxic effects of free iron. Collectively our findings provide a new dimension to the iron export mechanism where cell surface GAPDH recruits Apo Tf and facilitates the iron export in conjunction with ferroportin.

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Exit of the intracellular pathogen *Nematocida parisii* from *C. elegans* intestinal cells.

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Microsporidia comprise a diverse phylum of fungal-related pathogens that infect a broad range of hosts, including insects and humans. As obligate intracellular parasites, microsporidia are dependent on their hosts for replication. Although they likely usurp many host processes, very little is known about the mechanisms of pathogenesis used by these ubiquitous microbes. Recently we found that the microsporidia *Nematocida parisii* reorganizes the host *C. elegans* actin cytoskeleton and terminal web prior to making a non-lytic exit from host cells. We found that *N. parisii* spores exit from the apical side of intestinal cells into the lumen, and that they are free of host membrane after exit. By studying how *N. parisii* exits from cells while minimizing damage to the host we expect to learn about intestinal cell biology and intracellular trafficking as well as key mechanisms of microsporidia transmission. Our previous studies indicated that actin was required for *N. parisii* exit from intestinal cells, but we did not know the underlying mechanism. We now show that actin forms distinct "coats" on spores localized near the apical membrane. The number of actin coats in an animal is positively correlated with the number of

spores that are shed. In addition, animals with actin-coated spores are more contagious than animals without actin-coated spores. Thus, we believe that actin-coated spores are exiting from the host, and we are developing methods to image this process *in vivo*. A candidate RNAi screen of small GTPases revealed that members of the endocytic recycling pathway including *rab-5*, *rab-10*, *rab-11*, *cdc-42* and *ced-10* are required for efficient actin spore coat formation as well as spore exit. Furthermore, spore exit requires core components of the exocyst complex. So far, spore exit strongly resembles "kiss and coat" exocytosis, where membrane fusion is thought to trigger actin polymerization that stabilizes large cargo during the exit process. In this way, microsporidia appear to usurp host processes in order to proceed through their life cycle and exit non-lytically from host cells. These findings provide some of the first cell biological insights into microsporidia pathogenesis and may also offer insight into the fundamental biology of intestinal cells. Future work will focus on how microsporidia hijack the endocytic recycling pathway in order to traffic to the apical cell surface, as well as characterizing membrane topology during spore exit.

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Adaptor protein Sorting Nexin 17 facilitates human papillomavirus trafficking and infection.

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The Human Papillomavirus (HPV) L2 capsid protein plays an essential role during the early stages of viral infection, but the molecular mechanisms underlying its mode of action remain obscure. Using a proteomic approach we have identified the adaptor protein, Sorting Nexin 17 (SNX17) as a strong interacting partner of HPV-16 L2. This interaction occurs through NPxF/Y phosphotyrosine-binding domain, which is present in all papillomavirus (PV) types analysed. Using mutants of HPV-16 L2 defective for SNX17 interaction, or siRNA ablation of SNX17 expression we demonstrate that the interaction between L2 and SNX17 is crucial for viral infection. Furthermore, we show that SNX17 is essential for infection with multiple PV types from Alpha, Beta, Delta, Kappa and Pi genera, indicating an evolutionary highly conserved viral entry mechanism. Loss of the L2-SNX17 interaction results in enhanced turnover of the L2 protein and decreased stability of the viral capsids, and concomitantly there is a dramatic decrease in the efficiency with which viral genomes transit to the nucleus. Indeed, using a range of endosomal and lysosomal markers we show that capsids defective in their capacity to bind SNX17 transit much more rapidly to the lysosomal compartment. These results demonstrate that the L2-SNX17 interaction is essential for viral infection and facilitates the escape of the L2-DNA complex from the late endosomal/lysosomal compartments. Moreover, partial nuclear localisation of SNX17 during an HPV-16 infection suggests that SNX17 is also involved in the transport of the L2-DNA complex to the nucleus. These studies reveal a novel role for SNX17 in aiding viral infection processes, and raise the possibility that normal cellular recycling pathways are perturbed as a result of HPV infection.

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A Nibbling Mechanism for the Clathrin-mediated Recycling of Secretory Granule Membrane after Exocytosis.

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pTIRFM studies from our laboratory have revealed that most fused granule membranes do not undergo rapid endocytosis in chromaffin cells, but rather remain in the plasma membrane with varying degrees of curvature for many seconds. In this study we examine the subsequent fate of the chromaffin granule membrane. Immunocytochemistry of the granule membrane protein dopamine beta hydroxylase (DBH) revealed that DBH remained punctate on the surface for at least 30 minutes, but that the intensity of the puncta decreased over time. Other granule proteins (e.g., VMAT2, VAMP/synaptobrevin, synaptotagmin), some of which have clathrin-adaptor binding domains, also remained associated with these puncta. An analysis of the distribution of DBH puncta intensities over time suggested that the granule membrane components are gradually removed from the cell surface via a nibbling process, rather than via a single discrete event as in rapid endocytosis. Live cell TIRFM imaging of overexpressed GFP-labeled clathrin light chain or dynamin2 provided evidence that both proteins begin to accumulate at fusion sites within 10 seconds of release of granule contents. To directly demonstrate consummated endocytosis, a fixable fluid phase marker (Alexafluor cadaverine) was used to label endocytic vesicles in conjunction with antiDBH. In the absence of stimulation, cadaverine was taken up by constitutive endocytosis into vesicles which did not contain DBH. Following a 30 second depolarization, a second population of vesicles, containing both cadaverine and DBH, appeared. The presence of antiDBH did not alter constitutive cadaverine uptake. A role for endogenous dynamin in the nibbling process was demonstrated using the membrane-permeant dynamin GTPase inhibitor dyngo4a. Dyngo4a largely blocked the decrease in DBH puncta intensity over time, while having no effect on the constitutive uptake of cadaverine. In addition, overexpression of a GTPase-deficient mutant of dynamin2 (dyn2K44A) also prevented the loss of DBH puncta from the surface. We conclude that secretory granule membrane components remain associated on the plasma membrane following fusion and are internalized together in small increments via a clathrin- and dynamin-dependent process we term nibbling. These results explain the size mismatch between chromaffin granules (~300 nm diameter) and clathrin-coated vesicles (approximately 100 nm).

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Endosomes, Lysosomes, and Lysosome-related Organelles

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Lysosome-related organelle biogenesis protein complexes interact with the WASH complex.

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Biogenesis of lysosome-related organelles requires the targeting of specific membrane proteins and soluble cargoes to maturing organelles. One mechanism for this targeting is the recruitment and concentration of cargo into nascent vesicles by adaptor protein complexes, such as the

adaptor protein complex-3 (AP-3). Mutations to AP-3 and related protein complexes including the BLOC-1 complex cause the Hermansky Pudlak Syndrome, which is characterized by hypopigmentation, platelet dysfunction, and pulmonary fibrosis. Adaptor proteins are recruited to membranes from the cytosol in part by specialized phospholipids, making lipid kinases key regulators of membrane traffic. One such regulator is the phosphatidylinositol-4-kinase type IIa (PI4KIIa), which is known to bind and regulate the function of both AP-3 and BLOC-1 complexes. In order to search for upstream and downstream regulators of this pathway we designed an antibody that binds to PI4KIIa at its coat-interaction motif and thereby predominantly recognizes a subset of PI4KIIa that is not engaged in coat interactions. Using this antibody, we isolated PI4KIIa protein complexes by immunoaffinity chromatography coupled with *in vivo* isotope tagging of the cell proteome and mass spectrometry (SILAC). We identified several regulators of the actin cytoskeleton, including the WASH complex, an Arp 2/3 activator, and the RhoA guanine exchange factor RhoGEF1. We confirmed these interactions by independent immunoaffinity chromatography experiments, sucrose velocity sedimentation, and deconvolution immunofluorescent microscopy. We conclude that PI4KIIa, a key enzyme regulating cytosolic coats required for lysosome-related organelle biogenesis, interacts with RhoGEF1 and the WASH complex. Future work will test the hypotheses that the WASH complex is required for AP-3- and/or BLOC-1-dependent cargo sorting and vesicle fission for lysosome-related organelle biogenesis.

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Monoubiquitination of syntaxin 3 regulates its basolateral endocytosis and sorting to intraluminal vesicles in MDCK cells.

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Syntaxins are a family of membrane fusion proteins involved in nearly all intracellular membrane trafficking pathways. In polarized epithelial cells, syntaxin 3 (Stx3) functions in apical membrane fusion. A small population of Stx3 is sorted to the basolateral membrane, only to be rapidly internalized. Stx3 also localizes to multivesicular bodies (MVBs) and lysosomes, although their trafficking to and function at these sites is currently unknown. In Madin-Darby canine kidney (MDCK) cells, we show that monoubiquitination at a conserved, polybasic, juxtamembrane region of Stx3 induces its internalization from the basolateral plasma membrane and subsequent trafficking to early endosomes. We use a Rab5 mutant to enlarge early endosomes and observe Stx3 in intraluminal vesicles (ILVs). We also show that Stx3 is present in exosomes purified from mammalian cell culture and human urine.

Trafficking of Stx3 to endosomes is prevented when lysine residues in the polybasic region are mutated to arginines and ubiquitination is prevented. We show that the Stx3-5R mutant is delayed in its rate of internalization from the basolateral plasma membrane and is also excluded from ILVs in enlarged early endosomes. Consequently, nearly none of Stx3-5R is found in exosomes isolated from mammalian cells expressing the mutant. Altogether, our data show that monoubiquitination on Stx3 is a signal for endocytosis and sorting to intraluminal vesicles. This suggests that ubiquitination on some SNARE proteins may be a general pathway for their distribution throughout the cell.

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Syndecan-syntenin-ALIX regulate exosome biogenesis.

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Syndecans are essential for fine-regulating signaling events between cells¹. The extracellular heparan sulfate (HS) chains of the syndecans attract several signaling molecules, such as morphogens, to cell surfaces, but the mechanisms of their regulatory functions remain largely unknown.

Our work describes a new mechanism for exosome biogenesis and MVB formation. We have found that, through the PDZ protein syntenin², a well-known syndecan cytoplasmic adaptor, syndecans are connected to ALIX³, an auxiliary component of the ESCRT machinery, implicated in vesicular budding, and marker of small signaling vesicles released by cells, known as 'exosomes'.

In the study, we explore whether the syndecan-syntenin-ALIX connection might regulate the biogenesis of exosomes, impacting on the cellular release of morphogen signals. The results obtained show that interfering with syndecan-syntenin-ALIX reduces exosome production by affecting the biogenesis of MVBs and that syndecan-syntenin-ALIX complexes control the composition of a specific population of exosomes, potentially transferring specific information between cells⁴.

The establishment of a direct link between syndecans and exosomes, as a novel mechanism of morphogen spreading, may help explain the role of HS in the diffusion of morphogenetic signals, in particular how lipid-modified morphogens travel long-range. Syndecan-syntenin exosomes might also participate in the biology of HS-associated pathological processes, including neurodegenerative and oncogenic diseases.

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Endo-lysosome trafficking is critical for maintaining membrane integrity in polarized retinal pigment epithelial cells.

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Rapid plasma membrane repair is a ubiquitous, highly conserved response crucial for cell survival. Pore-forming toxins or the terminal complement complex C5b-9 blast holes in membranes, induce calcium influx and activate signaling pathways that compromise cell function and can result in cell death. Assembly of sub-lytic C5b-9 membrane attack complexes (MAC) on the plasma membrane of retinal pigment epithelial (RPE) cells contributes to the pathogenesis of age-related macular degeneration (AMD), the most common cause of blindness in older adults. Mechanisms that limit sub-lytic MAC activity include: cell surface GPI-anchored complement regulatory proteins CD55 and CD59 that inhibit specific steps of MAC formation; elimination of assembled MAC by secretion into exosomes or by endocytosis and subsequent lysosomal degradation; and rapid resealing of pores by the exocytosis of late

endosomes and lysosomes. Here, we investigated how RPE membrane integrity is maintained after exposure to calcium ionophores (ionomycin), pore-forming toxins (streptolysin O) or sub-lytic C5b-9 deposition. Live imaging of polarized primary RPE cells using spinning disk/TIRF microscopy showed that a short exposure to ionomycin or sub-lytic MAC results in rapid exocytosis of late endosomes and lysosomes expressing GFP-CD63 or GFP-LAMP2, indicating that these organelles participate in RPE membrane repair. Pore formation induces endocytosis of CD55-GFP, suggesting that toxin and MAC removal from the plasma membrane occurs by internalization into endosomes that also contain complement regulatory proteins. Calcium influx also accelerates the secretion of sub-micron membrane vesicles called exosomes from RPE cells. We next investigated membrane repair in cells containing the retinal lipofuscin fluorophore A2E, a hallmark of AMD that causes cholesterol accumulation in late endosomes and lysosomes. Late endosomal cholesterol levels are critical for regulating organelle motility and for sorting GPI-anchored proteins. In RPE cells with A2E, surface biotinylation and immunofluorescence analyses show that recycling of CD59 to the plasma membrane is decreased. A2E also inhibits the tubulo-vesicular trafficking of GFP-CD63 and GFP-LAMP2, prevents lysosome exocytosis and alters exosome secretion in response to ionomycin or C5b-9. Our results indicate that membrane integrity in the RPE is maintained by the concerted actions of CD55, CD59 and late endosome-lysosome trafficking in both the exocytic and endocytic pathways. Lipofuscin/A2E interferes with membrane trafficking routes that involve late endosomes and lysosomes in the RPE. These data provide mechanistic insight into how lipofuscin accumulation decreases complement regulatory protein function and compromises the ability of the RPE to maintain membrane integrity, both of which likely contribute to a chronic inflammatory environment in the outer retina.

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Rules for ESCRT-mediated protein sorting.

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Multivesicular bodies (MVB) are mature endosomes that internalize a portion of the limiting membrane containing membrane proteins into the compartment, thus forming intraluminal vesicles (ILVs). This invagination process is catalyzed and directed away from the cytosol by cytosolic protein complexes called ESCRTs (Endosomal Sorting Complexes Required for Transport). Ubiquitin is the only well established signal for proteins to be sorted into ILVs by ESCRTs but there are reports of other sorting signals that are not well understood. The internalized lipids and proteins are turned over in the vacuole/lysosome. The ESCRT pathway is responsible for the downregulation of integral membrane proteins and for shutting down cellular signals by sequestering the signaling membrane proteins away from the cytosol. The pathway consists of several proteins that can be broadly divided into 2 categories; (1) the ESCRT-machinery proteins themselves and (2) the cargo proteins that get sorted into ILVs. ESCRTs and cargo proteins are very similar in many aspects (both get ubiquitinated and bind other ESCRT proteins) but unlike cargo proteins, ESCRTs predominantly stay outside the MVBs and are used for several rounds of vesicle formation. It is not clear how the ESCRT pathway differentiates between the ESCRT-machinery and the cargoes. It is even more surprising that ESCRTs don't enter MVBs considering the fact that they bind phosphatidylinositol-3-phosphate (PI-3P), a lipid that enters MVBs. Indeed, a few studies have reported sorting of ESCRTs into MVBs under special circumstances (regulated degradation of ESCRTs by sorting into MVBs; identification of ESCRTs in exosomes that are derived from MVBs; identification of ESCRTs in HIV virions that use ESCRTs to egress from the host cell plasma membrane; and packaging of the mutant ESCRT-I into MVBs in a strain lacking an ESCRT-I subunit, *mbv12*). From the

perspective of cargoes, it is not clear what the requirements are to be sorted into ILVs. In this study, we address two important questions; (1) what prevents ESCRTs from entering ILVs during normal MVB formation? and (2) what makes an efficient cargo for the ESCRT pathway. We used artificial fusion protein mimics for ESCRTs and cargoes to probe for the rules that govern their behavior. Our unbiased approach led us to conclude that cargo proteins have to; (1) associate with the endosomal membrane, (2) bind any ESCRT protein (including ESCRT-III that has no clear role in cargo sorting thus far) (3) and lose the interaction with ESCRTs before ILV formation. We also discovered a simple underlying mechanism that differentiates the ESCRTs from cargoes based on interaction strength. We propose a tug-of-war model where in ESCRTs get recycled back into cytosol by virtue of their strong interactions with other ESCRTs. When this inter-ESCRT interaction is weakened, their interaction with the membrane wins over to pull them into the ILVs.

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The yeast Alix homolog, Bro1, functions as an ubiquitin receptor for protein sorting into multivesiculated endosomes.

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Sorting of endocytosed membrane proteins into luminal vesicles of late endosomes/multivesicular bodies (MVB) is an important step for their vacuole/lysosomal degradation. This step is mediated by the ESCRT multicomponent apparatus as well as associated factors such as Vps4, the AAA-ATPase that catalyzes ESCRT complex disassembly and release, and the accessory protein Bro1 that recruits deubiquitinating enzyme Doa4 to ESCRT-III so that ubiquitin can be removed from cargo after cargo sorting. ESCRT-0, -I, and -II proteins contain ubiquitin-binding domains, which recognize ubiquitinated cargo to promote its entry into MVB luminal vesicles.

Here we show that Bro1 together with ESCRT-0 may work as a receptor for selective sorting of ubiquitinated cargos targeted for endosomal degradation. We found synthetic genetic interactions of BRO1 with ESCRT-0, arguing that Bro1 works at a step similar to ESCRT-0, which serves as a central ubiquitin receptor for MVB sorting. We found that Bro1 binds ubiquitin via its middle V-domain. In addition, the V-domains of other Bro1-like proteins including HD-PTP, Alix, and RIM20 could bind both K63-linked polyubiquitin and monoubiquitin. To understand structural basis of Bro1 V-domain interaction with ubiquitin, we solved the X-ray crystal structure of the protein complex. The yeast Bro1 V-domain was found to fold in a V-shaped two three-helix bundle, similar to the Alix V-domain. The structure revealed that the V-domain has a major ubiquitin-binding region centered along its N-terminal helix. The residues involved in interaction with ubiquitin were confirmed using a series of NMR paramagnetic relaxation experiments. Using both X-ray diffraction and NMR data, we built a model for how Bro1 V-domain binds ubiquitin that was confirmed with mutagenesis experiments. Mutants of Bro1 lacking the ability to interact with ubiquitin were defective in sorting ubiquitinated cargo membrane proteins, but only when combined with additional mutations in ESCRT-0 that compromised its biochemical interactions. Together these data suggest that Bro1 can work early in the MVB sorting process to recognize ubiquitinated cargo and implies that other Bro1 family members may perform a similar function.

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Vps9 drives the formation of class E compartments in yeast by causing hyper-activation of Vps21.T. Shideler¹, G. Odorizzi¹; ¹MCDB, University of Colorado, Boulder, CO

Membrane trafficking through the endocytic pathway is regulated by Rab5 GTPases that promote fusion of incoming transport vesicles with early endosomes as well as homotypic fusion of early endosomes with one another. How Rab5 activity is regulated during the maturation of early endosomes into late endosomes is unknown. In *Saccharomyces cerevisiae*, Vps21 is the ortholog of human Rab5A. Vps21 is activated by the Guanine nucleotide Exchange Factor (GEF) Vps9. The aim of this study is to understand how Vps9 regulates Vps21 in the context of endosomal maturation.

Ubiquitinated transmembrane proteins targeted for lysosomal degradation are directed to endosomes, where they are sorted into intraluminal vesicles (ILVs) by the Endosomal Sorting Complexes Required for Transport (ESCRTs). The formation of ILVs occurs during endosomal maturation. Late endosomes filled with ILVs are known as multivesicular bodies (MVBs).

Disruption of ESCRTs in yeast blocks MVB biogenesis and, instead, leads to the formation of class E compartments, which consist of endosomes that have aberrantly flattened and stacked with one another. Recently, we showed that the formation of class E compartments is driven by hyperactive Vps21. Here we show that Vps9 is also required for class E compartment biogenesis, indicating that Vps9 is the GEF driving Vps21 hyperactivity in ESCRT-mutant cells.

Vps9 is composed of an unstructured N terminus, a conserved Vps9 domain, and a ubiquitin-binding CUE domain. We use confocal fluorescence microscopy to show that the CUE domain alone localizes to endosomes but is not necessary for endosomal localization of Vps9. By electron microscopy, we see that Vps9 lacking the CUE domain supports class E compartment formation. Therefore, we speculate that ubiquitin-binding reinforces Vps9 localization at endosomes but is not required for its activity toward Vps21.

The Vps9 ortholog in *C. elegans* is RABX-5, which appears to be down-regulated upon being displaced from endosomes by SAND-1. In yeast, the SAND-1 ortholog is Mon1. We see by confocal fluorescence microscopy that loss of Mon1 in yeast causes both GFP-Vps9 and GFP-Vps21 to accumulate at endosomes. By electron microscopy, we show that loss of Mon1 causes MVBs to enlarge, potentially as a consequence of continued Vps9 activation of Vps21. We propose a model where Vps9 is displaced by Mon1 after the completion of ILV cargo sorting by ESCRTs.

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Effects of ESCRT component knockout on MHCII compartments in dendritic cells.A. De Mazière¹, S. van Dijk¹, J. Ma², I. Mellman², J. Klumperman¹; ¹Department of Cell Biology, University Medical Center Utrecht, Utrecht, Netherlands, ²Genentech, South San Francisco, CA

Major histocompatibility complex class II (MHCII) molecules of dendritic cells (DCs) present antigenic peptides to T-lymphocytes to generate a cellular immune response. In immature DCs, MHCII molecules are loaded with antigenic peptides by the assistance of the chaperone DM. This occurs in MHCII compartments (MIICs), which are specialized late endosomes/lysosomes. In MIICs, the majority of MHCII is localized on intraluminal vesicles, where it is destined for degradation. It has been hypothesized that the ESCRT complexes are responsible for sorting of ubiquitinated MHCII to the intraluminal vesicles of MIICs. Upon maturation of the DCs,

ubiquitination is downregulated and the large majority of the MHCII molecules accumulates at the plasma membrane.

In the present study, the role of Hrs (ESCRT-0), and of Tsg101 (ESCRT-I) in the localization of MHCII molecules was investigated by immunoelectron microscopy. In immature DCs derived from mice with a conditional knockout (KO) for either Hrs or Tsg101, MHCII was distributed as in wild type (WT) DCs, with, in the MIICs, a similar percentage of MHCII molecules present on the intraluminal vesicles. After maturation of the DCs by 16 h stimulation with LPS, the majority of MHCII molecules in the Hrs KO DCs shifted to the plasma membrane, similar as in WT cells. In the Tsg101 KO DCs this shift also occurred, however, part of the MHCII was found on aberrant clusters of membranes (vesicles and cisterns), resembling class-E compartments described in yeast and mammalian cells. The vesicles and cisterns were surrounded by a dense matrix, which sometimes showed periodic densities. They were accessible for endocytosed BSA-gold and positively labeled for Invariant chain (Ii), indicating an early endosomal origin. In addition, the vesicle clusters were positive for ubiquitin and p62, suggesting that they were involved in the process of autophagy. In conclusion, our results suggest that Hrs and Tsg101 are not necessary for the sorting of MHCII to the intraluminal vesicles of MIICs, but in stimulated cells the absence of Tsg101 gives rise to a class-E like compartment.

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Characterization of PCSK9 trafficking reveals a novel lysosomal transport complex.

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Proprotein convertase subtilisin/kexin type 9 (PCSK9) interacts directly with the LDL receptor (LDLR) and targets it for lysosomal degradation. It has been shown that inhibiting PCSK9-LDLR interactions effectively blocks degradation of LDLR, thus, PCSK9 has emerged as a promising therapeutic target for lowering LDL cholesterol. Furthermore, it has been hypothesized that LDLR binding is required for PCSK9 internalization, making PCSK9 an ideal target for antibody therapeutics since there would be minimal expected target mediated clearance of anti-PCSK9 antibodies. We previously employed this reasoning to identify and develop an anti-PCSK9 antibody, J16, which completely blocks PCSK9 binding to LDLR. Surprisingly, these studies revealed that J16 is degraded in a PCSK9 dependent manner, suggesting that PCSK9 is internalized and trafficked to lysosomes regardless of its direct interaction with LDLR.

In this study, we confirmed that J16 does not alter PCSK9 trafficking, rather, exogenously added PCSK9 bound to J16 is still endocytosed and routed to lysosomes. Thus, a direct interaction with LDLR is not required for PCSK9 trafficking, and the mechanism by which PCSK9 diverts LDLR to lysosomes remained an important and open question. We hypothesized that lysosomal targeting of PCSK9 occurs via protein interactions on the luminal side of the membrane. We therefore sought to identify PCSK9 binding partners which could target PCSK9 to lysosomes. In doing so, we discovered a novel, pH dependent interaction between PCSK9 and the amyloid precursor-like protein 2 (APLP2). Interestingly, we found that PCSK9 and LDLR are trafficked along the same endo-lysosomal route as APLP2, and that recombinant LDLR and APLP2 can be complexed in a PCSK9 dependent manner at endosomal pH. Our data also indicate that APLP2 plays an active role in post-endocytic targeting of PCSK9 to lysosomes, thereby directly regulating PCSK9 trafficking and function.

Based on our observations, we propose that PCSK9 is involved in a novel lysosomal transport complex which would allow it to degrade multiple targets, including anti-PCSK9 blocking antibodies, by the same mechanism. Indeed, our data support a model in which PCSK9 bridges LDLR to APLP2, and APLP2 then mediates targeting of the trimeric complex to lysosomes.

Intriguingly, this system allows for a soluble messenger from either a local or distant source to modulate what has previously been considered a cell autonomous process.

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A Model System to Investigate Antibody Bipolar Bridging Mediated by gE-gI, a Herpes Virus Fc Receptor.

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The Herpes Simplex Virus 1 (HSV-1) glycoproteins (gE and gI) bind the Fc region of human immunoglobulin G (hIgG), which allows HSV-1 infected cells to escape recognition by host Fc receptor-mediated effector functions. HSV-1 gE-gI can further undermine the immune system by participating in antibody bipolar bridging (ABB), a process by which the antigen binding fragments of the IgG bind an antigen while the Fc binds to gE-gI. The fate of a gE-gI/IgG/viral antigen ABB complex is unknown. In this study, we engineered an in vitro ABB system to determine whether a gE-gI/IgG/viral antigen complex is internalized and targeted for degradation in host cells. This system involves HeLa and HEK293T cells transiently expressing HSV-1 gE-gI and an HSV-1 antigen, gD, which is normally localized at the cell surface of HSV virions and infected cells. gD was fused to the Dendra2 fluorescent protein. We constructed two forms of a monoclonal anti-gD antibody: one in which the Fabs were fused to a hIgG1 Fc, which can bind to gE-gI via its Fc and to gD via its Fabs, and one in which the Fabs were fused to a mouse IgG2a Fc, which can bind to gD but not to gE-gI. A hIgG against an irrelevant antigen was used as a control. Using 4D-time-lapse confocal fluorescence imaging, we demonstrated that surface antigens such as HSV-1 gD are internalized only in ABB permissible conditions and internalization is dependent upon gE-gI. Internalized hIgGs, bound or not to a surface viral antigen, were targeted into lysosomal compartments, and analysis of enriched and immunopurified Dendra2 tagged lysosomal compartments show degraded HSV-1 gD.

These results suggest that gE-gI plays an active role in clearing the infected cell surface of both host IgG and viral antigens, providing HSV-1 with a mechanism to evade immune responses. Knowledge gained from this study can guide new strategies for development of HSV-1 based oncolytic virotherapies.

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Drebrin E, an actin binding protein, regulates post-endocytic fate of apical proteins in intestinal cells.

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Intestinal epithelial cells are highly polarized and exhibit a complex architecture with a columnar shape, a specialized apical surface supporting microvilli organized in a brush-border. These microvilli are rooted in a dense meshwork of acto-myosin called the terminal web. We have shown recently that Drebrin E, an F-actin-binding protein, is a key protein for the organization of this terminal web and that Drebrin E is required for the columnar cell shape and the organization of microvilli in Caco-2/TC7 cells (human colonic cells) (Bazellères et al., J. Cell Sci., 2012). Now, to better understand how Drebrin E regulates the columnar epithelial phenotype we have investigated the levels of apical membrane proteins such as DiPeptidyl-Peptidase IV (DPPIV), Sucrase-Isomaltase (SI) or Alkaline Phosphatase (ALP) in Drebrin KD cells. All these apical proteins showed a marked decrease expression level while basolateral proteins do not, indicating that a specific step in apical membrane biogenesis or trafficking is affected. To

identify this step we have studied the subcellular localization of DPPIV, SI and ALP which was strikingly modified. Instead of being mostly present at the apical surface DPPIV, SI and ALP were accumulated inside a large sub-apical compartment and we showed, both by confocal and electron microscopy, that this compartment is related to lysosomes. Furthermore, by using endocytic assays we demonstrated that the enrichment of DPPIV in these lysosomes originates from apical endocytosis. We thus believe that Drebrin E is acting on the apical recycling pathway in intestinal epithelial cells to divert it to lysosomes. Interestingly, the phenotype observed in Drebrin E Caco-2/TC7 cells is reminiscent to a pathology called microvillar inclusion disease (MVID) which is mainly due to Myosin V mutations. We are now investigating the potential link between these two pathways with the hope to better understand this pathology.

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Mapping interactions of Vps55 and Vps68 with trafficking machinery in *Saccharomyces cerevisiae*.

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Vps55 and Vps68 mutants cause endosomal sorting defects and large-scale genomic data suggest functional ties to trafficking machinery, specifically Arf1 and Gcs1. However, it is not known how Vps55 and Vps68 interactions with trafficking machinery contribute to the overall function of the Vps55/Vps68 complex. We hypothesize that Vps55 and Vps68 bind to one another and to components of the trafficking machinery at separate sites. We aim to map these sites of interaction using a protein complementation assay, the Optimized yeast Cytosine Deaminase (OyCD) assay. This method utilizes a positive and negative selection technique to select for either interacting or non-interacting pairs. Thus, we can select mutants that have lost interactions with one protein but maintain interactions with another. This approach eliminates many mutants that perturb interactions due to global misfolding and identifies point mutants at the site of interaction. Thus far, we have shown that OyCD is a sensitive assay, which can show interactions and non-interactions between the proteins of interest (Vps55 and Vps68) and query proteins. Additionally, we have shown specific interactions with Arf1 and Gcs1 proteins, which supports the link to transport machinery. We are currently in the process of screening mutants to select Vps55 mutants that have lost interaction with Arf1 but maintain interaction with Vps68. These mutants will be used in further experiments to understand more fully how this interaction contributes to the function of the complex. By mapping sites of interaction, we hope to determine the role of Vps55 and Vps68, thereby gaining understanding of basic protein sorting, and the defects in this process that cause disease.

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Regulating Sit1 Trafficking and Turnover by a Deubiquitinating Enzyme Ubp3.

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Sit1 is a yeast iron siderophore transporter that is translocated either to the plasma membrane in the presence of its substrate or to the vacuole in the absence of its substrate. Previous studies indicate that accurate intracellular targeting of Sit1 requires appropriate ubiquitination of the protein by Rsp5 ligase. Here, we examined the potential regulation of Sit1 trafficking by deubiquitinating enzymes. We find that disrupting either Ubp3 or its partner protein Bre5 resulted in mislocalization of Sit1. In the absence of substrate, Sit1 is mislocalized to the plasma membrane instead of vacuole in these mutants. Interestingly, Ubp3 interacts with Sit1 and ubiquitinated Sit1 is accumulated in the *ubp3Δ* mutants, suggesting the regulation of Sit1 by

Ubp3 may be direct. We propose that upon ubiquitination and subsequent internalization of Sit1 at the plasma membrane, further progression to the vacuole requires a deubiquitinating step carried out by the Ubp3-Bre5 complex.

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Uptake and trafficking of opsonized and non-opsonized silica particles during silica induced cytotoxicity.

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Silicosis is a fibrotic lung disease induced by prolonged exposure to silica dust. Alveolar macrophages clear inhaled silica particles by phagocytosis, but this causes the macrophages to die by apoptosis. One of the first abnormalities associated with silica uptake is the leakage of phago-lysosomal contents into the cytoplasm, which may be the trigger for activation of the apoptotic pathway. Our objective is to understand silica particle uptake into phagosomes, endo-lysosomal (EL) fusion, and how the resulting leakage causes subsequent apoptosis. Silica particles were coated with protein (non-opsonized) or antibody (opsonized) and added to cultured alveolar macrophages labeled with a variety of fluorescent probes. Alveolar macrophages internalize opsonized and non-opsonized particles within 10 to 15 minutes. The opsonized particles are phagocytosed specifically by the Fc-receptor-mediated pathway, but the rapid uptake of non-opsonized particles suggests the use of an alternative pathway. In order to demonstrate EL fusion with phagosomes, macrophages were loaded with FITC-dextran (FD) prior to particle addition. Vesicles loaded with FD fused with opsonized or non-opsonized particle phagosomes, and in both cases the dye started to appear in the cytoplasm 30 minutes later. The same vesicle fusion events are observed with latex particles, however there is no leakage or cell death. This data indicates that silica causes phagosomal membranes to become permeabilized soon after internalization. However, it is not until about 4-6 hours later that activation of apoptotic markers can be measured.

To further define the molecular requirements for phago-lysosomal leakage and toxicity of silica particles, we have utilized Cos7 fibroblasts. Cos7 cells only internalize 10% of particles within 2 hours and continue to gradually internalize particles over the next 48 hours. Cos7 cells expressing the Fc-receptor internalize 70% of opsonized particles within two hours, while uptake of non-opsonized particles is still slow. Fusion of EL compartments with phagosomes occurs much slower in Fc-Cos7s than in macrophages. While this process takes only 80 seconds to occur in macrophages, it takes over 70 minutes in Fc-Cos7s. We are currently testing whether phago-lysosomal leakage also occurs in Cos7 cells. Of particular importance is whether the cells subsequently undergo apoptosis as a result of leakage. This data will allow us to explore what key steps in the uptake and trafficking of particles are important for the cytotoxicity of silica.

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Understanding the cellular mechanism of HDAC inhibitors for the treatment of NPC1 disease.

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Niemann-Pick type C type 1 disease (NPC1) is a rare but fatal neurodegenerative lysosomal storage disorder resulting in high levels of cholesterol accumulation in late endosomes (LE)/lysosomes (Ly) organelles in the cell. The NPC1 phenotype is caused by mutations in the

npc1 gene. Recently, histone deacetylase inhibitors (HDACi) have gained recognition in treating a number of non-cancer disorders. We have previously demonstrated that treatment with several HDACi significantly reduce cholesterol accumulation in the most commonly found I1061T mutation in the NPC1 patient derived cell lines (Pipalia et al., 2011). There are several cellular processes in NPC1 cells that may be affected by HDACi treatment. Our data indicates that NPC1 expression level is increased in the NPC1I1061T mutant cell line following HDACi treatment, and this might be responsible for correcting the NPC1 phenotype. However, the mechanism by which HDACi is enhancing NPC1 expression is not understood. We hypothesize that increased expression of NPC1 protein may be associated with protein folding chaperones in the endoplasmic reticulum (ER). It has also been reported previously that HDACi treatment causes induction of two chaperone proteins in a cancer cell line. We are currently testing the ER chaperones expression level after HDACi treatment in mutant NPC1 fibroblasts. We are determining the changes in expression level of Calnexin, BiP, HSF1, Hsp90, Hsp70, Hsp60, PDI and Hsp40 after HDACi treatment. Our preliminary results indicate that the expression levels of some of the ER chaperone proteins are altered after HDACi treatment. We are also measuring the NPC1 protein turnover rate in the absence and presence of one of the HDACi - suberoylanilide hydroxamic acid (SAHA) /Vorinostat. Determining how HDACi is correcting the NPC phenotype would lay the basis for targeted therapy based on genetic screening of NPC patients.

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Intracellular trafficking of human disease-causing prion protein mutants.

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Fatal, neurodegenerative, genetic prion diseases can be caused by specific mutations in the C-terminal globular domain (among other regions) of the human prion protein (PrP^c). These mutations are hypothesized to lead to the cellular accumulation of misfolded PrP species (including the transmissible scrapie or PrP^{sc} form). We have previously described the generation of a sub-population of misfolded PrP conformers in cells expressing several naturally occurring, disease causing human PrP C-terminal mutations. While a unifying theme among the C-terminal PrP mutants appears to be an intracellular accumulation of the misfolded PrP sub-population, potentially in acidic compartments, there is little known about how these mutants are selectively sorted in post-ER compartments. In addition, it remains to be shown how intracellular accumulation of misfolded PrP species might then lead to the downstream phenotype of neurodegeneration that is seen in these human diseases. To address these two specific goals, we have carried out a series of imaging analyses to definitely identify the specific cellular compartments that harbour the misfolded PrP sub-populations generated upon expression of C-terminal PrP mutants. We have examined the localization of C-terminal PrP mutants, as well as wildtype (WT) PrP and hydrophobic-domain mutants, in neuroblastoma cells with respect to various cellular organelle marker proteins, such as EEA1, LAMP1, Rab5, Rab 7 and proteins in the trans Golgi. Our confocal image analyses and Pearson's coefficient calculations for co-localization clearly show overlap of all PrPs examined with Golgi proteins as expected for those proteins expressed within the secretory pathway. Interestingly, we were able to show a subtle, but consistent increase in co-localization between LAMP1 and a C-terminal mutant, H187R, as compared to either WT or hydrophobic domain mutants. Upon treatment with inhibitors of lysosomal acidification, we see a further increase in this co-localization between misfolded H187R and LAMP1. This confirms our previous findings that the misfolded PrP subpopulation does indeed get sorted to lysosomes and can accumulate there if lysosomal function were compromised. Our data also point to accumulation of the C-terminal mutants within other intracellular vesicles, which may represent trafficking intermediates in the delivery of

the misfolded sub-population from the secretory pathway to lysosomes. Our ongoing experiments are determining whether accumulation of these PrP misfolded species within lysosomes leads to compromised function of these organelles. Such experiments would then allow us to ascertain the perturbation in cellular homeostasis that leads to neurodegeneration in human genetic prion diseases.

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Versatile roles of V-ATPase accessory subunit Ac45 in bone resorbing osteoclast formation and function.

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Vacuolar-type H⁺-ATPases (V-ATPases) are multisubunit proton pumps that acidify intracellular cargos and deliver protons across the plasma membrane of a variety of specialized cells, including bone-resorbing osteoclasts. In osteoclasts, V-ATPases functions in extracellular acidification a process that initiates the dissolution of mineralized bone matrix and crucial for osteoclastic bone resorption. While the importance of V-ATPases in osteoclastic resorptive function is well-defined, whether V-ATPases facilitate additional aspects of osteoclast function and/or formation remains largely obscure. Our labs research focus has been on the identification and characterization of novel subunits of the V-ATPase which impacts osteoclast function and potential V-ATPase inhibitors for therapeutic applications. In line with this focus, we have recently reported that the V-ATPase accessory subunit Ac45 participates in both osteoclast formation and function. Using a siRNA-based approach, we demonstrate that targeted suppression of Ac45 impairs intracellular acidification and endocytosis, both are prerequisite for osteoclastic bone resorptive function in vitro. Interestingly, knockdown of Ac45 also attenuates osteoclast formation owing to a reduced fusion capacity of osteoclastic precursor cells. In an effort to gain more detailed insights into the functional role of Ac45 in osteoclasts, we attempted to generate osteoclast-specific Ac45 conditional knockout mice using a Cathepsin K-Cre-LoxP system. Surprisingly, insertion of the neomycin cassette in the Ac45-Flox^{Neo} mice resulted in marked disturbances in CNS development leading to embryonic lethality thus precluding functional assessment of Ac45 in osteoclasts and peripheral bone tissues.

Vesicle Docking and Fusion

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N-ethylmaleimide sensitive factor hydrolyzes 12 ATP molecules to disassemble a single SNARE complex.

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SNARE proteins anchored in the vesicle and target membranes are required for the fusion of intracellular membranes. The energy for this process is thought come from the association of the cytoplasmic region of SNAREs into a stable four helix bundle. N-ethylmaleimide sensitive

factor (NSF) is an AAA+ ATPase that hydrolyzes ATP to disassemble the post-fusion SNARE complex. The interaction between NSF and the SNARE complex requires an adaptor protein, α SNAP. The mechanism by which ATP hydrolysis is coupled to SNARE disassembly is not known. The objective of this work is to understand how NSF utilizes energy to disassemble the SNARE complex.

Using spin columns to separate protein from radio-labeled nucleotides, we determined that NSF stably binds 12 ADP molecules in steady-state conditions, and that nucleotide hydrolysis is essential for this binding. We characterized the basic kinetics of NSF ATP hydrolysis, both in the absence and presence of α SNAP and neuronal SNARE proteins. The ATPase rate and catalytic efficiency of NSF increase substantially during interaction with α SNAP and the SNARE complex, indicating that the enzyme mechanism is altered during SNARE complex disassembly.

We used fluorescence-based methods to determine the rate at which NSF disassembles SNARE complexes, and by extension, the energy required for disassembly of a single SNARE complex. We determined that NSF hydrolyzes 12 ATP molecules to disassemble a single neuronal SNARE complex in pre-steady state conditions, and confirmed this value using catalytic constants determined from independent steady-state experiments.

Our findings demonstrate that sensitive measurements of nucleotide binding, hydrolysis and SNARE disassembly provide a greater understanding of the NSF mechanism, which may shed light on the modes of action of other members of the AAA+ ATPase family. Our findings offer detailed insight into the energy requirements of the membrane fusion process.

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Single SNARE Complexes Zipper in Three Distinct Stages.

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With the help of optical tweezers we directly observe and quantify the long-sought SNARE assembly intermediate in which only the membrane-distal N-terminal half of the four helix bundle is assembled. Our finding provides clear proof of the zipper hypothesis with the important and unexpected modification that zippering proceeds through three sequential binary switches, not continuously, in the N- and C-terminal halves of the bundle and the linker domain. The half-zipped intermediate is transiently stabilized by externally applied force that mimics the repulsion between apposed membranes being forced to fuse. This intermediate then rapidly and forcefully zippers, delivering free energy of 36 kBT to drive fusion. Complexin can clamp SNARE zippering and synchronize neurotransmitter release by further stabilizing the half-zipped intermediate.

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Regulation of SNARE complex assembly by the exocyst subunit Sec6.

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In eukaryotic cells, membrane-bound vesicles carry protein and lipid cargo between intracellular compartments, and between the cell surface and extracellular environment. A major unanswered question is how vesicle fusion is tightly controlled to prevent premature or

inappropriately localized membrane fusion. Two conserved families of proteins are required for correct vesicle fusion at the proper target membrane site – oligomeric tethering complexes and the SNARE proteins. The exocyst is the exocytic tethering complex that regulates polarized exocytosis, and is proposed to tether vesicles to their sites of fusion. Subsequently, the SNARE proteins mediate membrane fusion. Because SNARE proteins show little specificity for their binding partners, and are not always specifically localized to sites of membrane fusion, SNARE complex assembly must be highly regulated. Previous work from our laboratory demonstrated that, *in vitro*, the *Saccharomyces cerevisiae* exocyst subunit Sec6 directly binds the SNARE Sec9, and inhibits the formation of the Sec9-Sso1 SNARE complex. Sec6 and Sec9 also interact *in vivo*; therefore, we hypothesized that the interaction between Sec6 and Sec9 regulates the assembly of SNARE complexes, and is necessary for proper assembly of SNARE complexes at sites of exocytosis upon vesicle arrival. In order to elucidate the role of the Sec6-Sec9 interaction in SNARE complex assembly, we are selectively disrupting their interaction using the zero-length crosslinker EDC and mass spectrometry analyses to directly target the residues required for binding. We monitored the cross-linking reaction over a range of times, and quantification resulted in a rank ordered list of residues close to or in the binding site. A number of candidate residues were mutated, to either alanine or the opposite charge, and introduced into yeast as the sole copy of *SEC6* or *SEC9*. Mutations in several residues of both Sec6 and Sec9 resulted in temperature and media sensitive growth defects. Subsequent *in vitro* binding studies showed that an alanine substitution at one of the Sec9 sites caused only a modest decrease in the stability of the Sec6-Sec9 interaction, while the charge reversal mutation resulted in substantial destabilization of the Sec6-Sec9 complex. Therefore, a loss of interaction between the exocyst subunit Sec6 and the SNARE Sec9 leads to growth defects, likely due to a loss of SNARE complex assembly regulation.

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Structure of Vps33, a Key Regulator of Membrane Fusion.

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Intracellular trafficking involves the budding, directed movement, and fusion of vesicles with target membranes. SNARE proteins anchored in opposing membranes zipper into four-helix bundles, thereby exerting the force required to drive fusion between lipid bilayers. We have been interested in understanding how SNARE activity is regulated and enhanced *in vivo*. Many lines of evidence suggest that vesicle docking and fusion is orchestrated by a set of large protein complexes called MTCs (multisubunit tethering complexes). MTCs work in close collaboration with SNAREs and other elements of the vesicle trafficking machinery, notably Rab GTPases, which link trafficking to a regulated cycle of GTP hydrolysis, and a conserved family of SNARE binding proteins known as SM (Sec1-Munc18-like) proteins. Although several lines of evidence implicate MTCs as general regulators of fusion, they are large and complex, hampering efforts to understand their mechanisms of action. To address this deficiency, we have been studying an MTC called the HOPS (homotypic fusion and vacuole protein sorting) complex. Unusually, an SM protein, Vps33, is a stable subunit of the HOPS complex. HOPS binds the vacuolar Rab Ypt7, the Q-SNAREs Vam3 and Vam7, and both partially- and fully-formed SNARE complexes. These SNARE interactions are mediated by Vps33 and one or two other HOPS subunits. Through these and possibly other interactions, HOPS is able to tether opposing membranes and stimulate formation of *trans*-SNARE complexes, thereby promoting fusion. However, a molecular understanding of precisely how HOPS synthesizes so many inputs to produce a high-fidelity and physiologically competent fusion reaction remains elusive. As a first step toward characterizing the high-resolution structure of the HOPS complex, we have determined the x-ray structure of Vps33, and are using it as a blueprint to guide further structural and functional analysis.

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The Syntaxin1a N-peptide and LE 'open' mutation have no effect on the Munc18a-Syntaxin1a binding mode.

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In neurons, Soluble N-ethylmaleimide Sensitive Factor (NSF) Attachment protein Receptor (SNARE) proteins drive the fusion of synaptic vesicles to the plasma membrane through the formation of a four-helix SNARE complex. The neuronal protein Munc18a interacts with a closed conformation of the SNARE protein Syntaxin1a (Syx1a) and with assembled SNARE complex containing Syx1a in an open conformation. The N-peptide of Syx1a (1-24) has been implicated in the transition of Munc18a-bound Syx1a to Munc18a-bound SNARE complex, but the underlying mechanism is poorly understood.

Here, we report X-ray crystal structures of Munc18a bound to Syx1a with and without its native N-peptide (Syx1aΔN), and small-angle X-ray scattering data for Munc18a bound to Syx1a, Syx1aΔN, and Syx1a L165A/E166A (LE), a mutation thought to render Syx1a in a constitutively open conformation. We show that all three complexes adopt the same global structure, in which Munc18a binds a closed conformation of Syx1a. We also identify a possible allosteric connection between the Syx1a N-peptide and Munc18a domain 3a, providing mechanistic insight into the transition of closed-to-open Syx1a in SNARE complex assembly. While the role of the N-peptide in Munc18a-mediated SNARE complex assembly remains unclear, our results demonstrate that the N-peptide and LE mutation have no effect on the overall structure of the Munc18a-Syx1a complex.

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The birth of a membrane nanodomain - syntaxin clusters assemble from single molecules during secretory granule docking.

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Insulin is released by regulated exocytosis of secretory granules, and tethering/docking of the granules at the future release site is a critical step in this process. Granule docking depends on the plasma membrane t-SNARE syntaxin1a and its accessory protein munc18. Since syntaxin1a binds to granule proteins and forms clusters near docked granules, it may act as docking receptor for incoming granules. Here we used live cell imaging to test two simple predictions of this model: 1) syntaxin1a should cluster at the release site before a granule docks, and 2) syntaxin1a clusters should mark granules as docked and ready for exocytosis. Syntaxin1a formed small clusters near the majority of docked granules, which exhibited striking intensity variations with a period of a few seconds. The dynamic nature of the clusters was also evident from single molecule experiments indicating residence times of <1s at the cluster site. Surprisingly, when a granule arrived at the plasma membrane it did not dock at an empty syntaxin cluster. Instead, the cluster formed within seconds upon granule contact, and this correlated with the transition from a loosely tethered to a stably docked state. Conversely, spontaneous loss of the cluster predicted subsequent loss of the granule by undocking. All other tested proteins, including Munc13 and Munc18 arrived later than syntaxin1a. Granules did not recruit a deletion mutant of syntaxin1a that lacks the N-terminal Habc domain, and a soluble Habc fragment dose-dependently prevented cluster formation and granule docking. Stable

granule docking and association with syntaxin cluster was required for stimulation-evoked exocytosis, and sites of exocytosis were subsequently avoided by new granules attempting to dock. We conclude that granules induce the formation of their own docking site and that recruitment of syntaxin1a via its Habc domain is critical for docking. Syntaxin1a clusters form on a timescale consistent with rapid cellular signaling, which may be important for the short-term regulation of insulin secretion.

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Syntaxin binding protein 1 (STXBP1) modulates release of Weibel-Palade bodies from endothelial cells.

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Vascular endothelial cells contain unique large rod-shaped granules, called Weibel-Palade bodies (WPBs). These granules function as storage organelles for various haemostatic and inflammatory components, such as von Willebrand factor (VWF), chemokines and P-selectin. Controlled release of these components into the vascular lumen through WPB exocytosis is essential for maintaining vascular homeostasis in response to vascular trauma or stress. Secretory granule exocytosis is tightly controlled by signalling pathways that are linked to G-protein coupled receptors. One of the final steps in exocytosis involves the assembly of the so-called SNARE-complex. SNARE-complexes are formed by zipper-like proteins, VAMPs (or v-SNAREs) on vesicles and syntaxins (or t-SNAREs) on the plasma-membrane. Formation of the SNARE-complex promotes fusion of secretory vesicles with the plasma membrane and subsequent release of granule content into the lumen. SNARE-complex formation is controlled by proteins belonging to the syntaxin binding protein (STXBP) family. In this study we investigate the possible role of syntaxin binding protein 1 (STXBP1), also known as Munc18-1, in WPB exocytosis. We show that STXBP1 is expressed in endothelial cells. Interestingly, STXBP1 was also picked up in a proteomic screen in endothelial cells for interactors of synaptotagmin-like protein 4-a (Slp4-a), a Rab27A effector that has recently been implicated as a positive regulator of WPB release. Using siRNA mediated knock-down of STXBP1 expression we show that STXBP1 depleted cells exhibited a decrease in Ca²⁺-stimulated VWF release. We speculate that STXBP1 regulates WPB release through its interaction with Slp4-a, thereby docking WPBs to the plasma membrane and facilitating the release of haemostatic, inflammatory and angiogenic cargo from these organelles.

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Characterisation of the Weibel Palade body fusion pore using optical and electrochemical techniques.

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Regulated secretion of proteins and molecules from endothelial cells (ECs) is mediated by the exocytosis of secretory granules (SGs) called Weibel-Palade bodies (WPBs). Optical data indicate that WPBs can undergo a form of exocytosis that allows selective release of cargo molecules based on molecular size. Regulation of SG fusion pore dynamics is thought to underlie selective release of SG cargo, however the dynamics of the WPB fusion pore have not been quantitatively analysed. Using carbon fibre amperometry we have now determined the

properties and dynamics the WPB fusion pore during calcium-driven exocytosis. Initial biochemical and amperometric studies revealed that WPBs do not contain endogenous oxidisable molecules; therefore methods were developed to specifically load WPBs with suitable oxidisable molecules. Amperometry was performed in fura-2 loaded HUVEC expressing fluorescent WPBs to simultaneously monitor intracellular free calcium ion concentration changes and WPB exocytosis optically during stimulation. Current spikes were closely associated with WPB fusion events monitored optically. Current spike parameters comprised a 25-75% rise time, peak amplitude and decay time of 1.77 ± 1.37 ms, 49.24 ± 34.17 pA and 7.26 ± 7.75 ms respectively (mean \pm SD, $n = 617$ spikes). These values are comparable to those reported for SGs in other cell types (e.g. chromaffin cells), indicating that WPBs share similar processes controlling membrane fusion and mobilisation/release of oxidisable species. Approximately 50% of exocytotic events had pre-spike foot (PSF) signals indicating that a restricted fusion pore initially forms. In rare cases PSF signals showed step changes or fluctuations suggesting that during expansion the fusion pore may transition through different configurations before fully opening. Following characterisation of the WPB fusion pore under control conditions, factors which may affect the behaviour of the fusion pore were investigated, including the role of PM cholesterol. In line with previous studies, depletion of PM cholesterol led to a significant increase in the rate of fusion pore expansion from 1.65 ± 1.26 ms ($n = 409$) to 1.41 ± 0.92 ms ($n = 266$, $p = 0.014$) and a significant decrease in the duration of the lifetime of the restricted fusion pore from 5.41 ± 9.26 ms ($n = 253$) to 3.50 ± 4.53 ms ($n = 184$, $p = 0.025$), providing further evidence for the importance of cholesterol in shaping the exocytotic fusion pore.

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SNARE Complex Phosphorylation is Altered Prior to Mouse Sperm Acrosomal Exocytosis.

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Phosphorylation can alter activity of phosphoproteins including all three core SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins that have a critical role in intracellular membrane fusion events such as the acrosome reaction. During the final sperm maturation, known as capacitation, protein kinases and phosphatases are activated that may alter phosphorylation of SNAREs and other regulatory proteins. We hypothesize that during capacitation, SNARE protein phosphorylation changes, promoting SNARE complex formation in preparation for the acrosome reaction. To begin to test this hypothesis, we incubated mouse sperm in a specialized capacitating (dmKRBT) or non-capacitating (dmKRBT without BSA and – HCO₃) medium and detergent-extracted sperm protein. To isolate formed SNARE complexes, sperm protein was immunoprecipitated with a syntaxin antibody and subsequently run on 12% SDS-PAGE gels, followed by immunoblotting with syntaxin antibody to verify that the complexes contained syntaxin. Samples were not boiled prior to SDS-PAGE to maintain the integrity of the SNARE complex. To detect changes in total phosphorylation of syntaxin-containing (SNARE) complexes, immunoprecipitated complexes were separated on an SDS-PAGE gel that was subsequently stained with Pro-Q Diamond. We observed phosphoprotein staining in 75, 100, and 150 and 230 KD protein complexes. Immunoblotting with a syntaxin antibody demonstrated that each complex contained syntaxin and was presumably a SNARE complex. Quantitation of SNARE complex phosphorylation showed that after 30 min of capacitation, overall phosphorylation was higher in the 75, 100 and 150 KD bands. To detect tyrosine phosphorylation of syntaxin and associated proteins, the anti-syntaxin immunoprecipitates were blotted with a phosphotyrosine antibody. Tyrosine phosphorylation of SNARE complexes decreased after 15 min of capacitation time. These data demonstrate SNARE complex phosphorylation is a dynamic process during capacitation and suggest that phosphorylation may

regulate SNARE complex formation during capacitation in preparation for the acrosome reaction. This work was partially supported by a fellowship from COMSATS Institute of Information Technology, Pakistan and the University of Illinois.

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Regulated exocytosis in the exocrine glands: the multiple roles of the actomyosin complex revealed by intravital microscopy.

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The regulation and the dynamics of membrane trafficking events have been investigated primarily in in vitro models that often do not fully reflect the functional complexity found in a living multicellular organism. To address this issue we used intravital microscopy in the exocrine glands of live rodents (salivary glands, exocrine pancreas, and lacrimal glands) to investigate regulated exocytosis, a fundamental process in all the secretory organs. We found that upon stimulation of the appropriate GPCR, large secretory granules fuse with the apical plasma membrane, and gradually collapse without any evidence of compound exocytosis, as was previously described. Moreover, by using a series of selected transgenic mice, we showed that the driving force required to complete the collapse of the granules is provided by the sequential recruitment on the granule membranes of F-actin and two isoforms of nonmuscle myosin II, IIa and IIb. The recruitment is mediated by some membrane components of the apical plasma membrane and involved the small GTPase Septin 2. Our results provide information on the machinery controlling regulated secretion and show that intravital microscopy provides unique opportunities to address fundamental questions in cell biology under physiological conditions.

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FRET-based method to decipher the stoichiometry and structural assembly of bacterial ABC transporter involved in exporting endotoxins.

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The ATP-binding-cassette (ABC) transporters are membrane protein machineries and common to all living cells. ABC transporters utilize the energy of ATP hydrolysis to transport a broad spectrum of solutes across the membrane. Wzm (the transmembrane component) and Wzt (the nucleotide binding component) proteins constitute an ABC transporter that translocates the endotoxic polysaccharides through the inner membrane of the pathogenic bacterium *Pseudomonas aeruginosa*. To gain insights into the subunit stoichiometry and structural assembly of this ABC transporter, we reconstituted the ABC transporter in CHO cell lines. We expressed Wzm and Wzt proteins fused with green fluorescent protein (GFP2) or its variant, yellow fluorescent protein (YFP). The three-dimensional cellular localization of the expressed ABC components were constructed by stacking images of different sections of the cells utilizing a newly developed and spectrally-resolved two-photon microscope. We next used Fluorescence Resonance Energy Transfer (FRET) to obtain a quantitative understanding of the interaction between the ABC components by calculating the apparent FRET efficiencies for single pixels. When Wzm-GFP2 and Wzm-YFP were co-expressed, our FRET analysis indicated that Wzm self-associates within the cell membrane as an oligomer. This correlates with our biochemical characterization of membrane-extracted Wzm. When Wzt-GFP2 and Wzm-YFP were co-

expressed, our FRET analysis indicated that Wzm and Wzt interact at the inner membrane surface. This correlates with our in vitro studies that show Wzm and Wzt interact with each other. The distribution of FRET efficiencies were compared when Wzt-GFP2 and Wzm-YFP or Wzt-YFP and Wzm-GFP2 were co-expressed to study the stoichiometry of Wzm and Wzt in the ABC transporter. Based on FRET analysis, wzt protein assembles into a rhombus-shaped tetramer in the absence of Wzm, but changes its assembly into a square-shaped tetramer when associates with Wzm. By contrast, Wzm assembles into a square-shaped tetramer irrelevant to its association with Wzt. Based on our work, we propose a model for the ABC complex assembly. The combination of FRET analysis and biochemical approaches will lead to a comprehensive investigation of the structural assembly and subunit composition of this ABC transporter and ultimately other transporters.

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Enhancement of SNARE-mediated membrane fusion by synaptotagmin II requires Ca²⁺ and phosphatidylserine.

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Mast cells play a pivotal role in allergic responses. Antigen stimulation causes the elevation of intracellular Ca²⁺ concentration which triggers the exocytotic release of inflammatory mediators such as histamine. Recent researches revealed that SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins such as syntaxin-3, -4, SNAP-23 and VAMP-8 are involved in mast cells exocytosis. Although the exocytosis in mast cells is Ca²⁺-dependent, the target molecule of Ca²⁺ is not clear. Synaptotagmin is a Ca²⁺-sensor and regulates exocytosis in neuronal cells. However, the role of synaptotagmin II, the most abundant isoform in mast cells, in the exocytosis is still controversial. In the present study, we investigated the role of synaptotagmin II by liposome-based fusion assay.

SNARE proteins (SNAP-23, syntaxin-3, VAMP-8) and synaptotagmin II were expressed in E.coli and purified as GST-tagged or His-tagged fusion proteins. These SNARE proteins were incorporated into liposomes by a detergent dialysis method. Membrane fusion between liposomes containing VAMP-8 and liposomes containing SNAP-23 and syntaxin-3 was monitored by fluorescence resonance energy transfer between fluorescent labeled phospholipids.

Ca²⁺ did not show any effects on SNARE-mediated membrane fusion, but Ca²⁺ enhanced the fusion in the presence of synaptotagmin II. Interestingly, synaptotagmin II inhibited SNARE-mediated membrane fusion in a dose-dependent manner in the absence of Ca²⁺. However, this inhibitory effect was lost by the addition of Ca²⁺, and the membrane fusion was induced depending on the Ca²⁺ concentration. Next, we investigated the interaction of synaptotagmin II with phosphatidylserine (PS). PS-containing liposomes interacted with synaptotagmin II in the presence of Ca²⁺, whereas liposomes made from phosphatidylcholine only did not interacted with synaptotagmin II even in the presence of Ca²⁺. The enhancement of SNARE-mediated membrane fusion by Ca²⁺/synaptotagmin II required PS as a membrane component. These results suggest that synaptotagmin II regulates membrane fusion of SNARE-containing liposomes involved in exocytosis in mast cells, and this regulation is dependent on synaptotagmin II, Ca²⁺, and PS.

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The membrane association of the GEF Mon1p is regulated by its phosphorylation and binding to PI3P.

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The maturation of early endosomes to late endosomes and lysosomes requires the exchange of several factors. Central to this conversion is the exchange of the early endosomal Rab5/Vps21p for the late endosomal Rab7/Ypt7p. This exchange is mediated in part by the nucleotide exchange factor Mon1p, which indirectly activates the GTPase activity of Rab5/Vps21 and subsequent removal from the membrane. Mon1p then aids in the recruitment of Ypt7p and activates it through the exchange of GDP for GTP. In this study, we investigated the recruitment and vacuole binding of the Mon1p-Ccz1p heterodimer. Initially, we found that Mon1p was reduced on vacuoles from strains lacking the phosphatidic acid phosphatase Pah1p. Vacuoles from *pah1Δ* strains also lacked Ypt7p, phosphatidylinositol 3-kinase Vps34p and its product phosphatidylinositol 3-phosphate (PI3P). Because the *C. elegans* homologue Sand-1 binds PI3P, we hypothesized that Mon1p-Ccz1p is recruited to vacuoles by PI3P. We found that Mon1p was competed off from vacuoles in the presence of the PI3P ligand FYVE. We also found that Mon1p is released from vacuoles over time and that the release is linked to its phosphorylation by the yeast casein kinase Yck3.

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Imaging the Intraciliary Dynamics of Signaling Receptors at Single Molecule Resolution.

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Primary cilia participate in the sensing of extracellular stimuli by concentrating transmembrane receptors and signaling intermediates within a confined space. While several lines of evidence suggest that IntraFlagellar Transport (IFT) trains are essential for the transduction of mating signals in *Chlamydomonas*, the general relationship between the IFT machinery, the intraflagellar/intraciliary dynamics of signaling receptors and signaling output has remained largely unexplored. To characterize the dynamics of individual signaling receptors in the ciliary membrane, we set out to label individual Somatostatin Receptor 3 (SSTR3, a ciliary GPCR) molecules in live cells. Our approach relies on the site-specific biotinylation of an acceptor peptide fused to the extracellular domain of SSTR3 and on the application of fluorescently labeled monovalent streptavidin at low concentration onto cells. Using single molecule imaging of SSTR3, we have found that:

1- SSTR3 spends more than 90% of its time inside cilia diffusing rather than undergoing active transport. The diffusive movements are rapid and inhibition of IFT only reduces the effective diffusion coefficient of SSTR3 inside cilia by a modest amount. 2- Unlike IFT trains which move processively from one end of the cilium to the other, single SSTR3 molecules only display directed movements for limited distances. The latching of SSTR3 on IFT trains appears to be transient and rapidly reversible. 3- Immobilizing membrane proteins with the multivalent lectin WGA does not affect the movement of IFT trains (visualized as foci of GFP-IFT88 fluorescence) inside cilia.

Together, these studies suggest that the coupling between the IFT machinery and its cargoes is labile and dispensable for IFT train motility. Cargoes (at least transmembrane cargoes) behave as passengers randomly hopping on and off moving trains.

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Role of the small GTPase Cdc42 in renal ciliogenesis and cystogenesis.

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Background: Primary cilia have been strongly implicated in the pathogenesis of autosomal dominant polycystic kidney disease (ADPKD), the most common potentially lethal genetic disorder, and cilia disruption results in large cysts that destroy the kidney. In renal tubule cells, we showed that the highly conserved eight-protein exocyst complex, which is involved in targeting and docking vesicles carrying membrane proteins, localized to, and was necessary for ciliogenesis (Zuo et al, Mol Biol Cell, 2009); that sec10 knockdown phenocopied pkd2 knockdown in zebrafish (Fogelgren et al, PLoS Genetics, 2011); and that the small GTPase Cdc42 interacted with exocyst Sec10 to regulate ciliogenesis in MDCK cells (Zuo et al, J Biol Chem, 2011).

Methods: Given the importance of Cdc42 for cell function, to study how cdc42 affects ciliogenesis and cystogenesis *in vivo*, we used two different animal models and techniques: antisense morpholinos (MOs) to knockdown cdc42 in zebrafish, and the Cre-Lox system to knockout Cdc42 in a kidney-specific manner in mice. Zebrafish embryos were injected with cdc42 start-site antisense MOs. Cdc42 renal tubule cell-specific knockout mice were generated by breeding Cdc42 floxed (fl) and Ksp-cadherin Cre (Cre^{ksp/+}) mice.

Results: cdc42 morphants developed pericardial edema, short tail, small eyes, abnormal cilia, left-right patterning defects, and glomerular expansion that phenocopied sec10 and pkd2 morphants. Co-injection of small amounts of cdc42 and sec10 MOs, which individually had no effect, together resulted in an abnormal phenotype, suggesting that cdc42 and sec10 act in the same pathway. To generate Cdc42 kidney-specific knockout mice, female Cre^{ksp/+} mice were first crossed with male Cdc42^{fl/fl} mice. 7 female Cre^{ksp/+}-Cdc42^{fl/+} mice were then backcrossed against 2 male Cdc42^{fl/fl} mice, and 55 pups were evaluated from P11 to P23. Of the 55 pups: 16 Cdc42^{fl/+}, 20 Cdc42^{fl/fl}, 18 Cre^{ksp/+}-Cdc42^{fl/+} living mice, and only 1 dead Cre^{ksp/+}-Cdc42^{fl/fl} (P18) kidney-specific knockout mouse, were identified by PCR genotyping. From E16.5 to P8, 41 pups were studied. Of the 41 pups: 17 Cdc42^{fl/+}, 9 Cdc42^{fl/fl}, 7 Cre^{ksp/+}-Cdc42^{fl/+}, and 8 Cre^{ksp/+}-Cdc42^{fl/fl} kidney-specific knockout mice were identified. By histology, there were increasing cysts identified from E16.5 to P8, with the kidneys of P8 Cre^{ksp/+}-Cdc42^{fl/fl} mice replaced by small cysts. The kidneys themselves were not grossly increased in size, and the cystic kidneys were reminiscent of a nephronophthisis phenotype.

Conclusions: In zebrafish, cdc42 knockdown results in a ciliary phenotype, and cdc42 acts synergistically with exocyst sec10. Cdc42 kidney-specific knockout in mice leads to an early postnatal death apparently due to kidney failure from polycystic kidney disease. Taken together, these data indicate that Cdc42 is essential for normal cilia function and nephrogenesis *in vivo*.

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A novel stimulatory activity for a Rab GTPase in membrane fusion.

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Previous work has suggested that the principal function of the yeast vacuolar Rab GTPase Ypt7p is to recruit its effector, the HOPS/Class C Vps complex, to membranes. Here, it is demonstrated that Ypt7p has an activity beyond HOPS complex recruitment for membrane fusion. Reconstituted proteoliposomes made from lipid mixtures containing cardiolipin mix their lipids in a reaction that requires the HOPS complex, but not Ypt7p. Ypt7p is, however, required for lipid mixing of proteoliposomes lacking cardiolipin. Moreover, Ypt7p is still required for lipid

mixing even when the concentration of HOPS complex in lipid-mixing reactions is adjusted such that proteoliposomes with or without Ypt7p bind to equal amounts of HOPS. Ypt7p therefore must stimulate membrane fusion by a mechanism in addition to recruitment of HOPS to the membrane. This is the first report of such a stimulatory activity – that is, beyond bulk effector recruitment – for a Rab GTPase.

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Soluble SNAREs can function as transcription regulators.

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SNARE proteins are well established as the machines that accomplish membrane fusion in all intracellular membrane trafficking pathways in eukaryotes. Syntaxins, a conserved family of SNARE proteins, contain a C-terminal transmembrane anchor that is required for their membrane fusion activity. Here we show that syntaxin 3 (Stx3) functions as a nuclear regulator of gene expression in addition to its known function in membrane fusion at the plasma membrane. Stx3 undergoes proteolytic cleavage that releases it from the membrane. The cleavage site was identified as the most highly conserved residue among all eukaryotic syntaxins. In addition, we show that alternative splicing leads to a novel soluble isoform, termed Stx3S, that lacks the transmembrane anchor. These soluble forms of Stx3 bind to the nuclear import factor RanBP5 and target to the nucleus. Stx3S is endogenously highly expressed in human kidneys and several cell lines. A functional co-activator trap screen led to the identification of several transcription factors that interact with - and are regulated by - Stx3S. Among them is the ETS transcription factor ETV4 that is co-activated by Stx3S. Stx3S stimulates the expression of MMP-1, an endogenous ETV4 target gene. Stx2 and Stx4 undergo similar cleavage events as Stx3 suggesting that nuclear signaling by soluble syntaxins may be an ancient, conserved signaling pathway that may communicate information from cytoplasmic membrane trafficking events to the nucleus.

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Elucidating the Role of Phosphatidylcholine Transfer Protein (PCTP) in Tetrahymena Conjugation.

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The ability of membranes to fuse to one another is fundamental for many processes including tissue morphogenesis, virus transmission, and secretion. As a result, much work has sought to identify the molecular determinants that regulate membrane fusion. Membrane lipid content appears to play an important role in regulating membrane curvature during membrane fusion. Recently Kurczyk et. al, demonstrated that depletion of phosphatidylcholine (PC) (a curvature-resisting lipid) and increased levels of phosphatidylethanolamine (PE) (a high curvature-inducing lipid) coincided with the appearance of fusion pores during conjugation in *Tetrahymena Thermophila*. This data highlighted the potential role lipid mediated membrane curvature in fusion pore formation but these results also left many outstanding questions, including what are the mechanisms that might lead to rapid local changes in local PC or PE concentration in the specialized zone between conjugating cells. Using the *Tetrahymena* gene expression database, we identified a protein phosphatidylcholine transfer protein (PCTP), which has been shown to regulate PC transfer between the plasma membrane and endomembranes. Importantly, PCTP was expressed during the period in which fusion pores are being formed and PC is being

removed from the conjugation junction. We hypothesized that PCTP is required for the removal of PC during fusion pore formation and therefore proper conjugation. To test this hypothesis, we used a small molecule inhibitor (compound A1) of human PCTP on mating *Tetrahymena*. First, we found that compound A1 strongly inhibited the ability of *Tetrahymena* to form mating pairs (an early step in the conjugation process). This effect appeared to be due to the lack of clustering by adhesion receptors required for *Tetrahymena* pairing. Since pairing precedes fusion pore formation, we performed a time course to determine the specific effects of PCTP inhibition on fusion pore generation. We found that addition of compound A1 could inhibit pair formation both after adhesion receptors have clustered and more importantly could induce pairs to come apart if added immediately prior to the onset of fusion pore formation. Moreover, the ability of compound A1 to inhibit pairing diminished greatly if added two hours after mixing or more specifically, after fusion pores have been formed. This suggests that PCTP is required before and/or during pore formation but not after fusion pores have already been formed. Future experiments are aimed at determining the localization of PCTP using PCTP-GFP *Tetrahymena*. We are also in the process of generating PCTP knockout *Tetrahymena* to determine whether this will mimic the effects of compound A1. We believe these results provide a potential mechanism for the regulation of membrane lipid content during membrane fusion. Supported by ASCB-MAC, HHMI and the Consortium for Faculty Diversity at Carleton College.

Signaling Networks Governing Cell Migration I

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LOV-TRAP: A broadly applicable, genetically encoded system to control protein activity with light through controlled sequestration at membranes.

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The kinetics and subcellular localization of protein activity is precisely controlled to regulate protein interactions and to create specific signaling networks. To study this important aspect of cell signaling, genetically encoded methods are being developed to control protein activity with light in living cells and animals (1-4). Here we describe a new approach that promises to be broadly applicable and is simple to apply. Through an in vitro selection from a protein domain library with more than 10^{13} unique sequences using mRNA display, we developed Zdark, a small protein based on the Z domain (5) that binds only to the dark state of the LOV2 domain from *Avena sativa* phototropin (Figure 1). The LOV2 domain undergoes a large, reversible conformational change induced by light between 400 and 500 nm. Zdark binds to the dark state of LOV2 with a K_d of ~ 100 nM, but shows no detectable binding to the lit state. In our new method (dubbed LOV-TRAP for LOV trapping and release of active protein) the LOV domain is anchored at an intracellular membrane (to date the mitochondrial and plasma membranes) and the protein of interest is fused to Zdark. In the dark, the Zdark-protein fusion is sequestered at a membrane where it cannot interact with its targets (here the mitochondria). Upon irradiation, LOV binding of the Zdark-protein conjugate breaks down, releasing the Zdark-protein fusion. Release is rapid (< 0.5 secs) and reversible. We have demonstrated this for repeated light/dark cycles and have introduced mutations to vary the kinetics of return to the dark state. To date, we have completed caging of VAV2, Rac1 and RhoA. Progress with other targets will be

described, as will the use of the approach to control protein dimerization. Crystal structure and NMR data will be presented to illuminate the Zdark-LOV interaction. Applications of LOV-TRAP in studies of cell motility will be described.

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Rac1 induces PCK-dependent myosin IIA heavy chain phosphorylation to regulate association with focal adhesions and cell migration.

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Cell migration requires coordinated assembly of focal adhesions and contraction in the actomyosin cytoskeleton. The small GTPase Rac1 is critical to cell migration through its known functions in regulation of focal adhesion and actin cytoskeletal assembly dynamics, but its role in regulation of myosin II is not known. Myosin II dynamically assembles into minifilaments at the leading edge of migrating cells, and PKC-mediated phosphorylation in Ser 1916 in the non-helical tail is one of the main regulators. We hypothesized that Rac1 may regulate myosin II minifilament assembly dynamics during cell migration via downstream regulation of PKC and Ser 1916 phosphorylation. To test this, we analyzed the effects of Rac1 activation on the phosphorylation and dynamics of myosin IIA in U2OS cells. We found that transfection of active Rac1 (Rac1V12) induced PKC- and integrin-dependent myosin IIA phosphorylation on Ser 1916. Live cell imaging of GFP-myosin IIA revealed that Rac1 activation promotes rapid assembly, motion, and turnover of myosin IIA minifilaments, as well as perpendicular orientation to the leading edge, resulting in its accumulation specifically in focal adhesions. To determine the role of Ser 1916 phosphorylation on myosin IIA dynamics and localization, we expressed phosphomimetic (S1916D) and non-phosphorylatable mutants (S1916A) of myosin IIA. This showed that phosphorylation is critical to the Rac1-induced rapid assembly and turnover of myosin IIA minifilaments as well as to the focal adhesion association of myosin IIA. Thus, Rac1 acts as a master regulator of cell migration by coordinating actin assembly-mediated protrusion, adhesion, and actomyosin contraction dynamics.

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The small GTPase Rap1 promotes movement rather than strengthens cell-cell adhesion in cancer cells responding to insulin-like growth factor I.

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It is widely assumed that the activation of the Ras oncoprotein-related protein 1 (Rap1) is necessary and sufficient to mediate its downstream signaling. However, how active Rap1 regulates seemingly opposite cellular processes from maintaining adhesion to promoting movement remains puzzling. Here we tested the hypothesis that the labile function of Rap1 is cell context-dependent. Rap1 was examined in MCF-7 human breast cancer cells either

hypersensitive to insulin-like growth factors (IGFs) due to overexpression of the wild type IGF-I receptor or insensitive to these cues due to expression of a dead kinase mutant of the IGF-IR. In the IGF-sensitive cells, IGF-I treatment induce the translocation of active Rap1 from E-cadherin based contacts to the actin-enriched motile protrusions in conjunction with relaxing adhesion and promoting movement of the cells. The pretreatment of cells with cAMP-Epacs activators of Rap1 (forskolin and 8-pCPT-2'-O-Me-cAMP) did not rescue the loss of intercellular adhesion induced by IGF-I. At the biochemical level, transient rather than sustained activation of Rap1 by IGF-I appeared to be critical for the onset of cell movement. Ligand-bound autophosphorylated IGF-IR signaled to C3G, CRK SH3-binding guanine nucleotide exchange factor (GEF) to excite GTP-loading (activation) of Rap1. Subsequent internalization of the IGF-IR coincided with the gradual hydrolysis of the bound GTP leading to inactivation of Rap1. Blocking receptor-mediated endocytosis with dansylcadaverine and dynasore prolonged Rap1 activation but did not trigger cell movement. Moreover, expression of the constitutively active mutant V12Rap1 by itself was insufficient to induce movement in IGF-insensitive cells. When IGF-IR signaling was "off", basal activity of Rap1 was required to hold up cell adhesion. Forcing sustained inactivation of Rap1 by over expressing RapGTPase activating protein (RapGAP) or silencing C3G had a detrimental effect on filamentous actin and adhesion in all cells regardless of their sensitivity to IGF. Our novel findings reveal previously unidentified role of C3G-Rap1 signaling in the IGF-IR – actin axis promoting cell movement. We conclude that the ability of Rap1 to promote cell movement is critically dependent on IGF-IR signaling. Whether this mechanism can be extended to other receptor tyrosine kinases remains to be seen.

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Myopodin-induced cell migration is regulated by a non-canonical RhoA/ROCK-dependent and myosin-independent pathway.

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More than 80% of patients with invasive prostate cancer have deletions within their myopodin gene, suggesting that myopodin is a reliable predictive marker of prostate cancer metastasis. Five human myopodin (hMYO) splicing variants, each with a unique amino and/or carboxy-termini, have been identified. Biochemical studies have also revealed that myopodin is an actin-binding protein. Since actin rearrangement is an essential process involved in cell migration, it has been postulated that myopodin might affect cancer metastasis by modulating the actin cytoskeleton. However, this hypothesis has not been directly tested and there is no information on the role of the different myopodin isoforms in cancer cell migration. To address these deficits, we ectopically expressed the myopodin isoforms in invasive prostate cancer cells (PC3). Results indicate that all isoforms enhance the chemokinetic activity of PC3 cells, a form of non-directional cell migration. Immunohistochemical studies revealed that the myopodin isoforms induced distinct actin structures in the cell body, and disruption of these structures by genetic or chemical means significantly impaired cell migration, suggesting the myopodin-induced actin structures are directly involved in cell migration. Cell migration requires myosin-mediated actin fiber contraction in order to retract the trailing edge and propel the cell body forward. Interestingly, knockdown of myosin or inhibition of myosin contractibility with blebbistatin did not affect myopodin-induced cell migration, indicating myopodin enhances cell migration via a myosin-independent pathway. However, myopodin-induced migration requires the activation of the upstream effectors of myosin, i.e. RhoA and ROCK. Furthermore, myopodin rescued the tail-retraction defect in myosin-knockdown PC3 cells. Taken together, our data suggest that myopodin may functionally replace myosin to promote cell migration through its ability to induce actin bundles and via a non-canonical RhoA/ROCK-dependent, myosin-independent pathway.

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Rnd3/RhoE is down-regulated in hepatocellular carcinoma and controls cellular invasion.

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Hepatocellular carcinoma (HCC) is the main primary malignancy of the liver and one of the most common and aggressive cancer worldwide, causing 500,000 to 1 million deaths annually. HCC diagnosis is often done at advanced stages of the disease when curative solutions, mostly based on surgery, can no more be proposed. Therefore the development of new molecular targets for therapy, drugs and treatment strategies are needed.

From micro-array data analysis, we noticed the significant down-regulation of Rnd3 expression in HCC. Rnd3, also called RhoE, belongs to the RhoGTPase family and is atypical, since in contrast to other GTPases, it is always in its active GTP-bound form. Here, we address the role of Rnd3 in HCC development.

Rnd3 down-regulation was validated by quantitative real time PCR in a hundred independent tumors. Moreover, Rnd3 down-expression was confirmed using immunohistochemistry on tumor sections and Western blot on human tumor and cell line extracts. Rnd3 expression was significantly lower in invasive tumors with satellite nodules. Overexpression and silencing of Rnd3 in Hep3B cells led to decreased and increased 3D cell motility, respectively. The siRNA-mediated down-regulation of Rnd3 expression induced loss of E-cadherin at cell-cell junctions that was linked to epithelial-mesenchymal transition through the up-regulation of the zinc finger E-box binding homeobox protein ZEB2 and the down-regulation of miR-200b and miR-200c. We further analyzed the pathways involved in Rnd3 knockdown-induced invasion and found that tumor hepatocyte invasion occurred in an MMP-independent, and Rac1- and Cdc42-dependent manner.

In conclusion, Rnd3 down-regulation provides an invasive advantage to tumor hepatocytes suggesting that RND3 might represent a metastasis suppressor gene in hepatocellular carcinoma.

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Scaffold proteins coordinate small GTPase activation in HGF stimulated motility.

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Epithelial cells are largely immotile under normal circumstances, but become motile during development, repair of tissue damage and during cancer metastasis. Both normal and pathologic epithelial migration is stimulated by growth factors. Hepatocyte growth factor (HGF) induces many epithelial cells lines to begin moving. A number of small GTPases that are activated downstream of HGF and are required for HGF-induced movement. We have found that two scaffold proteins, CNK3 and GRASP, coordinate the activation of ARF6 and Rac in response to HGF. CNK3 binds to the ARF-GEF cytohesin 2/ARNO and is required for the activation of ARF6 in response to HGF. GRASP binds directly to both cytohesin 2 and to the Rac-GEF Dock180. This interaction promotes the activation of Rac 1 in response to ARF6. Knockdown of either scaffold inhibits HGF stimulated migration of MDCK cells. These two scaffolds work together to build a ARF-to-Rac signaling module that stimulates epithelial migration.

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Distinct spatiotemporal coordination of Rac1 and RhoA activities in migrating carcinoma cells revealed by experimental multiplexing of FRET sensors.

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Cell migration consists of cycles of protrusion and adhesion at the front, and detachment and retraction at the rear. Signaling crosstalk among different Rho GTPases is thought to play a significant role in coordinating these cellular behaviors spatially and temporally. Previously, genetically-encoded, Förster resonance energy transfer (FRET)-based biosensors were employed to study such dynamics, but left much to be desired specifically in better dynamic range and precise quantification. Here we report the result of coupling of activation-dependent effector binding with dimerization of fluorescent proteins in a new set of sensors of Rho GTPases, namely Dimerization-Optimized Reporters for Activation (Dora) of Rho GTPases. To provide an instrument-independent comparison, the FRET efficiencies of Dora sensors were determined to be 0.80/0.16, 0.68/0.14, and 0.69/0.20 (on/off) for Rac1, cdc42, and RhoA respectively, significantly improved from the published Raichu-Rac1 (0.50/0.28) and FLAIR-RhoA (0.54/0.46). Furthermore the Dora design of sensors were extended with dimerizing orange and red-emitting FRET pair of fluorescent proteins, and to broader signaling targets such as H-Ras, Rab11, and Protein Kinase A. These technical breakthroughs enabled us to experimentally resolve the spatiotemporal dynamics of Rac1 and RhoA activations in the same cell. In contrast to what was reported in mouse embryonic fibroblasts, MCF-7 breast cancer cells showed closer temporal correlation of protrusion with Rac1 activation, with RhoA activation lagging behind. Rac1 activation was absent from retracting tails. Rather than promoting it, RhoA activation appeared to stall or slow down protrusion at the front where it spatially overlapped with Rac1 activation. This occurred synchronously with transient, marked activation of RhoA and retraction at the rear. Blocking downstream signaling of RhoA with the Rho kinase inhibitor Y27632 disrupted the polarized distribution of active Rac1 and resulted in the rapid activation of Rac1 peripheral to active RhoA, indicating that RhoA antagonizes Rac1 at the front.

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Specific Cell-Permeant Cdc42 Inhibitor Prevents GTPase Activation and Cellular Functions.

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Cdc42 activation controls key cellular process including cytoskeletal remodeling, cell division, signal transduction, and vesicular transport. Dysregulated Cdc42 activity contributes to sporadic and genetic diseases ranging from cancer to immunologic disorders. We report the characterization of the first known small molecule inhibitor of Cdc42. The compound was identified through high throughput screening (HTS) of the Molecular Libraries Screening Center Network library of over 200,000 compounds. The compound acts as an allosteric guanine nucleotide binding inhibitor in a dose-dependent manner both in vitro and in cell based assays. The compound was highly specific for Cdc42 with no inhibitory activity against Rho and Rac GTPases. Structure activity analyses identified key structural determinants required for specific

Cdc42 binding. Cell based studies show the inhibitor is not cytotoxic, prevents GTPase activation, and blocks Cdc42-dependent filopodia formation and cell migration. Disease relevant applications are illustrated through the inhibition of hantavirus infection and integrin activation. Together the data identify a novel, allosteric Cdc42 specific inhibitor with utility for studies of in vitro mechanism and cell-based function.

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Regulation of CXCR4 Receptor Trafficking, Signaling, and Function by Arf6.

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CXCR4, a chemokine receptor essential for select neuronal, cardiovascular, and hematopoietic cell migration in response to stromal cell-derived factor-1a (SDF, CXCL12), has emerged as a critical player during breast cancer cell metastasis. Dysregulated CXCR4 on nonmotile primary tumor cells confers an aberrant migratory capacity and promotes the invasive homing to distal SDF-expressing organs. This has led to the vigorous pursuit of factors that regulate the SDF-CXCR4 axis. We demonstrate here that ADP-ribosylation factor 6 (Arf6) regulates cell surface levels of CXCR4 specifically upon activation with SDF. Expression in HEK293 cells of either wild type Arf6 or an active GTPase-deficient Arf6 mutant (ARF6-QL) maintains CXCR4 at the cell surface upon SDF stimulation, whereas an inactive GDP/GTP exchange-deficient Arf6 mutant (ARF6-TN) or Arf6 siRNA promotes CXCR4 loss from the cell surface. Consistent with these data, receptor cell surface levels are similarly altered following modulation of endogenous Arf6 by EFA6, an Arf6-selective guanine nucleotide exchange factor (GEF), or by ACAP1, an Arf6-selective GTPase activating protein (GAP). In addition to regulating SDF-dependent CXCR4 cell surface levels, Arf6 is activated downstream of CXCR4 and regulates SDF-dependent ERK signaling in both HEK293 cells expressing heterologous CXCR4 and MDA-MB-361 metastatic breast cancer cells expressing endogenous CXCR4. Specifically, Arf6 modulates agonist-dependent ERK activity in membranes and in response to SDF gradient stimulation. Furthermore, Arf6 modulates SDF gradient-dependent migration of highly invasive MDA-MB-231 metastatic breast cancer cells. Taken together, these data demonstrate Arf6 regulation of the SDF-CXCR4 axis, and have implications on the dysregulation of CXCR4 observed in breast cancer metastasis.

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The role of an R-Ras/ARF6 signaling module in hepatocyte growth factor (HGF) dependent migration

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In response to hepatocyte growth factor (HGF), epithelial cells show increased migration, as well as display branching morphogenesis in 3D matrices. ARF6 is one small GTPase that acts downstream of HGF leading to cortical actin rearrangements. It is known that ARF guanine nucleotide exchange factor (GEF) cytohesin-2/ARNO binds to scaffold IPCEF1. Upon transfection of Madin Darby canine kidney (MDCK) epithelial cells, ARNO causes a motility phenotype very similar to cells treated with HGF. We recently found that IPCEF1 is actually the C-terminal half of a scaffolding protein called CNK3 [1]. We also showed that the C-terminal CRAC domain of CNK3 associates with ARNO and is required for ARF6 activation downstream of HGF [1]. CNKs have been shown to be involved in signaling downstream of Ras GTPases. A unique R-Ras effector, RLIP76 has been shown to associate with ARNO and links R-Ras to

ARF6 activation [2]. Here, we show that CNK3 associates with RLIP76 via its C-terminal half. In addition, the RhoGAP domain of RLIP76 is needed for co-immunoprecipitation with CNK3. Immunofluorescence staining shows that CNK3 and RLIP76 co-localize. Using split-Venus bimolecular fluorescence complementation microscopy, we show that CNK3 is a shared partner between ARNO and RLIP76 suggesting that they could be apart of the same complex. Finally, we provide evidence that R-Ras does not modulate the interaction between CNK3 and RLIP76. We hypothesize that the interaction between CNK3/RLIP76 is required for HGF-dependent ARF6 activation and migration. Assessing the degree to which the RLIP76/CNK3 module promotes HGF-dependent migration is an important step in understanding the machinery cells need become motile. Elucidating HGF-induced motility can help us grasp developmental processes, epithelial mesenchymal transition (EMT), and metastasis.

[1] Attar, MA., et al. 2012. *Experimental Cell Research* 318: 228-237

[2] Goldfinger, LE., et al. 2006. *JCB* 174: 877-888.

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Microenvironment for hypoxia and interaction with endothelial cells control homing activity of placenta-derived mesenchymal stem cells via alpha4 integrin and Rho signaling.

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Mesenchymal stem cells (MSCs) are powerful source for cell therapy in the degenerative diseases. The homing activity of MSCs is one of important factors to enhance the therapeutic effect when they are transplanted into target tissues or organs. The characteristic capable of homing of MSCs is similar to invasion of cancer. Rho family, which is a migration factors, has been known to be regulated by integrin family in several cancers. However their mechanisms in MSCs were not known. Therefore, the objectives of this study were to compare the expression of markers related to integrins signaling in normal fibrotic cell (WI-38) and placenta-derived mesenchymal stem cells (PDMSC) depend on normoxia and hypoxic conditions. In addition, the correlation between homing activity of MSCs and alpha4 integrin and Rho signaling was evaluated. The invasion activities of PDMSCs exposed to hypoxic condition were significantly increased those of WI-38 and those of normoxia ($p < 0.05$) by Transwell invasion assay. Expressions of integrin $\alpha 4$ were increased both in WI-38 and PDMSCs under hypoxic condition compared to normoxia. Down-regulation of integrin $\alpha 4$ by siRNA in PDMSCs and WI-38 were shown to decrease their invasion activities. Interestingly, Rho family-related makers such as Rho A and ROCK1 were significantly increased in PDMSCs under hypoxic condition compared to normoxia ($p < 0.05$). Furthermore, the invasion activity of PDMSCs was decreased by Rho kinase inhibitor treatment (Y-27632) and by co-cultured with human umbilical vein endothelial cells (HUVEC) in ex vivo system; otherwise, invasion ability of WI-38 was not changed excepting WI-38 co-cultured with HUVEC under hypoxia. Although there are no difference in invasion ability of WI-38 in normoxia by Y-27632 treatment, invasion abilities of PDMSCs co-cultured with HUVEC were significantly increased ($p < 0.05$). Taken together, alterations of alpha4 integrin and Rho signaling by hypoxia and interaction with endothelial cells could be regulating the homing activity of PDMSCs.

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Role of microtubules in neutrophil polarity and migration in live zebrafish.S. Yoo¹, P-Y. Lam¹, M. R. Eichelberg¹, L. Zasadil¹, W. Bement¹, A. Huttenlocher¹; ¹University of Wisconsin Madison, Madison, WI

Microtubules control cell motility by positively regulating polarization in many cell types. However, how microtubules regulate leukocyte migration is not well understood, particularly in living organisms. Here we exploited the zebrafish system to study the role of microtubules in neutrophil migration *in vivo*. The localization of microtubules was visualized in motile neutrophils using various bioprobes, revealing that, in contrast to *in vitro* studies, the microtubule organizing center is positioned in front of the nucleus in motile neutrophils. Microtubule disassembly impaired neutrophil wound attraction but enhanced F-actin polarity based on the distribution of stable and dynamic F-actin. Microtubule depolymerization inhibited polarized PI(3)K activation at the leading edge and induced rapid, PI(3)K independent motility. Finally, we show that microtubules exert their effects on neutrophil polarity and motility at least in part by the negative regulation of both Rho and Rac activity. These results provide new insight into the role of microtubules in neutrophil migration in a living vertebrate and show that the motility of these professional migratory cells are subject to distinctly different rules than those established for other cell types.

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Semaphorin3A and Ephrin-A1 Suppress the NGF-Enhancing Effects on Breast Epithelial Cell Migration.M. D. Wallace¹, S. Gehler¹; ¹Biology Department, Augustana College, Rock Island, IL

The vast majority of all cancer deaths are a consequence of tumor metastases to other regions of the body. However, the molecular mechanisms underlying metastases are not well understood. In some cancers, neurotrophins and axon guidance molecules, all of which were originally characterized in the nervous system, have been shown to have varying effects on cell motility. We demonstrate that nerve growth factor (NGF) selectively enhances the migration of malignant, but not normal, breast epithelial cells, consistent with previously published results (Dolle et.al, 2003). Secondly, using a scratch assay, both semaphorin3A (Sema3A) and ephrin-A1 inhibit malignant breast epithelial cell migration by approximately 25% and 29%, respectively. However, there was no change in cell proliferation following exposure to either Sema3A or ephrin-A1. In the nervous system, Sema3A and NGF have been found to share common signaling pathways to regulate nerve cell growth and growth cone dynamics (Dontchev and Letourneau, 2002; Ben-Zvi et al., 2008). Since breast cancer cells have been shown to respond to NGF, Sema3A, and ephrin-A1 through paracrine and autocrine signaling mechanisms *in vitro* and *in vivo* (Dolle et.al, 2003; Pan et al., 2009; Wykosky and Debinski, 2008), we sought to determine whether Sema3A or ephrin-A1 could suppress the NGF-enhancing effects on breast epithelial cell migration. While NGF enhanced migration by approximately 62%, simultaneous treatment with Sema3A + NGF inhibited migration by 60%, similar to Sema3A treatment alone. Similar effects were observed with simultaneous treatment using ephrin-A1 + NGF. These results indicate Sema3A and ephrin-A1 were able to overcome the enhancing effects of NGF, and suggest Sema3A, ephrin-A1, and NGF share common signaling pathways to regulate motility of breast epithelial cells. Understanding the interactions between these molecules *in vitro* will lead to a better understanding of the complexities of tumor cell motility mechanisms *in vivo*.

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CSPG4-NEDD9 interaction promotes migration, invasion, and growth of breast cancer cells.

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Triple negative (TN) breast cancers are defined by a lack of expression of estrogen, progesterone, and HER2 receptors. It is widely recognized that TN breast cancers have a poorer prognosis than any other subtype of breast cancers. Given the lack of effective targeted therapies for TN breast cancer patients, understanding of the mechanisms of growth and invasion of these tumors will provide insight into developing novel approaches to lower the mortality from TN breast cancer. CSPG4 (chondroitin sulfate proteoglycan 4) gene is expressed on various cell types including breast cancer and melanoma cells and promotes cell migration and invasion. In this study, we demonstrated that statistical analysis (ANOVA) of The Cancer Genome Atlas (TCGA) project gene expression data (<https://tcga-data.nci.nih.gov/>) suggests that tumors from patients with basal subtype breast cancer had significantly higher CSPG4 expression than tumors from patients with other subtypes. Since TN subtype is generally overlapping with basal subtype, these results support the notion of CSPG4 as a key molecule for promoting TN breast cancer invasion and metastasis. We further demonstrated that NEDD9 was co-immunoprecipitated with CSPG4 but not with CD44 or β 1-integrin in MDA-MB-231 cells, suggesting that CSPG4 and NEDD9 form a molecular complex for facilitating tumor migration, invasion, and growth. Overexpression of NEDD9 in a TN breast cancer cell line, HCC38, significantly enhanced migration and invasion. Conversely, suppression of NEDD9 expression by siRNA significantly inhibited migration and invasion of MDA-MB-231 cells, suggesting that NEDD9 plays a key role in promoting invasion and metastasis of TN breast cancer cells. These results provide key insights for not only understanding mechanisms of TN breast cancer progression but also suggest the CSPG4-NEDD9 complex as a potential drug-target for patients who develop this subtype of breast cancer. The views expressed in this article are those of the author and do not reflect the official policy of the Department of Defense, or U.S. Government.

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GnRH Induces RUNX2 Expression Via ERK And AKT Signaling: Possible Involvement In The Regulation Of Trophoblast Invasion?

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Gonadotropin-releasing hormone (GnRH) is expressed in first trimester placenta and exerts pro-invasive effects on extravillous trophoblast cells *in vitro*. The RUNT-related transcription factor RUNX2 has been reported to regulate matrix metalloproteinase (MMP)2/9 as well as an integrin ligand osteopontin expression and is often associated with invasive phenotypes. Thus, our aim was to investigate the effects of GnRH on RUNX2 expression and examine the role of RUNX2 in regulating trophoblast invasiveness.

GnRH receptor, RUNX2, MMP2/9 and osteopontin were detected at invasion sites of human first-trimester placenta by immunohistochemistry. RunX2 and osteopontin are co-expressed in the invasive extravillous trophoblastic cells. Treatment of an immortalized human extravillous trophoblast cell line (HTR-8/SVneo) with GnRH resulted in time- and concentration-dependent increases in RUNX2 mRNA and protein levels. Pre-treatment with the GnRH receptor antagonist Antide attenuated the effects of GnRH on RUNX2 mRNA and protein levels.

Treatment with GnRH elevated phospho-ERK and phospho-Akt protein levels in HTR-8/SVneo cells. Moreover, GnRH-induced increases in RUNX2 protein levels were attenuated by co-treatment with a MEK inhibitor (PD98095) or a phosphoinositide 3-kinase inhibitor (LY294002). Small interfering RNA (siRNA) was used to examine the contributions of RUNX2, MMP2, MMP9 and osteopontin to HTR-8/SVneo cell invasiveness. Transwell Matrigel or collagen I mediated HTR8/SVneo cell invasion was reduced following knockdown of RUNX2, MMP2, MMP9 and osteopontin. Importantly, down-regulation of RUNX2 alone significantly reduced basal MMP2, MMP9 and osteopontin mRNA and protein levels.

Our results suggest that GnRH acts via its receptor to induce ERK and Akt phosphorylation which contributes to elevated RUNX2 expression. GnRH-induced RUNX2 expression may enhance the invasive capacity of trophoblasts by modulating the expression of MMP2, MMP9 or osteopontin.

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Effect of pituitary tumor transforming gene -1 (PTTG1) on the activities for invasion and proliferation of trophoblast cells.

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Pituitary tumor transforming genes (PTTGs), which are isolated from pituitary tumor cells play important roles in malignant transformation as well as several biological process such as cellular growth, development, DNA repair, metabolism and invasion. Invasion of trophoblast is a pivotal role in placentation, decidualization and successful implantation. It is regulated by numerous factors including growth factors, adhesion molecules, matrix metalloproteinases (MMPs), and hypoxia. However, role of the PTTG1 in trophoblast was unknown. Therefore, the objectives are to analyze the expression of PTTG1 in trophoblast and the effect of PTTG1 on activities for invasion and proliferation of trophoblast. Also, the function of PTTG1 in trophoblast was evaluated by the down-regulation of PTTG1 using siRNA. Expression of PTTG1 in trophoblast was localized into nucleus of the cells. Their expressions were significantly decreased in dose dependently when they were exposed to siRNA-PTTG1 treatment for 48 hours ($p < 0.05$). In addition, the numbers of invasive cells were significantly decreased in trophoblast treated with siRNA-PTTG1 compared to control ($p < 0.05$). Especially, invasion abilities of HTR-8 were significantly decreased by 200 and 400pM/ml of siRNA-PTTG1 ($p < 0.05$). Furthermore, down-regulated PTTG1 in trophoblast induced to change the expressions of markers related to adhesion molecules such as integrins and Rho signaling. However, there are no difference proliferations of trophoblast by FACS analysis. These results suggest that alteration of PTTG1 could be regulate invasion of trophoblast.

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Dynamin 2 Potentiates Migratory Tumor Cell Invasion through a Novel Stabilization of the Oncogenic Rac1 GEF, Vav1.

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Metastatic invasion represents a complex synergy of cell motility, adhesion, and matrix remodeling. A cytoskeletal enzyme recently implicated in neoplastic invasion is the large GTPase Dynamin 2 (Dyn2). Dyn2 is markedly upregulated in pancreatic cancer, is a potent activator of metastatic migration, and is required for Rac1-mediated formation of lamellipodia. However, the mechanisms by which Dyn2 participates in these processes are unclear. Here we demonstrate that Dyn2 promotes Rac1 activation in pancreatic cancer cells through a direct interaction with the Rac1 guanine nucleotide exchange factor (GEF) Vav1. Vav1 is ectopically

expressed in over half of human pancreatic cancers to increase tumor cell survival and invasion. Disruption of the Dyn2-Vav1 interaction impairs Rac1 activation and subsequent lamellipodia formation and cell migration. Surprisingly, inhibition of the Dyn2-Vav1 interaction results in a dramatic reduction in Vav1 protein stability, resulting in diminished Rac1 activation. In the absence of Dyn2 binding, Vav1 is targeted to the lysosome for degradation via an interaction with the cytoplasmic chaperone Hsc70. Importantly, a specific mutation of Vav1 near its Dyn2-interactive C-terminal SH3 domain prevents Hsc70 binding, resulting in a stabilization of Vav1 levels. Thus, Dyn2 binding prevents the interaction of Vav1 with the Hsc70 chaperone and thus prevents the targeting of this oncogenic GEF to the lysosome. The effects of Dyn2 on Vav1 protein levels and Rac1 activation are independent of Dyn2 enzymatic activity and function in endocytosis. These findings represent a completely novel function for both Dynamin and Vav1. The fact that these proteins are aberrantly co-expressed at high levels within a majority of pancreatic tumors supports the concept that these proteins interact to increase Vav1 stability and subsequently promote an oncogenic outcome. Supported by NCI RO1 CA104125 to MAM.

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Arpin, a Novel Protein that Inhibits the Arp2/3 Complex at the Leading Edge, Steers Cell Migration.

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The Arp2/3 complex generates branched actin networks that power protrusion of the leading edge in migrating cells. The small GTPase Rac controls Arp2/3 activation at the leading edge through the Wave complex. Here we present the identification of a novel protein that inhibits the Arp2/3 complex and its role in steering cell migration. By bioinformatics, we retrieved an uncharacterized protein containing an Acidic motif at its C-terminus, a characteristic motif of Arp2/3 activators. A combination of in vitro assays, revealed, however, that this protein inhibits the Arp2/3 complex by competing with the activators. We called this protein Arpin as a tribute to A.G.'s PhD supervisor and as a good mnemonic for Arp2/3 inhibition. Surprisingly, the Arp2/3 inhibitory protein Arpin localizes to the leading edge of migrating cells, where actin is polymerized. Rac activates Arpin and RNAi-mediated depletion of Arpin induces lamellipodia to protrude faster, consistent with its inhibitory role on Arp2/3 complex activity. Arpin depleted cells generated from an invasive breast carcinoma cell line, as well as Dictyostelium discoideum amoeba inactivated for the orthologous Arpin gene, migrated more than the controls, because of increased directionality. To confirm this role, we performed a gain-of-function experiment in fish keratocytes, which migrate in a persistent manner. Microinjection of purified zebrafish Arpin into keratocytes resulted in lamellipodial instability and in a turn of the migration direction. The function of the Arp2/3 inhibitory protein Arpin in steering cell migration is thus conserved and is likely to be important for cancer progression and metastasis formation. Indeed, an immunohistochemical analysis of human breast samples revealed that Arpin is well expressed in breast epithelia but that its expression is lost in most invasive carcinoma.

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Regulation of collective cell migration and intercellular force transmission by cell-cell junction proteins.

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In a wide variety of migratory processes, including embryonic morphogenesis, wound healing, and some forms of epithelial tumor invasion, cells migrate collectively. Collective motion of epithelial cell sheets involves physical forces that are generated by the actomyosin cytoskeleton and transmitted across intercellular junctions. The physical network of adhesion proteins that supports force transmission between motile cells remains poorly understood, however. To identify the proteins that are involved in intercellular force propagation and to study their regulation of collective cell migration within an epithelial cell sheet (MCF10A), we used siRNAs to target 20 genes encoding for structural proteins of cell-cell junctions. By using polydimethylsiloxane (PDMS) cell micropatterning combined with Traction force Microscopy, Monolayer Stress Microscopy and Particle Image Velocimetry, we systematically mapped the effect of depleting cell-cell adhesion proteins on cell-matrix forces, cell-cell forces and cell velocities. Our data show that cell velocities and intercellular forces are regulated not only by Cadherin-Catenin complexes, as commonly assumed, but also by proteins traditionally associated with tight junctions. By combining mechanical and gene expression data we provide a mechano-genetic network that captures the regulation of collective cell migration by intercellular adhesion proteins.

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Visualizing and manipulating focal adhesion kinase regulation in live cells.

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Focal Adhesion Kinase (FAK) is essential for cell migration, and plays an important role in tumor metastasis. However, the complex inter-molecular and intra-molecular interactions that regulate FAK activity at the focal adhesion remain unresolved. Recent studies using conformational FRET sensors to probe an important regulatory interaction between the FERM and kinase domains of FAK have yielded conflicting results. Here, we report on FAK FRET sensors using a new technique termed Systematic Protein Affinity Strength Modulation (SPASM). SPASM involves the expression of a single polypeptide with two proteins or protein domains separated by a modular ER/K linker. Changes in affinity between the proteins are quantitatively measured using a genetically encoded FRET pair on either side of the ER/K linker and manipulated by varying the length of the helix. We demonstrate that SPASM FRET sensors are more consistent in reporting changes in the strength of the FERM-kinase interaction than conformational sensors. Further, changes in the ER/K linker length were used to systematically vary the strength of the FERM-kinase interaction. Systematic control of the FERM-kinase interaction both *in vitro* and at live cell focal adhesions was evident in measurements of FRET, kinase activity, and Y397 phosphorylation in multiple cell lines. Control of this key phosphorylation event translated directly into control over cell migration in wound-healing assays. Our findings suggest that the FERM-kinase interaction is spatially patterned *in vivo*, in line with a recent report on the layered architecture of the focal adhesion. We also report on the use of our toolbox of SPASM FAK sensors to understand the impact of the tumor microenvironment on the FERM-kinase interaction. Specifically, cancer cells have been shown to maintain an alkaline intracellular pH (~ 7.5) compared to normal cells (6.8-7.2). SPASM FAK sensors reveal that the FERM-kinase interaction is sensitive to pH, with an enhanced auto-inhibitory interaction

between the FERM and kinase domains at acidic pH. The use of the SPASM technique quantitatively demonstrates, for the first time, a pH dependent change in a protein interaction at a macromolecular structure in live cells. The FERM-kinase interaction was weakest at pH 7.5 with a steady increase at lower pH, peaking at a 3-fold increase at pH 5.8. The enhanced FERM-kinase interaction at acidic pH in live cells was accompanied by reduced Y397 phosphorylation. Our findings provide a potential mechanism for enhanced FAK activity in the tumor microenvironment.

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Cytoskeletal SPECC1L is a novel modulator of cell adhesion, cell motility, actin stability and PI3K-AKT signaling.

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Orofacial clefts are among the most frequent birth defects, affecting one child in eight hundred, in the U.S. alone. Previously, we identified *SPECC1L* (sperm antigen with calponin homology and coiled-coil domains 1-like) as the first gene mutated in oblique facial clefts (ObFC). *SPECC1L* is a novel cytoskeletal 'crosslinking' protein, which interacts with both the actin and microtubule cytoskeletons. *SPECC1L* deficiency in *Drosophila*, zebrafish and mammalian cells results in migration and adhesion defects, revealing a potential mechanism underlying ObFCs [Saadi *et al.*, AJHG 2011]. We now report that *SPECC1L*-knockdown (kd) cells show cell-adhesion (HEK-293T and U2OS osteosarcoma) and cell migration (U2OS) changes **only upon confluence**. While confluent 293T kd cells fail to adhere to any extracellular matrix (ECM), confluent U2OS kd cells show a more selective defect by not adhering to poly-D-lysine substrate. In addition, the *SPECC1L*-kd cells appear drastically elongated upon reaching confluence, in contrast to cuboidal control cells. The average length/width ratio for *SPECC1L*-kd cells (2.94) compared to control cells (1.99) is significantly longer ($p < 10^{-8}$, $n=100$). More important, our initial results using live-imaging and Particle Image Velocimetry analysis indicate that, upon confluence, kd cells move faster **individually**, but are less coordinated **collectively** with the correlation length of sheet motion being 52 μm vs 45 μm for control and kd cells, respectively ($p < 0.0005$). Thus, our previous report of poor performance of *SPECC1L*-kd cells in wound-repair assays is not due to lack of individual motility, but rather their **inability to move collectively**. We are now extending analysis to mixed populations of cells, to test whether kd cells change shape and move less coordinately when surrounded by control cells and vice versa. At the molecular level, antibody array and Western blot analyses of lysates from *SPECC1L*-kd 293T and U2OS cells show a down-regulation of PI3K-AKT signaling, which plays a central role in mediating signals from the ECM, growth factors and cytokines. Several members of the pathway, including pan-AKT, FAK, GSK3 β , p53 and cofilin are down-regulated, while the p120 inhibitory subunit of PI3K is up-regulated. Reduced expression in kd cells of cofilin, which is required for actin turnover, also explains the marked increase in F-actin staining in *SPECC1L*-kd U2OS cells. Lastly, preliminary data from transient transgenic shRNA-based kd of *Specc1l* in mouse shows a range of embryonic developmental defects. We will now confirm whether PI3K-AKT signaling and neural crest cell migration are perturbed in these embryos. Together, these findings begin to explain the cellular and molecular role of *SPECC1L* in facial morphogenesis and malformation.

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Mechanism of integrin mediated Arg kinase activation.

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Abl family kinases, which include the mammalian Abl and Arg, act downstream of integrin receptors to coordinate changes in cell shape and movement. Although integrin-mediated adhesion to the extracellular matrix stimulates Abl and Arg kinase activity, the mechanism by which integrins interface with and activate these kinases is unclear. Abl family kinases are held in an inactive auto-inhibited conformation, in which the SH3 and SH2 domains form an inhibitory lock on the kinase domain. It is believed that activators of these kinases disrupt this configuration, allowing the kinase to assemble into a kinase-active configuration. We provide evidence that integrin beta1 directly activates Arg via a multistep mechanism that includes: 1. An initial, direct binding event between the Arg kinase domain and a lysine-rich region of the beta1 cytoplasmic domain, 2. A subsequent phosphorylation event at the membrane proximal tyrosine residue of beta1 mediated by Arg, 3. A stabilizing interaction between the phosphorylated beta1 cytoplasmic domain and the Arg SH2 domain. Together, these interactions act to robustly activate Arg kinase activity.

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Phosphorylation of the actin bundling protein L-plastin by Mst1 regulates T cell polarization and migration.

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A mouse forward genetics screen using ENU mutagenesis was performed to identify genes critical for T cell function. One mutant line was found to have T cell lymphopenia due to a recessive mutation in the sterile 20-like S/T kinase Mst1. In drosophila and non-immune cells, Mst (hippo) activity regulates organ size by promoting apoptosis and restricting proliferation. However, Mst1 deficiency in mice results in inefficient migration of mature T cells, attributed to defective integrin clustering and adhesion. Mst1 has been defined to be a downstream effector of the small GTPase Rap1. Through association with the adapter RapL, Mst1 is recruited to active Rap1 in proximity to integrins. The ability of Mst1 to activate and phosphorylate integrins or components of the inside-out signaling cascade remains an open and important question. We used phosphoproteomics of T cell receptor (TCR) and chemokine stimulated primary CD4 helper T cells to identify Mst1 substrates. Here, we report Mst1-mediated phosphorylation of the actin bundling protein L-plastin (L-fimbrin), a regulator of actin cytoskeletal rigidity. Using TIRF microscopy, we show that Mst1 phosphorylation directly alters F-actin bundling by L-plastin. Given that L-plastin deficiency in T cells resembles Mst1 deficiency, we propose that L-plastin is a major Mst1 effector in the establishment of T cell polarity and migration in response to TCR and chemokine stimulation, required for thymic egress. Future studies will evaluate the ability of L-plastin containing phospho-mimetic substitutions to rescue L-plastin or Mst1 deficiency in T cell function.

Chemotaxis and Directed Migration I

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Priming of Natural Killer cells following Transendothelial Migration mediated by specific Cytokines and Chemokines.

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Natural Killer (NK) cells must undergo transendothelial migration to locate and kill cancer or virally infected cells. This process is mediated in part by chemokines and cytokines that once secreted at the site of inflammation or tumorigenesis, activate the vascular endothelial cells, thus creating a homing signal for the NK cells. We have observed that when NK cells undergo transendothelial through vascular endothelial cells that have been activated with specific cytokines (TNF- α and TGF- β) and the chemokine (SDF-1 α), the NK cells become primed. This priming results in a more rapid response with respect to signaling downstream of the NK activation receptor, NKG2D and the integrin LFA-1. This priming effect also promotes a more rapid response by the actin and microtubule cytoskeletons during NK-mediated cytolytic activity. Taken together, transendothelial migration that is mediated by specific cytokines and chemokines serves as a priming mechanism for Natural Killer cells and thus leads to enhanced and more rapid cytotoxicity.

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The diabetic environment induces stable intrinsic changes in inflammatory cells that alter migratory responses.

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Inflammatory cell migration is critical to efficient tissue repair and regeneration. Chronic inflammation is characterized by excessive numbers of inflammatory cells present in the injured tissue and is a common characteristic of diabetes-induced non-healing wounds. Although inflammatory cells are present in wound tissue of diabetic patients and mouse models in excessive numbers, it is not clear whether this is due to altered chemotaxis-induced recruitment or aberrant retention of these cells. In order to model wound-induced recruitment and migration, we performed in vitro assays to analyse these behaviors in diabetic-derived versus non-diabetic derived primary monocyte/macrophages and granulocytes. Here we show that CCR2 and CxCR2 chemokine receptors are dysfunctional in diabetic-derived inflammatory cells. Interestingly, although diabetic-derived inflammatory cells express similar levels of these receptors, they display attenuated chemotactic responses. Moreover, diabetic-derived cells show abnormal cell polarization. To determine whether this may reflect a more general migratory defect, we performed scratch wound assays and analysed migration into the 'wound' zone using live imaging. Although diabetic-derived macrophages migrated randomly, they failed to close the 'wound' due to a lack of persistent directionality. In addition, several lines of evidence demonstrate that these defects persist outside of the diabetic environment, indicating the induction of stable cell-intrinsic changes.

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Role of iPLA2 in the regulation of the endosomal recycling of integrin and chemotactic motility of microglia.S-H. Lee¹, N. Sud¹, N. Lee¹, C. Y. Chung¹; ¹Pharmacology, Vanderbilt University Medical Center, Nashville, TN

Microglia are the immune effector cells that are rapidly activated in response to even minor pathological changes in the central nervous system. It has been reported that microglial activation is accompanied by the alteration of integrin expression. Inflammatory cytokines increased expression of $\alpha 4\beta 1$, $\alpha 5\beta 1$, and Mac-1 integrins on microglia but changes of integrin expression upon ADP (a chemoattractant for microglia) stimulation remain unknown. We, in this study, investigated if ADP can induce the alteration of integrin species on cell surface, leading to changes in chemotactic ability on different ECM. FACS scans and surface biotinylation assays showed that ADP stimulation induced a significant increase in the expression of $\alpha 6$ integrin, but not $\alpha 5$ on the surface of microglia cells. Examination of microglia motility on different ECM revealed that cells have greater motility on laminin than fibronectin upon ADP stimulation, presumably due to the increased expression of $\alpha 6$ integrin. Our recent study demonstrated that knock-down of Ca⁺⁺-independent phospholipase A2 (iPLA2) specifically inhibits the trafficking of c-Src through recycling endosomes. Given that iPLA2 activity is required for the regulation of recycling endosome trafficking, we tested if there is any change of the expression level of integrin subunits in iPLA2 knock-down cells. Integrin endocytosis assay revealed that iPLA2 activity is required for the recycling of $\alpha 6$ integrin back to the plasma membrane. Time-lapse microscopy also clearly revealed the essential role of iPLA2 activity for the recycling of $\alpha 6$ integrin from the endosomal recycling complex to the plasma membrane via recycling endosomes. The alteration of integrin-mediated adhesion may regulate the extent of microglial infiltration into the site of damage by controlling their chemotactic ability.

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Role of Cortactin Homolog HS1 During Transendothelial Migration of Natural Killer Cells.S. Mukherjee¹, O. Mooren¹, J. A. Cooper¹; ¹Cell Biology and Physiology, Washington University School of Medicine in St. Louis, St. Louis, MO

Natural killer (NK) cells provide immune protection by recognizing and killing target cells that lack MHC class I protein, often including cancer cells and virus infected cells. However, before NK cell can perform its functions, it has to traverse the barrier of endothelial cells lining the blood vessel wall. The process of transendothelial migration is complex and poorly understood in NK cells. NK cell motility is initiated by environmental signal, which promotes its attachment to the vascular endothelial layer and subsequent trans-cellular migration. Mechanism of transendothelial migration involves tight regulation of adhesion molecules, chemokines and cytoskeletal regulators. Thus, the molecules, which can integrate between signaling pathways and cytoskeletal regulation, may play essential function in this process.

We hypothesize that HS1 is a potential candidate in regulating NK cell transmigration as it is involved in signaling and actin assembly in NK cell and T cell activation. Our data indicate that depletion of endogenous HS1 protein by siRNA or shRNA technique in NK cells diminishes its ability to transmigrate across the endothelial cell monolayer during transendothelial migration assay and this transmigration event can be rescued by transfecting with shRNA insensitive HS1 construct. It has been established that, in NK cells, phosphorylation of HS1 Tyr residue at position 397 is required for adhesion to the integrin ligand ICAM-1, whereas phosphorylation of Tyr378 is important for chemotaxis. Moreover, a recent study has reported that phosphorylation of HS1 Tyr222, Tyr378 and Tyr397 is required for its interaction with Arp2/3 and efficient

chemotaxis in neutrophil. Currently we are investigating the role of phosphorylation on all of these Tyr residues in NK cell transendothelial migration.

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Loss of Arp2/3 complex does not inhibit fibroblast chemotaxis, but does trigger an NF- κ B-dependent secretion of chemokines and growth factors with autonomous and non-autonomous effects on migration.

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Chemotaxis is critical for numerous physiological processes such as wound healing, morphogenesis, and immune response, as well as being important for diseases such as metastatic cancer. While this process has been intensively studied in amoeboid cells, it is much less well understood in mesenchymal cells such as fibroblasts. Recently, our group showed that fibroblasts depleted of the Arp2/3 complex showed normal chemotactic responses, but were deficient in the ability to respond to gradients of extracellular matrix cues (haptotaxis). In these experiments, PDGF- a physiological chemoattractant for fibroblasts was used. Contrary to these results, another group recently showed that the Arp2/3 complex is required for EGF chemotaxis in fibroblasts. We repeated the chemotaxis assay with our depleted cells using EGF and observed that chemotaxis to this ligand was also intact. To address whether RNAi depletion was sufficient to completely abrogate Arp2/3 function, we tested the chemotaxis of fibroblasts derived from a conditional knockout mouse for the Arpc2 (p34) subunit of Arp2/3 and observed that these cells had intact chemotactic responses. One possible explanation for these differences is the design of the microfluidic chemotaxis chamber used by each group. Our chamber has continuous media exchange, while the Ibidi chamber used by the other group was sealed. Based on RNA-seq-based expression profiling of our Arp2/3-depleted cells, we discovered that loss of Arp2/3 leads to the up-regulation of many genes encoding secreted proteins such as chemokines, growth factors and proteases. Using immunofluorescent staining and ELISA, we have confirmed this up-regulation at protein level for a subset of these genes. Interestingly, most of the up-regulated secretory factors are regulated through the NF- κ B pathway. Indeed, we have found that the NF- κ B activity in Arp2/3-depleted cells was up-regulated and that inhibiting NF- κ B reversed the increased expression of secreted factors. To test whether the secreted factors produced by the Arp2/3-depleted cells could affect chemotaxis through signaling crosstalk, we repeated these experiments using conditioned media in our continuous flow chambers. Conditioned media harvested from Arp2/3-depleted cells blocked EGF chemotaxis, but conditioned media from control cells did not. Thus, our findings indicate that Arp2/3 loss does affect chemotaxis, but in an indirect way involving altered NF- κ B dependent gene expression. We have also identified the upstream signaling events leading from Arp2/3 loss or inhibition to NF- κ B activation involving the canonical MEKK3-IKK pathway.

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Reconstitution of *in vivo* macrophage-tumor cell pairing and streaming motility on one-dimensional micro-patterned substrates.

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In mammary tumors, intravital imaging techniques have uncovered an essential role for macrophages during tumor cell invasion and metastasis mediated by an epidermal growth factor

(EGF) / colony stimulating factor-1 (CSF-1) paracrine loop. It was previously shown that tumor cells in mammary tumors in mice exhibit high velocity migration on extracellular matrix (ECM) fibers. These cells form paracrine loop-dependent linear assemblies of alternating host macrophages and tumor cells known as “streams”. Here, we confirm by intravital imaging that similar streams form in close association with ECM fibers in mammary tumors derived from a highly metastatic patient-derived primary tumor cell, TN1 and in mammary tumors derived from MDA-MB-231, a human breast cancer cell line. To understand the *in vivo* cell motility behaviors observed in streams, an *in vitro* model of fibrillar tumor ECM utilizing adhesive 1D micropatterned substrates was developed and imaged by time-lapsed wide field microscopy. MTLn3 breast carcinoma cells on 1D fibronectin or type I collagen substrates migrated with higher velocity than on 2D substrates and displayed enhanced lamellipodial protrusion, cell velocity and persistence upon local interaction and pairing with bone marrow-derived macrophages (BMMs). Inhibitors of EGF or CSF-1 signaling disrupted this interaction and reduced tumor cell velocity and protrusion, validating the requirement for an intact paracrine loop. Both MTLn3 and MDA-MB-231 cells were capable of co-assembling into linear arrays of alternating tumor cells and macrophages that resembled streams *in vivo*, suggesting the stream assembly is cell autonomous and can be reconstituted on 1D substrates. Our results validate the use of 1D linear micropatterned substrates as a simple and defined approach to study fibrillar ECM-dependent cell pairing, migration and relay chemotaxis with high spatial resolution as a complementary assay to imaging of tumors in the living animal by intravital microscopy. Potential future applications include high-throughput screening for novel motility-related anti-metastasis drugs.

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Dynamic myosin II organization regulates front-rear coupling, cell shape determination, and efficient chemotaxis in neutrophil-like HL60 cells.

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Myosin contraction at the rear is known to be necessary for proper cell migration, though little is known about how myosin dynamics at the rear are coupled to protrusion at the leading edge. Here, we study migration of neutrophil-like HL60 cells expressing fluorescently labeled myosin II with high spatiotemporal resolution, both in uniform environments and in light-induced gradients of chemoattractant.

We find that the degree of rearward polarization of myosin II is determined by cell speed and myosin diffusivity. As the cell accelerates, myosin molecules are swept backward to concentrate at the rear in the cell frame of reference. Hyperphosphorylation of myosin II light chain decreases the average myosin mobility and leads to enhanced rearward polarization. Cells with rearward-polarized myosin II have a more convex shape of the rear and switch polarity less frequently than cells where myosin II is evenly distributed. The shape determination of the rear can be explained by a “Graded Radial Retraction” model.

In addition, myosin II localization is highly dynamic and correlates with changes in whole-cell movement. In a persistently moving cell, cycles of protrusion at the front are tightly coupled to simultaneous contraction at the rear, followed 10 seconds later by transient local increases in myosin II and F-actin concentration. In a turning cell, these myosin II “flashes” localize at the outside of the cell and correlate with left-right shape asymmetry and with periods of increased

angular velocity. The dynamics we observe are consistent with a model of self-reinforcing recruitment of contracting myosin II followed by myosin-triggered F-actin disassembly.

Finally, we demonstrate that proper phosphoregulation of myosin light chain is important for efficient chemotaxis in HL-60 cells. Using both siRNA and pharmacological perturbations, we find that hypophosphorylation of myosin II leads to front-rear decoupling, a long tail phenotype, reduced speed and frequent switching of polarity. Conversely, hyperphosphorylated myosin II becomes less dynamic and less efficient to reorient the cells in a light-controlled switching gradient of chemoattractant.

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Cooperative dynamics of actin nucleators in epithelial cell protrusion.

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Arp2/3 and several members of the formin family of actin nucleators have been shown to coexist at the leading edge of migrating cells where they drive cell protrusion by promoting actin filament assembly. Although their individual biochemical functions are well studied, the functional interactions between these various types of nucleators are poorly understood because of the spatiotemporal heterogeneity and transience of actin dynamics during cell migration events like protrusion. Here, in order to characterize the coordination of several actin nucleators *in situ*, we develop a novel local image sampling and 'event-averaging' approach. We measure the local fluorescence of HaloTag fusions to actin nucleators in small sampling windows moving with the leading edge. These local fluorescence time series data are then aligned using protrusion events such as protrusion onset or maximal protrusion velocity as common timing references. This allowed us to average the fluorescence over many sampling windows and extract from highly heterogeneous and noisy signals systematic trends in the kinetics of protein recruitment and further molecular variables. Our analysis shows that the formin mDia1 is recruited to the leading edge before protrusion onset along with components of nascent adhesions whereas Arp2/3, the formin mDia2, and VASP, and cofilin increase the rate of actin assembly after protrusions. Concurrently, we observe a steady increase in local traction forces, which are initiated at the cell edge with the formation of nascent adhesions and translocate backwards with adhesion maturation. Formin inhibition leads to significant reduction of lamellipodial Arp2/3. Our data is consistent with a model where mDia1-activation leads the way into a new protrusion, while Arp2/3 binds to mDia1-mediated actin filaments and nucleates actin filament growth in branched networks. Further analysis of the system dynamics using Arp2/3 inhibitor suggest that the reinforced actin polymerization by Arp2/3 and other actin regulators after protrusion onset supports edge advancement against increasing tension from the cell boundary.

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Directional persistence of migrating cells requires Kif1C-mediated stabilisation of trailing adhesions.

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Directional cell migration requires the establishment and maintenance of long-term differences in structure and function between the front and rear of a cell. Many cell types require the microtubule cytoskeleton to support cell polarity and guide directional migration. To do this, microtubules themselves are arranged asymmetrically in migrating cells with the majority

growing towards the front of the cell, thereby supporting front-biased processes. Cell migration is viewed as a cyclic process of protrusion at the cell front and retraction of the cell rear, with protrusion towards an attractant determining migration direction of the cell. In the absence of external guidance cues, however, symmetry breaking occurs at the rear.

Here we show that the cell rear is also important for directional stability of cell migration. We find that the microtubule motor Kif1C contributes to directionally persistent cell migration primarily through stabilization of an extended cell rear. Kif1C accumulates at the tip of cell tails shortly after tail formation and dispersal of Kif1C correlates to tail retraction. We find that Kif1C-mediated transport of $\alpha 5\beta 1$ -integrins is required for the proper maturation of trailing focal adhesions and resistance to tail retraction. Tail retraction correlates to and precedes changes in migration direction. Furthermore, stabilisation of cells tails through inhibition of myosin II activity suppresses the Kif1C depletion phenotype and results in longer-lived tails and higher directional stability of migrating cells. Taken together these findings indicate that the maintenance of an extended, tense cell tail facilitates directional persistent cell migration. We propose a rear-steering mechanism whereby the counterforce originating from a well-anchored tail serves to maintain directionality of the force-generating leading edge of the cell.

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Chemotaxing amoeboid cells migrate by switching between modes with distinct adhesion and contractility dynamics.

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Chemotaxis, or directed cell migration, is involved in a broad range of biological phenomena, ranging from the metastatic spreading of cancer to the active migration of neutrophils during wound healing or in response to bacterial infection. Chemotaxing Dictyostelium cells adapt their morphology and speed to external conditions such as the stiffness and adhesive properties of their substrate. The mechanism, by which they control both their shape and speed, still remains largely unknown. Using Fourier Traction Force Microscopy (FTFM) to measure the spatiotemporal evolution of shape and traction stress dynamics during migration, we construct traction tension kymographs. The kymographic representation of the traction stresses allows us to determine, for the first time, how the formation and disassembly of adhesions are coupled with the generation of axial and lateral traction stresses. We show that wild-type cells control their motility by switching between two motility modes with distinct adhesion and contractility dynamics. In the “Stepping-Stepping” mode, the adhesion sites remain stationary while the body moves forward by periodic axial contractions. The back adhesions break after new frontal adhesions are formed. In the “Stepping-Gliding” mode, the cell reduces the magnitude of the traction stresses, increases the frequency of axial contractions and its migration speed, and keeps the frontal adhesion stationary while sliding the back adhesion forward. These two modes are not conserved when cells move on highly adhesive poly-L-lysine coated substrates, where cells alternate between a “Nearly Stationary” mode, characterized by strong lateral contractions and extremely low migration speed and a “Gliding-Gliding” mode where multiple weak and transient adhesions are formed, which are gliding forward as the cell moves by barely adhering at the substrate. Finally, we found that cells with cytoskeletal crosslinking defects (*mhcA* and *abp120* cells), move by developing increased lateral contractility implementing distinct motility modes, which slightly differ from those observed in wild-type cells.

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ForC lacks formin activity but is required for chemotaxis and multicellular development of *Dictyostelium* cells.

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Diaphanous-related formins (DRFs) are known to drive nucleation and elongation of unbranched actin filaments downstream of Rho GTPase signaling pathways. They are characterized by the Diaphanous inhibitory domain (DID) in their N-terminal region and its counterpart – the Diaphanous auto regulatory domain (DAD) at the C-terminus. *Dictyostelium discoideum* ForC qualifies for this class of formins, but in spite of the presence of DID and DAD homology domains, it lacks the canonical proline-rich formin homology domain 1 (FH1) that recruits profilin-actin complex for subsequent filament assembly by the FH2 domain. Of note, ForC is not a pseudo gene since the genetic knockout of ForC strongly impairs late development. The phenotype of the mutant is characterized by the presence of aberrant fruiting bodies, as opposed to control, which forms single-stalked structures. Immunoblotting with specific antibodies revealed that ForC is absent in vegetative cells, becomes detectable after 4-5 h of starvation, which is defined as the onset of early aggregation, and peaks around 9-12 h. Fluorescence microscopy of cells expressing a GFP-tagged N-terminal ForC fragment showed that ForC transiently accumulates in the leading edge, suggesting that ForC may play a role in cell migration. Consistent with the expression profile, we observed no defects in random migration of vegetative mutant cells. Strikingly, however, chemotaxis towards a source of cAMP after 6 h of starvation was entirely abolished. Time-lapse phase contrast microscopy revealed though, that ForC-null cells can still aggregate albeit strongly delayed. Moreover, we observed an entire failure of phototaxis. Together, this suggests ForC to be critically involved in migration at the single cell level as well as multicellular aggregates during *Dictyostelium* development. Since ForC cannot be expressed in *E. coli* for biochemical analyses, we purified a C-terminal half of ForC containing the putative FH2 domain from *Dictyostelium* cells. Of note, ForC-FH2-C did neither inhibit nor stimulate actin polymerization as assessed by actin pyrene assays, suggesting that ForC lacks formin activity. Thus, ForC may have evolved from a genuine formin to become a developmentally expressed modulator of the actin cytoskeleton regulating cell migration.

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Model of Capping Protein and Arp2/3 Complex Turnover in the Lamellipodium Based on Single Molecule Statistics.

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Capping protein (CP) and Arp2/3 protein complex regulate actin polymerization near the leading edge of motile cells. They assemble near the edge of the lamellipodium, undergo retrograde flow, and dissociate into the cytoplasm as single subunits or as part of actin oligomers. To better understand this cycle, we modeled the kinetics of CP and Arp2/3 complex in the lamellipodium using data from prior single molecule microscopy experiments [Miyoshi et al. JCB, 2006, 175:948]. In these experiments speckle appearance and disappearance events corresponded to assembly and dissociation from the F-actin network. We used the measured dissociation rates of Arp2/3 complex and CP (0.048/s and 0.58/s, respectively) in a Monte Carlo simulation that includes particles in association with F-actin and diffuse in the cytoplasm. We explored the effect of slowly diffusing cytoplasmic pool to account for a big fraction of CP with diffusion coefficients as slow as 0.5 $\mu\text{m}^2/\text{s}$ measured by single molecule tracking [Smith et al. Biophys. J.,

2011,101:1799]. These slowly diffusing species could represent severed actin filament fragments. We show that such slow diffusion coefficients are consistent with prior FRAP experiments by Kapustina et al. [Cytoskeleton, 2010, 67:525] who fitted their data using larger diffusion coefficients. We also show that the single molecule data are consistent with FRAP experiments by Lai et al. [EMBO J., 2008, 28:986] who found that the Arp2/3 complex recovers more quickly at the front of the lamellipodium as compared to the back. We discuss the implication of disassembly with actin oligomers and suggest experiments to distinguish among mechanisms that influence long range transport.

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Mathematical modelling of protrusion generation and dynamics.

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Many cells generate protrusions repeatedly or even in an oscillatory manner. At the same time, existing lamellipodia may exhibit shape oscillations with an amplitude comprising almost the whole protrusion depth. We investigate the mechanism of this type of dynamics by mathematical modelling. We use a model which has been verified by explaining the force velocity relation of fish keratocytes. We find an oscillation mechanism agreeing with recent experimental observations. With that mechanism, oscillations of the signaling pathways upstream of nucleation promoting factors or other actin binding molecules are not required to obtain oscillatory protrusions.

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Actin filament elasticity and retrograde flow shape the force-velocity relation of motile cells.

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Cells migrate through a crowded environment during processes such as metastasis or wound healing, and must generate and withstand substantial forces. The cellular motility responses to environmental forces are represented by their force-velocity relation, which has been measured for fish keratocytes but remains unexplained. We present a mechanism quantitatively explaining the cell's force-velocity relation; and its changes upon application of drugs that hinder actin polymerization or actomyosin based contractility. Even pN opposing forces slow down lamellipodium motion by three orders of magnitude. At larger opposing forces, the retrograde flow of actin accelerates until it compensates for polymerization, and cell motion stalls. Subsequently, the lamellipodium adapts to the stalled state. Elastic properties of filaments close to the lamellipodium leading edge and retrograde flow shape the force-velocity relation. Our results shed new light on how these migratory responses are regulated, the relation of polymerization forces to elastic and viscous forces in the lamellipodium, and on its mechanics and structure.

Signaling Scaffolds and Microdomains

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Signal molecules anchoring of proliferation and differentiation genes by A-Kinase Anchoring Protein (AKAP) 95.

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A-kinase anchoring protein 95 (AKAP95) is a DNA-binding zinc finger protein that in addition to binding cAMP-dependent protein kinase, binds other signaling molecules such as protein phosphatase 1. AKAP95 is a nuclear matrix protein and also interacts with chromatin modifiers such as histone deacetylase (HDAC) 3, suggesting it may contribute to functionally compartmentalize chromatin and orchestrate fundamental nuclear processes such as replication or transcription. We are testing the hypothesis that AKAP95 constitutes an anchoring platform integrating information from signaling pathways involved in proliferation and differentiation with regulators of gene expression such as transcription factors and chromatin remodeling factors. To this end, we characterize by genome-wide approaches genomic sites occupied by AKAP95 in human mesenchymal stem cells (MSCs) and their transformed counterparts, using for this mesoderm-derived osteosarcoma U2OS cells. We focus our attention on proximal and distal regulatory sequences for cell cycle control and differentiation genes. We intend to identify co-occupying gene regulatory factors and address the role of AKAP95 in recruiting and maintaining these factors at the AKAP95 occupancy sites. We expect to characterize chromatin regulating complexes anchored by AKAP95 at genomic loci involved in oncogenic transformation and establish a new role for the major nuclear AKAP, AKAP95, in the regulation of proliferation-associated chromatin processes.

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P2Y receptors trigger loss of cortical gravin/PKA localization through calcium and PKC mediated signaling.

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Gravin (AKAP12), a multivalent A-kinase anchoring protein, anchors PKA and likely other signaling proteins to the plasma membrane through a subcellular targeting domain which includes N-myristoylation and three downstream polybasic regions rich in positively charged amino acids. Although gravin is postulated serve as a scaffold for diverse signaling complex at the plasma membrane, reports demonstrate that pharmacological activation of PKC or $[Ca^{2+}]_i$ increase causes the loss of cortical gravin through its translocation into the cytosol. However, the signaling effects exerted by gravin redistribution are not understood, nor has this response been demonstrated to occur through receptor mediated signaling. We postulate that receptor mediated signaling events which trigger PKC and/or $[Ca^{2+}]_i$ consequently alter subcellular PKA activity through the translocation of gravin. To assess this, we examined the effect of Ca^{2+} -elevating agents on gravin and gravin-dependent PKA distribution in cells expressing EGFP- or EYFP-tagged gravin constructs. Ionomycin and thapsigargin each initiated rapid gravin redistribution into the cytosol, an event which was inhibited by Ca^{2+} chelation using BAPTA-AM. Treatment of cells coexpressing gravin-EYFP and PKA RII-ECFP with Ca^{2+} -elevating agents also resulted in PKA redistribution, but only when gravin was present. Consistent with this, treatment with ATP in a Ca^{2+} free medium also resulted in rapid gravin redistribution in concert with a rise in $[Ca^{2+}]_i$, suggesting that this change is mediated by purinergic P2Y receptors. ATP also caused PKA redistribution in cells coexpressing gravin and PKA constructs. However,

inhibition studies using BAPTA-AM and bisindolylmaleimide indicated that PKC activation also contributed to ATP mediated changes in gravin distribution. Our studies confirm that receptor mediated signaling events involving activation of PKC and $[Ca^{2+}]_i$ increase causes gravin redistribution and support the hypothesis that these changes alter subcellular PKA activity. For future studies, we have developed an assay for measuring real-time changes in PKA activity with a FRET biosensor known as A-Kinase Activity Reporter 3 (AKAR3). Both membrane-localized and cytosolic AKAR3 are now being used to understand the role of AKAP12 translocation in compartmentalized PKA signaling.

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14-3-3 ζ is required for glucose homeostasis, lipid metabolism, and adipogenesis.

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Insulin is a critical hormone that regulates metabolism through multiple signaling pathways. It has been proposed that molecular adaptors, such as members of the 14-3-3 family, coordinate and integrate signaling cues to facilitate insulin's effects on cell survival, adipogenesis, lipid metabolism, and glucose uptake. However, this hypothesis has not been tested in vivo. To examine the in vivo role of 14-3-3 proteins in metabolism, we utilized knockout (KO) mice lacking 14-3-3 ζ , an isoform, which we and others have implicated in insulin signaling in vitro. 14-3-3 ζ KO mice were significantly smaller than littermate controls at weaning, but were indistinguishable from wildtype mice by 12 weeks. 14-3-3 ζ KO mice showed a significant delay in their ability to clear a glucose load (2g/kg). This glucose intolerance was due to significant insulin resistance, as assessed by insulin tolerance tests (1.5U/kg). Insulin resistance in 14-3-3 ζ KO mice was associated with 2-fold increases ($p < 0.05$) in fasting and random-fed insulin levels and 1.5-fold increased β -cell mass. Serum triglycerides were significantly higher in KO mice, and analysis of lipogenic genes in white adipose tissue showed significantly decreased transcript expression of hormone-sensitive lipase, fatty acid synthase, and acetyl-CoA carboxylase. Together, these in vivo data indicate that 14-3-3 ζ KO mice have many of the hallmarks of early type 2 diabetes. To further investigate the molecular mechanisms by which 14-3-3 proteins regulate insulin signaling, NIH3T3 fibroblasts were first transfected with a plasmid containing difopein, a pan 14-3-3 inhibitor. Loss of 14-3-3 function did not affect insulin-stimulated Akt phosphorylation, but attenuated activation of the MAPK signaling pathway. siRNA-mediated knockdown of 14-3-3 ζ recapitulated the effect of difopein on insulin signaling. As loss of 14-3-3 ζ was associated with altered lipid homeostasis, we next examined if the ζ isoform was involved in adipogenesis. siRNA-mediated knockdown of 14-3-3 ζ in 3T3-L1 pre-adipocytes resulted in a dramatic block of differentiation, as measured by Oil Red-O staining. Quantitative PCR analysis demonstrated that 14-3-3 ζ was required for expression of mature adipocyte markers, such as Ap2, PPAR γ , GLUT4, hormone-sensitive lipase, fatty acid synthase, AND SREBP-1c. Taken together, these findings demonstrate, for the first time that 14-3-3 ζ isoform is required for insulin signaling and glucose homeostasis in vivo. Furthermore, the ζ isoform is required for adipogenesis and may explain the defects in lipid metabolism observed in the knockout mice. These data suggest the possibility of modulating insulin sensitivity and obesity by targeting 14-3-3 adapter proteins.

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Pseudopodial pyruvate kinase M2 regulates senescence via the RhoGEF Tiam1 in metastatic cancer cells.

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Epithelial mesenchymal transition (EMT) promotes cancer cell invasion and metastasis and also overrides cellular senescence, a failsafe mechanism that limits cellular transformation. How cellular transformation, an early event in cancer progression, is controlled by a late stage process such as EMT remains unclear.

Here we identify 13 pseudopod-enriched proteins, including the YWHAE 14-3-3 ϵ scaffolding protein and pyruvate kinase M2 (PKM2), a key regulator of cancer cell metabolism, and show that all are required for tumor cell migration and invasion. While knockdown of five pseudopod-enriched proteins in metastatic human cancer cells induce expression of epithelial markers (i.e. E-cadherin), knockdown of the eight other pseudopod proteins (including PKM2 and YWHAE) increase cell spreading and expression of senescence markers (p53, p16, SA- β -Gal). Pseudopod protein reversal of EMT is associated with reduced RhoA activity, increased Rac1 activity and increased expression of the RhoGEF, Tiam1, while induction of senescence is associated with reduced Rac1 activation and loss of Tiam1 expression. The EMT- and senescence-associated pseudopod proteins form distinct complexes and senescence-associated YWHAE interacts directly with both PKM2 and TIAM1. Peroxide-induced senescence induces selective loss of senescence-associated pseudopod proteins and Tiam1, but not EMT-associated pseudopod proteins. Furthermore, expression of a constitutively active form of Tiam1 prevents senescence induced by either peroxide treatment or knockdown of senescence-associated pseudopod proteins. This suggests that pseudopod protein control of Tiam1 maintains the balanced RhoA and Rac1 activity that limits senescence and promotes EMT.

PKM2 regulation of senescence via Tiam1 control of Rho GTPase activity defines a novel role for this cancer-associated metabolic enzyme in cancer progression. Based on the critical role described for pseudopod proteins, acquisition of motile and migratory ability therefore represents a common mechanism linking senescence override and EMT in cancer progression.

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Proteins of the Ubiquitin Proteasomal System regulate the function of the MAPK1/2 pathway scaffold - Shoc2.

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The mitogen-activated protein kinases (MAPK1/2) signaling pathway is critical in normal human development as it controls cell proliferation, differentiation, migration and apoptosis. When these processes are perturbed, malignant transformations, cardiovascular pathologies and RASopathies (genetic syndromes) can develop. The MAPK1/2 pathway is a complex signaling interconnected network with large numbers of dynamic protein interactions and diverse biological outcomes. A significant part of how biological specificity is generated depends on scaffold proteins. Scaffold proteins are key players that control protein-protein interactions, compartmentalize incoming MAPK1/2 signals at particular cellular locations, and regulate accessibility to substrate, thereby delivering signaling specificity. Hence, scaffold proteins not

only have a huge impact on the biological behavior of the MAPK1/2 pathway, but they essentially determine the functional outcome of the signaling.

While studying the mechanisms of MAPK1/2 signal propagation, we discovered that the Ras-RAF binding scaffold protein Shoc2 is recruited to the surface of late endosomes. Shoc2 is a critical MAPK1/2 pathway regulator that accelerates MAPK1/2 signaling. Moreover, germline deletion of Shoc2 in mice results in early embryonic lethality due to severe defects in heart development. We showed that Shoc2 interacts with several proteins of the Ubiquitin Proteasomal System (UPS), and PSMC5 is among them. PSMC5 is an AAA (ATPase Associated with various cellular Activities) ATPase and a part of the 19S proteasomal subunit. In this study, we identified the functional properties of PSMC5 and the key structural elements mediating the interaction between Shoc2 and PSMC5. RNAi depletion of PSMC5 resulted in increased levels of Shoc2 protein and subsequent increase in MAPK1/2 activity upon EGFR activation. This suggests that interaction of Shoc2 with PSMC5 plays an important role in Shoc2 function as a positive regulator of MAPK1/2 signaling. We also demonstrated that fluorescent protein-tagged Shoc2 and PSMC5 expressed in cells are recruited to a subset of multi-vesicular bodies (MVB)/late endosomes containing Rab7. Moreover, increased translocation of Shoc2 to MVB affected Shoc2 protein levels and its function as a MAPK1/2 signal modulator.

In this study, we identified a novel non-proteolytic function of PSMC5 in regulating MAPK1/2 signaling. We determined that PSMC5 interaction with Shoc2 is part of the cellular machinery by which Shoc2 is targeted to the endocytic compartment. Our results suggest that spatio-temporal mechanisms regulating the MAPK1/2 pathway involve recruitment of Shoc2 to the MVB/LE compartment.

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Identification of a Novel Function of the Clathrin-Coated Structure at the Plasma Membrane in Facilitating GM-CSF Receptor-Mediated Activation of JAK2.

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It is well known that ligand-binding to the high-affinity GM-CSF receptor (GMR) activates JAK2. However, how and where this event occurs in a cellular environment remains unclear. Here, we demonstrate that clathrin-, but not lipid raft-mediated endocytosis is crucial for GMR signaling. Knockdown expression of clathrin heavy chain or intersectin 2 (ITSN2) attenuated GMR-mediated activation of JAK2, whereas inhibiting clathrin-coated pits or plagues to bud off the membrane by the dominant negative mutant of dynamin enhanced such event. Moreover, unlike the wild-type receptor, an ITSN2-non-binding mutant of GMR defective in targeting to clathrin-coated pits or plagues (collectively referred to as clathrin-coated structures [CCSs] here) failed to activate JAK2 at such locations. Additional experiments demonstrate that ligand treatment not only enhanced JAK2/GMR association at CCSs, but also induced a conformational change of JAK2 which is required for JAK2 to be activated by CCS-localized CK2. Interestingly, ligand-independent activation of the oncogenic mutant of JAK2 (JAK2V617F) also requires the targeting of this mutant to CCSs. But JAK2V617F seems to be constitutively in an open conformation for CK2 activation. Together, this study reveals a novel functional role of CCSs in GMR signaling and the oncogenesis of JAK2V617F.

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Caveolin-1 expression attenuates PKC activation in human melanoma cells.A. V. Phan¹, R. W. Baer¹; ¹Physiology, A. T. Still University, Kirksville, MO

Caveolin-1 (cav-1) is a membrane protein that is involved in structural integrity, vesicle transportation, and signal transductions. Depending on the type of cancer, cav-1 may have either a pro- or anti-metastatic effect. In melanoma, cav-1 is believed to be anti-metastatic. Our goal was to characterize possible mechanisms by investigating the effects of increasing cav-1 expression on PKC-alpha (PKC) activation and activation of putative pro-metastatic, downstream, proteins (ERK, JNK and AKT). Wild-type BLM and 1205Lu melanoma cells were each transfected to create corresponding stable, cav-1 expressing lines. BLM and 1205Lu wild-type and BLM and 1205Lu cav-1 expressers were treated with 10 ng/ml PMA to stimulate PKC. After 0, 1, 5, 15, 30, 60, and 120 min of treatment, 50 µg of each whole cell extract was run on SDS page gels and subjected to immunoblotting for analysis of total and phosphorylated proteins using IR detection (Licor Odessey). Time courses for each cell line were repeated at least in triplicate. In the BLM cell line, PMA stimulation caused an increase in PKC phosphorylation that peaked at 5 min. Peak phosphorylation of ERK subsequently occurred at 30 min and JNK's at 120 min. In the BLM cav-1 expressers, PMA treatment caused little stimulation of PKC phosphorylation over the 2 hr period, and the overall PKC activity was 36% less than wild-type. ERK's peak activity did not occur until 120 min, and JNK activity or activation was no longer detectable. AKT activity did not change with PMA stimulation in either wild-type or cav-1 expressers, but basal activity was 172% more in cav-1 expressers than wild-type. In the 1205Lu cell line, PMA stimulation caused little additional PKC phosphorylation over 2 hr. Peak phosphorylation of ERK occurred at 5 min, and peak phosphorylation of JNK occurred at 30 min. In the 1205Lu cav-1 expressers, PKC activity remained constant; but overall PKC activity was 42% less than wild-type. Peak phosphorylation of ERK occurred at 60 min and JNK activity or activation was also no longer detectable. AKT activity did not exhibit an activation curve in either wild-type or cav-1 expressers, but basal activity was 167% more in cav-1 expressers than wild-type. The decreased PKC activity with cav-1 expression is consistent with cav-1 being an anti-metastatic protein. Peak phosphorylation of ERK and JNK follows peak phosphorylation of PKC-alpha suggesting that their increased activation is in part related to PKC activation. We conclude that cav-1 expression decreases PKC protein activity, and the activation of putative pro-metastatic downstream proteins. PKC inhibition is one probable mechanism by which cav-1 expression induces anti-metastatic behavior in melanoma.

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Reconstitution of PSD-95 Scaffolding Activity Suggests Anomalous Diffusion at the Membrane.U. B. Choi¹, M. E. Bowen¹; ¹Physiology & Biophysics, Stony Brook University, Stony Brook, NY

Scaffold proteins organize signal transduction by binding multiple partners within a signaling pathway. This shapes the output of signal responses as well as providing specificity and localization. The Membrane Associated Guanylate Kinases (MAGUKs) are scaffold proteins at intracellular junctions where they localize cell surface receptors and link them to downstream signaling enzymes. Much of what we know about MAGUK scaffold protein function has been inferred from the binding sites discernible in their primary sequence. Distinguishing physiological binding partners has proved challenging. Observational studies of the MAGUK PSD-95 have identified more than 50 different ligands for a protein that only contains five binding sites. Understanding scaffolding is complicated by the molecular diversity of different protein isoforms functioning together in vivo, which stymies knock out approaches. Reconstitution provides a

simplified system where inferred knowledge can be put to the test. We have achieved the first in vitro reconstitution of multi-protein scaffolding using purified components. We have recapitulated the native geometry of cellular junctions by using single molecule approaches to watch protein interactions at a membrane surface with video microscopy. As expected from theory, scaffolding activity is highly dependent on the scaffold concentration. We found that the scaffold becomes self-competitive at high concentration. Although pairwise interactions with the scaffold are weak and transient, a soluble scaffold is able to raise the effective concentration of other soluble proteins near the membrane surface. Our results suggest that scaffolds may not function by creating stable multi-protein complexes as expected. Instead, scaffolding may simply increase the probability of two proteins being in close proximity without them interacting directly. The scaffold presents a kinetic trap that hampers free diffusion away from the target receptor. This may be sufficient to achieve proper localization without locking any individual components in place.

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Reconstruction of MAPK signaling cascades using PDZ-based synthetic scaffold proteins.

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Scaffold proteins are thought to play a critical role in flux control of signaling pathways by tethering signaling proteins into active complexes. However, the mechanism by which scaffold proteins trigger designated signaling responses is still unclear. As a bottom-up approach, we designed synthetic scaffolds to replace Ste5 scaffold for rewiring of the mating MAPK pathway in the budding yeast, *Saccharomyces cerevisiae*. The synthetic scaffolds based on PDZ domains with binding affinities for Ste11 (MAPKKK), Ste7 (MAPKK), Fus3 (MAPK), and plasma membrane led to the assembly of kinase complexes at plasma membrane, which is sufficient to trigger the mating MAPK responses. We also found that signaling plasticity is still manifested at the upper tier of MAPK pathway and the signaling specificity is determined in part by scaffold assembly. Modular characteristics of Ste5 and the synthetic scaffold complexes, and the plasticity of mating signaling suggest that signaling pathways might have evolved via recombination of simple yet modular protein domains. Artificial rewiring of signaling pathways using synthetic scaffolds allows for engineering of cell signaling pathways with noble physiological properties.

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Fas-associated factor 1 activates c-Jun N-terminal kinase to promote oxidative stress-induced necrotic cell death.

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Oxidative stress-induced necrotic cell death is implicated in the pathogenesis of various diseases. However, its signaling pathway is yet to be clearly defined. In this study, we investigated FAF1's role in the oxidative stress-induced necrotic cell death. FAF1-deficient mouse embryonic fibroblast (MEF) cells were resistant to necrotic cell death elicited by H₂O₂. Moreover, restoration of FAF1 in FAF1-deficient MEF cells recovered their sensitivity to H₂O₂.

These data indicate that FAF1 is essential for oxidative-stress induced necrotic cell death. FAF1 activated JNK, a key regulator of oxidative stress-induced necrosis, via physical interaction with upstream regulators of JNK signaling, RIP1 and TRAF2, upon oxidative stress. Furthermore, depletion of FAF1 markedly suppressed sustained PARP-1 activation, a downstream phenomenon of JNK activation. Finally, we examined severity of pancreatic tissue damage in the mouse acute pancreatitis model. Pancreatic tissue damage was significantly attenuated in FAF1-deficient mice when compared with that of the wild type mice. Taken together, our data implicate FAF1 as a key mediator of oxidative stress-induced necrosis. [This research was supported by the grant (KDDF-201202-10) from the Korea Drug Development Fund, and by the grant (2011-0031223) from the National Research Foundation at the Ministry of Education, Science and Technology in Korea.]

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Role of monocytic thrombomodulin in inflammation.

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Objective: This study aims at investigating whether monocytic thrombomodulin (TM) mediates lipopolysaccharide (LPS)-induced signaling pathways and inflammatory response.

Methods: Biotinylated LPS pull-down assay was used for investigating the binding of LPS and monocytic TM. LPS-induced inflammatory response was analyzed in TM-knockdown monocytic cells. Coimmunoprecipitation and immunofluorescence assays were used for evaluating the interaction between TM and CD14/Toll-like receptor 4 (TLR4)/myeloid differentiation factor-2 (MD-2) complex in primary macrophages. We also established the myeloid-specific TM-deficient mice for investigating the role of TM in experimental sepsis models.

Results: TM knockdown in human monocytic cells attenuated LPS-induced signaling pathways and cytokine production. Coimmunoprecipitation and immunofluorescence assays showed that monocytic TM interacted with the LPS receptors, CD14 and TLR4/MD-2 complex, indicating that it binds to LPS and triggers LPS-induced inflammatory response by interacting with the CD14/TLR4/MD-2 complex. The myeloid-specific TM-deficient mice displayed improved survival which resulted from the attenuation of septic syndrome, including reduced systemic inflammatory response and less bacterial dissemination after *Klebsiella pneumoniae* infection or cecal ligation and puncture surgery.

Conclusion: We conclude that monocytic TM is a novel component in the CD14/TLR4/MD-2 complex and participates in the LPS-induced signaling pathways and inflammatory response.

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Identification of IF4γ2 as a Novel Contributor to Anthrax Toxin Entry into Cells.

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Anthrax toxin endocytosis is mediated by two receptors, tumor endothelial marker 8 (ANTRX1/TEM8) and capillary morphogenesis gene 2 protein (ANTRX2/CMG2). The receptor-binding component of the toxin is protective antigen (PA), and the toxic components are edema factor (EF, an adenylyl cyclase) and lethal factor (LF, a metalloprotease). To harm cells, EF raises cAMP to abnormal levels, and LF cleaves certain mitogen-activated protein kinase kinases. PA has the central role of delivering EF and LF to cytosol, and is therefore pivotal in anthrax toxin intoxication. Here we report identification of initiation factor 4 gamma 2 (IF4γ2) as

an accessory player in anthrax toxin entry. We identified IF4 γ 2 by the yeast two-hybrid system as a candidate protein that may interact with the cytoplasmic domain of CMG2. The relevance of IF4 γ 2 to anthrax toxin entry was assessed by cytotoxicity assays following siRNA-mediated down-regulation of IF4 γ 2. These studies revealed that reduced IF4 γ 2 levels result in a degree of resistance to the toxin. Further, Western blotting with anti-PA antibody revealed that cells expressing less IF4 γ 2 show reduced PA binding. Binding studies with fluorescently labeled PA gave similar results. These studies show that IF4 γ 2 is a player in anthrax toxin entry into cells, and that it influences toxin endocytosis at the level of PA binding to receptors.

Signaling Receptors (RTKs and GPCRs) I

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Normal CFTR inhibits a pro-inflammatory IL-1R-TACE-EGFR pathway in airway epithelial cells.

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Objective: Cystic fibrosis (CF) airway cells lack normal CF transmembrane conductance regulator (CFTR) protein and produce increased amounts of the potent neutrophil chemokine interleukin (IL)-8. Multiple stimuli have been shown to induce IL-8 production via metalloprotease TACE-dependent activation of an epidermal growth factor receptor (EGFR) signaling pathway. Here we propose the novel hypothesis that lack of normal CFTR increases constitutive IL-8 production via a TACE-EGFR pathway. Methods/Results: We examined this hypothesis in airway epithelial (NCI-H292, NHBE) cells containing normal CFTR treated with the CFTR-selective inhibitor CFTR-172, and in airway cells containing mutant CFTR (IB3 cells) versus matched cells containing wild-type CFTR (C38 cells). CFTR-172 induced IL-8 production markedly in the NCI-H292 and NHBE cells. Pretreatment with the EGFR inhibitor AG1478, an EGFR neutralizing antibody, or with the TACE inhibitor TAPI-1 suppressed CFTR-172-induced IL-8 production completely in both cell types. In the NCI-H292 cells, CFTR-172 induced two phases of EGFR phosphorylation (EGFR-P) that involved TACE: the first EGFR-P led to the production, release and subsequent binding of IL-1 α to IL-1R on the cell surface, stimulating the second EGFR-P and downstream IL-8 production. Ligand binding to IL-1R also contributed to CFTR-172-induced IL-8 production in the NHBE cells, indicating that IL-1R leads to CFTR-172-induced IL-8 production in multiple cell types. In CF (IB3) cells, IL-8 production and steady state EGFR-P levels were greater than in wild-type CFTR (C38) cells. Pretreatment with AG1478, an EGFR neutralizing antibody, or with TAPI-1 decreased IL-8 and EGFR-P levels in both the IB3 and C38 cells, but IL-1R blockade decreased these responses only in the IB3 cells, indicating that IL-1R activates TACE-dependent EGFR-P and IL-8 production in IB3 cells but not in C38 cells. Conclusions: The lack of normal CFTR increases constitutive IL-8 production in airway cells via IL-1R-dependent activation of a TACE-EGFR signaling pathway. These findings suggest novel targets for the treatment of airway neutrophilic inflammation in diseases characterized by decreased or abnormal CFTR expression.

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Conformational Coupling Across the Plasma Membrane in Activation of the EGF receptor.

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How the epidermal growth factor receptor (EGFR) activates is incompletely understood. The intracellular portion of the receptor is intrinsically active in solution, and to study its regulation we measured autophosphorylation as a function of EGFR surface density in cells. EGFR escapes inhibition only at high surface densities, but deletion of the extracellular module shows that the transmembrane helix and the intracellular module suffice for constitutive activity at low densities. The intracellular module is inactivated when tethered on its own to the plasma membrane and fails to dimerize, as determined by fluorescence cross-correlation spectroscopy. NMR and functional data indicate that activation requires an N-terminal interaction between the transmembrane helices, which promotes an antiparallel interaction between juxtamembrane segments and release of inhibition by the membrane. We conclude that EGF binding removes steric constraints in the extracellular module, allowing activation through N-terminal association of the transmembrane helices.

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Intracellular sodium fine-tunes EGFR trafficking by a HDAC6 and tubulin acetylation-mediated feedback loop.

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Epidermal growth factor (EGF) regulates proliferation and/or migration in diverse cells by triggering molecular cascades including cytoskeletal reorganization, cell cycle progression and gene expression. It is well known that endocytosis of activated EGF receptor (EGFR) and its subsequent delivery to specific endocytic compartments through a microtubule-dependent process are required to terminate EGF signaling. Here we show that intracellular trafficking of EGFR vesicles is regulated by the acetylation status of microtubules and that EGF-induced sodium influx modulates this process. EGF treatment of DAOY cells induced tubulin acetylation which was significantly delayed under low-sodium conditions. Similarly, increased sodium influx induced by ionophores or by blocking the Na,K-ATPase activity resulted in increased tubulin acetylation. Blocking sodium influx by low-sodium conditions not only altered tubulin acetylation but also the EGF-induced turnover of EGFR and the trafficking of EGF-positive vesicles suggesting that intracellular sodium might be involved in modulating EGFR signaling. Moreover, increased intracellular sodium reduced the activity of HDAC6, the predominant HDAC family member that regulates tubulin acetylation. Knockdown of HDAC6 reversed the effects of sodium replacement in EGF-treated cells indicating that HDAC6 inactivation by sodium influx may be involved in modulating tubulin acetylation and EGFR trafficking. Together our data suggest a novel pathway by which EGF modulates EGFR trafficking through sodium influx/HDAC6 inactivation/tubulin acetylation.

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Cell shape can dynamically process information during signal flow in regulatory pathways.

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Shape is often an indicator of cell health. The role of cell shape in signaling is not well understood. We determined if cell shape could process information during signal transduction at the plasma membrane. Using analytical approaches and numerical simulations we studied elliptical shapes since neoplastic transformation often results in cells that are spindle shaped. Mathematical analyses showed that with increasing eccentricity of the cell, receptors diffusing evenly in the plane of the membrane accumulate transiently at regions of high curvature upon binding ligand. This inhomogeneous distribution of activated receptors is periodic and follows the Mathieu function. This transient inhomogeneity arises from local balance between reaction and diffusion of the soluble ligand and membrane-bound diffusion of the receptor. Numerical simulations for the receptor pathways show that these transient microdomains of activated receptors in the membrane amplify signals to downstream protein kinases. For the growth factor receptor pathway, change in cell shape from circle to ellipse results in a nearly two-fold increase in activated MAP-kinase in the nucleus.

The model predictions were tested experimentally using patterned cells. Experimental measurements of receptor density and diffusivity show that the EGF receptor activation and diffusion is dependent on the local curvature. Additionally, elliptical cells show a transient spatial inhomogeneity in the activation of signaling components when compared to circular cells in vitro.

Thus, cell shape and growth factor signaling can form a multi-scale positive feedback loop that could contribute to the maintenance of the transformed state.

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Regulation of Na,K-ATPase β -subunit in TGF- β_2 -mediated epithelial-to-mesenchymal transition in human retinal pigmented epithelial cells.

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Proliferative vitreo retinopathy (PVR) is associated with extracellular matrix membrane (ECM) formation on the neural retina and disruption of the multilayered retinal architecture leading to distorted vision and blindness. During disease progression in PVR retinal pigmented epithelial cells (RPE) lose cell-cell adhesion, undergo epithelial-to-mesenchymal transition (EMT), and start to deposit ECM leading to tissue fibrosis. The EMT process is mediated via exposure to vitreous cytokines and growth factors such as TGF- β_2 . Previous studies have shown that Na,K-ATPase is required for maintaining a normal polarized epithelial phenotype and that decreased Na,K-ATPase function and subunit levels are associated with TGF- β_1 -mediated EMT in kidney cells. In contrast to the basolateral localization of Na,K-ATPase in most epithelia, including kidney, Na,K-ATPase is found on the apical membrane in RPE cells. We now show that in RPE cells EMT is as well associated with altered Na,K-ATPase expression. TGF- β_2 treatment of ARPE-19 cells resulted in a time-dependent decrease in Na,K-ATPase β_1 levels while Na,K-ATPase α_1 levels, Na,K-ATPase activity, and intracellular sodium levels largely remained unchanged. Knockdown of Na,K-ATPase β_1 was associated with a change in cell morphology

from epithelial to mesenchymal and induction of EMT markers such as α -SMA and fibronectin, suggesting that Na,K-ATPase β_1 may be a potential contributor to TGF- β_2 -mediated EMT in RPE cells. Na,K-ATPase β_1 subunit was down regulated at the RNA level during TGF- β_2 signaling and this effect was independent of its known transcriptional regulator, Snail. Analysis of the 1141bp Na,K-ATPase β_1 promoter revealed a putative hypoxia response element (HRE). HIF-1 α levels were upregulated by TGF- β_2 even under normoxic conditions. HIF-1 α bound to the Na,K-ATPase β_1 promoter and blocking HIF-1 α binding to HRE blocked the TGF- β_2 mediated Na,K-ATPase β_1 decrease suggesting that HIF-1 α may play a potential role in Na,K-ATPase β_1 regulation during EMT in RPE cells.

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The physical basis behind achondroplasia, the most common form of human dwarfism.

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Fibroblast growth factor receptor 3 (FGFR3) is a receptor tyrosine kinase that plays an important role in long bone development. The G380R mutation in FGFR3 transmembrane domain is known as the genetic cause for achondroplasia (ACH), the most common form of human dwarfism. Despite many studies, there is no consensus about the exact mechanism underlying the pathology. To gain further understanding into the physical basis behind the disorder, we use quantitative imaging FRET to characterize the dimerization of wild-type FGFR3 and the ACH mutant in plasma membrane-derived vesicles. We demonstrated a small, but statistically significant increase in FGFR3 dimerization due to the ACH mutation. We also measured the activation of wild type and mutant FGFR3 in mammalian cells using Western blots, and analyzed the activation within the frame of a physical-chemical model describing dimerization, ligand binding and phosphorylation probabilities within the dimers. The data analysis demonstrated that FGFR3 activity in achondroplasia is increased due to increased dimerization and increased probability for phosphorylation of the unliganded mutant dimers. As achondroplasia is a heterozygous disorder, we also investigated the formation of FGFR3 heterodimers in cellular membranes. We accomplished that by designing an FGFR3 construct that lacks the kinase domain, and we monitored the formation of inactive heterodimers between this construct and wild-type and mutant FGFR3. The formation of the inactive heterodimers depleted the pool of full-length receptors capable of forming active homodimers and ultimately reduced their phosphorylation. By analyzing the effect of the truncated FGFR3 on full-length receptor phosphorylation, we demonstrated that FGFR3 WT/G380R heterodimers form with lower probability than wild-type FGFR3 homodimers at low ligand concentration. These results further our knowledge of FGFR3-associated bone disorders.

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Spatial Organization of EphA2 and its effects on internalization and signaling in living cells.

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Our research focuses on the spatial reorganization of receptors and ligands that occurs at the cell-cell interface during juxtacrine signaling. We have developed a unique experimental

platform in which live cells are interfaced with supported lipid bilayers to create a simplified and controllable system that recapitulates essential aspects of the cell-cell contact. To probe how protein organization in the membrane influences signaling in the cell, we pattern supported lipid bilayers into microscale corrals that restrict membrane protein oligomerization to microclusters and then measure changes in cell signaling. For instance, when MDAMB231 breast cancer epithelial cells expressing the receptor tyrosine kinase EphA2 are placed on a supported lipid bilayer containing the EphA2 ligand, ephrinA1, the receptors bind the ligands and the complex reorganizes into large clusters. Applying spatial perturbations to disrupt the reorganization of EphA2-ephrinA1 clusters results in alterations of downstream signaling events. In particular, we found that recruitment of the metalloprotease ADAM10 to the clusters is inhibited, indicating that EphA2 signaling is sensitive to perturbations in the spatial organization of the ligand. ADAM10 has been implicated in the cleavage and subsequent endocytosis of other ephrin ligands, suggesting that ADAM10 may be necessary for the efficient endocytosis of EphA2-ephrinA1. Therefore, our research is currently probing the role of endocytosis in context of how EphA2 is spatiomechanically sensitive. Here, we report that the spatial organization of EphA2-ephrinA1 does alter the internalization of the receptor and ligand. We also found that Pitstop2, a small molecular inhibitor of clathrin-mediated endocytosis (CME), negatively effects ephrinA1 endocytosis, indicating that CME is important in EphA2-ephrinA1 internalization. The implications of these observations for spatial regulation of EphA2 signaling will be further discussed.

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Soluble MerTK release helps regulating POS phagocytosis by Retinal Pigment Epithelial cells.

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In the eye, cells from the retinal pigment epithelium (RPE) exert a daily rhythmic phagocytic function that is essential for retinal health and vision by photoreceptor cells. Mer tyrosine kinase (MerTK) receptors at the apical surface of RPE cells are activated at the time of the morning phagocytic peak and required to internalize bound photoreceptor outer segments (POS). When alphavbeta5 integrin receptors and their ligands MFG-E8 are absent, retinal phagocytosis loses its rhythm but is still present, thus implying that MerTK can be activated independently from this pathway. We previously showed that a soluble form of MerTK (sMerTK) is released in the conditioned media (CM) of mouse J774 macrophages and rat RPE-J cells during phagocytosis. Moreover, in vivo sMerTK levels slightly fluctuate along the light:dark cycle peaking just before and after phagocytosis in the interphotoreceptor matrix (IPM). In addition, MerTK ligands Gas6 and Protein S are both present in the IPM but their in vivo role is still debated. Therefore, we investigated the influence of known ligands on RPE phagocytosis and examined if sMerTK could downregulate POS uptake by RPE cells in vitro. We challenged J774 and RPE-J cells with POS in serum-free medium with various doses of either ligand alone or in combination. We then analyzed sMerTK protein levels present in the CM of RPE cells using immunoblots and quantified the corresponding phagocytic activity using FITC-labeled POS. All MFG-E8 doses increased phagocytosis and amplified MerTK cleavage. Protein S had a similar effect in a dose-dependant manner albeit to a lower extent. In contrast, increasing doses of Gas6 decreased phagocytosis and exerted an antagonist effect even when used in combination. Interestingly, these effects seem RPE-specific as J774 macrophages sharing the same phagocytic machinery responded moderately and equally to all ligand/s. Next, we tested if sMerTK could impede POS phagocytosis using 2 approaches. First, POS resuspended in CM did not change RPE cells phagocytic intake. Then, we stimulated sMerTK release from the cell surface by preincubating

cells with POS or LPS, shown to stimulate sMerTK release in macrophages. In these conditions, both POS binding and uptake were decreased, the effect being more marked when protein neosynthesis was blocked using cycloheximide. Last, RPE phagocytic activity slightly increased when MerTK cleavage was blocked using an inhibitor specific for the potential MerTK protease Adam17. Taken together, these data strengthen our hypothesis that MerTK is implicated in controlling RPE circadian phagocytic activity aside from its internalization role.

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5-lipoxygenase plays a central role in TLR4-mediated monocyte adhesion to endothelial cells through an increased expression of Mac-1 on monocytes.

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Recent evidence has demonstrated an important role of toll-like receptors (TLRs) in atherogenesis. In particular, TLR4 has been shown to participate in the pathogenesis of atherosclerosis by the increased formation of macrophage foam cells as well as by monocyte activation. However, the role of this pathway in monocyte adhesion to vascular endothelial cells, an early event in macrophage foam cell formation, is still unclear. To investigate the underlying mechanisms involved in the TLR-mediated adhesion of monocytes to endothelial cells, we determined the expression of adhesion molecules on monocytes stimulated with the TLR4 agonist, lipopolysaccharide (LPS). Among the various adhesion molecules on monocytes, the Mac-1 expression were exclusively increased, which subsequently led to the increased adhesion of monocytes to endothelial cells. Moreover, the role of Mac-1 in LPS-induced monocyte adhesion to endothelial cells was confirmed by blocking antibody with Mac-1. Furthermore, The LPS-induced expression of Mac-1 and monocyte adhesion were attenuated by the inhibition of 5-lipoxygenase (5-LO) pathways with MK886, a 5-LO inhibitor, and U-75302, a LTB4 receptor antagonist as well as in monocytes from 5-LO^(-/-) mice. Moreover, an en face immunohistochemistry of endothelial surface revealed a marked increase in monocyte adhesion to the aortic endothelium in LPS-treated mice. This was also significantly attenuated in 5-LO^(-/-) mice treated with LPS, confirming a role of 5-LO in monocyte adhesion to endothelial cells induced by TLR4 activation in in vivo study. These results suggest a potential role of 5-LO-derived leukotrienes in TLR4-mediated monocyte adhesion to endothelial cells through Mac-1 expression. Collectly, these data suggest that 5-LO plays a central role in TLR4-mediated monocyte adhesion to endothelial cells through the increased expression of Mac-1 on monocytes, thus contributing to the initiational process of atherosclerosis.

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Oxysterols are allosteric activators of the oncoprotein Smoothed.

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Oxysterols are a class of endogenous signaling molecules that can activate the Hedgehog pathway, which has critical roles in development, regeneration and cancer. Though the majority of small molecule Hedgehog modulators identified to date appear to bind to overlapping sites on the seven-pass transmembrane protein Smoothed, oxysterols do not show this behavior. A complicating factor in identifying the mechanism by which oxysterols activate Hedgehog signaling is that as amphipathic molecules, they can transduce cellular signals directly through a

protein target or indirectly through effects on membrane properties. To answer this question, we took a chemical approach and synthesized the enantiomer and an epimer of the most potent oxysterol, 20(S)-hydroxycholesterol. Using these molecules, we show that the effects of oxysterols on Hedgehog signaling are exquisitely stereoselective, consistent with the hypothesis that they function through a specific protein target. Pharmacological analysis and affinity chromatography with a novel oxysterol analog suggest that these molecules target Smoothed at a site distinct from the canonical site that binds inhibitors such as cyclopamine and agonists such as SAG. Our work suggests that Smo may be susceptible to regulation by small molecules at multiple binding sites and provides a generally applicable framework for probing sterol signaling mechanisms.

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Visualizing the efficacy of GPCR signaling in live cells.

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G-protein coupled receptors (GPCRs) bind a variety of ligands to initiate a diverse array of cellular responses. Several recent structural and biophysical studies have shown that the same GPCR can adopt distinct structural conformations in response to different ligands. However, these structural conformations exhibit subtle differences that do not explain the different signaling efficacies of distinct ligands acting on the same GPCR. In this report, GPCR efficacy in live cells was captured using a new genetically encoded technique termed systematic protein affinity strength modulation (SPASM). SPASM involves the expression of a single polypeptide with two proteins or protein domains and a FRET pair separated by a modular ER/K linker. Varying the length of the ER/K linker systematically controls the effective concentration of the interaction. SPASM allows for expression of GPCR and G α (domains) in equal amounts and at an engineered effective concentration. We report on the application of SPASM to two prototypical GPCRs, opsin and β 2-adrenergic receptor (β 2-AR). SPASM was used to measure the interaction between each GPCR and either full length G α or a peptide derived from the G α c-terminus (α 5 helix). Active conformations of both GPCRs, that are known to initiate downstream signaling, showed an enhanced affinity for the c-terminus of the primary G α (Gat – opsin; Gas – β 2-AR). Activity state was altered using agonist (β 2-AR – isoproterenol (ISO); opsin – maturation with 9-cis-retinal), mutagenesis (opsin – K296A and E113Q; β 2-AR – D130N and CAM) or inverse agonist (β 2-AR – metoprolol or ICI118,551 (ICI)). The gain in affinity of the active GPCR for the primary G α c-terminus was directly reflected in measurements with G α chimeras (Gas/t and Gat/s). Sensor readout was a direct measure of the efficacy of the ligand in precipitating a known downstream cellular response. Specifically, live-cell ISO dose stimulation curves of the β 2-AR- α 5 sensors showed a characteristic sigmoidal FRET response with an EC50 consistent with the low affinity state of β 2-AR in the ternary complex model. Competition of ISO with ICI showed sigmoidal inhibition of FRET with EC50 consistent with ICI binding affinity. Sensors that probe the GPCR interaction with the G α c-terminus reported ligand efficacy more consistently than those with full-length G α . As an independent measurement of GPCR activation, FRET measurements directly correlated with rate of second messenger (cAMP) accumulation. Further, varying ER/K linker length systematically controlled the rate of cAMP accumulation without the need for site-directed-mutagenesis of either GPCR or G α . Taken together, our studies strongly support the use of SPASM to examine signaling efficacy and selectivity of any GPCR.

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Dynamic monomer-dimer equilibrium as a general property of the class A GPCR: detection by single-molecule imaging.

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Class-A G-protein-coupled receptor (GPCR) includes about 85% of GPCR or ~700 different GPCRs. Previously, by developing new methods based on single-molecule imaging, we succeeded in fully characterizing the two-dimensional (2D) dimer-monomer equilibrium of a class-A GPCR, formyl peptide receptor (FPR) (Kasai et al. JCB 2011). This was the first determination ever of the dissociation equilibrium of any molecules in the membrane. Here, we applied this method to another prototypical class-A GPCR, beta2-adrenergic receptor (B-AR), and found that B-AR also undergoes similar dynamic monomer-dimer equilibrium, with a 2D monomer-dimer equilibrium constant of 1.6 (3.6) copies/square micron, a dimer dissociation rate constant of 12.6 (11.0) /s [dimer lifetime of 80 (90) ms], and a dimer formation rate constant of 7.9 (3.1) /[copies/square microns]/s (FPR values in parentheses). Physiological expression levels of B-AR (FPR) have been reported to be 16 -260 (2.1) copies/square micron [46,000 - 740,000 (6,000) copies/cell]. Using the determined equilibrium constant, it is expected that, at any moment, 75 -92% of B-AR would exist as dimers, whereas ~42% of FPR would. However, it is critically important to realize that these are transient dimers of 80-90-ms lifetimes, and the difference comes from their monomer lifetimes due to different expression levels, i.e., 0.49 -8 ms for B-AR and 154 ms for FPR. These results suggest that class A GPCRs are under comparable dynamic monomer-dimer equilibrium and this might play a role in signal transduction and/or its regulation. Previously, Hern et al. (PNAS, 2010) reported rapid dimer dissociation of M1 muscarinic receptor, another class-A GPCR. Taken together, these results suggest that class-A GPCRs generally undergo dynamic equilibrium between monomers and homo-dimers. Because no homology exists between different GPCRs, this infers that each GPCR is individually evolved to be capable of forming transient dimers, suggesting the dynamic interconversion between monomers and dimers may be critical for the function of class-A GPCRs.

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Global GPCR dimerization screening using AdBiFC assay system in human cells.

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G protein-coupled receptors (GPCRs) are the largest class of cell surface receptors that mediate numerous physiological responses to various extracellular stimuli. GPCRs are believed to exist as monomers, but emerging evidences suggest that GPCRs may form dimers (or higher-order oligomers) leading to an alteration of their signals. Here, we developed an adenovirus-based bimolecular fluorescence complementation (AdBiFC) assay system, combining a gateway-compatible high-throughput technology and an adenovirus-mediated gene delivery system, to visualize protein-protein interactions in living human cells. To identify and profile the GPCR dimerization, we generated recombinant adenovirus libraries in which 164 of individual GPCR are C-terminally tagged with either the N- or the C-terminal fragment of a yellow fluorescent protein variant Venus, and performed a large-scale BiFC assay using a high-content screening analyzer. From this binary interaction assay, we identified over 1,200 GPCR dimeric combinations including 26 homodimerizations. Furthermore, we developed a β -arrestin

recruitment assay using AdBiFC technique to monitor GPCR activation, which can be readily adapted to potential drug screening in living cells. Overall, our AdBiFC assay system provides a platform for analyzing global GPCR interactions and may prove highly useful for studying the pathophysiology of GPCR dimerizations.

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A single Lys residue on the first intracellular loop modulates the ER export and cell-surface expression of α 2A-adrenergic receptor.

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Export from the ER represents an initial step in intracellular trafficking of G protein-coupled receptors (GPCRs). However, the underlying molecular mechanisms remain poorly understood. We have previously demonstrated that a highly conserved Leu residue on the first intracellular loop (ICL1) is required for exit of several GPCRs from the ER. Here we found that, in addition to Leu64 residue in the ICL1, the neighboring positively charged residue Lys65 also modulates the cell-surface transport of α 2A-adrenergic receptor (α 2A-AR). Mutation of Lys65 to Ala, Glu and Gln significantly attenuated, whereas mutation of Lys65 to Arg strongly augmented α 2A-AR expression at the cell surface. Consistent with the effects on the cell-surface expression of α 2A-AR, mutation of Lys65 to Ala and Arg produced opposing effects on α 2A-AR-mediated ERK1/2 activation. Furthermore, confocal microscopy revealed that the α 2A-AR mutant K65A displayed a strong intracellular expression pattern and was extensively co-localized with the ER marker DsRed2-ER, suggestive of ER accumulation. These data provide the first evidence indicating an important function for a single Lys residue on the ICL1 in the ER export and cell-surface expression of α 2A-AR. These data also suggest that the ICL1 may possess multiple signals that control the cell-surface targeting of GPCRs via distinct mechanisms.

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Identification of Ubiquitin-binding domain containing proteins that regulate trafficking and signaling of the yeast G-protein Gpa1.

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G-proteins and their associated receptors form the largest class of proteins that receive and transduce chemical and sensory signals. Regulation of G-proteins therefore, is critical to appropriate cellular responses. G Proteins are comprised of three subunits- α , β and γ . The yeast $G\alpha$ subunit, Gpa1 is subject to both poly and mono-ubiquitination. Each modification is carried out by different ubiquitinating enzymes and has different consequences for G-protein activity. While polyubiquitination is a signal for Gpa1 degradation, monoubiquitination results in Gpa1 endocytosis and delivery to the vacuole.

However, the specific ubiquitin binding domain (UBD) containing protein machinery that targets monoubiquitinated Gpa1 and mediates its endocytosis and trafficking is not known. We conducted a microscopy-based screen of 40 UBD gene deletion mutants to identify those in which Gpa1 trafficking to the vacuole was impaired. We also performed a parallel screen to identify UBD gene deletion mutants that disrupt endocytosis and trafficking of the monoubiquitinated receptor. Comparing the results from the two screens, we identified four UBD proteins that are needed specifically for proper trafficking and endocytosis of the Gprotein but not of the receptor. These four UBD's had no effect on trafficking of a Gpa1 mutant that cannot be ubiquitinated. Our analysis reveals novel G protein-binding proteins and, more broadly, the functional consequences of G protein monoubiquitination and endocytosis.

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sFRP1 Potentiation vs. Inhibition of Wnt3a/[beta]-catenin Signaling: Mechanistic and Structure-Function Analysis.

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Wnt signaling regulates a variety of cellular processes including proliferation, motility and polarity. Many of these activities are mediated by the Frizzled (Fzd) family of seven-pass transmembrane receptors, which bind Wnts via a conserved cyteine-rich domain (CRD). Secreted Fzd-related proteins (sFRPs) contain an amino-terminal, Fzd-like CRD and a carboxyl-terminal, heparin-binding netrin-like domain (NTR). Previous studies identified sFRPs as soluble Wnt antagonists that bind directly to Wnts and prevent their interaction with Fzds. However, subsequent observations suggested that sFRPs and Fzds can form homodimers and heterodimers via their respective CRDs, and that sFRPs can stimulate signal transduction. Here, we present evidence that sFRP1 either inhibits or potentiates the Wnt3a/[beta]-catenin pathway, depending on its concentration and the cellular context. sFRP1 has biphasic activity in a SuperTopFlash (STF) reporter assay in HEK293/STF cells. The potentiating effect in HEK293/STF cells was mimicked by CRDsFRP-1, while CRDsFRP-1 did not inhibit Wnt3a activity even at micromolar concentrations. In contrast, sFRP1 only inhibited Wnt3a/[beta]-catenin signaling in L929 fibroblasts. Interestingly, when L929 cells were engineered to stably express Fzd5 but not Fzd2 or Fzd4, sFRP1 enhanced Wnt3a/[beta]-catenin signaling at nanomolar concentrations. Future experiments will explore the molecular mechanisms responsible for potentiation vs. inhibition of Wnt/[beta]-catenin signaling by sFRP1.

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Mapping spatial approximations between residues contributing to the helix N-capping motif of secretin and distinct residues within each of the extracellular loops of its receptor using cysteine trapping.

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Secretin family peptides contain key determinants for biological activity and receptor specificity in their amino-terminal regions, however current understanding of how this region interacts with its receptor is unclear. Within the peptide amino terminus is a helix N-capping motif that has been postulated to contribute directly to its biological activity, while also stabilizing the carboxyl-terminal helical region of the peptide that docks within the receptor amino-terminal domain. This helix-helix interaction is poised to direct the peptide amino terminus toward the receptor core. In secretin, the N-capping motif includes residues Phe⁶, Thr⁷ and Leu¹⁰. The current work was designed to use the powerful cysteine trapping approach to systematically explore spatial approximations for each of these residues with those of the secretin receptor. Probes were developed in which cysteines were incorporated into each of these positions within secretin. Each of these analogues was a full agonist for cAMP, although it bound with lower affinity than the natural hormone. For the targets, secretin receptor mutants were prepared in which natural residues in each of 61 positions throughout the tops of transmembrane segments and extracellular loop (ECL) regions were also replaced with cysteines, and these were expressed in COS cells. After ligand binding under conditions permitting spontaneous formation of disulfide bonds, the patterns of covalent labeling were distinct for each of the three probes. The position 6 probe predominantly labeled multiple residues in the carboxyl-terminal half of ECL2 and amino-terminal half of ECL3, while the position 7 probe only labeled a single residue within the carboxyl-terminal region of ECL2 and another residue at the beginning of ECL3. The position 10 probe did not efficiently label any of the residues in any of these regions. Sites of disulfide formation were quite distinct for these N-capping motif residues than for analogous experiments

recently performed with position 2 and 5 probes. Utilizing this battery of spatial approximation data is helpful to orient the amino-terminal region of secretin toward its receptor and to provide new insights into the molecular basis for specificity and biological activity.

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Desensitization of Melanin-concentrating Hormone-Mediated ERK Signaling Despite Poor MCHR1 Internalization.

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Melanin-concentrating hormone (MCH) receptor 1 knockout mice have limited incidence of diet-induced obesity. This makes the MCH signaling pathway a potential pharmacological target to fight human obesity. MCHR1 is a G-protein coupled receptor (GPCR) that activates multiple signaling pathways, including ERK phosphorylation. Overstimulation of GPCR signaling is a hallmark of many diseases. Likewise, inadequate desensitization of MCH signaling could potentiate the obese phenotype. GPCR desensitization typically involves agonist-induced internalization of activated receptors, and subsequent degradation or receptor recycling. Our initial aim was to determine whether MCH signaling desensitizes. In order to measure this we maximally stimulated MCHR1-transfected BHK-570 cells with 100 nM MCH for 10 min, then following three washes in serum-free media and a 30 min recovery period, cells were stimulated again. Western blots of lysates for phosphorylated-ERK and total ERK were performed. ImageJ was used to normalize activation levels. MCH was unable to signal a second round of ERK signaling unless we waited 70 minutes, indicating that the MCH signaling pathway is desensitized during this period. We hypothesized that MCHR1 internalization was responsible, however when MCH was added to cells, no visible redistribution of MCHR1 was detectable using fluorescence microscopy. We tried a more sensitive assay, a cell-based ELISA, and only measured a 15% loss of surface MCHR1 after 30 min of MCH treatment. Live-cell experiments conducted with rhodamine-MCH and MCHR1-eYFP transfected cells support these conclusions. We tested the hypothesis that beta-arrestins and/or GRKs were limiting factors in preventing agonist-mediated endocytosis of MCHR1. Only overexpression of beta-arrestins-1 and -2 showed significant gains. We conclude that MCHR1 can undergo receptor-mediated endocytosis, but the fraction of available receptors on the plasma membrane does not account for the extensive loss of ERK signaling observed. This suggests that MCHR1 mediated ERK signaling desensitizes while MCHR1 is at the plasma membrane, rather than via removal of the receptor from the cell surface. Future experiments are aimed at determining whether this desensitization is homologous or heterologous.

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Melanin-concentrating Hormone Receptor 1 in CHO-K1, SH-SY5Y and 3T3-L1 Cells: A Pathway to Primary Cilia.

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Obesity results when caloric intake exceeds metabolic needs over an extended period of time. The condition predates heart disease and diabetes – two pathologies that diminish the quality of life and increase risk of premature death. Melanin-concentrating hormone (MCH) acts via a G protein-coupled receptor on the plasma membrane of neurons to stimulate appetite, and adipocytes to stimulate feedback secretion of leptin. Dysregulation of MCH signaling is hypothesized to be a contributing factor in select appetite disorders. Little is known about how cells regulate MCH receptor signaling however plasma membrane localization of MCHR1 has been implicated as a contributing factor. We previously reported that caveolae enhance MCH

signaling in CHO cells. This, together with recent reports of MCHR1 localization to primary cilia in hypothalamic neurons led us to hypothesize that organization of MCHR1 in the plasma membrane might be important to MCH function in other cell types as well. When VSVg-tagged MCHR1 is transiently expressed in CHO-K1 cells, receptor can be found on the plasma membrane, but occasionally localizes to two punctate dots near the nucleus, particularly after serum deprivation. We next studied two endogenously-expressing cell lines: human SH-SY5Y neuroblastoma cells and murine 3T3-L1 pre-adipocytes, which both responded to MCH by activating ERK. Using immunostaining, diffuse plasma membrane-localized MCHR2 was detected in SH-SY5Y cells. Although MCHR1 plasma membrane expression was confirmed in both SH-SY5Ys and 3T3-L1s, both cell types harbored distinctive MCHR1 patterning; SH-SY5Ys had clusters of MCHR1-positive vesicles and 3T3-L1 cells revealed MCHR1-positive primary cilia during differentiation. Together, these results suggest trafficking of MCHR1 to a centrosomal-location prepares receptor for entry into primary cilia, and that a role for primary cilia in the regulation of receptor signaling may be more widespread than originally thought. Future experiments will be aimed at determining the role that this localization plays in the regulation of MCH signaling.

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G Protein-coupled Receptor Kinase 2 Promotes CCR7 Internalization.

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C-C Chemokine Receptor 7 (CCR7) plays central roles in targeting T cells, breast tumors and activated dendritic cells to the lymph nodes during an immune response. Two ligands, CCL19 and CCL21 activate CCR7 and are thought to regulate this migration. To migrate, cells must sensitize/desensitize in response to differing ligand gradients. Others and we have shown that in human T cells, CCR7 rapidly desensitizes by internalizing 80% of the surface CCR7 in response to stimulation with CCL19, but not to CCL21. In addition, we have shown that activated CCR7 promotes redistribution of arrestin-3, a protein that binds to phosphorylated G protein-coupled receptors, to the nucleus. To determine the mechanism regulating the internalization and arrestin-3 redistribution we examined the roles of G protein-coupled receptor kinases human embryonic kidney (HEK-293T) cells. We found that comparing the four ubiquitously expressed GRKs, only GRK2 and GRK3 promoted differential internalization of CCR7 or redistribution of arrestin-3 to the nucleus. In addition, the kinase function of GRK2 mediated arrestin re-localization. Since GRK function is cell type dependent, we examined CCR7 internalization in primary wild-type (WT), GRK2^{+/-}, and GRK3^{-/-} T cells. WT cells internalized 36% of CCR7/CCL19 and 11% of CCR7/CCL21, while GRK2^{+/-} T cells internalized 57% of CCR7 in the presence of either CCL19 or CCL21. GRK3^{-/-} cells failed to internalize CCR7 in the presence of either ligand. To define a physiological significance of GRK regulation we characterized the effect of GRK2 and GRK3 on T cell migration. We found that WT murine T cells migrated to both CCR7 ligands, GRK2^{+/-} cells only migrated in the presence of CCL19, and GRK3^{-/-} cells failed to migrate to either ligand. Taken together, these results indicate CCL21- and CCL19-induced internalization of CCR7 and redistribution of arrestin-3 is dependent upon the expression of GRK2 and GRK3, and that T cell migration to CCR7/CCL21 or CCR7/CCL19 is dependent on GRK3.

Rho-Family GTPases

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Toward unraveling the structure and molecular basis of ferrous iron transport of feo from *klebsiella pneumonia*.

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Feo from *K. pneumoniae* is a unique type of ferrous iron transporter for bacteria. It consists of three proteins, FeoA, a small SH3-domain like protein; FeoB, a large protein consisting of a cytosolic N-terminal G-protein domain and a C-terminal transmembrane domain that may serve as a Fe²⁺ permease; and FeoC, a small putative transcriptional factor. The objective of the present study is to unravel the molecular basis of Fe²⁺ transport by Feo. Using X-ray crystallography and NMR we have solved the structure of *Kp*FeoA, the intracellular domain of *Kp*FeoB (*KpN*FeoB), *Kp*FeoC and *KpN*FeoB/*Kp*FeoC complex. In the structures, a canonical G-protein domain (G domain) is followed by a helical bundle domain (S-domain), which despite its lack of sequence similarity between species is structurally conserved. In the nucleotide-free state, the G domain's two switch regions point away from the binding site. This gives rise to an open binding pocket whose shallowness is likely to be responsible for the low nucleotide - binding affinity. Nucleotide binding induced significant conformational changes in the G5 motif which in the case of GMPPNP binding was accompanied by destabilization of the switch I region. In addition to the structural data, we demonstrate that Fe²⁺-induced foot printing cleaves the protein close to a putative Fe²⁺-binding site at the tip of switch I, and we identify functionally important regions within the S-domain. Moreover, we showed that *Kp*FeoC assumed a winged-helix structure and it forms a tight complex with *KpN*FeoB. The crystal structure of *KpN*FeoB/*Kp*FeoC complex suggests that *Kp*FeoC may function as a regulator, in addition to its proposed role as a transcriptional regulator.

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Signaling Events in Protection of Insulin-Secreting Cells from Glucotoxicity-Induced Apoptosis during Knockdown of p21-Activated Kinase-1.

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The major cause for various cardiovascular complications in diabetes is the chronic hyperglycemia which is called as glucotoxicity resulting in damage to many tissues including islet β -cells. We found that knockdown of p21-activated kinase-1 (PAK1), a downstream effector of the small G-protein Rac1, did not mediate the adverse effects of Rac1 suppression on cytoskeletal organization and glucose-stimulated insulin secretion in INS-1 β -cells. By contrast, PAK1 knockdown reduced β -cell demise during glucotoxicity. Exposure of INS-1 cells to high (20 or 30 mM) glucose for 2-3 days activated PAK1, while knockdown of PAK1 by siRNA transfection could protect INS-1 cells from glucotoxicity-induced apoptosis, as assessed by flow cytometry and activation of caspases. Glucotoxicity caused subdiploidy apoptosis by shifting INS-1 cells from G1 to sub-G1 phase due to DNA fragmentation and diminished cyclin D1 protein mass. Meanwhile, an increase of caspase-8 and -9 activities occurred, followed by

marked caspase-3 activation peaking at 48 h, as assessed by the cleavage of specific fluorogenic substrates. PAK1 knockdown significantly attenuated glucotoxicity-induced caspase activation and apoptosis. Chronic high glucose also activated p38 mitogen-activation protein kinase (MAPK) and JNK but not p44/42 MAPK. However, PAK1 knockdown only reversed p38 MAPK activation upon glucotoxicity. Additionally, chronic high glucose markedly increased thioredoxin-interacting protein (TXNIP) which might protect against glucotoxicity-induced β -cell apoptosis. However, PAK1 knockdown did not enhance, rather modestly reduced TXNIP (assessed by immunoblotting) upon glucotoxicity. Furthermore, inhibition of p38 MAPK by a specific inhibitor of 10 μ M SB203580 could protect INS-1 cells from glucotoxicity-induced apoptosis and reduce the activation of caspases in INS-1 cells exposed to high glucose for 2-3 days. SB203580 also slightly diminished glucotoxicity-induced increase in TXNIP, similar to the effect of PAK1 knockdown. Our studies have demonstrated that PAK1 may play an important role in glucotoxicity-induced apoptotic death of insulin-secreting cells. Such protective effect seems not to be mediated by TXNIP; rather it is through attenuation of activation of p38 MAPK and subsequent activation of caspases.

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Comparative Responses to Stress Caused by Ionizing Radiation (Gamma and Ultraviolet) in HeLa Cells: Studying New Roles for Rac1 GTPases.

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Rac1 is a member of the family of small GTPases related to Ras, and comprise a group of signaling molecules acting as switches (from GDP to GTP state) to control a number of essential cellular functions (proliferation, migration, formation of lamellipodia, membrane ruffles and adhesion). This protein is overexpressed in tumorigenic cells as lung, breast and colon, and the variant constitutively active of Rac1, Rac1b, is naturally expressed in colon and breast cancer. Ionizing radiation causes several types of DNA lesions, like single and double strand breaks, pyrimidine dimers and 6-4 photoproducts usually repaired by homologous recombination, non-homologous end-joining, nucleotide excision, base excision and mismatch repair mechanisms. Thus, the overexpression of Rac1 in human cervical cancer cells associated to their radiosensitivity against different types of DNA damages, prompted us to this study, where we compare HeLa cells (human cancer cervix), mutants expressing dominant negative Rac1 (HeLa-Rac1-N17) and constitutively active Rac1 (HeLa-Rac1-V12) as to cell migration, survival, proliferation and DNA damage response after exposition to low doses of gamma radiation (0.5; 2 and 5Gy) and ultraviolet radiation types A (50 KJ/m²), B(80 J/m²) and C (4 J/m²). From survival and proliferation assays we observed that the dominant negative clone HeLa-Rac1-N17 was more sensitive to different doses of gamma radiation and the same was observed in treatments with the three types of ultraviolet radiation, despite of the fact that HeLa-Rac1-N17 showed a delayed DNA repair rate compared to HeLa-Rac1-V12 clone and HeLa cells. The constitutively active clone HeLa-Rac1-V12 was significantly even faster than HeLa cells in relation to DNA repair after exposition to ionizing radiation. The migration studies showed that cells with higher Rac1 activity migrate more than dominant negative clone after radiation treatments. In conclusion, these results demonstrate that Rac1 protein very likely plays important roles in DNA damage responses and repair mechanisms in human cancer cervix cells, and molecular mechanisms underlying cell death and survival are under investigation to explain these differential responses after damage caused by gamma and ultraviolet radiation. (Supported by FAPESP).

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Signaling in Context: Rac1 Activation Encodes for Increased Survival in 3D.

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Survival of single cells in a three dimensional (3D) environment is crucial for the formation of embryonic bodies and infiltration of tissues by isolated tumor cells. While it has been established that cells on two-dimensional (2D) substrates undergo higher rates of apoptosis compared to those in 3D, most studies on the mechanisms regulating cell survival have been performed on spread cells in 2D environments. To investigate the molecular mechanisms underlying enhanced cell survival in 3D, we assessed the effect of cell shape, matrix ligation, and integrin-dependent adhesion on the survival of isolated mammary epithelial cells (MECs) in compliant 2D and 3D environments. We found that apoptosis in 2D is linked to Rac1 binding of NADPH oxidase, which increases production of reactive oxygen species (ROS) and leads to matrix metalloproteinase (MMP) activation. In contrast, increased $\alpha 6 \beta 4$ integrin ligation of laminin in 3D environments leads to enhanced Arf6 activity through two guanine exchange factors (GEFs), ARNO and GEP100. Arf6 activity then promotes membrane and actin cytoskeletal remodeling, altering Rac1 activity in concert with increased EGF-mediated activation of Rac1 to reduce NADPH-mediated ROS production and to favor apoptosis resistance signaling through Pak1. These data suggest that matrix dimensionality enhances cell survival in 3D by acting through changes in cell shape, membrane tension, and cytoskeletal organization that in turn affect intracellular signaling. This work illustrates the importance of matrix dimensionality in determining cell behavior and fate and highlights the necessity of contextualized study of molecular signaling.

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Ypk1/2 kinases, yeast TORC2 effectors, regulate Rho1 GTPase via controlling phospholipid composition.

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The conserved target of rapamycin kinase complex 2 (TORC2) regulates cell polarity via Rho GTPases. However, the precise mechanism by which TORC2 regulates Rho is obscure. Because it has recently been shown that TORC2 is activated in response to plasma membrane (PM) stretching, uncovering the molecular link between TORC2 and Rho is critical to understand how cell polarity is maintained under membrane-stressed condition. Here we demonstrate that yeast Ypk1/2 kinases, major effectors of TORC2, regulate localization of Rho1 GTPase possibly via controlling phospholipid composition at inner leaflet of PM.

We found that overexpression of Rho1 rescues temperature-sensitive growth of *ypk1/2* (*ypk-ts*) mutant, suggesting Rho1 as a major downstream target of Ypk1/2. Rho1 controls cytoskeleton at growing bud cortex, whereas this localization was impaired in *ypk-ts* cells. Thus Ypk1/2 seem to promote Rho1 function by targeting it to growth sites.

How do Ypk1/2 recruit Rho1? We hypothesized that Ypk1/2 promote accumulation of phosphatidylserine (PS) at inner leaflet of bud PM, which in turn recruits Rho1, based on following known facts. (1) Ypk1/2 negatively regulate Dnf1/2-Lem3 phospholipid flippase complex. (2) In *LEM3* deleted cells, via unknown mechanism, PS accumulates at inner leaflet of growth sites. (3) PS recruits Cdc42, another member of Rho GTPases, to bud cortex.

We asked if PS recruits Rho1. Indeed, deletion of *CHO1* PS synthase resulted in impaired Rho1 localization at bud cortex. On the other hand, artificial accumulation of inner PS by *LEM3* deletion caused enhanced bud localization of Rho1. Thus PS accumulation is necessary and sufficient for Rho1 targeting to bud cortex. Possible mechanisms by which PS recruits Rho1 will be discussed.

In unstressed condition, phosphatidylinositol (4,5)-biphosphate (PIP_2) also plays an important role for Rho1 tethering to PM at growth sites. However, it has been shown that PIP_2 is downregulated when cells are exposed to membrane stresses such as hypo-osmotic shock or inhibition of sphingolipid biosynthesis, the conditions where TORC2 activation occurs. Therefore Rho1 targeting by TORC2-Ypk1/2 pathway via PS might be a compensation mechanism for PIP_2 loss. Consistent with this idea, *LEM3* deletion rescued Rho1 mislocalization in PIP_2 deficient mutant cells.

Based on these results, we propose that TORC2-Ypk1/2 pathway let PS substitute for PIP_2 in Rho1 recruitment to maintain cell polarity under membrane stress.

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Human rhinovirus activates of the small G-protein Rac attenuating the TLR3/IFN- α pathway while promoting the p38 pathway in human monocyte-lineage cells.

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Rhinoviral infections are a major cause of asthma exacerbations. Though productive rhinovirus infection occurs predominantly in the bronchial epithelial cells of the upper airway, monocytic-lineage cells are implicated in establishing the inflammatory microenvironment observed during rhinoviral asthma exacerbation. Human rhinovirus (HRV) is unique in that nearly genetically identical viruses bind either the intercellular adhesion molecule (ICAM)-1 or low-density lipoprotein (LDL) receptor. Within minutes of binding to its cellular receptors, HRV is capable of eliciting a signaling response in both epithelial cells and monocyte-derived macrophages. It is unclear whether this signaling response is important to the subsequent release of inflammatory mediators, particularly in cells not capable of supporting viral replication. We show here that the small molecular-weight G-protein Rac is activated in macrophages following the binding of HRV to either ICAM-1 or LDL-R. Following this observation, we sought to determine whether Rac activation during HRV exposure is merely a byproduct of receptor attachment or is a key player in mediating the inflammatory response. We demonstrate that inhibiting Rac resulted in the upregulation of toll-like receptor 3 (TLR3) in macrophages exposed to major- and minor-group HRV and resulted in increased release of interferon- α . Furthermore, inhibiting Rac in HRV-exposed macrophages attenuated activation of the stress kinase p38 and release of the proinflammatory cytokine CCL2, but inhibiting Rac did not affect release of the proinflammatory cytokine CCL5. These findings suggest that Rac is an important regulator in establishing the inflammatory microenvironment that is initiated in the human airway upon exposure to rhinovirus.

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Regulation of the Rac activator Tiam1 by aPKC phosphorylation.

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The establishment and maintenance of cell polarity is fundamental to many biological processes, both physiological and pathological. It is now understood that the Rho family GTPases with several groups of evolutionarily conserved polarity proteins, such as the Par complex, comprise the core of the intrinsic cell polarization machinery. We, and others, have shown that the Par complex, consisting of Par3, Par6 and aPKC, functionally interacts with the

Rac activator Tiam1, by direct interaction through Par3, to control cell polarization in various cell types and contexts. However, it remains unknown how the Par complex regulates Tiam1. Here, we report that Tiam1 is a substrate of aPKC. Phosphorylation sites were mapped to the inhibitory N-terminal region of Tiam1. We also found that this N-terminus binds to the adjacent PHnCCEx as well as the DPH domains in the C-terminus, which are responsible for Par3-binding and GEF activity, respectively; these interactions were abrogated by aPKC-mediated phosphorylation of Tiam1. In N1E-115 cells, overexpression of phosphomimetic mutants induced formation of dorsal ruffles more efficiently than either WT or non-phosphorylatable mutants. Additionally, Tiam1 phosphorylation was necessary for Cdc42-dependent lamellipodia formation requiring the interplay between Tiam1 and the Par complex. Further, Tiam1 is phosphorylated by aPKC during growth factor signaling in multiple cell types in a timecourse consistent with Rac activation. Thus, it is attractive to consider regulated intramolecular associations as a regulator of Tiam1 activity.

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A naturally occurring dominant-negative splice variant regulates the responsiveness of Galphaq-regulated RhoGTPase activators.

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RhoGTPases have a powerful influence on cell behavior and fate via regulation of transcription and cytoskeleton. Small GTPases such as members of Rho family are activated by exchange of bound GDP for GTP, which is mediated by guanine-nucleotide exchange factors (GEFs). Extracellular signals activate RhoGTPases via G protein coupled receptors. Galpha12/13-regulated GEFs are well studied, whereas less is known about the Galphaq-regulated neuronally-enriched GEFs implicated in schizophrenia and other diseases. We have found and characterized a novel Galphaq-regulated GEF splice variant. This variant is expressed in different cells and tissues. This variant is inactive as a Rho activator, but it also acts as a dominant negative inhibitor of related GEFs. Deletion analysis identifies the region sufficient for inhibition, which we reveal possesses an unexpected yet strong homophilic interaction which may be responsible for the inhibitory action. We propose a model whereby activation of Galphaq-regulated GEFs renders them competent to interact in trans with the inhibitory region of the variant. The variant may selectively target the activated GEF population, thereby sharpening the response curve to activation signals to generate a more all-or-none character than would otherwise occur.

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Fission yeast Rga7 is a Rho2 GAP that regulates cell integrity and morphology.

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Rho GTPase-activating proteins (GAPs) are negative regulators that inactivate Rho GTPases. Rho GAPs are involved in the regulation of critical biological responses in eukaryotic cells such as cellular morphogenesis. Rga7 is one of the nine GAPs present in fission yeast. Rga7 localizes to the cell division site and to the cell tips. Mutant cells lacking Rga7 display aberrant morphology: cells are wider than wild-type, they have cytokinesis defects with higher percentage of cells having one or more septa and a percentage (13%) of dead cells in the population. We have found that Rga7 is a GAP for Rho2-GTPase which acts upstream of Pmk1, a MAPK involved in the regulation of fission yeast cell integrity and cell separation. However,

elimination of Rho2 only partially suppresses the cell death observed in cells lacking Rga7 and does not affect the cytokinesis phenotype. On the other hand, elimination of Pmk1 almost completely suppresses cell death caused by the lack of Rga7 and increases the proportion of cells with separation defects during cytokinesis. Two hybrid screening detected physical interaction between Rga7 and Rho2 and between Rga7 and Pek1, the MAPK kinase which acts as an activator of the Pmk1 MAPK signaling. All together, these data suggest that the function of Rga7 in fission yeast integrity and cytokinesis is performed through the modulation of the MAPK pathway, in both Rho2 dependent and independent ways.

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Investigating Rho GTPase Pattern Formation During Single-Cell Wound Healing: The Role of RhoGAP1/8.

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The ability to respond to physical damage is an essential and evolutionarily conserved feature of single cells and tissues. The Rho family of GTPases has been shown to coordinate the cytoskeletal rearrangements needed to reestablish the integrity of the plasma membrane and underlying cortex in response to cell damage. The precision with which Rho and Cdc42 are activated and maintained in distinct activity zones throughout the healing process suggests the involvement of multiple Rho GTPase regulators at the wound. To date, a candidate screen for guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) involved in regulating Rho GTPase activity at the wound has identified a single wound regulator, Abr. By continuing the candidate screen, we have identified two additional GAPs, RhoGAP1 and RhoGAP8, as potential wound regulators. eGFP-tagged RhoGAP1 localizes between the zones of active Rho and Cdc42 in wounded *Xenopus laevis* oocytes where it may be acting as an enzymatic barrier to prevent Rho GTPase activity zone mixing during wound healing. Targeting is mediated by its C-terminal region which contains both a GAP domain and a proline-rich region. Further, overexpression of RhoGAP1 results in a reduction in active Rho at wounds. We have also discovered that RhoGAP1 and RhoGAP8 colocalize at wounds which, in addition to previously reported evidence of their interaction, suggests they may form a complex to regulate Rho GTPase activity.

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DLC1 regulation by PP2A and Mek2 alters its RhoGAP activity.

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Actin remodelling is essential to many dynamic cellular processes such as morphogenesis, motility, differentiation and endocytosis. These changes are controlled by Rho GTPases that cycle between the active GTP- and inactive GDP-bound forms, which in turn are tightly regulated by guanine nucleotide exchange factors (GEFs), GTPase activating protein (GAPs) and the guanine nucleotide dissociation inhibitor (GDIs). Deleted in Liver Cancer-1 (DLC1), is a bona fide tumor suppressor GTPase activating protein (GAP) acting preferentially on Rho. It is a multi-domain protein, consisting of N-terminal SAM domain, C-terminal START domain and the catalytic RhoGAP domain. This allows for its interaction with diverse cellular proteins, including FAK, Tensins and Talin, all of which are focal adhesion-associated proteins, as well as other scaffolding, regulatory proteins such as 14-3-3, EF1A1, and S100A10. As such, the tumor

suppressive function of DLC1 can be mediated in a GAP-dependent or GAP-independent manner. Interestingly, DLC1 also contains a serine-rich region which is a phosphorylation hot-spot and is thought to be modified downstream of several potential kinases such as Akt, RSK and PKC/PKD. Despite all these, the nature of DLC1s activation and inactivation remains largely unknown. Here we show that PP2A and Mek2, which are ubiquitous for regulation of many growth-related kinase signalling, can regulate the activation and inactivation of DLC1. Specifically, DLC1-PP2A interaction follows for a unique Mek2-dependent binding profile accompanied by changes in the active RhoA levels. The physiological significance and impacts on cell spreading and migration will be discussed.

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Analysis of a Rho GTPase centered interactome reveals complex regulation of neurite outgrowth.

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Rho GTPases are key regulators of the actin, adhesion, and microtubule dynamics that allow complex morphogenetic events such as neurite outgrowth to occur. Rho GTPases are molecular switches that cycle between an active and an inactive state, which is regulated by two protein families: the guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs). Upon activation, Rho GTPases interact and regulate the activity of different effector proteins contributing to cellular responses. Classical loss of function experiments, using dominant negative (DN) constructs, have suggested that the Rho GTPases Rac1 and Cdc42 regulate neurite extension, whereas RhoA controls growth cone collapse and neurite retraction. Based on our recent analysis of the neurite proteome (1), we used a bioinformatic approach to identify a potential Rho GTPase centered, neurite-localized interactome. This interactome consisted of a surprisingly high number of GEFs, GAPs and effectors as well as upstream and downstream interactors (220 proteins total). To explore the significance of this complexity we performed an RNA interference screen. Intriguingly, loss of function of different components of this interactome led to more subtle phenotypes as compared to Rho GTPase DN mutants. A more complete understanding of these phenotypes required timelapse analysis of the neurite outgrowth dynamics and high resolution analysis of the F-actin and microtubule cytoskeleton. For example, RhoA knock down (KD) leads to longer neurites because of loss of growth cone collapse events. High resolution, quantitative analysis of the growth cone also reveals that RhoA loss of function leads to an increase in F-actin content in filopodia. Surprisingly, Cdc42 loss of function also leads to a loss of growth cone collapse, but unlike RhoA, exhibits diminished F-actin content in filopodia. Loss of function in response to KD of different GEFs, GAPs and effectors affected specific morphodynamic cell behaviors (neurite initiation, elongation, collapse, etc.), but also had an impact on subtle filopodium function (F-actin content, filopodia number, etc). These results are consistent with a scenario in which different signaling modules consisting of specific GEFs, GAPs, Rho GTPases and effectors co-operate to regulate specific neurite subfunctions in time and space to fine tune the neurite outgrowth process. This is in accordance with the complexity of spatio-temporal Rho GTPase signaling recently uncovered using FRET biosensors (2). Quantitative computer vision techniques will be necessary to appropriately analyze these complex phenotypes and have the potential to produce integrated models of spatio-temporal Rho GTPase signaling.

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Central role of the exchange factor GEF-H1 in TNF- α -induced sequential activation of Rac, ADAM17/TACE and RhoA in tubular epithelial cells.

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The Rho family small GTPases, RhoA and Rac, are key regulators of the cytoskeleton, and affect a variety of vital cellular functions, including growth, adhesion, polarity and migration. We have previously shown, in tubular epithelial cells, that the pleiotropic pro-inflammatory cytokine Tumour Necrosis Factor- α (TNF- α) activates RhoA through the Guanine Nucleotide Exchange Factor GEF-H1. Activation of GEF-H1 requires phosphorylation on its Thr 678 residue by the Extracellular Signal Regulated Kinase (ERK). ERK in turn is stimulated by TNF- α -induced transactivation of the Epidermal Growth Factor Receptor (EGFR), which is a key step in mediating RhoA activation, and cytoskeleton and junction remodelling. The aim of the current study was to explore the mechanisms through which TNF- α activates the EGFR.

We performed our studies in the proximal tubule epithelial cell line LLC-PK1. Using an enzyme activity assay with a fluorogenic substrate we show that TNF- α stimulates TACE/ADAM17. TACE silencing using specific siRNA prevented TNF- α -induced activation of the EGFR/ERK pathway. In search for the underlying mechanism, we found that TACE activation is mediated by the MAP kinase p38, which in turn is activated through the small GTPase Rac. Using affinity pull down assays to follow Rac and GEF-H1 activation (GST-PBD and GST-Rac (G15A) pull downs, respectively), we found that TNF- α activates Rac through the exchange factor GEF-H1. Thus, TNF- α stimulates both Rac and RhoA through the exchange factor GEF-H1. However, although EGFR and ERK-dependent phosphorylation at the T678 site of GEF-H1 is a prerequisite for stimulation of RhoA, it is not required for Rac activation. In contrast, GEF-H1 mediated-Rac activation is upstream from the TACE/EGFR/ERK pathway and requires phosphorylation at the S885 site on GEF-H1. Thus, the Rac- and RhoA-specific exchange activities of GEF-H1 are regulated through different phosphorylation sites. Moreover, GEF-H1-mediated Rac activation controls the EGFR/ERK pathway and the downstream RhoA activation. Together these findings provide a novel mechanism to explain the hierarchical activation of Rac and RhoA by TNF- α . Such a mechanism could be key in coordinating GEF function and fine-tuning Rac and RhoA activation.

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High-Content Combinatorial RNAi Screens Reveal the Regulation of Cytokinesis by Rho-family GTPase Signalling Networks in Drosophila.

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There is currently little understanding of the architecture and dynamics of the signalling networks that coordinate the dramatic cytoskeletal rearrangements underpinning cytokinesis. While classical genetic screens have identified several core cytokinesis regulators, signalling networks are robust to single genetic perturbations, suggesting more genes can be discovered. We have shown that double RNAi screens can be used to identify novel regulators of diverse cellular processes that are not apparent in single genetic screens (Bakal et al, 2008; Arias-Garcia et al, 2012). Here I have developed a quantitative, high-throughout combinatorial screen to isolate novel regulators of cytokinesis.

In *Drosophila* cells, inhibition of the Rho-family GTPase Rho1 or its upstream activators (RacGAP50C and Pebble) results in cytokinesis failure and the generation of multinucleate cells

due to defects in contractile ring assembly and ingression. I have completed double RNAi screens using libraries targeting *Drosophila* Rho-family GTP Exchange Factors (GEFs) or GTPase Activating Proteins (GAPs), as well as kinases/phosphatases in wild type cells or those sensitised with Rho1, RacGAP50C, or Pebble RNAi, totalling 3,264 genetic combinations. Within the screens each gene is targeted in duplicate or quadruplicate by 2-5 independent dsRNA amplicons. High throughput microscopy collects 40-160 images (30-90 cells per image) per treatment, and image analysis algorithms are used to assign a quantitative multidimensional signature describing cell shape to each combination. Measurement of multiple (9) morphology features allows regulators of cell shape to be identified independent of cytokinesis defects.

Here I will discuss how using combinatorial screening I have identified RhoGAP16F and RhoGAP54D as putative regulators of cytokinesis. Neither RhoGAP16F or RhoGAP54D was identified in single knockdown screens as contributing to cytokinesis. However inhibition of RhoGAP16F increased the number of multinucleate cells in Pebble or RacGAP50C deficient backgrounds. Conversely, knockdown of RhoGAP54D weakly suppresses the cytokinesis failure that occurs following inhibition of Pebble or RacGAP50C by RNAi. Both genes are poorly characterized, but we have previously used computational methods to predict that RhoGAP16F and RhoGAP54D are GAPs for Rac-type GTPases (Nir et al., *Genome Res* 2010). We hypothesize that these genes may normally play a role in regulating Rac-driven cortical tension whose inhibition impacts cytokinesis only when Rho1 levels are suppressed (Loria et al, 2012).

Analysis of kinase/phosphatase screen data is ongoing. Extensive genetic and biochemical validation of all screen hits will then be performed. Finally, one goal of these studies is to generate a comprehensive network model describing the architecture and dynamics of signalling during cytokinesis.

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Rho A signaling contributes to simvastatin-induced osteogenesis in bone marrow mesenchymal stem cells.

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Statins, 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, reduce cholesterol synthesis and prevent cardiovascular disease. They also have been found to inhibit prenylation of Rho proteins in recent decade. Previous reports showed that statins inhibited protein prenylation and decreased the active form of RhoA in osteoclasts. Others reports indicated that statins inhibited protein prenylation but increased the active form of RhoA in human erythroleukemia cells. Therefore, the role of statins regulates RhoA activity remains unclear. Rho GTPases act as molecular switches to regulate mesenchymal stem cell differentiation. Previous study showed that transfected constitutively active-form of RhoA into human mesenchymal stem cells (hMSCs) which leded osteogenesis. On the other hand, dominant negative RhoA leded adipogenesis. Rho GTPases play a significant role in regulating cytoskeletal dynamics and have been shown to be crucial for cell proliferation and differentiation. According to the description above, we want to investigate whether simvastatin (SIM) induced osteogenesis through RhoA signaling and further altering the actin cytoskeleton contributes and increasing the cellular tension within BMSCs. For all experiments, mesenchymal stem cells, D1, were treated with or without simvastatin (SIM) in osteo-induction

medium. The SIM 0.5 and 1 μ M appeared to alter cytoskeletal organization at day 2. And SIM 1 μ M enhanced the osteogenic gene expressions of Runt-related transcription factor 2 (Runx-2), bone morphogenetic protein 2 (BMP-2), Alkaline phosphatase (ALP), osteocalcin (OC) and dose-dependently increased the mineralization at day 5 in D1 cells culture. We further found that SIM 1 μ M sustainably increased active-form RhoA level at day2 and day3. In order to investigate the role of RhoA in osteogenesis, we transfect the constitutive active and dominant negative form RhoA in D1 cells. After transfect the constitutive active form RhoA increased the effect of mineralization rather than transfect dominant negative form RhoA. Blebbistatin, selectively inhibits the ATPase function of nonmuscle myosin II, 10 μ M significantly decreased the SIM-induced mineralization at day 5 in D1 cell culture. We summarized that SIM enhanced osteogenesis through activation of RhoA signal. And tension released by Blebbistatin significantly inhibited SIM-induced osteogenesis. We suggest RhoA signaling might contribute to SIM-enhanced osteogenesis in mBMSCs

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Mechanical Force on ICAM-1 Leads to RhoA-Mediated Cytoskeletal Changes in Endothelial Cells.

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RhoA-mediated cytoskeletal rearrangements in endothelial cells (ECs) play an active role in transendothelial cell migration (TEM), a normal physiological process in which leukocytes are recruited from the blood circulation into tissues. While much has been learned about RhoA signaling pathways downstream from ICAM-1 in ECs, little is known about the consequences of the tractional forces that leukocytes generate on ECs as they migrate over the surface before TEM. We have found that after applying mechanical forces to ICAM-1 clusters, there is enhanced RhoA signaling and cellular reinforcement compared to ICAM-1 clustering alone. Using the RhoA activity assay, we have determined ICAM-1 clustering alone leads to ~1.75 fold increase in RhoA activity over untreated cells, whereas the addition of force leads to ~2.5 fold higher RhoA activation over control. There is a ~21.5 fold increase in myosin light chain phosphorylation with force over control compared with a ~1.5 fold increase over control with clustering alone. We observe a 26% increase in RhoA-dependent cellular stiffening measured by pulling on ICAM-1 clusters with magnetic tweezers. We have confirmed the cytoplasmic tail of ICAM-1 is required for the observed findings. These changes may assist in downstream leukocyte TEM. Further exploring how ECs respond to forces generated by leukocytes on ICAM-1 will contribute to our understanding of inflammatory diseases where leukocyte TEM correlates with disease progression, such as atherosclerosis, ischemic cardiomyopathy, and ischemia reperfusion injury.

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Rho/Rho kinase signaling is involved in cAMP-regulated amylase release in rat parotid acinar cells.

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Rho family of proteins is a member of Ras superfamily and functions as molecular switches cycling between a GDP-bound inactivated state and GTP-bound activated state. The active Rho has been demonstrated to be involved in cell functions including migration, growth and differentiation. In rat parotid acinar cells, stimulation of β -adrenergic receptors provokes an

increase in intracellular cAMP level followed by the activation of protein kinase A (PKA) without an increase in intracellular Ca^{2+} , resulting in exocytotic release of amylase. We investigated the involvement of Rho in cAMP-dependent amylase release. A protein band ADP-ribosylated by *Clostridium botulinum* C3, a well known inhibitor of Rho function via ADP-ribosylation of Rho, was detected in rat parotid acinar cells. The same protein band was crossreacted with anti-RhoA antibody. C3 exoenzyme blocked the cAMP-induced amylase release in streptolysin O-permeabilized acinar cells. RhoA was activated by the β -agonist isoproterenol in a time-dependent manner. The isoproterenol-induced RhoA activation was inhibited by the inhibitor of PKA. The inhibitor of Rho kinase, which is activated by active Rho, Y-27632, partially inhibited the isoproterenol-induced and dibutyryl cAMP-induced amylase release. F-action coating of secretory granules observed in isoproterenol-stimulated cells was disturbed by the treatment of Y-27632. These observations suggest that RhoA/ROCK signaling is involved in exocytosis of amylase induced by β -adrenergic receptor activation in rat parotid acinar cells.

Regulatory and Noncoding RNAs

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Identification and Characterization of Novel Genes Required for miRNA Activity in *Drosophila melanogaster*.

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MicroRNAs (miRNAs) are small (~22nt), non-coding RNAs essential for homeostasis and development in plants and animals. Primary miRNAs are transcribed from genomic DNA and sequentially processed into single-stranded mature miRNAs. Mature miRNAs interact with the miRNA-induced silencing complex (miRISC) and bind to the 3'UTR of target mRNAs via Watson-Crick base pairing, to silence target messages. Functional mature miRNAs are regulated at multiple stages of biogenesis and maturation. While several key molecules in the miRNA pathway have been elucidated, identification and characterization of regulatory and downstream factors is ongoing.

To discern the molecular mechanisms of miRNA function *in vivo*, a forward genetic screen was conducted using *Drosophila melanogaster* to identify novel genes required for miRNA activity. EMS mutagenesis was followed by the isolation of mutants with disrupted miRNA activity as visualized by a change in the expression of an eye-specific GFP reporter fused to the 3'UTR of the *Bearded* gene, which is regulated by multiple miRNAs in the *Drosophila* eye. Mitotic clones were generated in the *Drosophila* eye using FLP/FRT, and mutants with altered levels of GFP fluorescence relative to background (and hence putative altered miRNA activity) were recovered. The screen yielded 25 novel alleles of core components of the miRNA pathway, including alleles of *Drosha*, *Pasha*, *Dicer-1* and *Ago1*. The isolation of previously characterized miRNA pathway genes demonstrates the efficacy of our approach. Additional sensors of miRNA activity reveal unique requirements for sensor silencing. In addition to the identification of 25 novel alleles of core components of the miRNA pathway, ~20 mutant lines demonstrating altered miRNA sensor expression were isolated. Our lab is identifying genes whose alteration leads to the disruption of miRNA-mediated silencing using a combination of recombination mapping with molecularly defined P-element insertions and deficiency mapping. The role of these genes in miRNA activity will be characterized through the analysis of miRNA pathway intermediates in our mutant lines.

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MvU620 plays a role in the release of transcripts from storage and prevention of degradation during development of the male gametophyte of *Marsilea vestita*.C. M. Van der Weele¹, S. M. Wolniak¹; ¹Cell Biology & Molecular Genetics, University of Maryland-College Park, College Park, MD

The male gametophyte of *Marsilea vestita* starts its development from a single cell stored as a dried microspore. Upon hydration the gametophyte initiates a spatially and temporally precise developmental program that first goes through a division phase in 4 hours to make 7 sterile cells and 32 spermatids. When the divisions are completed, development switches to a maturation phase in which each spermatid forms a complex motile apparatus. The development of the gametophyte is completed in 10-11 hours with the release of multiciliated male gametes. Previous research has shown that development in the gametophyte does not rely on new transcription but utilizes RNA that is stored in the dry microspore and that RNA processing is necessary for translation essential for development. Furthermore, the polyamine spermidine was shown to play a role in the release of transcripts from storage, and uncontrolled release caused aberrant development. MvU620 is a RNA binding protein with a single RRM1 domain that binds to U1A/U2B and has sequence homology to Rbp1. In RNAi knockdowns, a dominant phenotype is the lack of proper maturation after the division phase has ended. A reduction of acetylated alpha tubulin, lack of a microtubule ribbon, fewer and mislocalized basal bodies as well as misshapen or collapsed nuclei are indicative of a multifaceted role for MvU620 in post-transcriptional regulation. MvU620 has at least 4 splice forms, and RT analysis, morphological analysis after RNAi knockdowns, and *in situ* hybridizations with probes for shared or isoform-specific sequences showed temporal and spatial differences in abundance and distribution among isoforms. QPCR analysis on RNAi knockdowns showed that one of the shorter isoforms is regulating the presence of a longer isoform, perhaps through alternative splicing. QPCR analysis of several transcripts affected by MvU620 proteins showed an increase in abundance in the knockdown at 2 hours but reduction at 4 hours, which indicates an early release from storage but also precocious degradation. PCR analysis on RNA isolates showed the presence of antisense DNA matching these transcripts. Deep sequencing analysis of untreated spores at different times suggest that this antisense DNA becomes detectable with the appearance of MvU620 transcript isoforms. MvU 620 encodes a small family of RNA-binding proteins that play multiple roles in the post-transcriptional regulation of development in these rapidly maturing spermatids. Supported by NSF grant 0842525 to SMW.

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Alternative polyadenylation of Hsp70.3: Identification of regulatory RNA binding proteins.L. Smith¹, W. Paulding², A. Roessler¹, A. Gabanic¹, M. Kelley¹, L. Haar³, M. McGuinness³, W. K. Jones³, M. Tranter¹; ¹Division of Cardiovascular Diseases, University of Cincinnati College of Medicine, Cincinnati, OH, ²Dept. of Biology, Xavier University, Cincinnati, OH, ³Dept. of Pharmacology & Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH

Heat shock protein 70.3 (Hsp70.3) expression increases in response to cellular stress and plays a cytoprotective role. It has been shown that increased expression of Hsp70.3 in the heart is protective against ischemia/reperfusion injury (myocardial infarction). Recent work by our lab shows that Hsp70.3 expression is controlled through coordinated post-transcriptional regulation by miRNAs and alternative polyadenylation (APA). APA-mediated shortening of the Hsp70.3 3'-untranslated region (3'UTR) facilitates increased protein expression following cell stress via enhanced mRNA stability, removal of regulatory miRNA binding sites, and enhanced polyribosome loading. In this work, we sought to identify the RNA binding proteins (RBPs) that mediate APA of Hsp70.3 through interactions with the 3'UTR of the Hsp70.3 mRNA. To do this,

the 3'UTR sequence of Hsp70.3 mRNA was randomly biotinylated using in vitro transcription and incubated with protein extract from HL-1 cardiac myocyte cells following control or treatment with one hour heat shock (HS); a condition shown to induce APA Hsp70.3 as observed in the in vivo heart. RNA bound proteins were then co-precipitated with the biotinylated RNA using avidin coated magnetic beads and identified using mass spectrometry. Our results identified a total of 45 known RBPs interacting with the Hsp70.3 3'UTR. Of these, 26 RBPs were found to associate with the Hsp70.3 3'UTR during both control and HS conditions, including RBPs previously indicated to play a role in APA and/or mRNA degradation as well as RBPs whose function remains unknown. In addition, 9 unique RBPs were found to bind only under control conditions, whereas 10 unique RBPs were found to bind only after HS stress. These results represent an important first step in characterizing the proteins that associate with the Hsp70.3 3'UTR and allow for ongoing and future investigation into the functional role of the identified RBPs in APA and post-transcriptional control of Hsp70.3 gene expression. A mechanistic understanding of how these RBPs contribute to the regulation of Hsp70.3 gene expression will enhance our understanding of APA as a global modulator of gene expression.

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Identification and validation of miRNA-directed cleavage targets using omics approach.

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MicroRNAs (miRNA), discovered in *C. elegans*, are short non-coding RNAs that bind and regulate the expression of target mRNAs in plants and animals. *C. elegans* miRNAs bind to partially complementary sequences in the 3' untranslated region of the target mRNA, which results in translational repression through mRNA destabilization. The high-throughput sequencing of RNA cleavage fragments was performed to directly detect cleaved miRNA targets in *C. elegans*. Based on this analysis, *mir-249* was identified as a potential miRNA that regulates a *zk637.6* that is paralogous to *asna-1* (*zk637.5*) by cleavage mechanism with extensive, evolutionary conserved complementarity. Additionally, we validated *mir-249* directed cleavage of the *zk637.6* by a gene-specific 5' rapid amplification of cDNA ends and observed notable difference in expression of *zk637.6* in wild-type versus *mir-249* mutant *C. elegans* by quantitative real-time PCR. Furthermore, the expression of *zk637.6* was strongly dependent on the expression of *mir-249*, that is, the higher expression of *mir-249* in early stage results in stronger repression of *zk637.6* expression. These findings may lead to a better understanding of the biological roles of miRNAs in *C. elegans*. Funding Source: This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0022096), Republic of Korea. Correspondence: Chanseok Shin

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Expression of microRNAs 96 and 210 is associated with the production of fetal hemoglobin.

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Beta thalassemia (BT) is an inherited hematologic disease characterized by reduced or absent synthesis of β -globin chain with compensatory γ -globin chain production resulting in higher fetal

hemoglobin (HbF), excess of α -globin chains, hemolysis and ineffective erythropoiesis. MicroRNAs (miRs) have been described to participate in globin switching and can modulate transcriptional erythroid-specific regulators. Expression analyses of miRs 96 and 210 were performed on peripheral blood CD34⁺ cell cultures from healthy controls (CON, n=4) and nontransfused BT intermedia patients (n=4). Cells were cultured in a liquid system stimulating erythropoiesis and collected after 7, 10 and 13 days for extraction of miR, transcription to cDNA and analysis by q-PCR. During BT erythroid differentiation, overall expression of miR-96 was decreased, reaching significance on day 13 when compared to CON cultures (0.316 ± 0.042 vs. 0.051 ± 0.030 , respectively, $p < 0.05$). Expression of miR-210 increased during erythroid differentiation in both cultures, and on day 13 this expression was significantly higher in BT than in CON cultures (0.996 ± 0.086 vs. 1.970 ± 0.130 , respectively, $p < 0.05$). This data suggest that miR-96 and miR-210 could be associated with increased levels of HbF observed in BT patients, agreeing with studies showing that knockdown of miR-96 and overexpression of miR-210 are associated with increased production of γ -globin. We performed in silico analysis and found that miR-210 targets two regions of the *KLF1* mRNA, a critical transcription factor involved in globin switching by directly activating β -globin and indirectly repressing γ -globin gene expression (free energy miRNA:mRNA hybridization = -31.7 and -30.1 kcal/mol, significance threshold under -20 kcal/mol). Interestingly, in our BT cultures, *KLF1* expression was lower on days 10 and 13 when compared to CON cultures (1.159 ± 0.608 vs. 0.164 ± 0.087 and 1.360 ± 0.966 vs. 0.217 ± 0.081 , respectively, $p < 0.05$). A previous study showed that increased levels of *KLF1* can decrease γ -globin gene expression by inhibiting the interaction between the LCR (locus control region) and the γ -globin gene. Our data showing concurrent decrease of *KLF1* and miR-96 along with upregulation of miR-210 suggest that this regulatory mechanism may affect γ -globin expression. Taken together, these findings contribute to the understanding of HbF production and to the development of new therapeutic strategies for hemoglobinopathies. Financial support by FAPESP and CNPq/INCTS.

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An association between microRNA-155 and the Hp genotype in patients with sickle cell anemia.

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Sickle cell anemia (SCA) is characterized by chronic inflammation with a very variable immune response that depends on multiple genetic and environmental factors. Haptoglobin (Hp) is an acute-phase protein with immunomodulatory and antioxidant properties whose basic function is to bind to free hemoglobin in the plasma and protect blood vessels from its oxidative effects. Two codominant alleles (HP1 and HP2) result in three main genotypes/phenotypes (Hp1-1, Hp2-1 and Hp2-2), which correspond to proteins with distinct physical, chemical and functional characteristics. MicroRNAs (miRs) are post-transcriptional modulators of gene expression and their role in infection, inflammation and cell differentiation, proliferation and apoptosis has been investigated. miR-155 is involved in red blood cell differentiation and also plays an important role in inflammation and immunity; however, there is a dearth of information in the literature about the expression of these molecules in SCA. The aim of this study was to investigate the expression profile of miR-155 in granulocytes and determine plasma levels of cytokines IL-1 β , IL-6, IL-8 and TNF- α in SCA patients classified according to Hp genotype (Hp1-1, Hp2-1 and Hp2-2). Twelve patients of each Hp genotype (determined by allele-specific PCR), in steady state, were selected for the study. Cytokine plasma levels were determined by ELISA, and miR-

155 expression profile by q-PCR. The results showed that the miR-155 expression rate in granulocytes from patients with the Hp1-1 genotype was greater than the expression rate in Hp2-1 patients, which in turn was greater than that in Hp2-2 patients. The difference between the expression rates for Hp1-1 and Hp2-2 patients was statistically significant ($p=0.002$). Interestingly, plasma TNF- α levels were higher in the Hp1-1 group than in the others but did not reach statistical significance. There were no statistically significant differences in plasma IL-1 β , IL-6 and IL-8 levels between the different Hp genotypes. miR-155 is considered important for inflammatory activation of human myeloid cells and, when overexpressed in CD14⁺ cells in peripheral blood, can lead to down-regulation of SHIP-1, an inhibitor of inflammation, and an increase in production of proinflammatory cytokines such as IL-6 and TNF- α . In agreement with this, our results suggest that miR-155 may be related to Hp genotype and may play a role in the inflammatory response in SCA patients. Financial support: FAPESP/CNPq/CAPE

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Regulation of Human Growth Hormone Receptor Expression by MicroRNAs.

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MiRNAs are small (19-21nt) noncoding RNAs that play an important role in regulating gene expression primarily through targeting the 3'UTR of mRNAs and enhancing their degradation or inhibiting translation. In the last decade, a critical role for miRNAs has been demonstrated in many chronic diseases, including cancer.

The pleiotropic actions of Growth Hormone (GH) result from activation of its receptor (GHR) on target cells and the subsequent stimulation of multiple intracellular signaling pathways, leading to changes in gene expression, differentiation and metabolic activity. Clinical studies have associated over- or under-expression of GHR with effects on childhood growth, obesity, cancer and diabetes suggesting that GHR levels must be tightly regulated at every stage of life. Our previous studies have focused on 5'UTR (promoter) regulation of *GHR* gene expression. In the present study, we are mapping functional miRNA binding sites in the *GHR* 3'UTR.

We used multiple *in silico* prediction tools based on different algorithms to define putative miR-binding sites within the *GHR* 3'UTR and prioritized a subset based on conservation across several species, hybridization energy, published reports that link specific miRNAs to GHR-related physiological or pathophysiological activities, and the presence of parallel sites in GH/IGF axis-related genes. To test these sites, we created a Luc-*GHR* 3'UTR luciferase reporter vector and screened for miRNA activity: miR-16 ($p<0.05$), miR-202 ($p<0.01$), miR-129-5p ($p<0.01$) and miR-142-3p ($p<0.01$) showed significant inhibitory effects. Studies are ongoing to determine the specificity of their sites (site-directed mutagenesis) as well as to examine their effects on endogenous *GHR* mRNA (qPCR) and protein (western blot) expression levels. Future goals are to determine the role of these miRNAs in GHR-related pathophysiology and their potential use as therapeutic agents.

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Target RNAs Promote Release of Guide RNAs from Human Ago2.

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Argonaute proteins bind small guide RNAs to form the functional core of RNA induced silencing complexes (RISCs), which mediate the silencing of complementary target RNAs. The details of small RNA biogenesis and mechanisms for loading into Argonaute proteins are well-studied,

however much less is known about how small RNAs are removed from Argonaute and degraded. Here we show that highly complementary target RNAs induce the release of guide RNAs from human RISC by destabilizing the interaction between Argonaute2 (Ago2) and the bound guide strand. This “unloading” activity can be enhanced by mismatches between the target and the 5' of the guide RNA and attenuated by mismatches to the 3' end of the guide. The introduction of 3' mismatches leads to more potent silencing of abundant mRNAs in mammalian cells. These findings may help to explain why the 3' ends of mammalian microRNAs (miRNA) rarely match their targets, suggest a mechanism for sequence-specific small RNA turnover and offer mechanistic insight for design of improved siRNAs.

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The Crystal Structure of Human Argonaute2.

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At the core of every RNA-Induced Silencing Complex (RISC) is an Argonaute protein that mediates RNA interference (RNAi) in eukaryotes. The 2.3Å crystal structure of human Argonaute2 (Ago2), the human “slicer” enzyme, bound to a heterogeneous pool of guide RNAs reveals a bilobed architecture with the guide RNA cradled in a hydrophilic central cleft between the two lobes. Nucleotides 2-6 of the guide RNA are arranged in an A-form configuration exposed to solvent for target recognition. An isoleucine intercalates between bases 6 and 7 to introduce a kink that disrupts helical stacking, which may facilitate product release or target recognition. Tandem tryptophans bound in two binding pockets located within the PIWI domain represent a likely interaction surface for tryptophan rich cofactors. These studies provide valuable insight into the mechanisms of eukaryotic RNAi.

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Cyanobacterial DEAD-box RNA helicase: Autoregulation, RNA maturation and sRNA metabolism.

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DEAD-box RNA helicases are ubiquitous enzymes found in all domains of life that rearrange RNA secondary structure. They function in every aspect of RNA metabolism, frequently in response to abiotic stress. In the cyanobacterium *Synechocystis* sp. PCC 6803, expression of the cyanobacterial RNA helicase gene, *crhR*, is regulated in response to temperature fluctuation. Physiologically, CrhR inactivation dramatically affected the photosynthetic capacity and morphology of the mutant cells in response to temperature downshift. Analysis of the expression profile of the *crhR* dicistronic operon in wild type and Δ*crhR* cells indicated that CrhR autoregulates its own expression through a combination of transcript processing and temperature-dependent mRNA and protein stabilization mediated by dramatic temperature- and mutant-induced alteration of half-life. *crhR* inactivation resulted in unregulated accumulation of both transcript and protein while multiple transcripts detected in the mutant suggested defects in RNA processing and/or degradation. 5' RACE-RCA and immunoprecipitation analysis are ongoing to establish processing sites and potential RNases (RNase II/R, E, J) involved in transcript turnover and maturation. Furthermore, tiling arrays revealed that CrhR controls expression of a subset of small RNAs in *Synechocystis*. A model integrating the ability of CrhR in unwinding and annealing RNA with a role in gene regulation during environmental acclimation involving autoregulatory pathways and small regulatory RNAs will be presented.

Epigenetics and Chromatin Remodeling

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Promoting neocentromere assembly in human cells.

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In almost all eukaryotes, centromeres are propagated epigenetically. In order to understand how local sequence and chromatin features of the mammalian genome affect the creation and maintenance of a centromere we flanked a human centromere with loxP site-specific recombination sites by gene targeting. These sites are embedded within inactive selective markers that will be activated only upon successful Cre-mediated excision of the endogenous centromere of chromosome 4, creating a 170 megabase acentric chromosome. Stringent selection will allow for the recovery of centromere-depleted cells and for tracking the fate of the acentric chromosome. We anticipate one outcome to be the formation of neocentromeres. We will discuss the overall strategy and initial results of this assay in which we aim to determine the impact of chromosome context in the nucleation of human neocentromeres and to identify the key players involved in this process.

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The Sex-Determining Factor SRY Functions as a Male-Specific Genetic Modifier by Interacting and Modulating the Activities of Other Transcription Factors.

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The SRY gene on the Y chromosome is responsible for switching on the male sex development during embryogenesis. SRY is a dual-function transcription factor capable of simultaneously repressing and activating genes involved in ovarian and testicular differentiation respectively. Numerous SRY interactive proteins, capable of forming transcription complexes with different transcriptional regulatory functions, have been identified. Although SRY is normally expressed at the time of sex determination in the embryonic gonad, its ectopic expression in non-gonadal tissues has been documented. Hence, it could modulate the gene expression program and exert a male-specific effect(s) on the development and physiology of affected tissues.

Using proteomic and promoter characterization strategies, we demonstrated that SRY is capable of interacting with a variety of transcription factors, such as SP1, SF1, PAX3 and NKX2-1, in vitro and in vivo. Domain mapping showed that the HMG box of SRY is responsible for such interactions with various domains of these transcription factors. To evaluate the effects of such SRY interactions, we demonstrated that SRY is capable of stimulating the transcriptional activities of SP1 and SF1 on MAOA and SOX9 gene respectively. SRY interactions with PAX3 and NKX2-1 result in suppression of their transactivation of the RET promoter in cultured cells. Hence, SRY is capable of stimulating or repressing the transactivation of its interacting transcription factors; thereby modulating the latter's gene regulatory programs. Significantly, SRY is a member of a family of SRY-box containing (SOX) transcription factors, which harbor a conserved HMG box, a presumed DNA-binding domain. Our observations suggest that the HMG box could also function as a protein-protein interacting domain. Importantly, SRY could compete with other SOX transcription factors, via such protein-interacting domain, in their complex formation. Indeed, we demonstrated that SRY could compete with SOX10 on its binding with PAX3 and NKX2-1, and inhibit SOX10 exacerbation of the transcriptional activities of these transcription factors. The present observations, collectively, suggest that the Y-encoded SRY can serve as a genetic modifier capable of

modulating the activities of other transcription factors and their respective gene regulatory programs, thereby exerting a male-specific effect(s) on the development and physiology of the affected tissues.

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The Mof Acetyltransferase is Required for JIL-1 H3S10 Kinase Stability in *Drosophila* Males.

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Mof is a histone H4K16 acetyltransferase in both *Drosophila* and mammals. Mof, is a key component of the MSL (male specific lethal) dosage-compensation complex on the X-chromosome. However, Mof is also a member of the NSL (non-specific lethal) complex that is present in both sexes. The JIL-1 kinase localizes specifically to euchromatin interband regions of polytene chromosomes, is the kinase responsible for phosphorylation of histone H3S10 at interphase, and is up-regulated on the male X chromosome. However, it is not known how this localization is regulated or what causes JIL-1 to be enriched on the male X chromosome. To begin to address these issues and to study JIL-1's functional relationship to Mof we performed various combinations of double labelings of polytene chromosomes with JIL-1, Mof, H3S10ph, and H4K16ac antibodies. The results showed that JIL-1 and JIL-1 mediated H3S10 phosphorylation colocalize with Mof and H4K16 acetylation both on male and female chromosomes. We also performed RNAi-mediated knockdown of Mof using the Gal4-UAS system. Interestingly, we found that JIL-1 levels and localization were unaffected by Mof RNAi in females, but JIL-1 levels were substantially decreased in males. In contrast, loss of JIL-1 did not cause obvious changes to Mof or H4K16 acetylation localization or levels on polytene chromosomes. By analysis of a Mof partial loss-of-function mutant, *mof1*, we confirmed that a reduction of Mof acetyltransferase activity led to a substantial reduction of JIL-1 and H3S10 phosphorylation on polytene chromosomes in males. These levels could be restored by expression of Mof-LacI in the *mof1* mutant background indicating that the loss of JIL-1 indeed was caused by reduced Mof activity. Furthermore, applying a LacI tethering system we show that ectopic targeting of LacI-Mof induces enhanced H4K16 acetylation and recruitment of other MSL complex proteins, but not of JIL-1. Taken together, these results suggest that Mof acetyltransferase activity is necessary for stabilizing the JIL-1 kinase, but not for its chromosome localization in *Drosophila* males. Supported by NIH grant GM62916.

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Function of HP1 proteins as a component in kinetochore formation and its relation with chromosome instability.

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HP1 Family of proteins are involved in the formation and maintenance of chromatin higher order structure. In mammals there are known three isotypes (HP1 α , HP1 β and HP1 γ). Recently, it has been proposed that HP1 may play an important roll in inner centromere establishment, generated by its interaction with HMis12 complex, (HMis12C) which is relevant in kinetochore formation and microtubule recognition which ensure correct chromosomal segregation. However, alterations in chromatin structure or loss in H3K9 methylation lead to a reduction of

the protein presence and changes of HP1 proteins localization to heterochromatin followed by chromosome instability. It has not been studied if this is mediated by loss of recruitment of HMis12C to the kinetochore and which is its relation with chromosomal instability generation. Thus, the aim of this study is to determine if alteration of HP1 proteins is capable of reducing HMis12C recruitment to the kinetochore. We elaborated transfections of constructions of HP1-GFP for each isotype in HCT116 cells and performed time-lapse to observe localization along cell cycle by confocal microscopy; in addition, we treated cells with TSA 1 μ M to analyze changes in HP1 localization. We used ChIP assay in satellite alpha and satellite 2 to determine presence of H3H9me3, HP1 proteins, CENPA, and HMis12 in HCT116 transfected cells with HP1-GFP and with Jmjd2b to observe the effect of the loss of H3K9me3 to HMis12C incorporation. We found that each isotype presents a different localization at interphase, but HP1 α and β are present at the centromere at this phase, also this localization is highly dynamic in mitosis where HP1 α is removed and HP1 β is enriched at the chromosomes centromere. Treatment with TSA generates relocalization of HP1 proteins to pericentromeric chromatin and an increase of chromosome instability. Jmjd2b over-expression reduces HP1 presence at chromatin and also reduces HMis12 in mitosis. These results support another function of HP1 as a kinetochore partner leading to incorporation of HMis12 during cell division.

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A conformational switch in HP1 allows conditional activation and drives assembly of the minimal heterochromatin unit.

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HP1 proteins play a central role in the assembly and spread of H3K9-methylated heterochromatin. HP1-mediated heterochromatin is critical for diverse nuclear processes ranging from gene silencing to chromosome segregation. Yet how HP1 proteins assemble on their methylated nucleosomal templates and how the resulting HP1-nucleosome complex is regulated are poorly understood. Here we show that binding of the major *S. pombe* HP1 protein, Swi6, to methylated nucleosomes drives a switch from an auto-inhibited state to a spreading competent state. In the auto-inhibited state, a histone mimic sequence in one Swi6 monomer blocks methyl mark recognition by the chromodomain of another monomer. Auto-inhibition is relieved by recognition of two template features, the H3K9 methyl mark and nucleosomal DNA. Single-particle reconstruction of the Swi6-nucleosome complex by cryo-EM reveals the architecture of the spreading-competent state in which two unbound chromodomain sticky ends are exposed. The conditional activation of Swi6 uncovered here is reminiscent of other auto-inhibition controlled molecular assemblies such as actin nucleation and opens up a new class of mechanisms to explain the diverse functions of HP1 proteins.

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Single-cell epigenetics reveals that cellular variations in histone acetylation predict cellular phenotypic variations.

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Even isogenic clonal cells in culture dishes, which provide cells with a biochemically and biophysically controlled environment, tend to display extremely large variations in properties such as morphology and cytoskeleton organization. Here we developed, tested, and validated a

new assay to measure global acetylation on histones H3 and H4 of individual cells and simultaneously measure, in the same cells, a wide range of cell phenotypic properties, including cell and nuclear morphology, cytoskeletal content and organization, cell-cycle phase, and chromosomal organization. These measurements show that isogenic clonal cells of similar DNA content and same cell-cycle phase still display large variations in H3 and H4 acetylation and that these epigenetic variations predict phenotypic variations, in particular nuclear size and actin cytoskeleton content and organization, but not cell size or cell shape. To demonstrate its versatility, this assay is also used to quantify the complex interplay between cell cycle, epigenetics, and phenotypic variations following pharmacological treatments at single-cell resolution.

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Nuclear IGF-1 receptor phosphorylates histone H3 and recruits chromatin remodeling factors.

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Recent findings suggest that the insulin like growth factor 1 receptor (IGF-1R) can translocate from the plasma membrane into the cell nucleus. In the nucleus it was found to associate with regulatory genomic elements to induce transcription of oncogenes in highly malignant cells. Studies have also shown an enrichment of nuclear IGF-1R in highly aggressive tumours. The mechanism by which the nuclear translocation occurs and the mechanism of how nuclear IGF-1R mediates its function still remains elusive. In this study we aim to elucidate the role of nuclear IGF-1R regarding chromatin remodeling and its biological effect. We here show that nuclear IGF-1R associates with histone H3 and phosphorylates it in cancer cells. Phosphorylated histone H3 is recognized by histone acetyl transferases which acetylates the pre-phosphorylated histone H3. Acetylated histone H3 functions as a platform for the Brahma-related gene 1 chromatin remodeling factor where it can mediate its function. Taken together, this study show that IGF-1R can regulate histone modifications and chromatin dynamics in a more direct manner by phosphorylating histone H3. In contrast to previous studies, which focused on IGF-1R at the cell surface, we here show a new and novel function for IGF-1R in the cell nucleus. An increased knowledge about the role of nuclear IGF-1R would enable the development of novel cancer therapies.

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The N-terminal tail of the histone variant H3.3 is required for its early deposition into chromatin.

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Histone variant H3.3 is, in contrast to canonical H3.1 and H3.2, synthesized throughout the cell cycle and incorporated into nucleosomal chromatin in a replication-independent manner, notably at transcriptionally active sites. Despite recent identification of several H3.3 chaperones, the pathways of deposition of H3.3 into chromatin remain unclear. In our previous work, we have used transiently transfected primary cells to determine the behaviour of newly synthesized epitope-tagged H3.3. We show that exogenously expressed, newly synthesized H3.3 is recruited to promyelocytic leukemia protein (PML) bodies together with histone H4, in a DAXX dependent manner. PML bodies also contain other H3.3 chaperones including ATRX, ASF1A and HIRA, supporting a working model of DAXX-mediated targeting of (H3.3-H4) dimers to PML bodies, where (H3.3-H4) is available for deposition into chromatin by the distinct chaperones.

Here, we investigate the role of the N-terminal tail of H3.3 on the deposition of this histone variant into chromatin shortly after synthesis. We show that in the absence of the amino acid stretch (3-35), truncated exogenous H3.3 (referred to as H3.3[core]) is unable to incorporate into chromatin shortly after induction of expression. However, we find in fluorescence recovery after photobleaching experiments that H3.3[core] is targeted (together with H4) by DAXX to PML bodies, where it resides for several days as a highly mobile pool, whereas full-length H3.3 is fully incorporated into chromatin in a much shorter time frame. HIRA and ASF1A are also found at PML bodies enriched in H3.3[core]. Deletion of residues 3 to 26 does not affect the kinetics of H3.3 deposition into chromatin, narrowing a critical domain of the N-terminal tail of H3.3, containing post-translationally modifiable residues which may be important for chromatin deposition. Using deletion and single amino acid mutation analysis, we are scanning this region to identify the critical residue(s) necessary for deposition of H3.3 shortly after its synthesis, and the chaperones involved in this process.

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ATM-mediated phosphorylation of the chromatin remodeling enzyme BRG1 modulates DNA double-strand break repair.

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Although ATP-dependent chromatin remodeling complexes have been implicated in DNA double-strand break (DSB) repair and damage responses, the regulatory mechanisms that control their functions are largely unknown. Here, we show that ATM mediates the phosphorylation of BRG1, the catalytic ATPase of the SWI/SNF complex, at Ser-721 rapidly after DNA damage. Phosphorylated BRG1 binds gamma-H2AX-containing nucleosomes to form the repair foci. The Ser-721 phosphorylation of BRG1 is critical for binding gamma-H2AX-containing nucleosomes and stimulating gamma-H2AX formation and DSB repair. While the bromodomain of BRG1, which recognizes acetylated histones, is essential for DSB repair and transcription, the phosphorylation of Ser-721 is dispensable for transcriptional activity. This work establishes BRG1 as a novel and functional ATM substrate and reveals that the ATM-mediated phosphorylation of this chromatin-remodeling enzyme not only modulates DNA repair but also defines the specificity for DNA repair distinct from transcription.

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The SCF^{Slimb} ubiquitin-ligase regulates Cap-H2 levels to suppress condensin II-mediated nuclear reorganization.

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Condensin complexes play vital roles in chromosome condensation during mitosis and meiosis. Condensin II uniquely localizes to chromatin throughout the cell cycle, and in addition to its mitotic duties, modulates chromosome organization and gene expression during interphase. Mitotic condensin activity is regulated by phosphorylation, but mechanisms that regulate condensin II during interphase are unknown. Here, we report the first such mechanism: condensin II is inactivated when its subunit, Cap-H2, is targeted for degradation by the SCF^{Slimb} ubiquitin-ligase complex, and disruption of this process dramatically changes interphase chromatin organization. Inhibition of SCF^{Slimb} function reorganizes interphase chromosomes

into dense, compact domains and disrupts homolog pairing in both cultured *Drosophila* cells and *in vivo*, but is rescued by condensin II inactivation. Furthermore, Cap-H2 stabilization distorts nuclear envelopes and misregulates localization of Cid/CENP-A on interphase chromosomes. Therefore, SCF^{Slimb}-mediated down-regulation of condensin II is required to maintain proper organization and morphology of the interphase nucleus.

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Epigenetic control of the Osterix gene promoter activity during mesenchymal cell differentiation.

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Osterix (Osx) is a key regulator of osteoblast differentiation as it controls transcription of a number of bone-phenotypic genes. Here, we investigate epigenetic mechanisms that govern Osx gene transcription during the engagement of pluripotent mesenchymal cells to the osteoblast lineage. Epigenetic posttranslational modifications and binding of regulatory proteins to chromatin were determined by chromatin immunoprecipitation. DNA methylation was determined by cleavage with methylation-sensitive restriction endonucleases and immunoprecipitation of methylated DNA. Changes in Osx mRNA were assessed by RT-qPCR. The effect of the histone-deacetylase (HDAC) inhibitor TSA was verified by Western blot. We find that the Osx gene promoter in osteoblastic cells exhibits histone modifications normally associated with transcriptional activity (H3Ac/H3K4me3) together with minimal DNA methylation. In contrast, non-bone cells show histone modifications associated with transcriptional repression (H3K9me3/H3K27me3) and elevated DNA methylation. Interestingly, uncommitted pluripotent mesenchymal cells show a combination of both types of histone modifications (H3K4me1/H3K27me3), accompanied by reduced, but still significant, DNA methylation. Commitment of these cells to either bone or muscle lineages is accompanied by changes in chromatin modifiers including HDACs, histone-methyltransferases (HMTs) and DNA-methyltransferases (DNMTs) at the Osx promoter in tight correlation with the transcriptional status. DNMT1, DNMT3a, HDAC2, and HDAC4 are enriched during Osx silencing in muscle cells, whereas these proteins are released from the Osx promoter during osteoblast differentiation, together with demethylation of DNA. Also, inhibition of HDAC and DNMT activities in uncommitted mesenchymal precursors induces Osx mRNA expression. Thus, Histone and DNA modifications represent key regulatory mechanisms during control of Osx gene expression in mesenchymal-derived cell lineages, thereby controlling the osteoblastic cell fate choice.

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Identification and characterisation of monoallelic expression upon differentiation of mouse embryonic stem cells to neural progenitor cells.

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Monoallelic gene expression refers to the transcription of a gene from only one of its two homologous alleles. Well-characterised examples include X-chromosome inactivation and genomic imprinting, however a third category, random autosomal monoallelic expression, remains poorly understood. We performed an allele-specific RNA-sequencing screen in clonal

populations of hybrid mouse embryonic stem cells (ESC) and neural progenitor cells (NPC) to identify monoallelically expressed genes in both cell types.

We identified 138 genes that switched to a monoallelic gene expression pattern during ESC to NPC differentiation. The expressed allele was independent in each clone and was not coupled to the genotype or the parent of origin, consistent with a random choice of allele expressed. Candidate genes were validated by PCR amplification of exonic SNPs followed by Sanger sequencing.

The 138 monoallelic candidate genes were randomly distributed throughout the genome with no apparent clustering observed. Global analysis showed the distribution of transcript levels of monoallelic genes was not significantly different to overall gene expression levels, suggesting that allelic bias is not limited to low expression levels. Furthermore, quantitative real-time RT-PCR analysis of genes that were monoallelic in a subset of clones and biallelic in others, revealed no correlation between the number of active alleles and the total level of gene expression. This suggests that transcriptional compensation must take place, and that monoallelic gene expression is not a bona-fide mechanism to regulate the level of a particular gene product in a cell.

A key question is how this form of random autosomal monoallelic expression is maintained across cell divisions. Ongoing genomic analysis as well as molecular characterisation of the epigenetic modifications marking the active and inactive allele of monoallelic genes will be presented.

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DNA methylation in the miR-210 gene influences HIFa1 binding.

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microRNA-210 (miR-210) has been reported to involve in hypoxia-related events. Several studies suggested that miR-210 expression can be regulated by hypoxia-inducible transcription factors-1alpha (HIF-1a) during hypoxia. Furthermore, by using the bioinformatics analysis, the promoter of the miR-210 gene contains several CpG-rich regions. However, there are no reports about epigenetic regulation of miR-210 gene. We investigated the role and mechanism of miR-210 in the cardiovascular system. We first found miR-210 gene was upregulated in vascular smooth muscle cells (VSMC) and human umbilical venous endothelial cells (HUVEC) when they were exposed to oxidized low-density lipoprotein (oxLDL). By combining with 5-Aza-2'-deoxycytidine treatment or DNA methyltransferase 3b knockdown, we found that miR-210 gene expression could be epigenetically regulated by oxLDL. By using the bisulfite sequencing assay, the decreased methylation levels of miR-210 gene was identified in vitro and in vivo. This methylation change could affect the HIF-1a-regulated miR-210 gene expression. Furthermore, over-expression or knockdown of miR-210 influenced the ability of oxLDL-mediated VSMCs and HUVECs migration. According to the bioinformatic prediction, SPRED2 (an inhibition regulator in ERK activation) could be a major miR-210's target gene involved in cell migration. Our reporter assays using wild-type and mutant 3' UTR of SPRED2 gene confirmed that SPRED2 was a novel direct target of miR-210. Over-expression or knockdown of SPRED2 affected oxLDL-mediated ERK/c-Fos/MMPs pathways in cell migration. Altogether, the data suggest that epigenetic regulation of miR-210 gene and its effect on cell migration are involved in the cardiovascular diseases formation.

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X. laevis ISWI remodels nucleosomes through a random-walk.

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The chromatin remodeler ISWI from *Xenopus laevis* is capable of repositioning clusters of nucleosomes to create well-ordered arrays or moving single nucleosomes from the center of DNA fragments toward the ends without disrupting their integrity. Using standard electrophoresis assays we have monitored the ISWI-catalyzed repositioning of different nucleosome samples each containing a different length of DNA symmetrically flanking the initially centrally positioned histone octamer. We find that ISWI moves the histone octamer between distinct and thermodynamically stable positions on the DNA according to a random walk mechanism. By comparing the number of positions observed in experiments with different nucleosomes we have determined that the spacing between positions is ~12 bp, which is similar to the period of certain dinucleotide spacings in nucleosome positioning sequence on the DNA (~10 bp) and the spacing between histone:DNA contacts within the nucleosome core particle (~10 bp). Thus, we believe that the distinct species that we observe correspond to nucleosomes in which the histone octamer has been shifted relative to the periodicity of the positioning sequence by 1 set of histone:DNA contacts. Through the application of a novel spectrophotometric assay for nucleosome repositioning we were further able to determine the macroscopic rate of nucleosome repositioning by ISWI and an estimate of the efficiency at which ATP binding and hydrolysis are coupled to this activity.

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Regulation of HDAC2 by of the tyrosine kinase c-Abl, a new mechanism of regulation of the gene expression.

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The transcriptional regulation of genes is a dynamic process that involve the coordinated action of multiple regulatory proteins. A group of them, known as HDACs induce the deacetylation of histone for promote a more compact chromatin conformation leading to gene repression. HDAC2, a member of this family, had demonstrated to participate in the repression of genes during neurodegenerative process. Although the role of HDAC2 in gene repression is clear, the molecular pathways involved in this process are not yet clearly understood. In this respect, our laboratory has previously described that the tyrosine kinase c-Abl is crucial in the cell signaling in neurodegenerative process such as exists in Alzheimer's Disease. In this context, our results show, for the first time, that HDAC2 is modulated by the c- Abl tyrosine kinase, which is a potential mechanism to explain the role of HDAC2 in neurodegeneration.

We results show that c-Abl activity increases both protein levels and deacetylase activity of HDAC2, besides c-Abl regulates histone H3 acetylation levels. In the same line, treatments with Imatinib (c-Abl inhibitor) reduces both protein levels and activity of HDAC2. Interestingly, the treatments with Imatinib decreases the HDAC2 association to the promoter of the neuronal genes and increases the H3 acetylation levels. Besides, in the experimental Alzheimer Diseases (AD), we found in both primary culture of neurons treated with A β oligomers (oA β), as well as in the APP^{swe}/PSEN1 Δ E9 mice, an increase of protein levels as well as the enzymatic

activity HDAC2 which depend on c-Abl activity. In addition, c-Abl induces the tyrosine phosphorylation of HDAC2, a postraductional modification that has not been described previously for this protein, that is important in the regulation of activity of HDAC2. Based on these results, we suggest that c-Abl might regulate the neuronal gene expression through tyrosine phosphorylation of HDAC2.

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Chromatin structural changes by nuclear c-Abl-mediated tyrosine phosphorylation through histone deacetylation.

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Chromatin structure is dynamically changed by histone modifications, and chromatin dynamics is involved in transcriptional regulation. c-Abl tyrosine kinase is a proto-oncogene product and ubiquitously expressed. Cytoplasmic c-Abl plays important roles in cell proliferation, differentiation, and adhesion. c-Abl has three nuclear localization signals (NLSs) and translocates into the nucleus. However, the function of nuclear c-Abl is poorly understood. In this study, we examined the relationship between nuclear c-Abl and chromatin structural changes.

To examine whether nuclear c-Abl was involved in chromatin structural changes, cells were treated with adriamycin (ADR), which enhances nuclear translocation of c-Abl. The level of chromatin structural changes was significantly increased by ADR treatment and this increase was repressed by imatinib, an Abl inhibitor. Moreover, transfection with c-Abl induced chromatin structural changes. Intriguingly, the oncogenic Bcr-Abl also changed the chromatin structure. c-Abl- and Bcr-Abl-induced chromatin structural changes were enhanced by ADR, which promoted their nuclear translocation. Furthermore, NLS-c-Abl, which is tagged with fourth NLS to localize to the nucleus, induced drastic chromatin structural changes compared with intact c-Abl. These results suggest that nuclear c-Abl plays a role in chromatin dynamics.

Next, we examined the involvement of histone modifications in c-Abl-mediated chromatin structural changes. By immunofluorescence microscopy we detected repressive histone modifications including an increase in histone H3 trimethylated on lysine 9 (H3K9Me3) and a decrease in H3K4Me3, H3K14Ac (acetylated on lysine 14), and H4K16Ac in cells exhibiting chromatin structural changes strongly induced by NLS-c-Abl. Since the most prominent histone modification among them was a decrease in H4K16Ac, we examined the relationship of histone deacetylation with nuclear c-Abl-induced chromatin structural changes. A broad inhibitor of histone deacetylases, trichostatin A (TSA), inhibited both H4K16 hypoacetylation and chromatin structural changes induced by NLS-c-Abl. Inhibition of NLS-c-Abl by imatinib was found to block H4K16 hypoacetylation and chromatin structural changes. Furthermore, we observed repressed transcription of the RASSF1A tumor suppressor upon NLS-c-Abl expression. Repression of RASSF1A transcription is known to involve repressive histone modifications. Taken together, these results suggest that nuclear c-Abl-mediated tyrosine phosphorylation induces chromatin structural changes through histone deacetylation, which may affect gene expression.

In conclusion, we show that nuclear tyrosine phosphorylation by c-Abl regulates chromatin dynamics through histone deacetylation. The oncogenic Bcr-Abl-induced chromatin structural changes and nuclear c-Abl-repressed transcription of the tumor suppressor RASSF1A lead us to speculate that Abl-mediated chromatin structural changes are involved in tumorigenesis. Now, we are trying to identify the nuclear substrate of c-Abl and to clarify the mechanism of changing the chromatin structure.

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Emerin regulates chromatin architecture and gene expression during myogenic differentiation in cooperation with Histone Deacetylase 3 (HDAC3).

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Spatial organization of the genome is critical for maintaining cell-specific gene expression and ensuring proper cellular function. Changes in chromatin architecture during development and tissue regeneration are important for regulating proper spatial and temporal gene expression throughout differentiation. It is well established that repressed chromatin preferentially associates with the nuclear lamina and that dynamic association of gene loci with the nuclear lamina contributes to temporal regulation of gene expression. However, the molecular mechanisms underlying the formation and maintenance of repressive chromatin at the nuclear lamina, as well as the spatial organization of developmentally regulated gene loci, remain poorly understood. Here we show that the inner nuclear membrane protein emerin associates with loci containing important myogenic differentiation factors in mouse muscle stem cells, including *Myf5*, *MyoD* and *Pax7*. These loci are transcriptionally silent when localized at the nuclear lamina. During muscle differentiation, *Myf5* and *MyoD* loci move to the nuclear interior concomitant with their transcriptional activation, while the *Pax7* locus, which promotes stem-cell identity, shifts from the nuclear interior to the nuclear lamina upon its transcriptional repression. Localization of *Myf5*, *MyoD* and *Pax7* to the nuclear lamina requires emerin and HDAC3. We also found these chromatin architecture changes are dependent on emerin's activation of HDAC3, as loss of *Myf5* localization to the nuclear lamina can be rescued by treatment with a small-molecule activator of HDAC3. Collectively these data support a model whereby emerin facilitates repressive chromatin formation at the nuclear lamina by activating the catalytic activity of HDAC3. We hypothesize the formation or maintenance of this repressive environment at the nuclear lamina by emerin contributes to the coordinated temporal expression of myogenic differentiation genes through modulating chromatin architecture, ensuring proper myogenic differentiation during muscle regeneration. This model illustrates the importance of interactions between the nuclear lamina and epigenetic modifiers in regulating developmental transitions, and provides insight into the molecular mechanisms responsible for the impaired muscle regeneration characteristic of X-linked Emery-Dreifuss Muscular Dystrophy, which is caused by mutations in emerin.

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Polycomb-Group proteins (PcG) Ezh1 and Ezh2 differentially regulate PSD-95 gene expression in developing hippocampal neurons.

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Introduction: Polycomb-Group proteins (PcG) are key epigenetic regulators that can either silence or activate genes. Little is known about the role of PcG in controlling genes associated with neuronal plasticity. We analyzed the expression of PcG proteins, Ezh1 and Ezh2, during hippocampal development and their contribution in transcriptional regulation of the PSD-95 gene.

Material and Methods: mRNAs were analyzed by qRT-PCR and proteins by Western-blot at distinct development stages: rat hippocampal tissue (from E18 to P90) and cultures (from 2 to 20 DIV). Ezh1 and Ezh2 knock-down was realized using shRNAs lentiviral particles, electroporation by AMAXA® and Calcium Phosphate method. Chromatin Immunoprecipitation (ChIP) assays were realized in hippocampal tissue and quantified by qRT-PCR.

Results: We report that Ezh1 and Ezh2 are expressed in neurons and astrocytes, however, their expression levels depends on the developmental stage analyzed: Ezh2 expression is high during development and then declines, whereas Ezh1 expression persists with development. A switch in the association to PSD-95 gene promoter from Ezh2 to Ezh1 is also prominent during development, concomitant with specific changes in epigenetic marks. We also found that Ezh2 depletion in early neurons increased PSD-95 expression, whereas Ezh1 knockdown in mature neurons reduced expression of this gene.

Discussion: Our findings reveal differential roles for Ezh1 and Ezh2 in epigenetic regulation during neuronal development and on the PSD-95 gene promoter, suggesting an important role for PcG proteins in developmental-dependent neuronal plasticity.

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Maternal high-methyl diet alters DNA methylation in mammary tissues of female rat offspring.

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Prenatal and early neonatal nutrition is an important factor for growth and health in early and later life. It has been suggested that maternal diet affects metabolic processes in the mammary glands of offspring. Lipotropes (methionine, choline, folate, and vitamin B12) are methyl donors and cofactors essential for one-carbon metabolism, a process which provides methyl groups for biological methylation pathways. We hypothesized that a maternal lipotrope-additive diet (LAD) would alter global DNA methylation and methylation-related gene expression in the mammary glands of female rat offspring. Throughout gestation and lactation, dams were fed either a control diet (CD; AIN-93G) or LAD which contained 5 times more choline, folate, and vitamin B12, and 1.8 times more methionine. The female offspring were weaned onto the CD and organized into two groups based on the dams' diet. These female offspring were then mated, bred, and at weaning their mammary tissues were harvested for analysis of global DNA methylation and gene expression. The level of DNA methylation was significantly increased by 22.1% in the female offspring of LAD-fed dams ($P < 0.05$). There was also a tendency toward lower mRNA expression of DNA methyltransferase 1 in the LAD group ($P = 0.09$). Interestingly, protein expression of fatty acid synthase (FASN) was increased in the LAD rats. Our study demonstrates that supplementation of dietary lipotropes during pregnancy and lactation increases global DNA methylation and protein expression of FASN, suggesting that maternal high-methyl diet may modulate DNA methylation and metabolism in the mammary glands of offspring. Future studies are warranted to establish the putative mechanistic link responsible and its possible influence on the growth and health of offspring.

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Epigenetic silencing of key bone phenotypic genes during neuronal development.

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Runx2 and Osterix transcription factors are essential for bone development and osteoblastic differentiation as they regulate the expression of key bone lineage-specific genes. In osteoblasts, epigenetic mechanisms that lead to transcriptional activation of these genes have been described, but their silencing in non-osteoblastic cells remains to be elucidated. In neurons, the regulatory networks that may trigger transcription of osteoblast-specific genes are active, although are unable to promote bone-related gene expression. We aim to evaluate the contribution of epigenetic mechanisms during silencing of osteoblast-specific genes in neuronal cells. Hippocampal tissue from E18, P10, P30 and P90 rats was obtained to perform Chromatin Immunoprecipitation (ChIP) studies using antibodies directed against covalent histone modifications and chromatin modifying enzymes. It was found that Runx2 and Osterix promoter sequences exhibit enrichment of the repressive H3K27me3 epigenetic mark in all the stages analyzed. Additionally, we detect the presence of Polycomb-group proteins Ezh1 and Ezh2, binding in a developmental stage-dependent manner at Runx2 and Osterix promoters, correlating their expression patterns. We also find the heterochromatin-associated H3K9me3 epigenetic mark in the adult stage, tightly correlated with decreased histone H3 acetylation and enrichment of Histone Deacetylases HDAC1 and HDAC2. Finally, we find a strong DNA methylation pattern at bone-specific genes (e.g. Osteocalcin) that are expressed at late stages during bone formation. Together, our results support a model where epigenetic post-translational modifications ensure the silencing of non-neuronal genes during neuronal differentiation.

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Epigenetic regulation of PSD95 gene expression during hippocampal development.

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Introduction: Data from us and others indicates that increased expression of PSD95, the major anchoring protein for excitatory receptors at the post-synaptic membrane, is a key contributor for the limited plasticity in the adult brain. Here we establish a pattern of histone tail modifications that are associated with PSD95 expression in hippocampal tissue during development. Also, we investigated if PSD95 expression is controlled by methylation of its DNA promoter sequence.

Methods: E18-P10-P30-P90 to obtain mRNA, protein or chromatin. Western blot and qRT-PCR were conducted to determine the expression of PSD95 at the different developmental stages. Chromatin immunoprecipitation (ChIP) was performed using antibodies for activation or repression marks and quantified by qPCR with specific primers for the PSD95 promoter sequence. Genomic DNA was extracted and treated with bisulfite to define DNA promoter sequence methylation. Bisulfite converted DNA was sequenced to analyze methylation status.

Results: Expression of PSD95 mRNA and protein levels increases gradually with hippocampal development. Concomitant with enhanced PSD95 expression during development, active epigenetic marks are associated to this promoter in mature neurons. Repressive marks

associated to the PSD95 promoter are never detected. Methylation of the PSD95 DNA promoter sequence was also not present at any stage.

Conclusions: Expression of the PSD95 gene during hippocampal development is regulated by histone tail modifications on histones associated to its promoter, but not by methylation of the promoter DNA sequence. We were able to identify a specific pattern of histone tail modifications that correlates with PSD95 expression during hippocampal development.

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Tissue Development and Morphogenesis I

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Gastrulation in high-resolution: New insights into an important process of development.

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Gastrulation is a complex orchestration of movements by cells that are specified early in development. Until now, it was thought that lateral rearrangement of endoderm cells by convergent extension was the main contributor to sea urchin archenteron elongation. Our project characterizes, at high resolution, the repertoire of cellular movements contributing to the length of gut. We have performed cell transplantation to live image and analyze a subset of labeled endoderm cells in the optically clear sea urchin embryo. We have found that the endoderm cells that initially invaginate remain contiguous throughout extension, so that, if convergent extension is present, it is not a major contributor to elongation. We have also found, unexpectedly, that endoderm cells proliferate as they move to elongate the archenteron. Our descriptive studies of the cellular processes during gastrulation have allowed us to begin investigating their molecular control. The endomesoderm gene regulatory network (GRN) describes the cell fate specification of the future larval gut; however, the GRN does not describe specific cell biological events driving morphogenesis. We plan to dissect the transcriptional circuitry of the GRN responsible for the cell biological events, such as changes in polarity and adhesion, of gastrulation. Our ability to connect the endomesoderm GRN to the morphogenetic events of gastrulation will provide a framework for characterizing this remarkable sequence of cell movements in the simplest of deuterostome models at an unprecedented scale.

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The regulation of the organ morphology by cytoskeletal remodeling.

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All organs arise from their respective germs through reciprocal interactions between epithelium and mesenchyme in the developing embryo. Their morphologies are shaped by accumulation of spatiotemporally controlled cellular behaviors such as cell proliferation, differentiation and movement. In this study, we investigated how complex cellular behaviors were coordinated during morphogenesis by the quantitative kinetic analysis of developing tooth germ as an organ model.

We first took a long-term live imaging of developing tooth germ using the confocal laser microscopy, visualizing three-dimensional cell dynamics with the spatio-temporal pattern of

proliferation. We subsequently constructed four-dimensional analysis system of organ morphogenesis, which reproduced morphological changes and complex cell behaviors such as cell movements and divisions on the computer by cell tracking. The quantitative analysis using this system revealed that the spatial relative position of the cells hardly moved on the growth-arrested area, although the cells actively moved with cell division on high proliferative area, suggesting that cell proliferation and motility are orchestrated in developing tooth germ. We therefore investigated the molecular mechanism underlying these coordinated cell behaviors during tooth morphogenesis. Since cytoskeleton reorganization is essential for cell shape change, migration and mitosis, we focused on the regulatory mechanism via the cytoskeleton remodeling and found that a phosphorylated (inactivated) cofilin, which is a member of the actin depolymerization factor (ADF)/cofilin family, was localized in low proliferative area in tooth germ. We also found that these phosphorylated cofilins localized in low proliferative area of only tooth germ but also hair germ through development process. Fluorescence recovery after photobleaching (FRAP) analysis revealed that actin-filament assembly was inhibited at the regions where highly phosphorylated cofilins were localized. Furthermore, proliferation and migration of epithelial cells were significantly inhibited according to the elevation of phosphorylation level of cofilin.

These results suggested the possibility that the morphological changes in tooth germ development were controlled by the spatiotemporal coordinates of cell proliferation and cell movement via regulation of actin remodeling.

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Rab8a-regulated Wnt-Gpr177 Exocytic Trafficking and Stem Cell Niche Development in Mouse Small Intestine.

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Being a crucial morphogen driving mammalian tissue development, the scheduled secretion of Wnt ligands (or the wingless-type MMTV integration site family proteins) calls for high regulation. However, in contrast to the extensive studies done on Wnt downstream signaling pathways, relatively less is known about the molecular basis regulating Wnt ligand secretion in Wnt-producing cells. We have explored the role of Rab8a, a master regulator of intracellular protein endo- and exo-cytosis, in regulating Wnt transport and intestinal stem cell niche development. We demonstrate that in absence of Rab8a, the mouse intestinal crypts exhibit a phenotype indicating perturbed Wnt signaling. The Rab8a-deficient crypts are enlarged with an increased population of stem and transit amplifying cells. In contrast, Paneth cells are drastically reduced with ultrastructurally abnormal endoplasmic reticulum and secretory vesicles. Using a Wnt signal reporter, the Axin2-LacZ mice, we revealed a reduced Wnt-receiving zone in Rab8a-deficient intestinal crypts, indicating an affected gradient formation. We propose that Rab8a regulates Wnt secretion in the intestinal stem cell niche, putatively by recruiting the Wnt transporter GPR177 into a highly regulated secretory route. These data shed light on factors contributing to stem cell niche development and maintenance, and will open up new prospects in manipulating Wnt production that has translational potentials.

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Towards an understanding of the role of apical mitosis in pseudostratified epithelia.

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Pseudostratified epithelia feature a single layer of highly elongated cells, whose nuclei occupy positions all along the apico-basal axis, creating the stratified appearance. This cellular organization is prevalent in rapidly proliferating embryonic tissues during development of a

broad range of species. A hallmark of pseudostratified epithelia is interkinetic nuclear migration (IKNM). IKNM comprises of stochastic nuclear movements, punctuated by rapid migration towards the apical surface in G2. This results in apical localization of all mitoses. Despite extensive studies on mechanisms and kinetics of IKNM, one fundamental questions remains unanswered: What is the significance of the apical division for tissue development? Why do nuclei migrate towards the apical side prior to each division? One possible reason could be the apical localization of the centrosome, which serves as a basal body of the primary cilium in the interphases. We are therefore currently investigating whether apical centrosome location is necessary and/or sufficient for apical mitosis.

We found that until shortly before mitotic entry, the centrosome is attached to the apical surface via short, dynamic astral microtubules. Interference with this pool of microtubules leads to mispositioning of the centrosome towards more basal locations. Subsequently, chromosome condensation and nuclear envelope breakdown occur at more basal positions. Nevertheless, rapid apical migration of nuclei still takes place but under these conditions nucleus and centrosome travel together.

To understand whether centriole number plays a role in apical mitosis, we have decreased the amount of centrioles by inhibiting a centriole duplication pathway. However, this manipulation also does not abolish rapid apical migration. Cells still divide at apical locations, often featuring monopolar spindles, significantly increasing the time needed for successful mitosis. Additionally, centriole overduplication leading to extranumerary, non-apical centrosome-like structures does not inhibit apically directed IKNM.

In summary, we have shown that neither centrosomal localization nor centriole number influences rapid nuclear migration during IKNM. Restricting all mitoses to the apical side might therefore be needed to facilitate rapid, vertically oriented divisions that are not possible in case divisions would happen all along the epithelium. Thus IKNM may serve to sequester nuclei of different cell cycle phases and create a distinct germinal zone to the tissue. We are currently testing this hypothesis.

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Stimulation of angiogenesis and protection of vascular permeability by C-peptide *in vitro* and *in vivo*.

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Human C-peptide has a beneficial effect on the prevention of diabetic neuropathy, nephropathy, and vascular complications; however, its role in angiogenesis and in protection against increased vascular permeability in diabetic retinopathy remains unclear. Increased vascular permeability and defective angiogenesis due to the hyperglycemia-induced elevation of vascular endothelial growth factor (VEGF) contributes to diabetic retinopathy, a leading cause of blindness. We investigated the potential roles of C-peptide, in parallel with VEGF, in angiogenesis and vascular permeability using human umbilical vein endothelial cells (HUVECs) and streptozotocin diabetic mice. C-peptide induced migration and capillary-like networks formation of endothelial cells in dose-dependent manners, with the maximal effect at 0.5 nM, through pathways involving extracellular signal-related kinase 1/2, Akt, and nitric oxide. Consequently, C-peptide enhanced angiogenesis *in vivo* as demonstrated by the Matrigel plug assay. We also demonstrated the preventive role of C-peptide against VEGF-induced vascular permeability by inhibiting the generation of intracellular reactive oxygen species (ROS), ROS-dependent stress fiber formation, and the disassembly of the adherens junction protein VE-cadherin. Consistently, intravitreal injection of C-peptide prevented extravasation of FITC-dextran in the retinas of diabetic mice. Thus, our results suggest that C-peptide plays a novel

angiogenic role that may have significant implications in reparative and therapeutic angiogenesis in diabetes, and it protects against VEGF-induced vascular permeability in diabetes; this suggests that C-peptide replacement is a promising therapy to prevent diabetic retinopathy.

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Wounded cells drive rapid epidermal repair in the early *Drosophila* embryo.

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A striking feature of wound repair is that its efficiency decreases with age. In embryos, epidermal lesions heal fast and with no scars, whereas adult wounds heal slowly and with scar formation. In humans, the transition from rapid to slow wound repair occurs at the final stages of fetal development. To determine if a similar transition occurs in *Drosophila*, we quantified wound closure in the epidermis of early and late embryos (3 and 14 hours after egg laying, respectively). We developed computational image analysis tools to automate the identification and tracking of the wound margin in time-lapse movies, facilitating the analysis of a large number of samples.

Wound repair was significantly faster in the epidermis of the early embryo, where wounded structures sustained greater mechanical tension than in the late embryo. A contractile actin-myosin II ring formed around early and late wounds. In the early embryo, ring formation was followed by the assembly of dense actomyosin networks at the medial-apical surface of the wounded cells, in parallel with rapid wound contraction. Laser ablation demonstrated that medial actomyosin networks in the wounded cells were contractile and pulled on the wound margin. In the early embryo medial networks were disassembled as they contracted, whereas in the late embryo the total levels of actin and myosin remained constant during ring contraction. Taken together, our data indicate that wounded cells in the early embryo trigger a program of apical constriction that contributes to rapid wound closure. These experiments also suggest that actomyosin network architecture may determine the mechanisms of network contraction and the magnitude of the forces generated. We are currently using super-resolution microscopy, particle tracking and 3D image analysis to investigate the molecular architecture and mechanisms of assembly of the contractile networks associated with wound closure, as well as the association between mechanical changes in the epidermis and the transition from fast to slow wound repair in the *Drosophila* embryo.

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Laser scanning-based tissue autofluorescence/ fluorescence imaging (LS-TAFI), a new technique for analysis of microanatomy in whole mount tissues.

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Intact organ structure is essential in maintaining tissue-specificity and cellular differentiation. Subtle physiological or genetic variations lead to changes in microanatomy that if persist, would have functional consequences and could easily be masked by the heterogeneity of tissue response. Current techniques rely on histological sections requiring sample manipulation that

are essentially two-dimensional (2D). We have developed a method for 3D imaging of whole mount, un-sectioned mammalian tissues to elucidate distinct and detailed micro- and macro-anatomies in mammalian organs and embryos. We analyzed intact or dissected organ whole mounts with laser scanning confocal microscopy-based tissue autofluorescence/fluorescence imaging (LS-TAFI). We report clear visualization of microstructures within murine mammary glands and mammary tumors and other tissues without the use of immunostaining, and without probes or fluorescent reporter genes. Combining autofluorescence with reflected light signals from chromophore-stained tissues allowed identification of individual cells within 3D structures of whole mounted organs. This technique could be also useful for rapid diagnosis of human clinical samples.

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Investigating the role of Shroom: Rock signaling module in cellular morphogenesis.

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Cellular morphogenesis is critical for diverse cellular behaviors and processes like cell adhesion, migration, shape and polarity and defects in these processes lead to malignant human conditions. This cellular remodelling is brought about by proteins that modulate the dynamics and organization of the cytoskeleton in a spatially restricted manner. Of the vast number of proteins that act on the cytoskeleton, Shroom family members have been implicated in, or are required for, several biological processes. Shroom, a class of Actin-associated proteins have been implicated in regulating cell and tissue architecture via activation of non-muscle Myosin II. This activity is restricted to its evolutionarily conserved motif, Shroom Domain 2(SD2) that binds directly to the Shroom Binding Domain(SBD) of Rho-kinase(Rock). Rock then activates MyoII contractility by regulating the phosphorylation status of Myosin light chain and brings about change in cell shape. My project uses structural, biochemical and cell biological assays to dissect the molecular nature of the Shroom:Rock interaction. The structure of Shroom SD2 domain has been shown to be an antiparallel coiled-coil dimer. The surface exposed residues in Shroom SD2 play an important role in mediating Shroom:Rock binding whereas the residues buried interiorly are required for dimerization. SD2 mutants that fail to bind Rock or dimerize also fail to cause apical constriction in MDCK cells. In order to map this Shroom-Rock interaction, we have mapped the SBD of Rock to a stretch of 79 amino acids in the coiled-coil region of Rock. This sequence is highly conserved across species, with the most conserved amino acid residues being surface exposed in the SBD crystal. We have generated mutations in these highly conserved residues in Rock SBD and tested for disruption of binding to Shroom SD2 via in vitro binding assays and co-localization with Shroom in vivo. Six different Rock SBD mutants have been identified that disrupt Shroom binding in vitro and prevent colocalization with Shroom3 in vivo. Efforts to recreate these Rock SBD mutants in full length Rock are being made to conduct cell-based apical constriction rescue assays after siRNA mediated knockdown of RockI and RockII in MDCK cells. These results will be supplemented by the crystal structure of Rock SBD which will facilitate better understanding of the molecular nature of Shroom:Rock interaction and ultimately cell shape change.

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Embryo Scale Integration of Forces and Gene Patterns Controlling Tissue Morphogenesis.

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The shape of a developing organism is generated by the activities of its constituent cells: cell growth and proliferation, cell movements and cell shape changes. We are particularly interested in shape changes.

Much has been learned about how cell differentiation programmes determine the morphogenetic activity of the cell, and we know the subcellular protein networks that are the effectors of cell behaviour. However, cells do not act in isolation, and morphogenesis requires an integration of forces across neighbouring cell populations. In addition to the subcellular mechanisms, it is now necessary to understand the supracellular integration of forces if we want to understand morphogenesis at the whole tissue or organismal level.

We are studying a simple morphogenetic process, the formation of the ventral furrow in the *Drosophila* embryo, which is well understood in its genetics and cell biology. The cells that form the furrow are the major force generators that drive furrow formation, but the neighbouring cells must respond and may contribute. We show by quasi-simultaneous time-lapse imaging of multiple-angle views of the embryo that the neighbouring cells undergo specific shape changes.

We are now studying the interdependence of cell behaviours and their coordination. We will use genetic and mechanical manipulations to reveal the underlying control circuits.

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A new model for investigating the regulation of epithelial-mesenchymal transition and cell plasticity in vivo.

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During development, morphogenetic rearrangements are often accomplished through a remarkable cellular transformation known as epithelial-mesenchymal transition (EMT). During EMT, epithelial cells dramatically alter polarity, change shape and often become migratory. These changes in polarity and behavior occur without cell division and are often reversible, making EMT an excellent model of cellular plasticity. EMT is essential for normal development and is also a hallmark of tumor metastasis. Despite its importance, we still lack a comprehensive knowledge of the integrated cell biology involved in coordinating EMT. To address this I am studying an EMT-like event required for the final steps of *Drosophila* wing development. When a fly emerges from its pupal case the wings are folded and contain a bilayer of epithelial cells. Over the next few hours the wing expands and the epithelial cells undergo a transformation that closely resembles early EMT. Eventually, all epithelial cells are lost from the mature wing. Though this process differs from other EMT events in that the cells do not ultimately differentiate or proliferate and are not protected from death, this system nevertheless shows many of the hallmark cellular and molecular features of EMT. To understand whether *Drosophila* wing maturation can be used as a model to identify new regulators of EMT *in vivo*, I am using a combination of live imaging and genetic manipulation to further characterize the behavior and ultimate fate of these cells. I have confirmed through confocal analysis of fixed and living maturing wings that the epithelial bilayer is disrupted, with loss of adherens junctions preceding changes in membrane integrity, apical-basal polarity, and acquisition of motile cell morphologies. In addition, knock down of EMT-relevant gene products during wing maturation results in adult wings with easily scored visible defects that can be used

for primary forward genetic screening. Furthermore, GFP fusion proteins and immunostaining demonstrate that these defects correlate with failure to complete the epithelial transition. Taken together, these studies will identify new mechanisms regulating the cell biology of epithelial transition and cellular plasticity.

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CFTR expression in iPSCs differentiated to airway epithelium.

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Cystic fibrosis (CF), the most common genetic disorder in the Caucasian population, has over 1,000 new cases diagnosed each year in the US alone. While >1800 mutations may lead to CF, the most prevalent mutation (found in ~70% of all CF chromosomes) is a phenylalanine deletion at codon 508 in the CF transmembrane conductance regulator (*CFTR*) gene (delF508). The CFTR protein is a cAMP-dependent Cl channel found in the apical membrane of epithelial cells that regulates the Cl gradient at the cell surface. While the *CFTR* gene has been well characterized, little research has been done on the regulation of CFTR throughout development. The studies described will evaluate CFTR expression in an *in vitro* developmental system using induced pluripotent stem cells (iPSCs) that are directed to differentiate along an endodermal lineage-specific pathway. An expression vector, (pcDNA3-CF1-EGFP), comprised of a pcDNA3 backbone and EGFP under the regulation of a ~1.7kb CFTR promoter element was constructed. To test whether this construct was specific for CFTR expressing cell types, it was transfected into immortalized human airway epithelial cells (16HBE14o-) that are known to express CFTR and primary human airway fibroblasts (FHTF-18) that are negative for CFTR expression. A pcDNA3-EGFP with EGFP under the regulation of a CMV promoter was used as a positive control and untransfected cells as a negative control. Expression of the pcDNA3-CF1-EGFP construct was observed in 16HBE14o-, but not FHTF-18, while expression of pcDNA3-EGFP was observed in both cell lines. No expression was detected in untransfected cells. These data indicate that the pcDNA3-CF1-EGFP construct contains the necessary elements for cell specific expression of CFTR. Studies are now underway to evaluate the expression of CFTR in the directed endodermal differentiation of iPSCs.

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Regulation of lung branching morphogenesis by dynamic luminal fluid flows.

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Branching morphogenesis of the lung is dynamic, highly regulated, and stereotyped, leading to an architecture that is conserved within a given species and critical for survival. Whereas the architecture of the airways is optimized for efficient conduction of air, development occurs while the lumen is filled with liquid, which is pushed throughout the airways via periodic peristaltic contraction of smooth muscle. Clinical observations and large animal models suggest a critical role for the (dynamic) regulation of transmural pressure in the lung, with dysregulation resulting

in hypo- and hyperplastic airways. However, experimental limitations have prevented precise control of the mechanical environment during development to distinguish the effects of dynamic transmural pressure from those due to peristaltic fluid flow. To address these shortcomings, we designed a microfluidic device to culture and apply dynamically-controlled transmural pressures within murine whole lung explants. Lungs explanted from E12 mouse embryos were intubated and inserted into the microdevice, which consisted of two fluidic chambers - one that surrounded the organ (pleural chamber) and one that was continuous with the airways via the intubated trachea (luminal chamber). Luminal and pleural pressures were regulated dynamically and imaged in real-time over 48 hours of culture. Transmural pressure significantly altered both the branch morphology and spatial localization, organization, and amount of airway smooth muscle. In addition to using allometric analysis to quantify pressure-induced morphological changes during branching, separate experiments mapped tissue deformations and fluid flow within the lumen using fluorescent microspheres. Whereas small-scale local peristaltic movements ($\sim 1/\text{min}$) from airway smooth muscle have been documented, our timelapse-imaging studies also demonstrated significant systemic low-frequency long-duration contractions of the proximal airways ($\sim 0.1/\text{hr}$). We also found significant fluid flow into the lung end-buds, producing bud dilation followed by branch extension. These fluid movements and cyclic temporal pressure changes within the bud resulted in spatially non-uniform epithelial surface strains consistent with previously reported patterns of cell proliferation during branching morphogenesis. Our studies suggest that luminal fluid forces may be critical for sculpting the airway architecture, through regulation of spatial patterns of growth, tuning branch angles, lengths, and/or diameters, ultimately leading to enhanced convection of air through the mature airway tree. Our microfluidic approach complements existing molecular and genetic tools in the mouse to elucidate how the mechanical microenvironment regulates lung development. These studies indicate a previously unappreciated role for the flow of luminal fluid in the regulation of branching morphogenesis.

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Apical constriction initiates budding morphogenesis in the embryonic chicken lung: insights from computational modeling.

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During early lung development, the complex system of interconnected airways is formed via branching morphogenesis. Physical forces play an indispensable role in this process, as altered mechanical loads have been shown to drive abnormal branching. Moreover, defects in branching can lead to a host of congenital respiratory defects, so determining how this tree-like structure arises in the embryo is crucial to understanding these disorders. In the early chicken embryo, airway branching is monopodial; that is, new branches form laterally off of a parent branch or stem. Here, we characterized the physical and cellular mechanisms which drive bud formation in the avian lung. Using culture of lung explants *ex vivo*, we found that apical localization of both F-actin and phosphorylated myosin light chain (pMLC) accompanies the formation of new secondary buds along the primary bronchial tube. Moreover, inhibiting actomyosin contractility prevents new buds from forming, which suggests a possible morphogenetic role for apical constriction (AC). To test if AC alone is sufficient to drive the formation of new buds, we constructed a nonlinear, 3D finite element model of the lung epithelium and used it to simulate AC in a bud-forming region of the bronchial tube. To model active shape changes in the epithelium, we employed a continuum theory for finite volumetric growth. Our results suggest that AC can drive only the initial stages of bud formation, and is insufficient to generate the more fully developed bud geometries observed experimentally. Moreover, a gradient in AC (decreasing with distance from the bud tip) is necessary to match these initial bud geometries – a result consistent with our F-actin staining. Since the developing

lung epithelium is also highly proliferative, we included growth in our model to assess whether AC and growth together are sufficient to drive bud formation. We considered cases where growth is either (i) uniform along the tube or (ii) elevated in the bud-forming region, and either (i) isotropic or (ii) longitudinal. In each case, however, we were unable to match the fully developed bud geometries observed in the embryo, which may suggest a possible mechanical role for the surrounding mesenchyme during branching. Since localized cell proliferation is generally considered to be the primary driving force behind lung bud formation, our finding that AC (not growth) initiates bud morphogenesis constitutes a novel mechanism in the lung.

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A moving zone of actomyosin contractility drives epidermal zippering and neural tube closure in ascidian embryos.

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Neural tube closure is a key morphogenetic event in chordate development, whose failure leads to birth defects in humans, but its underlying mechanisms remain poorly understood. We are investigating the cytomechanical basis for neural tube closure in ascidians - basal chordates that share a similar body plan with vertebrates and form a simple neural tube with <100 cells. Ascidian neurulation occurs by neuroectoderm (ne) folding, followed by unidirectional "zippering" in which the neural folds and adjacent epidermis (epi) meet at the midline, then undergo junctional exchange (ne/epi \rightarrow ne/ne + epi/epi) in a posterior-anterior progression to form a simple tube beneath a continuous epidermal sheet. Combining time-lapse fluorescence microscopy and immuno-staining of fixed embryos, we show that active non-muscle myosin II is highly enriched within a localized contractile zone (CZ) just ahead of the moving zipper where individual ne/epi junctions undergo rapid and pronounced shortening. Laser ablation experiments reveal that junctional tension is high along the entire ne/epi boundary and highest in the CZ. Chemical inhibitors of RhoA/Rho Kinase prevents localized myosin activation in the CZ, and inhibitors of either Rho kinase or Myosin II activity prevent the increase in junctional tension, ne/epi junction shortening, zipper progression and neural tube closure. Kinetic analysis reveals that newly met epidermal cells remain associated with the zipper during junction exchange, become highly elongated as the zipper moves anteriorly and then release from the zipper and relax towards more isodiametric shapes. Individual release events are often associated with transient increases in zipper progression, suggesting that active contraction ahead of the zipper is balanced by resistance to cell deformations behind the zipper, which is dissipated by junctional exchange and cell shape relaxation. Consistent with this, we measured elevated tensions at elongated epi/epi junctions just behind the zipper, but not following cell shape relaxation. These data suggest, and computer simulations validate, a model in which a localized zone of actomyosin contractility provides the driving force, while junctional exchange and cell shape relaxation create the essential force asymmetry to produce unidirectional zipper progression. We highlight mechanical advantages associated with this mode of tissue closure and suggest that similar mechanisms may operate during the late stages of neural tube closure in other chordates, including humans.

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Involvement of cell polarity and cell migration in folding phenomena of epithelial sheets

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Epithelial cells form a three dimensional (3D) structure while keeping epithelial polarity, consisting of apical and basolateral surfaces. Canine kidney epithelial cells (MDCK cells) form a cyst structure when embedded in a collagen gel. On a collagen gel (2D environment), MDCK cells form a monolayer sheet. Our group found that MDCK cells migrate to change the morphology from an epithelial sheet to a cyst structure when culture environment is shifted from 2D to 3D by collagen gel-overlay. However, the process and the mechanism of the migratory cyst formation after gel-overlay are unclear. Therefore, this study aimed at observing the cyst formation from following two points; 1: Rho and Rac activity that is known as a key regulator for cell migration, 2: cell polarity that is reported about an important role for morphogenesis in a collagen gel.

3D live cell imaging was performed to observe the process of the cyst formation. As a result, an epithelial sheet showed collective migration, folding from the very edge of the sheet. In order to observe the contribution of Rho activity, cells were treated with Y-27632 that is an inhibitor for ROCK, a downstream effector of Rho. For inhibition of Rac activity, Rac1 inhibitor II (Z62954982) was used. Each inhibitor decreased the folding speed. To clarify the contribution of the cell polarity to the folding phenomena, cells were treated with TGF- β 1 that is a reagent to disturb apical-basal polarity and taken time-lapse images by using phase contrast microscopy. Compared with non-treated cells, TGF- β 1 treated cells did not showed folding behavior. They just moved randomly. In order to perturb the function of basolateral protein, cells were treated with AIB2, which is an inhibitor of integrin- β 1 known to localize at basolateral surface. The AIB2 treatment resulted in reduction of the folding speed.

Furthermore, the simulation model was built based on migratory force and cell polarity. The model showed that the weaker migratory force delayed the morphological change. In addition, the model did not display folding phenomena under the disruption of the polarity. These results indicate that the maintenance of cell migration and cell polarity is responsible for the cyst formation under the collagen gel-overlay.

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Foxg1 is necessary for thymic epithelial cell differentiation and survival.

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Thymic epithelial cells (TECs) are an essential component of the thymic microenvironment and are necessary for the production of normal T cells. Cortical (cTEC) and medullary TEC (mTEC) subtypes have different functions and distinct gene expression profiles. However, the transcription regulatory network controlling the specification and differentiation of TECs is poorly understood. We have identified additional transcription factors required for normal TEC differentiation and maintenance. Our gene expression analysis identified Foxg1 as a possible regulator of thymic epithelial cell differentiation. We have found that Foxg1 is expressed in TECs before the onset of TEC differentiation and continues to be expressed in them throughout fetal and postnatal development. Analysis of Foxg1 mutant mouse embryos revealed that TEC differentiation initiates normally but then becomes abnormal at E13.5-E14.5. In addition, levels of apoptosis are elevated in the fetal thymus of Foxg1 mutants. Foxn1, which is required for the initiation of TEC differentiation, is expressed throughout fetal development in Foxg1 mutant TECs suggesting that Foxg1 activity is not necessary for Foxn1 expression. Our results

suggest that Foxg1 may regulate TEC differentiation independently or in collaboration with Foxn1. Our genetic analysis supports a key role for Foxg1 in the development and survival of fetal thymic epithelial cells.

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A spatiotemporal blueprint for actomyosin assembly/disassembly during focal contractions in *C. elegans*.

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Actomyosin contractility plays many key roles during animal development by controlling changes in cell size and shape, migration, rearrangements and cell division. We are using the early *C. elegans* embryo as a model system to study a particularly dynamic mode of contractility known as pulsed or focal contractility, which is characterized by periodic cycles of actomyosin accumulation, contraction and disappearance. Focal contractility is used during many morphogenetic processes, including polarization in *C. elegans* embryos, ventral furrow invagination and germband elongation in *Drosophila* and convergent extension in *Xenopus*. Yet despite their apparent importance, the mechanisms that govern focal contractions remain unclear.

As an excitable event, pulsed contractility requires a combination of positive and negative feedback to start and terminate, respectively. One simple model - termed cortical instability - is that local stochastic accumulation of myosin drives local contraction of the cortex, thereby increasing local myosin concentration and favoring further contraction of the cortex. Alternatively, local accumulation of actin and myosin regulators under a pulsing master regulator might drive the local accumulation and dissipation of the actin and the myosin leading to the contraction. Distinguishing these possibilities requires measuring kinetics of F-actin and myosin turnover with very high spatial and temporal resolution. To this end, we developed methods that combine single molecule imaging and particle tracking to quantify local assembly, movement and disassembly of actin filaments and myosin motor minifilaments during focal contractions at very high spatial and temporal resolution.

Our results show that the focal contraction cycle can be separated into (1) an initiation phase that predates the contraction, and which correlates with a burst in actin assembly and myosin recruitment to the cortex, (2) a contraction phase, where actin and myosin are stabilized on the cortex and their movements undergo a transition from a rapid, uncorrelated to slow and directional, suggesting bipolar engagement of the myosin mini-filaments to the actin meshwork, and (3) a termination phase correlated with a burst in actin and myosin disassembly. These results show that focal contractions cannot be driven by a simple contractile instability – instead, they suggest that pulses are driven by phasic modulation of both actin and myosin assembly and disassembly by upstream regulators.

Knocking down cofilin, a regulator of actin disassembly, we observe an inhibition of contractility - demonstrating that actin turnover is critical to produce effective focal contractions – and while local pulses of actin concentration are retained, their dynamics is different. On the other hand, knocking down CYK-1, a formin actin nucleator, we observed larger, stronger contractions with temporal signatures similar to control.

Our work demonstrates that (1) the contraction is not required as a pacemaker but modulates the temporal dynamics of the pulses and (2) actin and myosin regulators tune the macroscopic properties – size, period, and intensity - of the focal contractions.

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Analysis of Caspase-dependent Signaling Mechanism on Myogenic Differentiation*K. Shimizu¹, T. Sawasaki¹; ¹Cell-free Science and Technology Research Center, Ehime University, Matsuyama, Japan*

To clarify the whole picture of caspase-dependent signaling mechanism, we developed the high-throughput protease substrate screening platform based on wheat germ cell-free protein synthesis system and molecular interaction detection technology (AlphaScreen). Using this system, we identified 30 protein kinases (PKs) as new substrates for caspase-3. Furthermore, we reported that the role of cleavage of TRB3, one of the substrate, under ER stress condition. It has been thus far shown that caspase activation accompanied by substrate cleavage is mainly involved in the execution of apoptosis, and yet at the same time is also increasingly being recognized as an important factor of regulation of cell differentiation. In this study, we investigated the effect of substrate PKs for caspase-3 on myogenic differentiation of C2C12 myoblasts as a model system. As a result, expression of myosin heavy chain, a late marker of myogenic differentiation, was increased by transient overexpression of two caspase-substrate PKs, suggesting that these PKs accelerate myogenic differentiation. Interestingly, cell death was observed in a large number of cells expressing one of the two PKs during the early phase of differentiation. Additionally, another PK induced process formation in C2C12 myoblasts and HeLa cells, and these cell mobility was increased. Now, we are investigating the crosstalk between caspase and these PKs signaling during the differentiation process.

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ENU-induced chemical mutagenesis reveals that choline kinase beta is an important regulator of osteoporosis.

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The maintenance of bone homeostasis requires a tight balance between bone formation and bone resorption by osteoblasts and osteoclasts. The molecular mechanism(s) underlying the fundamental activities of these cells still remains largely unclear. In search of novel molecules that potentially play an important role in bone homeostasis we screened a number of ENU-induced mutant mouse lines. We identify choline kinase beta, a kinase that phosphorylates the first reaction in the biosynthesis of phosphatidylcholine, as a novel candidate regulator of bone homeostasis. Choline kinase beta mutant mice exhibit an osteoporotic phenotype as evidenced by microCT and histological assessment. In vivo and in vitro analysis reveals elevated osteoclast numbers in the mutant mice. Furthermore, osteoclasts from choline kinase beta mutant mice exhibit increased resorptive activity compared to those of littermate controls. Interestingly, exposure to elevated extracellular calcium results in a significant increase in intracellular calcium in osteoclasts derived from control mice however, this response is significantly attenuated in osteoclasts derived from choline kinase beta mutant mice. This may account for the increased resorptive activity in osteoclasts derived from the mutant mice. Treatment with CDP-choline in vivo and in vitro reduces osteoclast numbers, thereby rescuing the osteoclast phenotype. In vitro assays show a reduction in bone mineralisation in osteoblast cultures derived from the bone marrow of mutant mice. Taken together, our data document, for the first time, that choline kinase beta plays an important role in bone homeostasis by regulating both osteoclasts and osteoblasts.

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Tissue-specific stiffening of embryos parallels myosin and matrix expression and dynamically matches cardiomyocyte function.

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Healthy heart function and structure, from the tissue to cellular scale, depends on the establishment of mature tissue mechanics. On the cellular scale, late embryonic, neonatal and adult cardiomyocytes have demonstrated mechanosensitivity to ECM elasticity [1], namely that contractile activity, longevity in culture, and electrophysiological function is optimized when the cells are cultured on substrates of physiological stiffness of ~10-20 kPa. However, the heart begins functioning long before this mature mechanical environment is established. Here we examine the effects of microenvironment mechanics on chick cardiomyocyte function during early development, embryonic day 2-4 (E2-E4). We use micropipette aspiration to measure an effective local Young's modulus for the developing heart tube, finding an average value of ~1-2 kPa at these earliest stages. We use quantitative Mass Spectroscopy to identify abundant structural proteins that most likely contribute to tissue mechanics. Primarily actomyosin contractile protein and collagen I expression parallels the mechanical stiffening of the heart tissue. Pharmacologically interfering with either actomyosin contractility or the collagen network with blebbistatin or collagenase, respectively, softens heart tissue, in a dose dependent manner in the case of collagenase. In addition, transglutaminase can be used to stiffen the collagenous ECM. Stiffening or softening the ECM of intact heart tubes should allow for the maintenance of functional cellular contractile machinery. Both the stiffened or softened heart tube strains less during contraction than in the untreated case, suggesting an optimum mechanics for myocardial contraction even at the earliest functional stages. The contraction wave along the heart tube propagates with a velocity proportional to the stiffness of the tissue. When we culture isolated E3-4 cardiomyocytes on collagen coated polyacrylamide gel substrates of different stiffnesses and allow them to beat spontaneously in culture, we find that the cells are best able to contract on substrates of ~1.5 kPa, consistent with the physiological stiffness of the tissue from which they were derived. As in the whole heart, the cells were also less able to contract on much softer substrates of 300 Pa, or stiffer substrates of 10 kPa or more. This further points to an optimizing mechanics or cardiomyocyte strain that matches the physiological stiffness of the myocardium throughout development.

[1] Majkut SF, and Discher DE. (2012). *Biomechanics and Modeling in Mechanobiology*. Doi: 10.1007/s10237-012-0413-8

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Mechanical Stretch Promotes Elastic Fiber Formation in Rat Aortic Smooth Muscle Cells.

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[Background] The elastic fiber system plays a principal role in the structural integrity and function of pulsating arteries. Vessels are exposed to various stresses by pulsatile and blood flow. These stresses are very important epigenetic factors for construction of vascular elastic fiber system through changes in extracellular matrix. Stretch is considered to change the elastic modulus, the expression levels of metalloproteinase-2 (MMP2), and phenotypes of vascular smooth muscle cells (SMCs). However, the relationship between mechanical stretch and elastic

fiber formation remains unknown. Therefore, our aim is to quantitatively evaluate the effect of stretch on elastic fiber formation. **[Methods]** The aortic SMCs of rat at embryonic days 21st were plated on a silicone chamber coated with fibronectin. The chamber was set on a pulse-motor-driven stretch machine (STREX, Japan), and stretched for 1day-14 days (60 cycles/min, extension rate: 10%). We assessed the elastic fiber formation, the expression levels of elastic fiber associated-genes and differentiation markers of SMCs in the stretched SMCs by immunostaining and qRT-PCR. **[Results]** Stretch promoted elastic fiber formation after 10days and 14days. The deposition of elastin was markedly aligned by stretch as the same alignment of SMCs. The expression levels of elastic fiber associated-genes such as elastin, lysyl oxidase, lysyl oxidase like protein 1, fibrillin1, fibulin4 and 5, latent TGF- β binding protein 1 and 2, TGF- β 1 were increased by stretch, although the timing of increasing these genes expression was different. Especially, the expression of lysyl oxidase like protein 1 mRNAs was increased earlier from 2 days after stretch. Stretch also increased the expression of MMP2 mRNA, but decreased the expression of tissue inhibitor of MMP 1, 2 mRNAs. Collagen \pm T α 1 and Collagen \pm V α 1 were also increased after stretch. qRT-PCR analysis showed that SMCs differentiation markers such as h-caldesmon, SM1 and SM2 were increased after 14day-stretch, whereas the inflammatory mediator interleukin-6 was immediately increased after 1day-stretch and rapidly decreased afterward. **[Conclusion]** Cyclic stretch promoted elastic fiber formation through the alignment of elastin and the repeat of deposition and decomposition by changing in the gene expression and differentiation of aortic SMCs.

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Role of Telethonin in Sarcomere Assembly and Maintenance.

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The N-terminus of telethonin forms a unique structure linking two titin N-termini at the Z-disc. While a specific role for the C-terminus has not been established, several studies indicate it may have a regulatory function. Using a morpholino approach in *Xenopus*, we show that telethonin knockdown leads to embryonic paralysis, myocyte defects, and sarcomeric disruption. These dystrophic defects can be rescued by expressing full-length telethonin mRNA in morpholino background, indicating that telethonin is required for myofibrillogenesis. However, a construct missing C-terminal residues (C^{del}) is incapable of rescuing motility or sarcomere assembly in cultured myocytes. We therefore tested two additional constructs: one where four C-terminal phosphorylatable residues were mutated to alanines (C^{Ala}) and another where terminal residues were randomly replaced (C^{mis}). Observations with these constructs support that the telethonin C-terminus is required for assembly, but in a context-dependent manner, indicating that factors and forces present *in vivo* can compensate for C-terminal truncation or mutation. Further to determine if the phosphorylatable residues at the telethonin C-terminus are required for sarcomere organization additional constructs with individual alanine substitutions and two constructs containing phosphorylation mimics were also tested. These results corroborate our previous finding that telethonin is required for sarcomeric organization and also demonstrate that phosphorylation at telethonin C-terminus may be essential for its functions.

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Roles of CCN2 in energy metabolism in chondrocytes.

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Purpose : CCN2, also known as connective tissue growth factor (CTGF), is a classical member of the CCN family and is composed of four modules that interact with various molecules, such as growth factors, membrane receptors, and extracellular matrix components. CCN2 acts as a multifunctional molecule and regulates extracellular signaling in the microenvironment. CCN2 is known to play a critical part in the development and regeneration of mesenchymal tissues. In CCN2-null mice, skeletal dysmorphisms and deficiency in cell proliferation and extracellular matrix production in osteoblasts and chondrocytes are observed. These findings suggest that CCN2 is involved in the basic metabolism in osteoblasts and chondrocytes. Therefore, we aimed to clarify the role of CCN2 in energy metabolism therein.

Methods: Murine chondrocytes from CCN2-null and wild type(WT) mice, and a human chondrocytic HCS-2/8 cell line were utilized. Total RNA was extracted from those cells for subsequent microarray and real-time RT-PCR analysis. Metabolites were also extracted from the cells by methanol for the metabolome analysis with a capillary electrophoresis-tandem mass spectrometry. Cellular ATP levels were quantified by a bioluminescent assay system.

Results: Initially, we extracted and analyzed the RNA from chondrocytes obtained from rib cartilage of CCN2-null and WT mice. The result of DNA microarray analysis showed that the lack of CCN2 increased the expression of several ribosomal protein genes and remarkably decreased ATP synthase subunit γ gene expression. Since ATP synthase is a critical mitochondrial molecule motor that supplies a major part of cellular ATP. We next comparatively quantified ATP content in chondrocytes from CCN2-null and WT mice. ATP bioluminescence assay showed that ATP concentration in CCN2-null chondrocytes was significantly lower than that of WT chondrocytes. Subsequently, after knocking down the CCN2 mRNA using CCN2 siRNA, we analyzed the expression of these genes in a human chondrocytic cell line HCS-2/8 by real-time RT-PCR. Indeed, we found that even by temporarily knockdown of CCN2 gene, the expression of ATP synthase subunit γ gene was also suppressed. Furthermore Cto uncover the effects of CCN2/CTGF defect on energy metabolism in chondrocytes, metabolome analysis was performed with CCN2-null and WT chondrocytes. In CCN2-null mice, the intracellular levels of ATP, acetyl CoA and several amino acids including Glutamine, Serine, Tryptophan were remarkably lower than those in WT mice.

Conclusions: These findings indicate that CCN2 plays an important role in the maintenance of energy metabolism system in chondrocytes by supporting the production of ATP synthase.

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Osteopontin Expression in Human Fetal Osteoblastic Cells Grown on a Chitosan Composite Biomaterial.

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Identification of a safe bone scaffolding material that would enhance the bone modeling process is needed. Interpretation and analysis of osteoblast cell biology in the presence of Chitosantrimethoxysilylpropyl Methacrylate (CTS) is the genesis of understanding the future potential of CTS bone scaffolding technology as an effective promoter of osteoblast function. We investigate human Fetal Osteoblast 19 (hFOB.19) cells in the presence of various concentrations of CTS. Analysis of cell viability, proliferation, as well as, identification of expression and localization of Osteopontin (OPN) was observed and recorded. MTT assay results suggest CTS directly enhances hFOB.19 proliferations in-vitro. Immunocytochemistry observations demonstrate OPN is localized in the plasma membrane. Increased concentration of CTS significantly increased hFOB.19 cell proliferation. OPN is localized throughout the plasma membrane of hFOB 1.19 cells. CTS proves to be an effective promoter of hFOB 1.19 function in-vitro.

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Delayed Bone Development in a Preeclampsia Mouse Model.

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Pre-eclampsia (PE), a disease associated with high blood pressure during pregnancy, is a devastating and potentially fatal process which can negatively impact both mother and fetus. Beyond simple hypertension, the disease has negative impacts on maternal kidney function as well as fetal development. PE is highly associated with intrauterine growth retardation (IUGR) which can also impact bone and cartilage development. In this study, we investigate the effects of this disease during fetal and post-natal bone development. It is currently unclear how PE affects cartilage and bone development, though angiogenesis may be one possible connection, particularly because vasculature is crucial during endochondral ossification. Recent studies have demonstrated a down-regulation of VEGF to be a contributing factor in the development of PE. This study has focused on a BPH mouse model which imitates human PE (the mice develop hypertension and proteinuria in the final trimester, which resolve postpartum). The fetuses born to these mice show a significant delay in bone development, supporting our hypothesis of an association between angiogenesis and bone development. Specifically, it appears that BPH mice experience a significant delay in endochondral ossification. Histological analysis showed that BPH mice demonstrate significantly less von kassa staining (and therefore less matrix mineralization) when compared to the C57, wild type (WT) at E18.5 and in newborns. Expression of aggrecan protein, a key component of the cartilage ECM framework, was dramatically decreased in BPH fetuses when compared to the WT. Link protein, an anchor protein for aggrecan in cartilage, was also substantially decreased in BPH compared to WT fetuses. Further histomorphometric analysis of the BPH growth plate demonstrated considerably thicker hypertrophic zones in the cartilage growth plate, indicative of delayed endochondral ossification. These elements, coupled with the decrease in von kassa staining, confirm delays in endochondral bone ossification in BPH compared to WT fetuses. Taken together, further studies

are warranted to demonstrate the mechanism involved in delayed bone formation in PE as well as possible therapeutic strategies for the treatment of this devastating disease.

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***Gpr177*, a novel locus for bone-mineral-density and osteoporosis, regulates osteogenesis and chondrogenesis in skeletal development.**

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Human genetic analysis has recently identified *Gpr177* as a susceptibility locus for bone-mineral-density and osteoporosis. Determining the unknown function of this gene is therefore extremely important to further our knowledge base of skeletal development and disease. The protein encoded by *Gpr177* exhibits an ability to modulate the trafficking of Wnt similar to the *Drosophila* Wls/Evi/Srt. Because of a critical role in Wnt regulation, *Gpr177* might be required for several key steps of skeletogenesis. To overcome the axial patterning defects with embryonic lethality associated with the inactivation of *Gpr177* in mice [1], conditional gene deletion is utilized to assess its functionality. Here we report the generation of four different mouse models with *Gpr177* deficiency in various skeletogenic cell types, including the mesenchymal cell, osteoblast and chondrocyte. The mesenchymal ablation of *Gpr177* severely impairs development of the craniofacial and body skeletons, demonstrating its requirement for intramembranous and endochondral ossification, respectively. Defects in the expansion of skeletal precursors and their differentiation into osteoblasts and chondrocytes suggest that Wnt production and signaling mediated by *Gpr177* cannot be substituted. Because the *Gpr177* ablation impairs the secretion of Wnt protein, we therefore identify their sources essential for osteogenesis and chondrogenesis. Contrary to the requirement of β -catenin signaling in the osteoblast precursors, our data show that the deletion of *Gpr177* in this population does not impair osteogenesis, suggesting that Wnt production from the undifferentiated mesenchymal cells is critical for Wnt signaling in the osteoblast precursors. In contrast, the deletion of *Gpr177* in either mesenchymal cells or chondrocytes causes similar skeletal deformities, implying that Wnt secretion from both cell types are required for chondrogenesis. These results indicate that the intercross of Wnt signaling between distinct cell types is carefully orchestrated and necessary for skeletogenesis. Our findings lead to a proposed mechanism by which *Gpr177* controls skeletal development through modulation of autocrine and paracrine Wnt signals in a lineage-specific fashion.

Reference

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Prenatal exposure to ethinylestradiol leads to the increase of premalignant lesions multiplicity and alters the morphologic pattern of male and female prostate of senile gerbils.

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Endocrine disruptors (ED) are substances that have the potential to alter the structure and function of the endocrine system, including prostate. The aim of this study was to analyze the behavior of senile male and female gerbil prostatic gland exposed to ethinylestradiol (EE - component of oral contraceptive) during the prenatal period. Pregnant female received 15 µg/Kg/day EE diluted in mineral oil (EE/PRE group), whereas control group received only mineral oil. When male and female pups completed 12 months the male ventral prostate (VP) and female prostate were removed and processed for paraffin embedding. The prostates were stained with HE for multiplicity analysis of prostatic intraepithelial neoplasia (PIN) (n=5 and p≤0.05) and Gömori's reticulin for prostatic stromal analysis. In addition, the sections underwent smooth muscle alpha actin immunofluorescence and ultrastructural analysis using electron microscopy. The male and female prostate of EE/PRE group presented an increase in the number of PIN compared with the respective control. The stromal analysis revealed derangements in reticulin and collagen fibers disposition, mainly in PIN regions of EE/PRE male and female prostate. Moreover, it was verified through immunofluorescence the absence of alpha actin immunoreaction in prostatic buds areas and increased immunoreactivity in PIN regions of EE/PRE male and female prostate compared to respective control group. The ultrastructural analysis of male VP in EE/PRE group showed an apparent increase in Golgi apparatus, osmiophilic deposits such as lipofuscins, basal membrane detachment and collagen system derangements. Regarding to EE/PRE female prostate, it was noted the presence of vesicle fusion with secretion accumulation, lipofuscins deposits and displaced basal nucleus. The exposure to EE during the prenatal period contributed for appearance of PIN and disturbance in stromal compartment of senile gerbil male and female prostate. The gerbil prostate presents spontaneous lesions with aging due to hormonal imbalance that occurs in both sexes at this phase. Furthermore, the cellular ageing tends to augment in response to EE. The prostatic synthesis was also altered. Therefore, the action of this synthetic hormone can interfere in functionality of these glands. Thus, it can be concluded that exposure to EE during critical period, such as prenatal, promotes premalignant lesions and development alterations in the male and female prostate gland, and may contribute for the emergence of aging prostatic diseases.

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Behavior of the contractile myonemes of the ciliate *Stentor coeruleus* during oral regeneration and division.

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The contractile myonemes of *Stentor* are linear fibers that run longitudinally over the entire length of the cone-shaped cell some distance under the cell membrane that are thicker at the posterior end and gradually become thinner as they progress anteriorly. These myonemes have been shown to be composed of centrion-related proteins and an antibody to these proteins has been used to show their arrangement in normal cells (Maloney et al, J. Euk. Micro. 2005). During oral regeneration, the new oral apparatus forms as a oral primordium (OP) on the ventral surface of the cell in the area where the myonemes are thinner and more mesh-like. Initially the basal bodies of the new OP form above the myonemes and the myonemes can be seen running

through this area. As the primordium lengthens, the myonemes now curve along the ventral surface of the primordium and do not appear to be continuous with the myonemes on the dorsal surface of the OP. Furthermore, some of the myonemes just ventral to the OP often branch, forming a V shape in this area. On the dorsal surface of the OP, the myonemes begin forming the frontal field of the OP where they become much more numerous and tightly packed and precise branches form at right angles so that the myonemes here form a very tight, precise mesh seen in the mature oral apparatus. Cell division in *Stentor* involves transverse fission of the cone-shaped cell and the formation of an OP for the new posterior cell in a manner almost identical to that occurring during oral regeneration. Changes in the myonemes near the OP were the same as seen during oral regeneration with one dramatic exception. At the point where the cleavage furrow would form, the myonemes above the region of the prospective furrow began to thicken considerably whereas those below the prospective furrow remained thinner. The area above the furrow will become the posterior end of the new anterior daughter cell and the myonemes in this area should be thick. This thickening of the myonemes initially began on both sides of the OP and then proceeded in opposite directions around the cell, just as the cleavage furrow would when it becomes apparent.

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Rac1 Activity Maintains the Human Tenocyte Phenotype.

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When cultured *in vitro*, human tenocytes undergo 'dedifferentiation', a process by which they change shape – lose their normal elongated phenotype – and stop producing collagen I. Previous work has demonstrated the involvement of the Rho GTPases, including RhoA and Rac1, in human connective tissue cell differentiation. We hypothesized that the process of human tenocyte dedifferentiation is caused by loss of activity of Rac1GTPase.

Human tenocytes were dedifferentiated via *in vitro* culture at low cell density. Human tenocytes were harvested from tendons of individuals undergoing finger revision amputation using IRB-approved protocols. Human tenocytes were plated at 5k cells/cm² and passaged *in vitro*. Tenocytes from passage 0, passage 2 and passage 8 were harvested after one week in culture and collected. With time in culture at low density, there is a change in tenocyte phenotype from elongated to polygonal. Furthermore, there is also decreased collagen I fibril formation, and decreased expression of collagen I and scleraxis in dedifferentiated tenocytes.

Inhibition of Rac1 activity via pharmacologic inhibition in human tenocytes resulted in dedifferentiation, cell shape changes and decreased collagen I production. Normal human tenocytes were treated with the Rac1 inhibitor NSC23766. The normal tenocyte phenotype was lost with Rac1 inhibition, as seen by changes in cell shape and decreased collagen I fibril formation. There was also decreased expression of collagen I and scleraxis with pharmacologic Rac1 inhibition.

When Rac1 activity was controlled in dedifferentiated human tenocytes using adenoviral infection of constitutively active Rac1, the tenocyte phenotype was regained, and collagen I production was increased. Human dedifferentiated tenocytes were infected with constitutively active Rac1, and harvested after one week and three weeks in culture. The human tenocyte phenotype is regained with Rac1 activation *in vitro*, as seen by resumption of elongated cell shape, increased collagen I fibril formation, and increased expression of collagen I and scleraxis

in those cells infected with constitutively active Rac1. These findings show that increased Rac1 activity underlies the maintenance of the normal human tenocyte phenotype *in vitro*.

We have observed the dedifferentiation of human tenocytes when plated at low density in culture. We have reproduced this phenotype via Rac1 inhibition, and have discovered that the normal human tenocyte phenotype can be potentiated in dedifferentiated tenocytes by increasing Rac1 activity. Knowledge of this molecular mechanism lends insight into strategies to improve connective tissue engineering, repair and regeneration.

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Abnormal tracheal cartilage formation in mice lacking Ca_v3.2 T-type Ca²⁺ channels.

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Ca²⁺ influx is crucial in initiating the differentiation of mesenchymal cells into chondrocytes, but which Ca²⁺ channels are involved remains uncertain. Here we show that the T-type voltage-gated Ca²⁺ channel Ca_v3.2 is essential for tracheal chondrogenesis. Mice lacking this channel (Ca_v3.2^{-/-}) develop congenital tracheal stenosis, as a consequence of incomplete formation of cartilaginous tracheal support. Conversely, forced Ca_v3.2 overexpression in ATDC5 cells leads to enhanced chondrogenesis, an effect that can be blunted by both a blocker of T-type Ca²⁺ channels and an inhibitor of calcineurin, suggesting that Ca_v3.2 is responsible for Ca²⁺ influx during chondrogenesis. Finally, tracheas from Ca_v3.2^{-/-} mice exhibit a reduction in Sox9 expression. Ca_v3.2-dependent Sox9 upregulation requires a putative NFAT binding site that we identified within the mouse Sox9 promoter. Our findings show that the signaling pathway underlying Ca²⁺-induced chondrogenesis involves the Ca_v3.2 T-type channel and is dependent on calcineurin and NFAT

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Salt-Inducible Kinase 3 is critical for chondrocyte hypertrophy during bone development.

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Salt-inducible kinase 3 (SIK3) belongs to AMPK family, but its physiological and functional role was so far poorly understood. To elucidate its biological function, we generated SIK3 deficient mice and found that SIK3 deficient adult mice presented systemic bone abnormality. The feature of skeletal malformation in SIK3 deficient mice was close to characteristic of that in rickets, but the concentration of phosphate and calcium in serum were almost normal compared with wild-type mice. Thus, we suspected that the cause of this bone malformation was bone in itself. Anatomical and histological analyses revealed that the bone inside was occupied by massive chondrocytes at adulthood and pointed out that the bone defect occurred since E14.5 stage, in which chondrocyte hypertrophy was almost blocked. Consistent with this phenotype, SIK3 expression was detected in cytoplasm of prehypertrophic and hypertrophic chondrocytes in wild-type embryos. By immunofluorescence imaging, we found that HDAC4, a crucial repressor of chondrocyte hypertrophy, remained in the nuclei in SIK3-deficient chondrocytes, but was localized in the cytoplasm in wild-type hypertrophic chondrocytes, suggesting that SIK3 was required for translocation of HDAC4 to quit its transcriptional repressive activity. In order to

confirm this hypothesis, we demonstrated that SIK3 directly formed complex with HDAC4 and SIK3 was required for anchoring HDAC4 in the cytoplasm, thereby releasing MEF2C, a crucial facilitator of chondrocyte hypertrophy, from suppression by HDAC4 in nuclei. As a gain-of-function approach, we also generated chondrocyte-specific SIK3 transgenic mouse. Although we could not see any phenotype in developmental and juvenile stage, we finally found the abnormal closure of growth plate and flatten face since 4.5 month-old age, suggesting that overexpression of SIK3 induced excessive consumption of chondrocytes. The SIK3-deficient phenotype in bone was rescued by this transgenic SIK3 expression. In conclusion, we here report an essential role for SIK3 in facilitating chondrocyte hypertrophy during skeletogenesis and growth plate maintenance.

Cell Fate Determination

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The Role of *Rx* in cell fate decisions during retinogenesis.

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During retinogenesis, retinal neuron cell birth occurs in two distinct temporal phases: early (during embryonic ages) and late (in the first ten postnatal days). Retinal neurons generated during the late phase are dependent on continued retinal progenitor proliferation. The transcription factor *Rx* is crucial for eye development, playing roles in both optic vesicle initiation and progenitor proliferation and is therefore necessary for late-born cells. Cone photoreceptors are early-born cells and therefore are not as dependent on continued progenitor proliferation. In mice with a conditional *Rx* deletion, we observed an absence of cone opsin expression in postnatal day 21 retinas, in addition to the loss of the late-born cell markers for Muller glia, bipolar cells and rod photoreceptors. In this study, we sought to explore the necessity of *Rx* on retinal cell fate early in retinogenesis. We hypothesized that *Rx* is essential for cone photoreceptor development in addition to being necessary for the formation of the late-born cells via its role in progenitor proliferation. Using embryonic retinas to explore the effects of *Rx*-deletion on early development, we used a panel of antibodies to assess cell fate changes in the embryonic retina. The results showed similar cell fate changes to those seen in adult tissue and activated caspase-3 labeling suggest that the changes were not due to cell death. This evidence suggests that *Rx* is necessary for cone cell fate decisions in the vertebrate eye.

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Functional rescue of PR null mammary epithelium by redirected testicular cells in vivo.

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Stem and progenitor cells in most adult tissues reside in specialized, highly regulated microenvironments called stem cell niches. We have previously shown that testicular, neural, bone marrow and cancer cells respond to the signals provided by the mammary niche (stroma, epithelial cells, local and extrinsic factors) and alter their cell fate to that of mammary epithelial progenitor cells. In an effort to determine if hormonal signals from progesterone receptor (PR) expressing mammary epithelial cells are required for this redirection, we mixed PR null epithelial cells with Wap-Cre/Rosa26reporter testicular cells from adult male mouse seminiferous tubules. The testicular cells were redirected to mammary epithelial cell fate and in the process rescued the functionally deficient PR null cells, allowing them to produce alveolar secretory structures in the chimeric outgrowths at parturition.. We identified redirected testicular cells by staining with

anti-Progesterone Receptor (anti-PR) antibodies. Positive cells were found both in ducts and in secretory structures. PR null outgrowths were uniformly negative for anti-PR staining. This demonstrates that PR null mammary tissue can redirect testicular cells to undertake mammary epithelial cell fates and in addition PR null testicular cells are redirected as well to mammary epithelial secretory cell fate by signals emanating from the PR-positive redirected wild type testicular cells.

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Regulated phosphorylation of the stem cell fate determinant Musashi controls cell cycle progression during development and differentiation.

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Musashi is an evolutionarily conserved, sequence-specific RNA binding protein that has been shown to play a critical role in promoting physiological stem and progenitor cell self-renewal. Musashi has also been implicated pathologically in the development and progression of neural, colon, breast and hematopoietic cancers. During self-renewal of mammalian stem cells, Musashi represses translation of mRNAs encoding inhibitors of cell cycle progression. However, during stem cell differentiation Musashi target mRNAs are translated. The underlying molecular mechanism controlling this functional switch in Musashi activity has not been established. In this study, we demonstrate that differentiation of rat embryonic neural stem cells and human SH-SY5Y neuroblastoma cells results in the rapid phosphorylation of Musashi on two evolutionarily conserved serine residues. Musashi phosphorylation is associated with de-repression and translation of Musashi target mRNAs. Expression of a Musashi phosphorylation mutant protein blocked differentiation and promoted inappropriate growth of SH-SY5Y cells under nutrient deprivation. Using a novel biological assay to directly assess Musashi function we have found that phosphorylation of Musashi is mediated by both cyclin-dependent kinase and MAP kinase signaling pathways. Taken together, our data indicate that regulated phosphorylation directs a functional switch in Musashi action by converting it from a repressor to an activator of mRNA translation to promote cell cycle arrest in response to environmental cues. The identified phosphorylation-dependent switch in Musashi function may present a novel regulatory node for therapeutic control of pathological stem cell proliferation.

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maternal KLF2 regulates the expression of early pan-ectodermal activator, Foxi1e, in *Xenopus* development.

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One of the first, and major, patterning events that takes place in all triploblastic embryos is the formation of the three primary germ layers. In the early *Xenopus* embryo, maternally encoded T-box transcription factor, VegT, is localized to the vegetal cytoplasm in the oocyte, and can initiate mesoderm and endoderm formation. But, much less is known about the formation of the ectoderm, which arises from the most animally located cells of the blastula. Previously we shown that Foxi1e is the first known early zygotic pan-ectoderm activating gene and another Forkhead box protein, Foxi2, is key activator for its expression and other epidermal genes' expressions. Despite having maternal activator, Foxi2, in entire ectodermal tissue, Foxi1e is only expressed in deep layer of presumptive ectodermal tissue, it suggests that the existence of potential repressor in superficial layer. Here, we report that a member of Kruppel-like factor

family, KLF2, as the maternal repressor for Foxi1e expression. This set of maternal activator/repressor (Foxi2/KLF2) will provide the mechanism of differential expression of Foxi1e.

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Testing a role for dHb9 expressing neurons in eclosion behavior in *Drosophila* by targeting the cell death gene, reaper.

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Introduction: The *Drosophila* nervous system undergoes structural changes during metamorphosis to accommodate new behaviors that are manifested by the adult. Among these changes is a drastic reduction of the abdominal ganglion. Our lab is interested in understanding motor remodeling, specifically how larval motor neurons are restructured to innervate adult specific muscles and how they contribute to adult-specific neural circuits. Since there are 34 motor neurons in each hemisegment, we use subset specific motor neuron markers to follow the fate of larval motor neurons through metamorphosis. dHb9 is an identity gene that labels motor neurons that project to mostly ventral muscles and some dorsal targets in the larva. Initial work has shown that dHb9 is expressed by several neurons in the anterior abdominal hemisegments and that these innervate segment specific muscles. Among the muscles innervated by the neurons are two that are known to affect the early adult behavior of eclosion (Kimura et al 1990), the act of emerging from the pupal casing. The objective of this study is to test the role of dHb9 expressing neurons in eclosion by expressing the cell death gene reaper. **Methods:** Using a dHb9-Gal4 driver, a UAS-reaper transgene will be targeted to dHb9 expressing cells during the entire period of metamorphosis to bring about elimination of the ventrally projecting neurons. Temporal control of reaper expression will be achieved by expressing a temperature sensitive Gal80 gene which blocks Gal4 function at permissive temperatures. The restrictive temperature will be deployed during the period of metamorphosis. The average time for normal fly to eclose at 25°C is 101 hours after puparium formation (APF). Therefore, flies that eclose between 98 and 103 hours APF will be classified as normal. Flies that eclose after this time range will be classified as late eclosers. The flies that develop in the puparium but do not eclose will be classified as non-eclosers. 100 animals of both the control and experimental types will be examined. At least 10 animals from each category will be dissected to determine (1) the number/pattern of dHb9 expressing cells, (2) presence/absence of neuromuscular junctions made on muscles in A1 and A2, with an emphasis on MFs 12 and 13. **Results:** Preliminary studies have shown that when the cell death gene, reaper was targeted to dHb9 expressing cells during the pupal phase, 36% failed to eclose (n= 38), a 3 fold deviation from control animals (12%, n=26). These data allow us to conclude that dHb9expressing neurons do contribute to the function of eclosion. **Future Directions:** (1) We anticipate that lack of eclosion/delay in eclosion will involve absence of/defects in dHb9 innervation of the eclosion muscles in A1 and A2. These attributes will be assessed using immunohistochemistry to detect dHb9 expression (anti-GFP), Innervation (anti-HRP) and Muscles (phalloidin). (2) The motor neurons and their terminals will also be followed during metamorphosis to analyze any developmental deviations in neuromuscular patterns (3) A more severe transgene, Diptheria toxin, which blocks protein synthesis will also be targeted to dHb9 expressing neurons. All these experiments are ongoing and the emerging data will be presented.

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Vexin (Vxn) is a novel neural-specific gene that regulates cell cycle exit downstream of proneural factors in Xenopus.

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We identified vexin (vxn; previously called sbt1) as a target gene for proneural basic helix-loop helix transcription factors during *Xenopus* development. Vxn is conserved across vertebrate species and encodes a novel protein with no known functional motifs. Vxn is transiently expressed as cells initiate differentiation in the *Xenopus* nervous system. In mouse, vxn is expressed in differentiating neurons and is maintained in subsets of postmitotic neurons, with high expression levels in the adult cerebral cortex.

To assess the function of vxn, we performed gain and loss of function experiments in *Xenopus*. Inhibition of Vxn translation by injection of antisense morpholino (MO) into cleavage-stage blastomeres blocked neuronal differentiation. Similarly, targeting vxn MO to retinal progenitors inhibited retinal neuron differentiation, resulting in an increase in Müller glia.

Conversely, overexpression of vxn causes enhanced neural differentiation, with expansion of the domains of N-tubulin positive neurons within the neural plate. Similarly, overexpression of *Xenopus* or mouse vxn in retinal progenitors promoted differentiation of early born retinal neurons, and also strongly cooperated with the proneural bHLH factor Atoh7 to promote neurogenesis.

Overexpression of vxn caused a reduction in mitotic cells in neurula stage embryos, as measured by phospho-histone H3 staining, and also caused an increase in expression of the cyclin-dependent kinase inhibitor p27Xic1. Morpholino knockdown of both vxn and p27Xic1 enhanced the block on neural differentiation in the *Xenopus* neural plate. The function of vxn during *Xenopus* neurogenesis is strikingly similar to that of Pak3, and we showed that vxn and Pak3 synergize to promote expansion of N-tubulin positive domains during primary neurogenesis, and that knockdown of both vxn and Pak3 enhanced the block on neural differentiation.

Both exogenously expressed epitope-tagged Vxn protein (mouse and *Xenopus*), as well as endogenous Vxn protein, localize to the cell membrane and the nucleus in *Xenopus*. By generating forms of Vxn tagged with either nuclear localization or nuclear export sequences we showed that Vxn must localize to the nucleus to promote cell cycle exit and neural differentiation.

We propose that vxn is expressed in progenitors as they initiate neuronal differentiation, and appears to regulate cell cycle exit downstream of proneural bHLH factors during *Xenopus* development.

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The role of fad104, a regulator of adipogenesis, in calvarial bone formation through BMP/Smad signaling.

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To elucidate the molecular mechanism of adipogenesis, we isolated numerous genes whose expression was increased at the beginning of adipogenesis. Among them, factor for adipocyte differentiation 104 (fad104) is a novel gene, which positively regulates adipogenesis. FAD104 is a transmembrane protein containing proline-rich region and nine fibronectin type III domains. In previous study, we reported that disruption of fad104 causes neonatal lethality due to lung hypoplasia including immaturity of alveolar type II cells. In addition, we found that fad104 negatively regulates the differentiation of MEFs into osteocytes. This result implied that fad104 was involved in bone formation, but the physiological role of fad104 in bone formation was not known. Therefore, we prompted to characterize the role of fad104 in bone formation.

Alizarin red S/Alcian blue skeletal staining revealed skeletal alteration in fad104-deficient mice. In calvarial bones, anterior fontanel closure was accelerated in fad104-deficient mice at E18.5. Furthermore, the femur was widened in fad104-deficient mice at E18.5. Next, we examined the role of fad104 for the differentiation of primary cultured mouse calvarial cells. In calvarial cells, the expression of fad104 was decreased in the early stage of differentiation. Deletion of fad104 enhanced calvarial cell differentiation. Moreover, adenoviral infection of adfad104 into fad104-deficient calvarial cells suppressed the enhanced ossification by disruption of fad104. These results indicate that fad104 inhibits calvarial osteoblast differentiation. Bone morphogenetic protein (BMP) plays essential roles in osteoblast differentiation. It is largely known that activation of BMP/Smad signaling pathway strongly promotes osteoblast differentiation. Therefore, we examined whether fad104 participated in BMP/Smad signaling pathway. In calvarial cells, disruption of fad104 enhanced the level of BMP2-induced Smad1/5/8 phosphorylation and the mRNA expression of Inhibitor of DNA binding 1 (Id1), a BMP target gene. Additionally, FAD104 overexpression attenuated the level of Smad1/5/8 phosphorylation and Id1 expression. In conclusion, our results demonstrate that fad104 inhibits BMP/Smad signaling pathway, and negatively regulates calvarial bone formation.

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Alkaline phosphatase expression in 3T3F44-2A adipocytes is related to gene regulation of lipid metabolism.

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The increasing worldwide prevalence of obesity and its related disorders, make of relevance the understanding of the molecular interconnections related to increased adiposity. Differentiation of adipocytes is a complex process in which preadipocytes undergo changes in gene expression that produce biochemical, morphological and metabolic transformations that culminate with the cell ability to store energy in form of triglycerides in organelles known as lipid droplets. Regulation of synthesis, storage and release of triglycerides is mediated by different mechanisms involving the expression of molecules with specific functions for fatty acids synthesis and degradation.

There are three types of alkaline phosphatase: tissue nonspecific (TNAP) or liver/bone/kidney, intestinal, and placental, which are encoded by different genes *akp2*, *akp3* and *akp5* respectively that have different physiological tissue specific functions. The activity of alkaline

phosphatase in 3T3L1 cell line and in murine and human adipose tissue has been described, but it is not clear if this activity is addressed only to *akp2* gene. By RTPCR we measured the mRNA levels of these enzymes in 3T3F44-2A cells and in mice adipose tissue; we found only the expression of *akp2*. Enzymatic activity assays showed that the activity of the enzyme alkaline phosphatase increases during differentiation into adipocytes of the 3T3F44-2A cells as has been shown for the sister cell line 3T3L1, reinforcing the idea of a possible role for this enzyme in the metabolism of adipocytes. In order to gain some insight we used the specific inhibitors of TNAP, levamisole and L-histidine. We found that inhibition of TNAP alters the expression and activity of the enzyme glycerol phosphate dehydrogenase, but has no impact in malic enzyme. Also, inhibition of TNAP modifies mRNA levels of the main lipogenic regulator PPAR γ 2 and other specific genes of lipid and carbohydrate metabolism. Our data demonstrate that the activity of alkaline phosphatase is involved in the regulation of the activity and gene expression of relevant metabolic molecules in adipose cells.

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Probing mammalian neural stem cell differentiation in vivo.

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Mammalian neurogenesis in the cerebral cortex requires the generation of millions of neurons originating from neural stem cells while maintaining an active population of undifferentiated progenitors. This necessitates a tightly controlled balance of symmetric and asymmetric neural stem cell divisions as well as subsequent manifestation of stem cell versus neuronal fates. Many cellular processes influence these crucial balances but systematic analysis of factors involved has been missing. Here we describe an approach to probe multiple genes in a parallel screening setting in vivo allowing for systematic analysis of mammalian neurogenesis. To this end we developed an effective viral delivery method to the developing murine neocortex targeting cycling neural stem cells lining the lateral ventricle. Comparison of viral constructs and promoters yielded a retroviral construct that is highly efficient in transducing stem cells and exhibits sustained expression in differentiated progeny. Retroviral preparations carrying short hairpin RNA (shRNA) targeting specific mRNAs as well as a fluorescent marker were used to follow the fate of neural stem cells and their progeny at embryonic day E13.5. Analysis followed 4-5 days post transduction by immunofluorescence, as well as separation of shRNA carrying neurons and stem cells using FACS. The goal of this setup is to quantify individual shRNAs in both populations in parallel using next generation sequencing. This will allow for characterization of both total stem cell and neuron pool size as well as their respective balance depending on individual shRNAs. Initial candidate shRNAs will target transcriptional and epigenetic factors as these are key to establishing differential cell fates. This systematic approach to characterize mammalian neurogenesis will deepen our understanding of this vital process.

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Molecular and biochemical responses of *Volvox carteri* to oxidative stress.

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Reactive oxygen species (ROS) can act as signaling molecules to cause physiological changes to gene expression and metabolism. ROS, including superoxide (O₂⁻) and peroxides, are also highly reactive molecules that cause oxidative damage to proteins, lipids and DNA. It has previously been shown that exposure of the multicellular, eukaryotic green alga *Volvox carteri* to heat shock conditions (42.5°C), which induces the onset of the sexual reproductive cycle, results in the production of ROS. Here we show that the ROS response to heat stress is paralleled by changes in photosynthetic metabolism, antioxidant enzyme activity and gene expression, and fluctuations in the elemental composition of cells. Metabolism, as measured by pulse amplitude modulated (PAM) fluorometry over two hours of heat stress, showed a linear decrease in the photosynthetic efficiency of *Volvox*. ROS quantification uncovered an increase in ROS in the culture medium accompanied by a decrease in ROS produced inside the *Volvox* colonies, suggesting an export mechanism is utilized to mitigate stress. Enzyme kinetics indicated an increase in SOD activity over time when cultures were heat shocked. Using X-ray fluorescence at the Stanford Synchrotron Radiation Lightsource, we show that these changes coincide with cell-specific import/export and intracellular redistribution of transition elements and halides. This suggests that the cellular metallome is also engaged in mediating oxidative stress in *Volvox* by modulating ROS signaling and developmental pathway decisions.

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Actin stress fiber disruption triggers adipocyte differentiation.

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Adipocyte differentiation at an early stage is directly regulated by gene expression of a master regulator such as peroxisome proliferator-activated receptor- γ (PPAR γ). On the other hand, the hallmark of adipogenesis process is the dramatic alteration in actin cytoskeleton as the structure of filamentous actin is converted from stress fibers to cortical actin. However, the correlation between actin cytoskeleton remodeling and PPAR γ -mediated adipocyte differentiation program still remains to be clarified. Here, we report that actin cytoskeleton remodeling itself acts as a trigger of adipocyte differentiation. Actin stress fibers were immediately disrupted prior to PPAR γ expression after adipogenic induction. The inhibition of actin stress fiber disruption by treatment with an cytoskeletal fixation agent phalloidin and transfection of an actin depolymerizing factor cofilin siRNAs resulted in suppressed PPAR γ expression and lipid droplet accumulation after adipogenic induction. In addition, the activation of RhoA protein, which is a prominent regulator of cytoskeletal dynamics, was rapidly reduced after adipogenic induction. We further found that ectopic expression of activated RhoA inhibited actin stress fiber disruption and adipogenesis, and these effects were recovered by treatment with the RhoA kinase inhibitor Y-27632. Moreover, addition of an actin polymerization inhibitor cytochalasin D (CytD) in active RhoA-expressing cells led to the disruption of actin stress fibers and, thereby rescued adipocyte differentiation. Amazingly, we found that treatment of Y-27632 alone or CytD alone is sufficient for causing adipocyte differentiation even without an adipogenic cocktail by mimicking actin cytoskeleton remodeling. Our findings strongly suggest that actin stress fiber disruption triggers adipocyte differentiation, which provide a new insight to the regulatory mechanisms of adipogenesis.

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Lgr5+ Supporting Cells Proliferation and Hair Cell Transdifferentiation in Response to Wnt/ β -Catenin and Atoh1 Expression in the Postnatal Mouse Cochlea *in vivo*.

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During the development of the inner ear, Wnt signaling plays a role in the formation of the otic placode, and Wnt-responsive cells are present in the postnatal quiescent cochlea and vestibular sensory organs. Lgr5 is a Wnt target gene that is expressed in actively cycling cells of the intestinal crypt and hair follicles of the skin, and lineage tracing shows that Lgr5+ cells can self-renew and give rise to the major cell types and derivatives of the aforementioned organs. Thus, Lgr5 has been proposed to be a stem cell marker. Lgr5 has been shown to be expressed in the inner ear during the first week of postnatal development. We hypothesize that these Lgr5+ supporting cells might represent the progenitor/stem cells of the postnatal mouse inner ear with potential for hair cell regeneration, and that Wnt/ β -catenin overexpression in these cells would result in cell fate switch to hair cells. To test this hypothesis we characterized the Lgr5-EGFP-IRES-CreER;Rosa-floxed-stop- β -catenin overexpressor mouse that can be induced to ectopically express β -catenin specifically in Lgr5+ cells. Following the induction at P0-P1, Lgr5+ cells medial to the inner hair cells divided and formed many isolated replication foci throughout the cochlea that persisted up until P21. The Wnt-responsive cells did not transdifferentiate into hair cells, as they do not express putative hair cell markers (Myosin7a or Calbindin), but retain the expression of putative supporting cell marker (Sox2), and Lgr5. We conclude that the Wnt/ β -catenin overexpression alone in Lgr5+ supporting cells leads to their division, but is not sufficient to promote their differentiation into hair cells. We are investigating whether the co-expression of Atoh-1, a hair cell differentiation factor, will promote the transdifferentiation of the proliferating Lgr5+ supporting cells into hair cells. Supported in part by NIH DC006471 (J.Z.), NIH DC008800 (J.Z.), NIH DC011043 (A.C), and NIH CA21765, the Office of Naval Research N000140911014 (J.Z.), and ALSAC of SJCRH. J. Z. is a recipient of The Hartwell Individual Biomedical Research Award.

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Inflammatory cytokines alter cell fate decisions during airway epithelial morphogenesis.

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The precise balance and distribution of different epithelial cell types is required to maintain tissue homeostasis. In the lung, perturbations of this balance are hallmarks of human respiratory diseases, leading to goblet cell metaplasia and enhanced mucus secretion in asthma and chronic obstructive pulmonary disease (COPD). In the conducting airway, the basal cell acts as the progenitor for both secretory and ciliated cells. To examine the mechanisms regulating cell fate decisions in the airway, primary human basal cells were grown in a three-dimensional matrix to generate 'bronchospheres' containing a single, central lumen surrounded by a pseudostratified layer of epithelial cells. During development of the bronchospheres, the basal cells differentiated into mucus-producing goblet cells and ciliated cells. We found that inflammatory cytokine treatment resulted in a skewing of basal cell differentiation towards a

goblet cell fate, culminating in enhanced mucus production. We conclude that inflammatory cytokines, such as IL17A, direct the fate of the airway basal cell towards a mucus-secreting goblet cell, thereby altering the composition of the airway epithelium to produce the goblet cell metaplasia described in many respiratory diseases.

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Clonal analysis of hematopoietic stem and progenitor cells marked by five fluorescent proteins using confocal and multiphoton microscopy.

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We demonstrate a methodology for tracing the clonal history of hematopoietic stem and progenitor cells (HSPCs) behavior in live tissues in four dimensions (4D). This integrates genetic combinatorial marking using lentiviral vectors encoding various fluorescent proteins (FPs) with advanced imaging methods. Five FPs: Cerulean, EGFP, Venus, tdTomato, and mCherry were concurrently used to create a diverse palette of color-marked cells. Imaging using a confocal/two-photon hybrid microscopy approach enables simultaneous assessment with high resolution of uniquely marked cells in conjunction with structural components of the tissues. Generation of a diverse palette of clone colors via co-transduction of HSPCs with five LeGO vectors, combined with new imaging and analysis technologies, allowed tracing of HSPC engraftment and hematopoietic contributions at a clonal level, in the bone marrow and in a wide variety of other tissues in transplanted mice. The unprecedented high resolution images from optical sections were used to computationally reconstruct 3D-patterns of great complexity to depths of 150-300 μm , elucidating biologically-interesting clonal reconstitution patterns. We demonstrate that confocal imaging can be combined with multiphoton microscopy, revealing complementary information from autofluorescent and second-harmonic-generating (SHG) structures. Furthermore dynamic 4D high-resolution imaging is achievable by using video-rate scanning, red-shifted FPs and longer wavelengths lasers. Live studies combining video-rate multiphoton and confocal time-lapse imaging in 4D demonstrate the possibility of dynamic cellular and clonal tracking in a quantitative manner. Our approach allows clear tracking of color-marked hematopoietic clones readily identifiable in tissues of recipient for extended periods of time, bringing a new tool for assessing the plasticity that HSCPs possess. Understanding different aspects of development of hematopoietic clones in an irradiated reconstituted animal is of clinical relevance for chemotherapy, transplantation and gene therapy.

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Cell Cycle Arrest is Required For Cell Invasion Through Basement Membranes.

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There is an intimate and well-established link between cell cycle arrest and the transcriptional control of the differentiation of discrete cell types. Emerging data suggests that the complex cell biological behaviors that occur during morphogenesis (e.g., EMT and convergent extension) also require a prolonged G1 gap phase. Cell invasion through the basement membrane is a poorly understood morphogenetic cellular behavior, which occurs during development, immune surveillance and cancer metastasis. To functionally dissect cell invasive behavior at single-cell resolution, we are using the simple *in vivo* model of anchor cell (AC) invasion that occurs during *C. elegans* larval development. The AC, a specialized somatic gonadal cell, undergoes a basement membrane transmigration event to connect the developing uterine and vulval tissues. In order to identify transcriptional regulators of AC invasion, we performed a tissue-specific

RNAi screen targeting 697 *C. elegans* transcription factors, identifying the TLX ortholog, NHR-67, as a new regulator of invasion. Strikingly, loss of NHR-67 results in multiple ACs that fail to invade. Prevention of cell cycle progression in NHR-67-depleted animals rescues the invasion defect, demonstrating that cell cycle arrest is required for invasion. Time-lapse microscopy shows that mitotic ACs lack invadopodia, subcellular structures within the AC that function to breach the BM. Additionally, dividing ACs show reduced expression of downstream markers of invasive differentiated ACs, including matrix metalloproteinases (MMPs). Our data indicates the requirement for the precise transcriptional control of a genetic program that links cell cycle arrest to the differentiation of the invasive phenotype.

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Complement C1q production by osteoclasts and its regulation of osteoclast development.

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C1q deficiency is the strongest known risk factor for systemic lupus erythematosus (SLE) but its endogenous cellular origin remains limitedly understood. Here we investigate its production by both cultured and endogenous bone osteoclasts. Blood monocytes were cultured with RANKL and M-CSF to generate osteoclasts and these cells expressed C1q mRNA and also secreted C1q protein. Intracellular C1q was detectable in developing osteoclasts at day 3 by Western blotting and was also detectable by flow cytometry. By immunofluorescence microscopy, C1q was preferentially detected in immature osteoclasts. By multiple detection methods, C1q expression was markedly increased after IFN γ treatment. By immunohistochemistry, C1q was also detected in endogenous bone osteoclasts. When osteoclasts were cultured on immobilized C1q, these cells exhibited 2-7 folds increases in the expression of signature osteoclast genes (TRAP, cathepsin K, calcitonin receptor, carbonic anhydrase II and NFATc1), suggesting an osteoclastogenic capability. This is the first report of C1q production by osteoclasts. Its ability to enhance osteoclast development implies reduced osteoclastogenesis in SLE patients as they often experience decreased C1q levels. This is consistent with the non-erosive nature of lupus arthritis.

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Bioelectric Control of Regenerative Patterning.

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Regenerative biology holds the potential for developing transformative biomedical approaches addressing birth defects, injuries and degenerative diseases. Biophysical signals are crucial but still poorly-understood regulators of cell shape, differentiation, migration, and proliferation in vivo. Thus, patterns of resting potential in non-neural cells in vivo comprise an important system of instructive information that guides the structure and function of organ systems during embryogenesis and regenerative repair. During normal *Xenopus* development, a striking hyperpolarization demarcates specific clusters of cells in the anterior neural field. Specifically altering these Vmem patterns results in malformed eyes. Moreover, manipulating Vmem pattern in non-eye cells induces formation of well-formed ectopic eyes, even well outside the anterior neural field, suggesting that bioelectric signals reveal novel aspects of tissue lineage

competence. These ectopic eyes are morphologically and physiologically similar to endogenous eyes, and we characterized a feedback loop by which the transcriptional controls of eye induction interact with the biophysics of ion flows in early embryonic cells. Likewise we report that patterned control of resting potential in the anterior neural field is critically important in *Xenopus* brain tissue patterning. By developing strategies for specific, molecular-level control of bioelectric signals *in vivo*, we also identified cryptic asymmetries in the development of eye and neural crest (linked to the main left-right asymmetry determining pathway). Our data reveal that gradients of V_{mem} in patterning tissues are a new, instructive signal in during development, and demonstrate techniques by which these biophysical regulators of growth and form can be characterized and functionally manipulated at molecular resolution. Learning to control bioelectric initiators of organogenesis offers significant potential for regenerative biomedical treatment of diseases and for the basic understanding of patterning in evolutionary developmental biology.

Chaperones, Protein Folding, and Quality Control I

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Unusual post-translational modification of GFP derivatives.

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We have already reported a novel post-translational modification on the BraC protein in mammalian cells including human cells. The modification consists of two types of modifier, a peptide modifier and its N-glycosylation. The modification did not occur when the C-terminal lysine of BraC protein was replaced to Alanine or Arginine. In addition to the C-terminal requirement, a C-terminal sequence of 4 amino acids, TXXK is required for the modification. Recently, we found another target of the modifier, a CFP derivative expressed in mammalian cells by pCMV-secCFPmcs3. The plasmid encodes CFP fused with a mouse secretion signal of MHC Class I H-2K^d on the N-terminus and a peptide coded by multi-cloning-site (mcs3) on C-terminus. To study the sequence requirements of modification, we mutated C-terminal glutamic acid to the other 19 amino acid and then all the CFP derivatives were expressed in COS-7 cells. The cells were cultured in the presence or absence of epoxomicin, and the cell-lysates were immune-precipitated with anti GFP antibody. The immune-precipitates were analyzed with western blotting using anti GFP antibody. The modification occurred when the CFP were the C-terminal derivatives of R, K, E, H, M, G and D. Whereas the modification efficiencies were different with each amino acids of C-terminus, the other CFP derivatives of C-terminus were never modified. These results indicated that the C-terminal amino acid of secCFPmcs3 affects the modification in a different manner from that of BraC protein. Then, we examined whether the secCFP derivative fused with TEVK of C-terminal sequence of BraC is modified or not. The CFP derivative was modified. The C-terminal 4 amino acids sequence might be essential for the modification. We report here the GFP derivative as a novel target of the modification.

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Systematic genetic interaction mapping of protein folding and stress response pathways in the human ER.

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Systematic mapping of genetic interactions is a powerful approach to gaining a comprehensive mechanistic understanding of complex biological processes, and has successfully been applied to the yeast endoplasmic reticulum (ER) and other areas of biology in microorganisms. However, systematic genetic interaction mapping has thus far not been applied to mammalian cells. A genetic interaction map of the human ER would likely yield important insights into mammalian-specific ER functions.

We have now developed an RNA interference-based platform that allows us to conduct highly quantitative genetic screens and genetic interaction mapping in mammalian cells. To probe human ER function, we used the bacterial subtilase cytotoxin (SubAB), which kills cells by specifically cleaving BiP/GRP78, the Hsp70 chaperone of the ER, and thereby inducing the unfolded protein response (UPR) and apoptosis. We have identified a large number of genes that modulate the response of human cells to SubAB: Host factors required for SubAB trafficking to the ER, many of which we also identified for other ER-trafficking toxins; factors contributing to protein load in the ER; a variety of chaperones and other protein folding factors; signaling components of the UPR; and previously uncharacterized factors. Surprisingly, factors implicated in other types of cellular stress responses also modulated sensitivity to subAB, hinting at possible cross-talk between different homeostatic and stress response pathways. We are now systematically mapping genetic interactions between these factors to understand how they interact in pathways, and in order to pinpoint putative functions for previously uncharacterized proteins.

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Alteration of Hsp70 co-chaperone levels suppresses amyloid-like protein proteotoxicity by increasing the cells capacity to sequester polyQ into benign aggregates.

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Protein quality control (PQC), led by molecular chaperones, is crucial for partitioning misfolded proteins towards refolding or degradation via the ubiquitin proteasome (Meacham GC, et al. Nat Cell Biol 2001; Nillegoda NB, et al. Mol Biol Cell 2010). Improper PQC can result in misfolded protein aggregation. Numerous human diseases, particularly neurodegenerative disorders, are characterized by the accumulation of amyloid-like protein aggregates containing the disease protein (Wolfe KJ, Cyr DM. Semin Cell Dev Biol 2011). While the Hsp70 system is the first line of defense against protein aggregation by facilitating refolding, more recent work suggests a protective role of chaperone dependent promotion of aggregation (Douglas PM, et al. PNAS 2008). Rather than being causative, the inclusions seen in protein aggregation diseases may actually be a cellular coping mechanism. However, what determines the fate of a misfolded protein remains unclear. To address the debate of harmful versus helpful aggregation, we studied proteotoxicity associated with expression of polyglutamine expanded Huntingtin (Htt103Q) in a yeast model. We carried out a high copy toxicity suppressor screen in order to find a link between suppressors of toxicity and an increase in Htt103Q aggregation. Excitingly, we discovered that elevation of Hsp70 co-chaperone levels promotes formation of protective higher order species of amyloid-like aggregates. Suppression of proteotoxicity by the Hsp70 co-chaperone correlated with the packaging of Htt103Q in the JUNQ and IPOD quality control compartments (Kaganovich D, et al. Nature 2008). Thus, sequestration of Htt103Q into

intracellular protein deposits decreases cytotoxicity, and the Hsp70 system plays an active role in aggregate formation. [Work funded by Grant Number F31NS074777 from the NINDS]

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Determination of the Intracellular, Surface, and Extracellular Localization of Hsp70s under Different Stress Conditions.

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The 70-kD Heat shock proteins (Hsp70s) are a family of molecular chaperones that play essential roles in stress response by promoting protein homeostasis. Members of the Hsp70 family are primarily localized in different subcellular compartments, including the cytosol, endoplasmic reticulum, and mitochondria. Apart from their primary intracellular locations, several Hsp70s have also been found in different locations within the cell, as well as at the plasma membrane and extracellular milieu. Although few stresses and pathophysiological conditions, like cancer, have been related with the re-localization of Hsp70s within the cell, their translocation to the membrane, and secretion from viable cells, the majority of these conditions remain largely unknown. To this end we studied the localization of four members of the Hsp70 family under different stresses using sub-cellular fractionation. These experiments revealed that under normal growth conditions all four Hsp70s were present in multiple fractions, as well as in the extracellular medium in varying amounts. In stressed cells, Hsp70s' amounts in each fraction were significantly different from the untreated cells. These results suggest that heat and ethanol stresses cause re-localization and differential membrane-anchorage and secretion of the four Hsp70s. Additional experiments using pharmacological manipulation of intracellular trafficking pathways are currently being performed to identify the molecular mechanism used by Hsp70s to achieve their translocation, membrane anchorage, and subsequent secretion. This study is the first step towards elucidating the biological importance of the re-localization and membrane occurrence of these chaperones under stress conditions.

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The Co-chaperone Hch1 regulates Hsp90 function differently than its homologue Aha1 and confers sensitivity to yeast to the Hsp90 inhibitor NVP-AUY922.

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The 90 kiloDalton heat shock protein (Hsp90) plays a critical role in supporting the aberrant proliferative and anti-apoptotic signaling observed in cancer cells. Together with its numerous co-chaperones, Hsp90 stabilizes the active but labile forms of many oncoproteins. Like other molecular chaperones, Hsp90 is an ATPase but it possesses a unique ATP binding fold that is specifically targeted by certain small molecules. These Hsp90 inhibitors are being aggressively developed as anti cancer agents as Hsp90 in cancer cells is more sensitive to them than Hsp90 in normal cells. The basis for this selectivity is not known but thought to be related to the multiprotein complexes that Hsp90 forms with its co-chaperones and substrates in cancer cells. Using yeast as a model system, we have discovered that the co-chaperone Hch1p is a key determinant of Hsp90 inhibitor drug sensitivity. We show that the deletion of *HCH1* confers resistance to Hsp90 inhibitor drugs in yeast. Hch1p is a close relative of another co-chaperone protein called Aha1p which is a potent stimulator of the very low ATPase activity of Hsp90. Aha1p is comprised of an N terminal and C terminal domain that interact with the Hsp90 dimer at two discrete sites. Hch1p has a high degree of sequence identity to the N terminus of Aha1p but lacks the C terminal domain necessary for full ATPase stimulation. Interestingly, a chimeric protein comprised of Hch1p fused to the C terminal domain of Aha1p (which should behave like

Aha1p as an ATPase stimulator) does not stimulate the Hsp90 ATPase activity suggesting that there are important differences between Hch1p and the Aha1p N terminus. Our analysis of the interaction of these two co-chaperones with Hsp90 revealed that they cause very different conformational changes in the Hsp90 structure that influence nucleotide binding. Domain swaps show that this difference can be traced to a small region of these co-chaperones and reveal the importance of nucleotide exchange in drug sensitivity. Despite the similarity in the sequence and structure of these two co-chaperone proteins, they define Hsp90 complexes with very different sensitivity to Hsp90 drugs.

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Structural and Mechanistic Investigation of the Hsp90 ATPase Stimulator Aha1.

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The 90kDa heat shock protein (Hsp90) is an essential ATP-dependent eukaryotic chaperone that is upregulated during times of stress and helps to fold substrate proteins, called client proteins. Many of Hsp90's clients are key components for the development and maintenance of malignant cells. Because of this, the selectivity of Hsp90 inhibitors makes ideal candidates for cancer therapy and some Hsp90 inhibitors, such as 17-AAG, have entered the first phase of clinical trials. Hsp90 acts within a highly regulated and structurally dynamic cycle that includes the hydrolysis of ATP, cochaperone proteins and clients. Cochaperones interact with Hsp90 help to regulate client activation and ATPase rate. The cochaperones Hch1p and Aha1p are highly conserved and interact with Hsp90 in similar ways yet Aha1p is a robust stimulator of Hsp90's ATPase rate but Hch1p is not. To investigate the difference between these cochaperones, I created 8 chimeric proteins where either domains or small loops located at the Hsp90-cochaperone interface are interchanged between Hch1p and the N terminal domain of Aha1p. I used ATPase assays to test these Aha1p-Hch1p chimeric cochaperones' ability to stimulate WT Hsp90's ATPase rate. These results provide insight into the mechanistic basis for Aha1p-mediated ATPase stimulation of Hsp90. Moreover, this work shows the importance of conformational changes in the Hsp90 middle domain and how they allosterically regulate ATPase activation in the N terminal domain.

Lotz, G.P., Lin, H., Harst, A., and Obermann W. 2003 Aha1 Binds to the Middle Domain of Hsp90, Contributes to Client Protein Activation, and Stimulates the ATPase activity of the Molecular Chaperone. *Journal of Cell Biology*, 19: 17228-17235.

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EMC2 Encodes a Putative Novel Hsp90 Co-chaperone in *Saccharomyces cerevisiae*.

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The open reading frame *YJR088C* encodes a protein named Emc2p (for ER membrane protein complex 2); *EMC2* deletion results in a constitutively upregulated unfolded protein response (UPR). Yeast deleted for *EMC2* also exhibit synthetic genetic effects with Hsp90 in two different published high-throughput approaches. Of interest, *EMC2* encodes a ~35 amino acid TPR motif, suggesting the possibility that it may act as an Hsp90 co-chaperone.

In order to assess whether Emc2p is an Hsp90 co-chaperone, we first tested whether yeast deleted for *emc2* exhibit Hsp90 substrate folding defects using the well-established substrate glucocorticoid receptor (GR), the folding and function of which is dependent upon Hsp90. We find that yeast lacking Emc2p fail to robustly fold GR, similar to what is observed in yeast lacking the known Hsp90 co-chaperone Sti1p.

If Emc2p is indeed an Hsp90 co-chaperone, we would expect to find a physical interaction between the two proteins. Affinity pulldown experiments demonstrate a specific interaction between Emc2p and Hsp90. Of note, this interaction does not require the TPR domain of Emc2p.

Altogether, our data demonstrate both a functional and physical interaction of Emc2p with the Hsp90 chaperone machinery, supporting its role as a putative novel Hsp90 co-chaperone.

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The co-chaperone Hch1p regulates sensitivity to heat shock protein 90 inhibiting drugs in yeast.

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The 90kDa heat shock protein (Hsp90) is a highly conserved molecular chaperone that plays a vital role in regulation of a specific subset of target proteins termed 'clients'. Hsp90 regulates its clients, many of which are oncoproteins, through a conformationally dynamic cycle involving ATP and co-chaperone proteins. Cancer cells depend on Hsp90 making them sensitive to Hsp90 inhibitors, such as geldanamycin and NVP-AUY922, which are known to target multiple signaling pathways involved in cancer progression. Within eukaryotic cells Hsp90 is found in two highly conserved isoforms, and thus components of the yeast and human Hsp90 systems are functionally interchangeable. Human Hsp90 α and the *Saccharomyces cerevisiae* Hsp90 gene, *HSP82*, share 60% amino acid identity, making yeast an excellent model organism. Using yeast genetics I have determined that deletion of the co-chaperone *HCH1*, but not its homologue *AHA1*, rescues two temperature sensitive Hsp90 mutants, A587T and G313S. Importantly, strains that express either of these two mutant forms of Hsp90 are hypersensitive to Hsp90 inhibitors. This sensitivity is reversed when *HCH1* is deleted. Overexpression of *HCH1* confers hypersensitivity to Hsp90 inhibitors in wild type yeast and *HCH1* deletion confers high resistance to these drugs. We conclude that the co-chaperone Hch1p plays an important role in regulating sensitivity to Hsp90 inhibitor drugs and regulates Hsp90 in a manner distinct from its homologue Aha1p.

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Analysis of dynamic complex formations in heat shock protein 90 mutants utilizing ATPase assays and immunoprecipitation.

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The 90kDa heat shock protein (Hsp90) is a highly abundant, ATP-dependent molecular chaperone essential for regulation of a specific set of substrate proteins called 'clients', many of which are oncoproteins. Dozens of co-chaperone proteins regulate Hsp90 by assisting in client-protein recruitment or release, as well as activating or inhibiting Hsp90's ATPase activity. The relationship between Hsp90 and its co-chaperones is of great clinical importance as drugs targeting these co-chaperones alter Hsp90 action and arrest anti-apoptotic and pro-proliferative signaling in cancers. Hsp90 acts in the context of a conformationally dynamic ATPase cycle. Mutants of Hsp90 can confer growth defects to yeast owing to impairments in specific conformational transitions. Importantly, these defects can be overcome by co-chaperone proteins. These Hsp90 mutants are useful tools to determine the precise roles that specific co-chaperone proteins play in the Hsp90 cycle. We have shown that yeast that express either of the two different mutants (A587T or G313S) are rescued via deletion of the co-chaperone *HCH1*. I have investigated the co-chaperone dynamics of these two Hsp90 mutants

enzymatically through ATPase assays and assessed complex formation by immunoprecipitation to elucidate why *HCH1* deletion mitigates their disrupted growth phenotype. Hch1p and its homologue Aha1p are ATPase stimulators that can be inhibited by the action of another co-chaperone, Sba1p. The G313S and A587T mutants have very different intrinsic ATPase activities and are not affected by co-chaperones in the same way. Analysis of complexes formed in vitro between these mutants and co-chaperone proteins shows that the in vivo phenotypes in yeast involve other regulatory factors. Despite being considered homologues, Hch1p and Aha1p play very different roles in the Hsp90 cycle.

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Defining the Hsp104 folding reservoir: novel prions and toxic, intrinsically aggregation-prone proteins.

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Hsp104 is a yeast protein that performs two distinct and biologically important functions. First, Hsp104 fragments yeast prions to ensure their inheritance through successive generations. Prions are infectious amyloid conformers that convert soluble copies of the same protein to the amyloid form. Second, Hsp104 rescues proteins that have aggregated due to age or environmental stress and returns them to the native form and function. However, few natural Hsp104 substrates are known other than a handful of prions. To define Hsp104 clients with prion or aggregation-prone characteristics we exploited an Hsp104-deficient yeast strain ($\Delta hsp104$). Hsp104 is not essential for survival under regular growth conditions, but confers massive selective advantages in environments that elicit protein-folding stress. $\Delta hsp104$ yeast have reduced longevity, decreased stress tolerance and cannot maintain prion states. Extended overexpression of some prion proteins is toxic in wild type (WT) yeast due to loss of wild-type protein function or sequestration of other essential proteins, but $\Delta hsp104$ cells cannot propagate prions and are thus resistant to prion toxicity. Hence, we hypothesize that if proteins are overexpressed to induce toxic prion formation they will only do so in WT yeast and not in the $\Delta hsp104$ yeast. In this way, we can identify prion candidates in an unbiased manner. In addition, we hypothesize that if Hsp104 buffers against the toxicity of intrinsically aggregation-prone proteins, then overexpression of these proteins will be toxic only in $\Delta hsp104$ yeast but not in WT yeast. To determine the clients of Hsp104, a genome-wide dosage lethality screen in WT and $\Delta hsp104$ yeast was used. In this screen, WT and $\Delta hsp104$ yeast were transfected with a library of plasmids, in which overexpression of each yeast gene is controlled by a galactose-inducible promoter. Genes that are 'hits' in our screen fall into three broad classes: (A) toxic in WT and $\Delta hsp104$, (B) toxic in WT but not $\Delta hsp104$ and (C) toxic in $\Delta hsp104$ but not WT. We hypothesize class B is enriched in Hsp104-dependent prions and class C is enriched in toxic, intrinsically aggregation-prone proteins. Further assays such as fluorescence microscopy, SDS-resistance, and domain mapping will be used to determine whether proteins are forming prions or amorphous aggregates.

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Conformational changes of Hsp104 revealed through Small Angle X-ray Scattering (SAXS).

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Hsp104 is a hexameric AAA+ disaggregase found in yeast. Hsp104 has two key roles *in vivo*; thermotolerance and the regulation of prions. After thermal or chemical stress Hsp104 has the ability, in collaboration with co-chaperones Hsp40 and Hsp70, to remodel cytosolic aggregates and return proteins to their soluble, native form. Its role in prion regulation is two-fold. In yeast, prions function as non-genetic heritable traits. Hsp104 is required for prion propagation from mother to daughter through nucleation and fiber fragmentation, but cures some prion phenotypes when overexpressed.

Hsp104 is a large, multidomain protein. Each monomer contains two AAA+ domains resulting in an oligomeric species containing 12 sites of ATP hydrolysis. To date, there are no high resolution structures of either monomeric or hexameric Hsp104, and little is known about how the protein changes in shape during the ATPase cycle and how it uses these changes to exert remodeling forces on a variety of substrates.

Here I present ongoing work that uses an in solution technique, SAXS, to study the conformational changes of the Hsp104 hexamer during the ATPase cycle. SAXS studies are well suited to the Hsp104 system due to the large size of the particle, the large available yield after purification, the monodispersity of the hexamer and the large changes that take place in the presence of different nucleotides.

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Potentiated Hsp104 variants antagonize diverse proteotoxic misfolding events.

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Aberrant protein folding is implicated in several devastating neurodegenerative diseases. Inclusions containing the proteins TDP-43 and FUS are implicated in some cases of amyotrophic lateral sclerosis (ALS), while amyloid fibers comprised of α -synuclein are implicated in Parkinson's disease. Hsp104, an AAA+ protein from yeast, functions in regulating the disassembly of amorphous aggregates as well as prions. There are no other proteins known that are capable of specifically disassembling and solubilizing amyloid. Though Hsp104 is highly conserved, it has no human homologue. Therefore, we have developed potentiated Hsp104 variants and applied them to disease models of TDP-43, FUS, and α -synuclein pathology. These potentiated Hsp104 variants dissolve the aggregates, return the proteins to their proper cellular location, and strongly suppress toxicity in each of these disease models at levels far greater than wild-type. Surprisingly, we have also found that at certain positions in Hsp104, generic mutations to nearly any class of amino acid yield a hyperactive protein capable of eliminating aggregates. Using pure protein biochemistry experiments, we have probed the biochemical basis for these variants' potentiated activity and found that they have an enhanced ATPase and translocation rate, and are capable of dissolving aggregates without requiring co-chaperone collaboration. These results reveal important new insights into the mechanism by which Hsp104 dissolves amyloid, and demonstrate that proteins that misfold in neurodegenerative disease can be reactivated to their native state.

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Heat shock protein expression induced by elevated seawater temperature in the larvae of the reef-building coral *Porites astreoides*.

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Many scleractinian (hard) corals are living at or near their upper temperature limits, and are negatively impacted by global increases in sea surface temperatures. While elevated seawater temperatures have been shown to affect the survival of corals, few have investigated the physiological response of corals exposed to thermal stress. One mechanism of this response is the production of heat shock proteins (hsps), which have been shown to elevate thermotolerance in many organisms. To evaluate the expression of hsps, larvae of the reef-building coral *Porites astreoides* were incubated in either ambient (27°C) or elevated (31°C) seawater baths. Larvae were removed from the baths following either 4, 24 or 48 hours of incubation and analyzed for the differential expression of hsps with the molecular weights of 16 and 60 kDa. Larvae exposed to elevated seawater temperatures had greater expression of HSP16 and HSP60 following 24 and 48 hours of incubation compared to their ambient counterparts. The results indicate that hsp production is induced by elevated seawater temperature and may increase the thermotolerance of those reef-building corals with the genetic capacity to synthesize them. This may have implications about the effects of rising seawater temperatures on the future generations of hard corals and the ecosystems they construct.

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The SIRT1 modulators AROS and DBC1 regulate HSF1 activity and the heat shock response.

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The heat shock response (HSR) is the cellular response to protein damaging stress and is critical in the management of denatured proteins. The HSR plays a role in a number of diseases including heart disease, diabetes, cancer, and neurodegenerative diseases. We note that SIRT1 has been indicated as an important regulator of the HSR in an HSF1-dependent manner. In addition, protein modulators AROS and DBC1 have been indicated as important regulators of SIRT1. We are interested in gaining a better understanding of the mechanism of SIRT1 regulation of the HSR. We have found that the transcriptional and translational levels of SIRT1 are not effected upon the initiation of the HSR and that changes in the activity of SIRT1 are responsible for HSR regulation in an HSF1-dependent manner. We found that heat shock (HS) results in a decrease in NADH levels, an increase in the NAD⁺/NADH ratio, and an increase in recruitment of SIRT1 to the *hsp70* promoter. We also found that protein modulators AROS and DBC1 have an impact on *hsp70* transcription, HSF1 acetylation status, and HSF1 recruitment to and activation of *hsp* promoters. Our results indicate that SIRT1 regulation of the HSR impacts the deacetylation of HSF1 through a direct interaction that is influenced by an increase in the NAD⁺/NADH ratio. Furthermore, AROS and DBC1 regulate the acetylation status and thus the activity of HSF1. AROS and DBC1 are now two new targets available for therapeutic regulation of the HSR.

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Cellular nucleic acid binding protein ZNF9 is required for stimulating translation of terminal oligo pyrimidine tract containing mRNAs in HeLa cells during recovery from heat shock.

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The mRNAs encoding poly (A) binding protein (PABP1), eukaryotic elongation factor 1A (eEF1A) and ribosomal protein S6 (RPS6) are members of the terminal oligo pyrimidine tract (TOP) containing family of mRNAs. Translation of these mRNAs is regulated by growth signals and usually encodes proteins involved in mRNA translation. Previous studies from our laboratory showed that translation of PABP1 mRNA is stimulated during recovery of HeLa cells from heat shock. It was shown that the presence of the 5' TOP *cis* element was required for the preferred increase of PABP1 mRNA translation during recovery from heat shock. In the studies reported here we showed that translation of eEF1A and RPS6 was similarly enhanced during recovery. Analyses of samples of *in vivo* cross linked RNA– protein complexes, immunoprecipitated by ZNF9 antibody, for the presence of specific mRNAs revealed that the cellular nucleic acid binding protein ZNF9 binds to TOP mRNAs. However, mRNA lacking the TOP element, such as, the β -actin mRNA was also bound to ZNF9. To further elucidate the role of ZNF9 in stimulation of TOP mRNA translation during recovery from heat shock, siRNA was used to prevent expression of cellular ZNF9. Our results show that depletion of ZNF9 prevented the preferred stimulation of PABP1, eEF1A and RPS6 expression during recovery from heat shock. However, the constitutive expression of either β -actin or PABP1, eEF1A and RPS6 in HeLa cells was not affected by the absence of ZNF9. Our results suggest that binding of ZNF9 per se to mRNA does not inhibit translation. It is rather likely that ZNF9 act as a general facilitator of mRNA translation. Perhaps modification of the interaction between ZNF9 with other protein partners is responsible for its preferred effect on all three TOP mRNAs studied here. Furthermore, our results suggest that different TOP sequence responds similarly to ZNF9.

Regulation of Aging

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Mitofusin 2 is necessary for maintaining axonal targeting in midbrain dopamine neurons.

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Defects in precise control of mitochondrial dynamics can contribute to increase of dysfunctional mitochondria in mammalian cells. This is believed to play a role in aging and neurodegenerative diseases, such as Parkinson's disease (PD). However the link to the pathophysiological relevance is not well established. Here we generated conditional knockout mice with disruption of *Mfn1* or *Mfn2*, two homologous large GTPases that mediate mitochondrial fusion, in midbrain dopamine (DA) neurons to study the importance of *Mfn1* and *Mfn2* in DA neurons *in vivo*. Loss of *Mfn1* in DA neurons is clinically unaffected. We find no differences in life span or spontaneous motor activity of *Mfn1* DA neuron-specific knockouts. In contrast, disruption of *Mfn2* in DA neurons cause a lethal phenotype accompanied with reduced body weight and defects in rearing activity at 5 weeks of age. In addition, *Mfn2* DA neuron-specific knockouts died before 7 weeks of age. By using a reporter mouse with tissue-specific expression of YFP targeted to the mitochondrial matrix (mito-YFP), we find that loss of *Mfn2* in DA neurons leads to fragmentation of mitochondrial network and enlarged mitochondria. These spherical

mitochondria have abnormal cristae structure and impaired respiratory chain function. Parkin is involved in clearance of dysfunctional mitochondria *in vitro*, we therefore performed stereotaxic injections of adeno-associated virus (AAV) encoding mCherry-Parkin into the midbrain of *Mfn2* DA neuron-specific knockouts. However, mitochondria in *Mfn2*-deficient DA neurons with defective respiratory chain function do not recruit virally expressed Parkin *in vivo*. Surprisingly, we find no loss of midbrain DA neurons in *Mfn2* DA neuron-specific knockout mice, whereas there is a severe loss of DA nerve terminals in striatum accompanied by altered DA homeostasis. Our results show that *Mfn2*, but not *Mfn1*, is indispensable for maintaining mitochondrial morphology and axon integrity of DA neurons *in vivo*. Future elucidation of the underlying molecular mechanism may provide a role of mitochondrial dynamics in PD with degeneration of striatal DA nerve terminals.

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Retention of aging factors in yeast mother cells is SAGA-dependent.

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Almost all organisms undergo aging, even uni-cellular organisms such as *S. cerevisiae*: each mother cell produces a limited number of daughter cells before it eventually dies. However, the daughter cells are born young, indicating that the aging factors are retained in the aging mother cell. One such aging factor are extrachromosomal rDNA circles (ERCs). ERCs, like all non-centromeric plasmids replicate in S-phase but preferentially stay in the mother cell, where they accumulate and cause aging.

The retention of ERCs in yeast mother cells is crucial to produce rejuvenated progeny, but still the mechanism ensuring asymmetric segregation of ERCs is poorly understood. Previous work from our lab proposed that ERCs are attached to nuclear pore complexes (NPCs) and thereby are subjected to a diffusion barrier in the outer nuclear membrane at the bud neck (Shcheprova *et al.* Nature 2008). To investigate how ERCs are retained in the mother cell, we screened for proteins involved in this process.

The SAGA complex is a histone acetyl transferase complex involved in transcriptional activation, transcription elongation and mRNA export by linking actively transcribed genes to NPCs via the TREX-2 complex. Remarkably, we found that retention of non-centromeric DNA circles and ERCs in the mother cell require both SAGA and TREX-2 activity. Non-centromeric circles were less frequently found at the nuclear rim in SAGA-deficient cells, no longer co-localized with NPCs and moved faster. This suggests that the SAGA complex anchors plasmids to NPCs. Consistent with this conclusion, the defect in circle retention observed in SAGA mutants was reverted by either expression of SAGA-NPC fusion proteins or by artificial anchoring of the circle to NPCs. Furthermore, SAGA deficient cells show extended lifespan or age in an ERC independent manner, supporting the hypothesis that they fail to accumulate ERCs.

Taken together our work proposes a new role of the SAGA complex in the retention of aging factors in yeast mother cells.

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Trehalose defines yeast longevity by modulating cellular proteostasis.

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The nonreducing disaccharide trehalose has been long considered only as a reserve carbohydrate. However, our recent study of how a lifespan-extending caloric restriction (CR) diet alters the metabolic history of chronologically aging yeast suggested that their longevity is

programmed by the level of metabolic capacity - including trehalose biosynthesis and degradation - that yeast cells developed prior to entry into quiescence. To investigate whether trehalose homeostasis in chronologically aging yeast may play a role in longevity extension by CR, we examined how mutations affecting trehalose biosynthesis and degradation impact 1) the age-related dynamics of changes in trehalose concentration; 2) yeast chronological lifespan under CR conditions; 3) the chronology of oxidative protein damage, intracellular reactive oxygen species level and protein aggregation; and 4) the timeline of thermal inactivation of a protein in heat-shocked yeast cells and its subsequent reactivation in yeast returned to low temperature. Our data imply that CR extends yeast longevity in part by altering a pattern of age-related changes in trehalose concentration. We established molecular mechanisms underlying the essential role of trehalose in defining yeast longevity by modulating cellular protein homeostasis throughout lifespan. Our findings suggest the existence of two lifespan checkpoints at which the intracellular level of trehalose defines longevity by modulating cellular proteostasis. At one of these lifespan checkpoints in post-diauxic phase, trehalose operates as an anti-aging compound that 1) stabilizes the native state of proteins; 2) reduces the formation of insoluble protein aggregates by shielding the contiguous exposed hydrophobic side chains of amino acids that are abundant in aberrantly folded proteins and promote their aggregation; and 3) protects cellular proteins from oxidative carbonylation by interacting with their carbonylation-prone misfolded and unfolded species. At another lifespan checkpoint in stationary phase, trehalose functions as a pro-aging compound that shields the contiguous exposed hydrophobic side chains of amino acids in aberrantly folded proteins. By competing with molecular chaperones for binding with these patches of hydrophobic amino acid residues, trehalose interferes with the essential longevity-extending process of chaperone-assisted refolding of aberrantly folded proteins.

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Avian Eggshell Membrane: Cell Biology-Based Innovation of Human Health (2) Type III Collagen, Small Heat Shock Protein Alpha B-crystallin and Mild Exercise.

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AlphaB-Crystallin (α B), a small heat shock protein, has chaperone activities for various substrates, including proteins constituting the cytoskeleton, such as actin; intermediate filament; and tubulin. α B is now used as an anti-inflammatory and neuroprotectant molecule for post-stroke (Arac et al, 2011). We have already identified α B as a protective molecule for slow muscle atrophy (Atomi et al, 1991; Sakurai et al, 2005). α B expresses in lens, heart, brain, muscle, and also in skin. Skin is the largest organ of the human body, interfaces to the environment and protect us from various life-threatening stimuli such as UV, heat, infection, and dry. Sirtuins are key molecules for metabolism, ageing, and diseases and induced expression of sirtuins expects to have health benefits but few studies in skin have been done. For more than 400 years, avian eggshell membrane (ESM) has been utilized for skin problems with burned skin as well as wound. ESM has a fibrous network mainly comprised of type I, V, and X collagens. We have found that water-soluble alkaline-digested form of ESM can stimulate type III collagen, decorin, and MMP2 expression in human dermal fibroblast cells (Ohto-Fujita et al, 2011). In this study, we are aim to evaluate in vivo function of the ESM and benefit of mild exercise that both contribute to skin health. Gene expression profiling of early wound healing stages were examined using ϕ 6 mm full-thickness skin grafted db/db mouse and found that eggshell membrane may contribute to scar-less healing through time-limited type III collagen

expression. Hairless mouse (Hos HR-1) were exposed either single administration of ESM containing supplement and/or single 30-min mild treadmill exercise. Preliminary results showed that both ESM and mild exercise contribute to induction of type III collagen, α B, and Sirtuins. Upstream gene cascade and possible cross-talks between ESM administration and exercise will be further investigated.

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**Avian Eggshell Membrane: Cell Biology-Based Innovation of Human Health (1)
Stimulation of Extra Cellular Matrix Genes and Sirtuins in Skin.**

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In Asian countries, avian eggshell membrane (ESM) has long been utilized as a Chinese medicine for recoveries from burns, injuries, and wound. Even nowadays, Japanese Sumo wrestlers use it for wound healing. Powdered ESM is on market and used as a supplement and it is popular among Japanese women because they feel improvements for various health problems. We previously reported that type III collagen, decorin, and MMP2 gene expressions of human dermal fibroblast cells cultured on the alkaline-solubilized eggshell membrane (ASESM) conjugated with 2-methacryloyloxyethyl phosphorylcholine polymer (PMBN) biointerface were significantly higher than those on type I collagen dishes or tissue culture dishes, where similar microenvironment to dermal tissue such as spared cell distribution (Ohto-Fujita et al, 2011). We also performed an evaluation study of topical ASESM application on both human and mouse and reported at 2011 annual meeting. Aged 20-65 years of women human subjects were tested. The elasticity of both arms was measured after 12 weeks. Topical 1% ASESM solution's significantly increases of elasticity shown as Cutometer parameters at forearms and upper arms. It is postulated that some factors in ASESM may stimulate dermis regeneration/rejuvenation. Effects were also evaluated in mouse skin. Extracellular matrix (ECM) gene expressions of dorsal skin of hairless mice (Hos HR-1, male 6 weeks) after 10 days application of 10% ASESM solution were examined. Significant increase in both major fibrous ECMs of type III collagens and elastin and MMP2 mRNAs were observed. Recently Sirtuins (SIRTs) are reported to be responding to the photodamage in human skin (Benavente et al, 2012). In this study we tested the expression of SIRTs 1-7 in mouse skin after ASESM application and found that SIRT3 mRNA were significantly higher. Possible signaling cross talks between ECM effects and sirtuin stimulation by ESM will be discussed.

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The role of KLOTHO protein in regulation of melanin synthesis and protection against oxidative stress in Retinal Pigment Epithelia.

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Purpose: Increased susceptibility of the Retinal Pigment Epithelium (RPE) to chronic oxidative stress has been implicated in the pathogenesis of age-related macular degeneration (AMD), the

leading cause of vision loss and blindness among the elderly. Melanin, the pigment synthesized in the RPE, is important for light absorption and for protection against oxidative damages. Therefore, sustaining normal levels of melanin synthesis is crucial for RPE functions. In this study, we are investigating the role of KLOTHO (KL) protein in the pigment synthesis and oxidative stress response of RPE.

Methods: Histology, Real-time PCR, siRNA, cAMP assay, Ca²⁺ imaging, Immunostaining, ELISA.

Results: We have observed significant loss of pigmentation in the RPE of *Kl*^{-/-} knockout mice by histological examination of their paraffin embedded eyes, suggesting that KL might play a role in promoting pigment synthesis. To further test this hypothesis, we treated the human RPE with KL protein at the physiological levels (100-200pM) and showed that KL significantly increases the expression levels of the key melalogenesis genes, Microphthalmia-associated Transcription Factor (*MITF*) and tyrosinase (*TYR*), the rate-limiting enzyme for controlling the production of melanin. Since *MITF* gene expression is regulated by its upstream cAMP response elements and *MITF* is a known transcriptional regulator of *TYR* gene, we measured the levels of cAMP in the presence of KL and have shown a 50% increase in cAMP concentration after 10 minutes incubation with KL. In addition, knocking down the expression of the endogenous *KL* gene of RPE by *KL*-specific siRNA, decreased the expression levels of both *MITF* and *TYR* genes by 80%. To explore the role of KL in the stress response of RPE, we generated an *in vitro* system by exposing RPE to the oxidative stress inducing agent tertiary-butyl hydroperoxide (tBH) for 2 hours, and measuring the expression of stress response genes. We have observed a significant increase in gene expression for crystallin alpha A (*CRYAA*), crystallin gamma S (*CRYGS*) and vascular endothelial growth factor (*VEGF*). We demonstrated that pretreatment with KL protects the RPE from tBH-induced stress, by reducing the expression of the above stress response genes to control levels.

Conclusion: Our current results, together with our previous findings that KL decreases VEGF secretion in RPE, strongly suggest that KL has an important role in the regulation of the melanin synthesis by increasing the endogenous cAMP levels and in protection against oxidative stress in RPE.

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Cell Biological Approaches to Test the Efficacy of Botanical Ingredients for Aging Skin - Method development.

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Skin is the largest organ in the human body as it provides a protective barrier against the environment which also defends us from external pathogens. It is an indicator of overall well being and health. In the recent times there is a high demand of using a non invasive technology in restoring “youthfulness” of the skin. Therefore, there is a need for a method that will help in testing efficacy of ‘antiaging’ ingredients. Outlined below, we identified novel approaches to better characterize ‘antiaging’ ingredients.

It is known that oxidative stress is a primary culprit in causing cutaneous aging. The first method we developed was to screen ingredients which resist oxidative stress. We coined this method as the ‘anti-stress’ assay. In this assay we induced aging of the human dermal fibroblast (HDF) cells with hydrogen peroxide, which simulates oxidative stress conditions. The ‘aged’ cells were

then treated with one of our botanical ingredients and the cell viability was measured by a fluorescent-based assay. We concluded that our ingredient protected HDF cells from oxidative stress and hence from known 'aging' processes.

Further, from the above experiment we investigated deeper into the molecular mechanism of this novel ingredient. We know Sirtuins, especially SIRT 1 is a longevity gene. This gene is also known as the 'youth protein'. Stimulation of this protein extends the lifespan in yeast and delays the onset of age-related diseases in mammals. We observed that our ingredient upregulated SIRT1 protein expression.

A good anti-aging ingredient must not only activate anti-aging parameters but also inhibit the "aging gene programming". An aging program in cells can be initiated by activation of NF κ B. Therefore, we hypothesized that an ideal antiaging ingredient should inhibit this pathway. Our ingredient inhibited NF κ B activity in a cell-based reporter assay.

Furthermore, we tested to see if the stimulation or inhibition of the 'aging' pathways modulates any response of the target genes which makes the skin youthful. We saw an increase in the gene expression of HAS and Ki67 by qPCR assay.

Another key cause of aging is the glycation of collagen, known to increase with age. The glycation process disrupts the collagen assembly and its networking capability making the skin appear stiff and aged. We showed that our ingredient also inhibited glycation.

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Mediation of metal-catalyzed oxidative damage by a Nutraceutical Formulation.

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As advances in medicine and nutrition have led to increased human longevity, diseases of aging have thus become more prevalent and have stimulated increased research interest. These diseases of aging include neurodegenerative disorders such as Alzheimer's Disease (AD) and Parkinson's Disease. A better understanding of the pathophysiology underlying these disease states and factors mediating their progression is critical. One such factor is the aggregation of amyloid beta, a normal protein which misfolds in AD and forms plaque deposits in the brain. A second factor is oxidative damage caused by excessive levels of free transition metals, which exist normally in human physiology but are toxic to neurons at high levels.

Recently, we developed a Nutraceutical Formulation (NF) which has been shown to improve cognitive function in people with AD, Mild Cognitive Impairment or even in normal aged individuals. The aim of this study was to determine the impact of exogenous iron and copper on the effectiveness of NF in neuronal cell culture at 2 hours to 7 days post-treatment, as well as the interplay between these metals and amyloid beta. Evaluation of cell cultures was accomplished using fluorescence microscopy, with dichlorofluorescein (DCF) used to detect oxidative damage and diamidino phenylindole (DAPI) used to determine cell death.

We determined that excessive exogenous metals, particularly iron, can increase oxidative damage to at least the same extent as amyloid beta in neuronal cell culture. NF counteracts this damage. Also, simultaneous exposure of cultured neurons to metals and amyloid beta produced a synergistic deleterious impact; ongoing research is examining if NF can counteract this

synergy. Future treatment of neurodegenerative diseases such as AD may include strategies to eliminate excess amounts of these metals while at the same time employing nutrients such as those found in NF to counteract neuronal damage.

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Sestrin2 modulates ROS-dependent cellular senescence through the NADPH oxidase 4 activation.

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Sestrin2 (Sesn2) is known to be involved in the maintenance of metabolic homeostasis and in aging through modulation of the AMPK-mTOR pathway. We show here that Sesn2 also plays a role in the cellular senescence in mouse embryonic fibroblasts (MEFs). Sesn2 knockout MEFs showed senescent-like morphology, including flattened, irregular shape and enlarged size, as well as senescence associated (SA)- β -galactosidase activity. Reactive oxygen species (ROS) were significantly increased in the Sesn2 knockout MEFs. While the levels of many antioxidant proteins including thioredoxin and 6-members of peroxiredoxins were not changed in the Sesn2 knockout MEFs, the level of NADPH oxidases 4 (NOX4), one of ROS generating enzymes, increased dramatically. Additionally, AMP-activated protein kinase (AMPK), a sensor of cellular energy, was activated in Sesn2 knockout MEFs. Pretreatment of Sesn2 knockout MEFs with an antioxidant N-acetyl-cysteine (NAC) reduced SA- β -galactosidase activity and inhibited AMPK activation. Therefore we suggest that the induction of NOX4, which causes ROS generation and subsequent AMPK activation, is a likely mechanism of cellular senescence resulted from the loss of Sesn2.

Cell Death

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Simultaneous analysis of DNA damage, cell cycle, and apoptosis using multiparameter flow cytometry.

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DNA double-strand breaks can be induced by a number of cellular events, including exposure to genotoxic agents, apoptotic DNA fragmentation, and normal cellular processes such as immunoglobulin gene recombination. Histone H2AX is phosphorylated on serine 139 in response to DNA double-strand breaks. Antibody-mediated detection of H2AX (pS139), also known as gamma-H2AX, has been shown to be a useful indicator of DNA damage, enabling the detection of double-strand breaks induced by genotoxic agents or by apoptosis. The topoisomerase inhibitors camptothecin and etoposide induce DNA double-strand breaks in cycling cells by two mechanisms. Both inhibitors have direct DNA-damaging effects, while also triggering apoptosis and the associated DNA fragmentation. To distinguish between direct versus apoptosis-initiated DNA damage, H2AX (pS139) was analyzed simultaneously with other indicators of cell status, including markers of cell cycle position and apoptosis. Conditions for cellular fixation and permeabilization were optimized to achieve the highest resolution of all markers of interest. The kinetics of H2AX phosphorylation, caspase-3 activation, and PARP cleavage were assessed following cellular treatment with camptothecin, etoposide, or TNF in the presence or absence of caspase inhibitors. Caspase-independent, early phosphorylation of

H2AX was induced by both camptothecin and etoposide but not by TNF. In contrast, all three cellular treatments induced late phosphorylation of H2AX in a caspase-dependent manner. The kinetics of the late H2AX phosphorylation corresponded closely with caspase-3 and PARP cleavage kinetics. Multiparameter flow cytometric analysis of H2AX (pS139), cleaved caspase-3, cleaved PARP, and DNA content provides a powerful tool for studies of normal cellular processes and responses to cytotoxic agents.

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Induction of Cellular Apoptosis and Necrosis following Manganese Oxide Nanoparticle Exposure in Neuronal Cell Cultures.

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Exposure to high levels of manganese (Mn) has been linked to a Parkinson-like disorder called manganism, also called Welder's disease. These exposure levels may result in irreversible neuronal damage to critical areas of the brain. The exact mechanism of neurotoxicity associated with high manganese exposure is unknown. In previous studies, we have shown that manganese oxide nanoparticles (Mn₂O₃) and ionic manganese containing compounds, manganese chloride (MnCl₂) and manganese acetate (MnAc), result in a dose-dependent increase in cytotoxicity and oxidative stress using human embryonic kidney cells (HEK 293). At the same comparative dose, Mn₂O₃ was more cytotoxic and induced a greater level of oxidative stress. In this study, we first addressed the role of dispersion and dissolution in the neurotoxicity of Mn₂O₃ nanoparticles. There was a significant reduction in the agglomeration of particles with the addition of fetal bovine serum, FBS (media alone 4194nm vs. media/serum 261nm). The dissolution rate of free Mn following the incubation of Mn₂O₃ nanoparticles in cell culture media for 48h at 37°C was minimal (<1%). Increasing concentrations of all Mn-containing compounds resulted in time- and dose- dependent increases in the number of apoptotic cells. At higher concentrations of Mn₂O₃, in comparison to MnCl or MnAc, we observed the accumulation of particles inside cells, alterations in cellular nuclei, and increased cellular necrosis. These studies suggest the toxicity of manganese containing compounds may not only be related to the release of free Mn but the physical properties of manufactured nanoparticles.

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Rapid Mitochondrial Depolarization Induced by Gambogic Acid in Cytotoxicity Studies

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Understanding and evaluation of drug induced cyto-toxicity requires evaluation of how treatments impact multiple cell health parameters. Gambogic acid, a xanthone from *Garcinia hanburyi*, is a compound with potent anti-cancer activity. It stimulates apoptosis and cell death in a number of cell lines however its mechanism of action is not well understood. In this study, we present results from the treatment of gambogic acid on Jurkat and HB Cells evaluated using multiple assays that helped assess mitochondrial potential changes, caspase 3,7 and MultiCaspase activity and phosphatidylserine expression using Annexin V binding, all being performed with inclusion of a cell death marker. Data from both dose response and time course studies were obtained and samples analyzed on the Muse Cell Analyzer. Our results demonstrate that gambogic acid induces rapid cell death in Jurkat, HB and HL-60 cell lines by induction of cellular apoptosis. Further, time course studies with Jurkat cells with gambogic acid demonstrate that a high proportion of cells exhibited rapid mitochondrial membrane

depolarization in less than 1 hr of treatment while apoptotic impacts such as Annexin V response, and cell death showed a slower rate of appearance for the same concentration of treatment. Apoptosis is soon followed by cell death in these treatments. Overnight treatment of Jurkat cells with low doses of gambogic acid demonstrated significant apoptotic impacts on Jurkat cells with negligible impact on cell cycle observed under the same conditions indicating apoptosis being the primary and initial impact on these cells. Treatment of HB cells with gambogic acid also resulted in significant apoptosis with pan-caspase and Caspase-3,7 and cell death activity being observed. Our studies suggest that gambogic acid induced apoptosis proceeds through significant and rapid impacts of the drug on the mitochondria which subsequently trigger other apoptotic and cytotoxic effects. Comprehensive concentration and time course studies with multiple apoptotic markers can thus provide valuable insights into mechanism of compound action.

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Structural and Functional Determinants of Toxicity in Spinal and Bulbar Muscular Atrophy.

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Polyglutamine-repeat disorders are part of a larger family of neurodegenerative diseases characterized by protein misfolding and aggregation. In spinal and bulbar muscular atrophy (SBMA), polyglutamine expansion within the androgen receptor (AR) causes progressive debilitating muscular atrophy and lower motor neuron loss in males. Histologically, the polyglutamine-expanded AR aggregates to form nuclear inclusions upon hormone binding. Soluble (pre-inclusion) aggregates of the AR are considered to be a toxic intermediate in the aggregation process. Determining the molecular criteria guiding the formation of soluble aggregates is fundamental to understanding SBMA pathogenesis.

Gender-specificity is unique to SBMA and is caused by the requirement of hormone (testosterone or dihydrotestosterone) for disease. Several events downstream of the hormone-bound AR, including an interdomain interaction between the amino- and carboxyl- terminus of the AR, nuclear localization, and acetylation are also necessary for toxicity. In order to gain further insight on nuclear events that may contribute to the formation of a toxic conformation of the polyglutamine-expanded AR, we sought to determine the role of AR DNA binding in SBMA pathogenesis. Based on differences in aggregate morphology - but not toxicity - between DNA binding-competent and DNA binding-deficient cells, we carried out extensive biochemical analyses to identify similarities between aggregated species that may be indicative of a toxic conformation. Utilizing SDS agarose gel electrophoresis (SDS-AGE), ultracentrifugation, and filter trap by cellulose acetate, we identified a heterogeneous distribution of AR oligomers that precede the formation of frank nuclear inclusions. Using tet-on AR112Q PC12 cells, we show that oligomers that form early in the course of hormone treatment are comprised of full-length AR and retain reactivity to the conformation-specific antibody 3B5H10. With time, we see a predominant shift towards faster migrating oligomers that are not reactive for 3B5H10. We further show that these 'late oligomers' are comprised of full-length, insoluble AR species; this conformational change directly correlates with an increase in nuclear inclusions, as detected by fluorescent microscopy. In an attempt to hone in on a particular conformation of AR species that may be correlative with toxicity, ongoing studies are focused on characterizing commonalities between DNA binding-competent and DNA binding-deficient cells. Identifying a common signature of soluble AR species will advance the development of targeted therapies applicable to a wide range of neurodegenerative diseases caused by protein misfolding.

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Effect of vitamin A on recovery of noise-induced hearing loss.

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To determine the relation between vitamin A deficiency and noise-induced tissue repair, groups of normal diet or vitamin A-deficient diet C57BL/B6 mice were exposed to a white-band noise for 3 hours at levels of 95 dB SPL. Auditory brainstem response thresholds were measured pre-exposure and several times post-exposure (1-30 days) to determine magnitude of threshold shift. Degree of hearing loss immediately after exposure to noise at all 4 frequencies (4, 8, 16, and 32 kHz) was similar in two groups. However, hearing recovery after noise exposure was different. Vitamin A-deficient diet mice were not recovered at 4 kHz frequency while normal diet mice were recovered at 4, 8, and 16 kHz frequencies. At 32 kHz frequency, two groups were recovered with similar level but not fully rescued.

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Mgrn1 regulates neuronal endosomal trafficking and functions in cellular defense against apoptosis.

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Mahogunin RING finger 1 (Mgrn1) is a ubiquitously expressed protein whose null mutation causes age-dependent prion disease-like spongiform neurodegeneration in mice. Moreover, depletion of Mgrn1 by pathogenic prion protein isoforms has been implicated in the pathogenesis of human prion disease. These findings highlight the importance of understanding the cellular function of this poorly characterized protein. We have shown that Mgrn1 is an E3 ubiquitin-protein ligase that regulates endosome-to-lysosome trafficking by ubiquitinating ESCRT-I component TSG101 in HeLa cells. Immunofluorescence confocal microscopic analysis reveals that endogenous Mgrn1 protein is associated with early endosomes in axons, dendrites, and synapses of mouse cortical neurons. We find that Mgrn1 null mutation causes accumulation of enlarged and clustered endosomes and lysosomes in adult brain tissue and cortical neurons from Mgrn1 deficient mice. Furthermore, our analyses reveal that loss of Mgrn1 leads to increased susceptibility of cortical neurons to oxidative stress induced apoptosis. Our findings support a role for Mgrn1 in the regulation of neuronal endosome-to-lysosome trafficking and cellular defense against apoptosis, and they suggest a pathogenic mechanism by which Mgrn1 null mutation causes spongiform neurodegeneration by impairing neuronal endosomal trafficking.

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Genetic Interactions Between BX11 and IRE1 in the Regulation of the Unfolded Protein Response and Cell Death in Yeast.

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Bax inhibitor-1 (BI-1) is an anti-apoptotic gene whose expression is upregulated in a wide range of human cancers. Studies in both mammalian and plant cells suggest that the BI-1 protein resides in the endoplasmic reticulum and is involved in the unfolded protein response (UPR) that is triggered by ER stress. We have been investigating the function of the yeast gene, BX11, which appears to be a homolog for mammalian BI-1. In previous studies, we showed that yeast cells from two different strain backgrounds lacking BX11 are more sensitive to heat-shock

induced cell death and ER-stress induced cell death. They also have lower viabilities in ethanol and 2% glucose, two known triggers of yeast programmed cell death. Finally, we noted that $\Delta bxi1$ cells have a decreased unfolded protein response as measured with a UPRE-lacZ reporter. More recently, we have created $\Delta bxi1 \Delta ire1$, $oeBXI1 \Delta ire1$, and $oeIRE1 \Delta bxi1$ double mutants that have allowed us to characterize the genetic interactions between these two genes in the regulation of the UPR and cell death in yeast. Our data suggests that both genes may work in different pathways to influence the physiology of yeast cells undergoing programmed cell death. [Our laboratory is supported by the following grants: NIGMS R15 GM094712, NSF MRI-R2 0959354, and a subcontract from NIH Grant 2 P20 RR016457 awarded to the Rhode Island INBRE Program, for undergraduate student training.]

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Non-canonical apoptotic caspase cleavage after glutamic acid is biologically relevant.

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The caspase proteases play a major role in initiating and executing apoptosis. Caspases were discovered in the mid 1990's and were quickly shown to have a unique and required preference to cleave after aspartic acid. Recent proteomics studies have identified close to 2000 apoptotic caspase substrates in human cells. However, caspase cleavages after aspartic acid represent only 30% of identified apoptotic cleavage events. Analysis of non-aspartic acid apoptotic cleavage events reveals a smaller set of ~250 cleavages after glutamic acid that are as significantly enriched as aspartic acid cleavages over untreated levels. The sequence logo for the glutamic acid cleavages shows enrichment for DEVE|[G/S/A], identical to the preferred executioner caspase substrate sequence. Kinetic, structural, and further substrate analysis confirm that caspase -3 and -7 do cleave after glutamic acid in cells.

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Depletion of either nuclear poly (A) binding protein (PABP) or the cytoplasmic PABP1 results in nuclear translocation of PABP4 and apoptotic cell death triggered by p53 phosphorylation.

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In vertebrates, poly(A) binding protein (PABP) is known to exist in five different isoform. PABPs are primarily cytosolic with the exception of the nuclear PABP (PABPN1), which is located in the nucleus. PABP1 binds to the 3' poly(A) tail of the mature mRNA and facilitates the recruitment of the ribosome to the mRNAs 5' end. PABPN1 binds to poly(A) of the nascent mRNA and regulates the polyadenylation process. The cellular function of other PABPs (PABP3, 4, 5) remains relatively uncharacterized. In this study we have investigated the effect of PABP1 and PABPN1 ablation on human cells. For this, we depleted PABP1 or PABPN1 using RNAi from both HeLa and HEK293 cells. In all cases the survival rate of transfected cells was severely affected. After 72 hours of transfection, 40-60% cells were found to undergo apoptosis by p53 phosphorylation at ser46. PABP1 depletion led to almost 50% reduction in cellular protein synthesis, whereas PABPN1 depletion did not have any effect on poly(A) tail length, transcription, or translation of mRNA. However, in both situations a unique mechanism of PABP homeostasis was observed. Notably two non targeted cytoplasmic PABPs, PABP4 and 5, expressions were altered in PABPN1 or PABP1 depleted cells. There was approximately 3 fold increase ($p \leq 0.05$) in the cellular abundance of PABP4 and PABP5 in PABP1 depleted cells. In PABP1 depleted cells PABP4 but not PABP5 was associated with the polyribosomes and co-immunoprecipitated with eIF4G. However, this homeostasis was not sufficient to prevent cell

death as the number of apoptotic cells further increased to 85% in 96 hour culture. In PABPN1 depleted cells, the level of PABP5 increased by 4-5 fold ($p \leq 0.05$); PABP4 level remained unaffected, however significant amount of the protein translocated to the nucleus (4-5 fold increase in nuclear PABP4). Furthermore, PABP4 was detected in the nuclear α -actin pre-mRNA of PABPN1 depleted cells by RNA immunoprecipitation studies. Nevertheless, like PABP1 depletion, PABPN1 cells also showed significant apoptosis in 96 hours culture suggesting PABP4 and PABP5 homeostasis were not sufficient to rescue the PABPN1 depleted cells. Our results suggest that PABPN1 may have a novel anti apoptotic role in mammalian cells.

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Protective role of C-peptide against hyperglycemia-induced endothelial apoptosis by regulating ROS-mediated TG2 activation in diabetes.

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C-peptide is a bioactive peptide with a potentially protective role in diabetic complications; however, its molecular mechanism of protection against cardiovascular damage due to hyperglycemia-induced apoptosis remains unclear. We investigated the protective mechanism of C-peptide against hyperglycemia-induced apoptosis using human umbilical vein endothelial cells and streptozotocin diabetic mice. High glucose (33 mM) induced apoptotic cell death in endothelial cells via sequential elevation of intracellular Ca^{2+} and ROS as well as subsequent activation of transglutaminase 2 (TG2). C-peptide (1 nM) prevented endothelial cell death by inhibiting protein kinase C- and NADPH oxidase-dependent intracellular ROS generation and by abolishing high glucose-induced TG2 activation, without affecting intracellular Ca^{2+} levels. Consistently, in the aorta of streptozotocin diabetic mice, hyperglycemia stimulated transamidating activity and endothelial cell apoptosis that was inhibited by C-peptide replacement therapy (35 pmol/min/kg) using osmotic pumps. In addition, C-peptide prevented hyperglycemia-induced activation of transamidating activity and apoptosis in the heart and renal cortex of streptozotocin diabetic mice. Thus, C-peptide protects endothelial cells from hyperglycemia-induced apoptotic cell death by inhibiting intracellular ROS-mediated activation of TG2. Furthermore, TG2 may be a promising avenue of therapeutic investigation to treat diabetic vasculopathies.

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Xenoestrogens, Bisphenol A (BPA) and 4-nonilphenol (NP), induce activity of ADAM17.

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Introduction: Plasticizers with estrogenic activity such as BPA and NP disrupt spermatogenesis by a still unknown mechanism. In our lab, we have shown that ADAM17, an extracellular metalloprotease capable of shedding the ectodomain of several membrane-anchored proteins like neuregulin [1], participates in physiological and genotoxic induced-germ cells apoptosis [2]. The aim of our work was to determine if apoptosis induced by xenoestrogens is dependent of ADAM17 activity.

Materials and Methods: Prostate cancer cell line LnCap were stably transfected with a vector encoding a fusion protein encompassing alkaline phosphatase (AP) and neuregulin β 1, (AP)-NRG β 1. ADAM17 sheddase activity was measure as the release of AP into the culture medium after incubation with BPA and NP, in the presence or absence of different metalloprotease

inhibitors. Active ADAM17 protein levels were evaluated by western blot in the different conditions. Cell death was evaluated by trypan blue incorporation, cleavage of Poly ADP ribose polymerase (PARP) by Western blot and active caspase-3-positive cells by immunohistochemistry.

Results: LnCap cells express ADAM17, but not NRG β 1, as evaluated by RT-PCR, Western blot and immunofluorescence. AP activity showed a robust increase after 3 h of treatment with 100 μ M BPA or 50 μ M NP compared with the vehicle, which was reduced by GW280264X (10 μ M), an ADAM17 inhibitor. In addition we observed a statistically significant increase of the processed active ADAM17 in the total protein extract.

Active caspase-3-positive cells were robustly increased after incubation with 50 or 100 μ M NP, and an increase in cleaved PARP protein levels (which is a substrate of caspase-3) was detected starting from 1 h after treatment. In addition, trypan blue positive cells were observed with 100 μ M NP. In order to study the pathway involved in ADAM17 activation we found that the p38 MAPK inhibitor PD169316 (5 μ M) and the calcium chelator BAPTA-AM (10 μ M) did not have any effect upon AP shedding after NP treatment.

Discussion: Our results show that xenoestrogens (BPA and NP) induce ADAM17 sheddase activity in LnCap cells, and suggest that this may be related to its role in apoptosis.

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Characterization of anti apoptotic protein family (Bax inhibitor and Lifeguard) in *Hydra vulgaris*.

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Hydra vulgaris belongs to the phylum Cnidaria with a very well conserved programmed cell death signalling pathway. Components of the apoptotic molecular pathway such as Bcl2-family members, caspases, caspase inhibitors (IAPs), APAF-1, TNF-receptor and FADD have been identified in hydra. However, two cytoprotective protein families, which consider playing important role in regulation of apoptosis pathways, remain to be investigated.

Our results revealed that two cytoprotective protein families with cell death suppressing function exist in hydra i.e., the Bax inhibitor protein family and the Lifeguard-family (LFG). These proteins are hydrophobic with 6-7 helix transmembrane domain structures and have been shown to be localized in membranes (Golgi, endoplasmic reticulum and nuclear membrane).

Moreover, Bax inhibitor1 is an integral endoplasmic reticulum -membrane protein and is shown to inhibit apoptosis induced by camptothecin when expressed in HEK293 cells. In situ hybridisation experiments show that it is expressed during both, oogenesis and spermatogenesis, where apoptosis pathways also play roles.

In addition, we have found three sequences encoding LFG proteins in Hydra i.e., LFG4 and two homologous of LFG1i. We cloned TMBIM4/LFG4 (Golgi anti apoptotic protein) and found it to be localized in Golgi and endoplasmic reticulum compartments. In situ hybridisation experiments indicate expression of LFG4 in endodermal epithelial cells. We are currently analyzing, whether it protects hydra cells from both intrinsic and extrinsic apoptotic stimulating factors.

Further studies on these proteins will provide more evidence about their role in regulating cell death pathways.

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The apoptosis inhibitor ARC alleviates the ER stress response to promote β -cell survival in diabetes.

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Type 2 diabetes involves both insulin resistance and “failure” of β -cells, the cells responsible for insulin secretion. An important component of β -cell failure is cell loss by apoptosis. ARC (Apoptosis Repressor with Caspase Recruitment Domain) is an inhibitor of apoptosis that is highly expressed in cardiac and skeletal myocytes and neurons. ARC possesses the unusual property of antagonizing both the extrinsic (death receptor) and intrinsic (mitochondria/endoplasmic reticulum (ER)) cell death pathways. We discovered that ARC protein is abundant in cells of the pancreatic islets, including >99.5% of mouse and 73% of human β -cells. Moreover, we found that deletion of *nol3*, encoding ARC, markedly exacerbates hyperglycemia and further impairs glucose tolerance in diabetic (*ob/ob*) mice, and this is accompanied by pancreatic islet disorganization and increases in β -cell apoptosis. ER stress is a major component of type 2 diabetes and an important trigger of β -cell death. Genetic gain- and loss-of-function studies in cultured cells demonstrated that ARC inhibits β -cell apoptosis elicited by the ER stressors tunicamycin, thapsigargin, and the free fatty acid palmitate. Unexpectedly, inhibition of cell death by ARC is mediated by suppression of the ER stress response, a novel function of ARC. Further analysis showed that ARC acts distal to ER stress sensors PERK and IRE1 α to suppress induction of CHOP, a transcription factor that activates both genes involved in resolving ER stress and genes involved in cell death. In pancreatic islet tissue stimulated with palmitate, depletion of ARC augments CHOP abundance and apoptosis, which is dramatically rescued by deletion of CHOP. Taken together, these data demonstrate that ARC is a previously unrecognized inhibitor of β -cell apoptosis in type 2 diabetes, and its protective effects are mediated through a novel mechanism involving suppression of the ER stress response pathway.

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Moderate ER stress induces p44/p42 kinase activation and an ITPR-dependent apoptosis.

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When unfolded proteins accumulate in the endoplasmic reticulum (ER), stress signaling pathways called the Unfolded Protein Response (UPR) are induced. Modeling of ER stress in cultured cells is often performed with high doses of poisons that arrest essential functions within the ER. In examining the potential implications of chronic ER stress signaling in organismal models, a modest ER stress would be anticipated as clinically appropriate. In this study, the ER was challenged with a 50-fold range of tunicamycin, an inhibitor of N-linked glycosylation.

Tunicamycin induced cellular apoptosis and stress signaling over a range of concentration from 20 nM to 1000 nM. Activation of the ER transmembrane protein IRE1 was measured by quantification of IRE1-mediated splicing of the XBP1 pre-mRNA. IRE1 activity increased with dose of tunicamycin, but was detectably activated at doses as low as 20 nM. GRP78 expression, another marker of ER stress signaling, was increasingly induced with dosage of tunicamycin exposure. Interestingly, apoptosis induced by moderate levels of ER stress was susceptible to inhibitors of the inositol trisphosphate receptor (ITPR), a key release point for ER Ca²⁺ stores. This susceptibility was not observed with doses of tunicamycin above 40 nM, suggesting apoptosis induced by moderate doses is controlled by ITPR signaling. Assessment of cytosolic protein kinase pathways revealed the p44/p42 kinases to be specifically induced at tunicamycin concentrations from 20-40 nM while the p38 kinase was increasingly stimulated with increasing tunicamycin dose. This work collectively reveals moderate ER stress to generate a distinct stress signal relative to more severe ER stress exposure.

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Nanoparticle accumulation in human alveolar cells induce reactive oxygen species and ER stress signaling.

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The signaling of cell stress in response to organelle dysfunction, toxin exposure, and mutation is complex; generating responses that can include adaptation, or in severe cases cellular apoptosis. Nanoparticles (20-100 nm diameter) have been shown to induced cell stress in lung cells, potentially identifying a cause of lung disease in areas with high levels of air pollution. This study examines the effect of carbon black (CB) and titanium dioxide (TiO₂) nanoparticles on stress signaling and apoptosis in cultured A549 human alveolar epithelial cells. Nanoparticles were prepared via sonication and physical disruption. Cellular accumulation in A549 was confirmed by microscopy and shown to result in a primarily perinuclear cellular localization. Cells exposed to CB and TiO₂ nanoparticles over a 25-100 ug/ml range of concentration displayed significant increases in cellular apoptosis, determined by nuclear morphology, over a period of 72 hours. We hypothesize that accumulated nanoparticles may generate stress signaling similar to that exhibited by cytosolic aggregates of misfolded proteins which generate endoplasmic reticulum (ER) stress. Mobilation of cellular reactive oxygen species (ROS) was rapidly induced by CB exposure, or by exposure to an ER stress-inducing toxin (tunicamycin, 400 nM). TiO₂ did not generate elevated ROS levels. An evaluation of stress signaling pathways induced by ER stressors showed TiO₂ to induce phosphorylation of eIF2 α , consistent with activation of the PERK pathway. Neither GRP78 expression, nor IRE1 α activity were induced by nanoparticle exposure. This work reveals that nanoparticle composition strongly influences the signaling pathways induced during exposure, and that select components of ER stress signaling pathways are induced by nanoparticle exposure.

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AGE-LDL affects the risk of foam cell apoptosis associated with hydrolysis of CE, corresponding to PC-FC Complex formation.

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[Objective] Phosphatidylcholine (PC)-cholesterol (FC) complex structures, a unique atheroma related antigen expressed on the surface of FC rich lipid droplets, associating with foam cell death. To elucidate the role of antigen in the development of atherosclerosis, the

relationship among **AGE(Advanced Glycation Endproduct)-LDL**, PC-FC complex, cellular FC transport and role of HDL in cultured foam cell were investigated.

[Method] Foam cells were prepared by incubation with various modified LDL for 24hr, then cultured up to 3 \6 days in the presence of LPDS +/- HDL and various inhibitors against ABC transporter, nCEase. PC-FC complex and Apoptotic cells were detected by immunofluorescent microscopy using specific monoclonal antibodies or FITC-AnnexinV. FC rich lipid droplet can be detected by Filipin staining.

[Results & Discussion]

Acetylated LDL(AcLDL) strongly induces PC-FC Complex in foam cells via FC-rich lipid droplet formation, however, HDL treatment can induce CE hydrolyse and FC export associated with foam cell survival. FC-rich particles once appeared in the AcLDL induced foam cells, HDL treatment leads to decrease these particles associating with foam cell apoptosis, whereas, the phenotype of the cell is significantly different in AGE-LDL induced foam cells. FC-rich lipid droplets were not reduced by HDL treatment in these cells. Probucol, a potent ABC transporter inhibitor leading reduce FC export, no significant difference can be detected on AGE-LDL induced foam cells after probucol treatment. Among two potent foam cell inducer, AcLDL and AGE-LDL, distinctive difference can be investigated. Once accumulated AGE-LDL can be hydrolysed in Late Endosome, however, the rate of accumulation and re-esterified to CE is proceeding slowly, comparing with Ac-LDL induced foam cells. HDL treatment might be affected cellular nCEase activity leading to cellular FC increase, in resulting foam cells expreed FC-rich lipid droplets, no significant difference PC-FC complex formation and foam cell death, whereas, amount of FC in the cells increased markedly.

These phenomena would be related to PC-cholesterol complex formation and foam cell death via cellular FC export. HDL played an important role in FC-rich lipid droplets and PC-FC complex formation.

These significantly relationship in AGE-induced foam cells, among the PC-FC complex formation, cellular FC export and foam cell apoptosis can reflect to development of atherosclerosis in thickened intima.

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Neurotoxicity of lovastatin is not associated with cholesterol reduction in human neuroblastoma cells.

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The brain is the organ with the highest cholesterol content which essentially comes from *de novo* synthesis. Epidemiological and experimental studies indicate that high cholesterol increases the susceptibility to neurodegenerative disorders such as Alzheimer Disease (AD). Several studies have analyzed the relationship between the use of statins, a class of drugs that lower serum cholesterol, and the onset of AD yielded conflicting result. Statins are inhibitors of the enzyme hydroxy-methylglutaryl-coenzyme A reductase (HMGCR), which catalyzes the rate limiting step in cholesterol synthesis. **Aims:** We have analyzed the effects of lovastatin a statin able to cross the brain blood barrier, in cholesterol levels and cell viability in human neuroblastoma cells. **Methods and Results:** To examine the effects of lovastatin on cholesterol content, we fist studied its effects on neuronal viability in the absence of added serum to the culture media or with low (2%) or normal content of serum (10%). Different doses of lovastatin (10, 5 and 2 μ M) induced 50-80% of cell death that was more prominent when serum was absent and cholesterol completely removed from the media. Addition of cholesterol 50 \cdot M did not protect from lovastatin toxicity. Moreover, added cholesterol showed to be more toxic under conditions of

normal serum content than in the absence of serum. Incubation of cells with lovastatin did not induce a reduction of endogenous cholesterol even if there was an important induction of the HMGCR. **Conclusions:** Lovastatin induced-neurotoxicity was not associated with inhibition of cellular cholesterol synthesis but increased when cholesterol was removed from de culture media.

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Selective & Inducible Proteolysis of the Apoptotic Substrate ICAD.

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Programmed cell death or apoptosis is essential for maintaining tissue homeostasis and plays a crucial role in a variety of disease pathologies including cancer, viral infection, and neurodegeneration. Apoptosis results in the elimination of cells coordinated by a family of cysteine-aspartyl specific proteases called caspases. A subset of caspases, termed executioners, selectively cleaves death substrates that result in the profound morphological transformations characteristic of apoptotic cells. The list of known death substrates continues to grow, however the functional consequences ensuing from proteolysis of individual substrates remains an unanswered challenge. We propose a novel protein engineering and genetic strategy to investigate loss of protein function as a result of proteolytic processing. Our approach incorporates a novel lenti-viral vector to simultaneously knockdown the endogenous substrate and replace it with a Nla Tobacco Etch Virus (TEV) susceptible allele. Site-specific proteolysis is controlled temporally through the utilization of a small molecule activated split-TEV protease. We are validating the functionality of our system with a classic example of caspase-mediated loss of function, inhibitor of caspase activated DNase (ICAD). ICAD is a mandatory folding chaperone and inhibitor of caspase activated DNase (CAD). Upon liberation from ICAD, CAD dimerizes leading to CAD mediated DNA cleavage. We demonstrate our approach utilizing ICAD as a model substrate by presenting functional ICAD knockdown, single isoform replacement, and inducible site-specific proteolysis. We discuss results from on-going experiments that suggest new molecular requirements for CAD mediated DNA cleavage during apoptosis.

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CtIP is Required to Initiate Replication Dependent Interstrand Crosslink Repair.

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DNA interstrand crosslinks (ICLs) are toxic lesions that block the progression of replication and transcription. CtIP is a conserved DNA repair protein that facilitates DNA end resection in the Double Strand Break (DSB) repair pathway. Here we show that CtIP plays a critical role during initiation of ICL processing in replicating human cells that is distinct from its role in DSB repair. CtIP depletion sensitizes human cells to ICL inducing agents and significantly impairs the accumulation of DNA damage response proteins RPA, ATR, FANCD2, γ H2AX, and phosphorylated ATM at sites of laser generated ICLs. In contrast, the appearance of γ H2AX, and phosphorylated ATM at sites of laser generated double strand breaks (DSBs) are CtIP independent. We present a model in which CtIP functions early in ICL repair in a BRCA1 and FANCM dependent manner prior to generation of DSB repair intermediates.

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Interferon regulatory factor-1 (IRF-1) and regulated in development and DNA damage response 2 (REDD2): interaction, localization and effect on cell cycle and apoptosis.*M. Gupta¹, P. C. Rath¹; ¹School of Life Sciences, Jawaharlal Nehru University, New Delhi, India*

IRF-1 is the first member of the IRF-family of transcription factors, involved in type I and type II interferon (IFN)-mediated signaling, regulation of cell growth, development of immune cells, immune response, tumor suppression, apoptosis and autophagy in mammalian cells. IRF-1 is a modular protein with an intrinsic disordered domain and it interacts with many other proteins. We show that GST-IRF-1 pull-down assay from mouse bone marrow cells (BMCs) and MALDI-TOF/MS analysis identified regulated in development and DNA damage response 2/DNA-damage-inducible transcript 4-like (REDD2/DDIT4L) protein, a negative regulator of mTOR, as a novel IRF-1-interacting protein. Moreover, coimmunoprecipitation and confocal microscopy showed association of cellular IRF-1 and REDD2 in the cytoplasm of mouse bone marrow cells as speckles in a punctate distribution pattern indicating large protein complexes. In vitro interaction of the two recombinant proteins showed that IRF-1 physically interacts with REDD2. IRF-1 and REDD2 were overexpressed in the human embryonic kidney (HEK-293) cells up to 3.5x ($p=0.023$) and 8.2x ($p=0.005$) folds higher respectively. REDD2-overexpression induced endogenous IRF-1 up to 2.5x fold but IRF-1-overexpression reduced endogenous REDD2 up to 0.75x fold. Hence, IRF-1 and REDD2 influenced each-other's gene expression. Cell cycle analysis showed that overexpression of IRF-1 decreased 22% of the cells in G0/G1 phase and increased 23% of the cells in S phase whereas overexpression of REDD2 showed no apparent effect. Overexpression of IRF-1 and REDD2 showed a 2.2x and 1.5x fold increase in the endogenous NF- κ B activity, which was reduced up to 1.2-1.7x fold when IRF-1 and REDD2 were coexpressed. In response to Lipopolysaccharides (LPS), IRF-1-overexpressed cells showed 8-13% reduction of cells in S phase and an increase of cells in G0/G1 phase by 10% whereas REDD2-overexpressed cells showed a 7% increase in cell death selectively only at low dose of LPS. However, LPS-treatment of IRF-1+REDD2 coexpressed cells increased the cells in G0/G1 phase by 8% and decreased the cells in S phase by 6-13% whereas at high dose of LPS, cell death was increased by 10%. In conclusion, IRF-1 interacts with REDD2 in BMCs, IRF-1 and REDD2 participate in cross-talk between the cytokine (interferon) pathway and the DNA damage response pathway for cellular regulations related to cell growth, proliferation and apoptosis.

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Differential regulation of poly(ADP-ribose) polymerases in cancer cells.*K. A. Krukenberg¹, C. H. Benes², J. Steen³, T. J. Mitchison¹; ¹Harvard Medical School, Boston, MA, ²Massachusetts General Hospital, Boston, MA, ³Boston Children's Hospital, Boston, MA*

Poly(ADP-ribose) (PAR) is an important but poorly understood post-translational modification. Poly(ADP-ribose) polymerases (PARPs) catalyze the addition of ADP-ribose onto acceptor proteins using NAD⁺ as a substrate. The function of PAR has been best studied in the context of the DNA damage response where PARPs are activated by DNA damage. Emerging evidence reveals broader roles for PAR in other essential cellular pathways including transcriptional control, apoptosis and inflammation. The role of PARPs in DNA damage has led to much interest in PARP inhibitors as chemotherapeutics, and multiple inhibitors are currently in clinical trials. The focus so far has been on using PARP inhibitors in combination with DNA damaging agents or in cancers deficient in DNA damage repair pathways (e.g. BRCA1/2 deficient cancers). Recent studies suggest that PARP inhibitors may also have utility in treating some cancers independent of DNA damage, e.g. Ewing's sarcoma. A weakness of previous work on PAR in cancer cells has been a lack of quantitative assays. PAR levels are typically inferred

from smears on western blots. We have developed an assay for quantifying PAR levels in cell lysates that has a large dynamic range and high selectivity. We have found that PAR levels vary widely between cancer cell lines, apparently independently of DNA damage responses. The differences in PAR levels appear to be largely attributable to differences in PARP1 activity. We are currently investigating mechanisms of PARP1 activation and the consequences for cell physiology and chemotherapeutic drug responses. Using proteomics we have identified several candidate regulators of downstream pathways, some of which are known oncogenes, that are selectively PARsylated in a cell line with high PARP1 activity. In a complementary approach we are investigating the relationship between PARP1 activity and drug sensitivity in several cell lines. Not only will this provide insight into the biological function of PAR, but PAR levels may also be useful as an indicator of which cancer cells respond best to PARP inhibitors or other treatments.

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DNA Damage Sensitivity of Developing Oocytes Is Regulated by a p63 Autofeedback Regulatory Loop.

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TAp63 α is an isoform of the p53 tumor suppressor gene and is highly expressed in the primordial follicle oocytes. Pre-dictyate stage oocytes generate hundreds of meiotic DNA double strand breaks for meiotic recombination. TAp63 α phosphorylation is essential for oocyte death after DNA damage. Ironically, however, many oocyte continue to live during meiosis I. The mechanistic details underlying the role of TAp63 α in DNA damage-induced cell death remain unclear. So, we focused on how TAp63 α regulates cell death in response to DNA damage.

p63 is low expressed in post-natal 0.5 (P0.5) oocytes which fail to undergo phosphorylation and cell death unlike P5.5 oocytes. ATM inhibitors block TAp63 α phosphorylation and abolishes cell death induced by DNA damage. Due to weak phosphorylation of TAp63 α in P0.5 oocyte we investigated whether protein phosphatase activity might downregulate TAp63 α phosphorylation. Calyculin A, which is a Ser/Thr phosphatase inhibitor, facilitated ATM-mediated TAp63 α phosphorylation in predictyate oocytes. We propose the balance between kinase and phosphatase activity determine oocyte sensitivity to DNA damage. DNA binding TAp63 α mutant fail to undergo phosphorylation. Furthermore, phosphorylation is linked to transcription involving an autofeedback regulatory loop. Also, we find that ATM plays a very important role in TAp63 α phosphorylation but is not the TAp63 α transcriptional target gene. Our results also suggest high expression of TAp63 α activates the autofeedback loop to augment TAp63 α phosphorylation and overcome phosphatase-mediated TAp63 α phosphorylation suppression.

Taken together, control of p63 level-dependent phosphorylation has the potential to be useful in cancer therapy as well as in radiation-induced infertility prevention in female cancer patients.

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p53 regulates the Ets transcription factor MEF/Elf4 via MDM2.

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Myeloid elf-1-like factor (MEF) or Elf4 is an ETS-related transcription factor that influences cell cycle progression. MEF is transcriptionally activated by E2F1, but is inhibited by p53 through E2F1-p53 protein interaction. While the transcriptional activation of MEF has been investigated in depth, its post-translational regulation is not well explored. By overexpressing MEF plasmid in human cell lines, here we show that MEF protein is suppressed by p53. MEF protein level was invariably higher in HCT116 p53 knockout (KO) cells and in p53 siRNA-transfected cells than in control cells. Consistently, p53 dose-dependently suppressed the transcription of MEF target

genes, lysozyme, perforin and interleukin-8. The stability of MEF was lower in HCT116 p53 WT than in KO cells, but MEF stability was enhanced by proteasome inhibitor MG132. By screening a number of E3 ligases regulated by p53, we found that MDM2 is involved in the effect of p53 on MEF. MDM2 interacts with MEF to influence its degradation. Together, these results indicate that p53 affects MEF protein level through MDM2. Our findings reveal another mechanism by which a cell proliferative factor, such as MEF, is modulated by p53.

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Bod1L regulates the dissociation of MDC1 from the site of double-strand DNA breaks.

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Double-strand breaks (DSBs) are often considered to be the most detrimental and destructive type of DNA damage in cells. Unrepaired DSBs can lead to gene deletions or mutations often linked with cancer. Here we show that the novel protein Bod1L, from the same family as the PP2A-B56 inhibitor Bod1, acts to modulate the localisation of MDC1 at DSB repair foci. In HeLa cells depleted of Bod1L by siRNA, we observed progression through mitosis with fragmented and acentric chromatin, likely to have arisen from unrepaired DSBs. Induction of DSBs in Bod1L depleted cells with the topoisomerase inhibitor Etoposide, led to normal γ H2AX localisation at the DSB foci, yet caused a significant increase in the levels of MDC1 at these sites. Treatment of HeLa cells for 18 hours with Etoposide followed by removal, produced DSB sites that slowly lost MDC1 over an 8 hour period. In cells depleted of Bod1L however, MDC1 was never lost but continued to increase at these sites over this period. In contrast to this increasing MDC1, no observable change could be seen in the accumulation of γ H2AX when compared with control. As Bod1L is related to the PP2A-B56 inhibitor Bod1, we next determined if Bod1L works through a PP2A-dependent pathway. Co-depletion of both Bod1L and the B56 regulatory subunit of PP2A rescued MDC1 accumulation at DSB foci and allowed MDC1 to recover following withdrawal of Etoposide. These results suggest that Bod1L is acting as a novel PP2A-B56 modulator, the function of which is critical in controlling the dissociation of MDC1 from the DSB repair foci to ensure proper resolution of DNA damage.

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Regulation of Dvl and PP2A by the Retinoblastoma protein in the MC3T3 cell line.

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Disruption of the retinoblastoma (Rb) pathway is observed in most human cancers. In addition to acting as a cell cycle repressor, Rb is a co-activator of differentiation-specific transcription factors such as Runx2, which is needed for osteoblast differentiation. As with the Rb pathway, the Wnt pathway also plays an important role in osteoblast differentiation. Given the observed inactivation of both pathways in some human cancers, we hypothesize that they are functionally linked. We propose that Rb inactivation results in de-regulation of the Wnt pathway. Using MC3T3 pRb+/+ and pRb-/- osteoblasts, we determined the expression levels of the Wnt pathway components PP2A and Dvl and performed a phosphatase assay to test PP2A activity to determine if these are affected by Rb. Experiments were performed in the presence and absence of the Wnt3a ligand. Also, histology analyses of normal bone and osteosarcomas were performed to determine if Rb regulates β -catenin's localization. qRT-PCR show a 0.4 fold-decrease in the Dvl and a 1.6 fold-increase in the PP2A mRNA expression levels in Rb+/+ relative to the Rb-/- cells ($p \leq 0.05$). Addition of Wnt3a abolished the changes in the Dvl levels but

produced a 0.4 fold-decrease in PP2A expression in the Rb+/+ relative to Rb-/- cells ($p \leq 0.05$). Immunoblots show no significant change in the protein levels of PP2A and Dvl when comparing Rb+/+ and Rb-/- cells, however, they revealed a significant increase in the phosphorylated-to-total Dvl ratio in the Rb+/+ cells. The phosphatase assay show a higher PP2A activity in Rb+/+ compared to Rb-/-cells ($p \leq 0.05$), but this change was abrogated by Wnt3a addition. Our preliminary histology results show that the absence of a functional Rb does not affect β -catenin's localization. In summary, our results show that Rb can regulate the expression and activity of several Wnt related proteins, and the addition of the Wnt3a ligand can modify this regulation. PP2A inhibits Wnt pathway by promoting β -catenin degradation. Therefore, the increase of the PP2A phosphatase activity in the presence of Rb suggests that Rb may be promoting β -catenin degradation, which subsequently inhibits Wnt pathway. This research was supported by grants from the National Center for Research Resources (G12 RR003050), National Institute on Minority Health and Health Disparities (8G12MD007579-27), National Cancer Institute (U56CA118809) from the National Institutes of Health, and ACS Grant 93-032-13.

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E-cadherin is a molecular switch for Caveolin-1 function in melanoma cells that synergizes in tumor suppression and blocks Caveolin-1-enhanced metastasis.

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The role of caveolin-1 (CAV1) in cancer development and progression is highly controversial. For example, results from this laboratory have shown that CAV1 dependent suppression of genes favoring tumor development requires E-cadherin. Moreover, CAV1 promotes focal adhesion turnover and migration of metastatic cells. How these contrasting observations relate to CAV1 function in vivo is unclear. Here, murine melanoma B16F10 cells, with low endogenous CAV1 and E-cadherin levels, were employed to evaluate how introducing CAV1 affects tumor growth and metastasis in syngeneic C57BL/6 mice and how co expressing E cadherin modulates CAV1 function in vivo. Augmented CAV1 expression in B16F10(cav 1) cells reduced subcutaneous tumor formation, but, when injected intravenously, enhanced metastasis compared with plasmid transfected B16F10(mock) or wild type cells. E-cadherin expression in B16F10(E cad) cells reduced both subcutaneous tumor formation and metastasis to the lung. Importantly, for B16F10(cav 1/E cad) cells, expressing CAV1 together with E-cadherin, tumor formation and lung metastasis of B16F10 cells were completely abolished. Thus, CAV1 is shown for the first time in a pre-clinical model to function both as a tumor suppressor and promoter of metastasis. Moreover, E-cadherin synergizes with CAV1 in suppressing tumor formation and, importantly, blocks the ability of CAV1 to promote metastasis. These observations identify E-cadherin as a molecular "switch" that determines whether or not CAV1 can promote B16F10 metastasis. Consistent with these findings, expression profiling analysis of data available for a large number of melanoma lines suggests that elevated CAV1 and reduced E cadherin are associated with melanoma transition from a proliferative phenotype to an invasive one.

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B-type cyclins exert concurrent oncogenic properties through distinct aneuploidization mechanisms.

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Cyclin B1 and B2 are part of a signature of overexpressed genes that predicts chromosomal instability and poor clinical outcome in multiple human cancers. However, whether and how these mitotic cyclins might drive aneuploidization and malignant cell transformation when overexpressed is unknown. To address these questions, we generated transgenic mice that overexpress cyclin B1 or B2. Here, we show that both of these transgenic strains exhibit high rates of aberrant chromosome segregation, with cyclin B1 overexpression inducing chromatin bridges and cyclin B2 overexpression inducing lagging chromosomes. At the molecular level, cyclin B1 overexpression precludes full activation of separase upon chromosome bi-orientation, resulting in anaphase onset prior to complete cleavage of cohesin molecules that hold sister chromatids together. In contrast, cyclin B2 overexpression perturbs spindle geometry rather than timely activation of separase. The observed spindle geometry defect is caused by precocious splitting of centrosomes in early mitosis, which we find is due to hyperactivation of Plk1 by Aurora A. Importantly, both cyclin B1 and B2 transgenic mice develop a broad spectrum of spontaneous tumors and form increased numbers of colon tumors on a APC^{Min} genetic background. Consistent with the observation that lung tumors are highly prevalent in both transgenic strains, we find that cyclin B1 and B2 transcript levels are frequently elevated in human lung tumors. Together, these data reveal that cyclin B1 and B2 overexpression causes aneuploidization through entirely distinct mechanisms of chromosome missegregation and suggest that overexpression of B-type cyclins is causally implicated in the pathogenesis of human cancer.

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The involvement of MCT-1 oncoprotein in inducing mitotic catastrophe and nuclear abnormalities.

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Centrosome amplification and chromosome abnormality are frequently identified in neoplasia and tumorigenesis. However, the mechanisms underlying these defects remain unclear. We here identify that MCT-1 is a centrosomal oncoprotein involved in mitosis. Knockdown of MCT-1 protein results in intercellular bridging, chromosome mis-congregation, cytokinesis delay, and mitotic death. Introduction of MCT-1 oncogene into the p53 deficient cells (MCT-1-p53), the mitotic checkpoint kinases and proteins are deregulated synergistically. These biochemical alterations are accompanied with increased frequencies of cytokinesis failure, multi-nucleation, and centrosome amplification in subsequent cell cycle. As a result, the incidences of polyploidy and aneuploidy are progressively induced by prolonged cell cultivation or further promoted by sustained spindle damage on MCT-1-p53 background. These data show that the oncoprotein perturbs centrosome structure and mitotic progression, which provide the molecular aspect of chromosomal abnormality in vitro and the information for understanding the stepwise progression of tumors under oncogenic stress.

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Association of Lin28 Expression and Tumorigenesis in Human Wilms' Tumor Cell Lines.

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LIN28 is an evolutionarily conserved RNA binding protein that plays an essential role in regulating developmental timing in *Caenorhabditis elegans*. Recently, many reports have shown the effect of LIN28 in stem cell function, cell differentiation, cell proliferation and tumorigenesis. In mammals, LIN28 is highly expressed in embryonic stem cells and in early embryogenesis. One of the major targets of LIN28 is the Let7 microRNA family. By binding to the pri/pre Let-7 microRNA genes, LIN28 prevents the maturation of this miRNA family and thus enables the translation of genes that are suppressed by Let-7 miRNAs. Therefore, LIN28 is capable of regulating translation and stability of oncogenes including K-Ras, C-Mys and many others. In cancer cells, LIN28 is up-regulated as an oncogene, which promotes transformation and tumor progression. It has been shown by our lab that ~15% of human cancers are linked to the expression of LIN28A or its paralog LIN28B. In addition, some reports show that knocking-down LIN28A reduces cancer cell viability and cell growth *in vitro*. We have shown that LIN28B is expressed in samples of Wilms' tumor cells. In Wilms' tumor cell lines, *in vitro* LIN28B-knockdown significantly decreased cell proliferation compared with control cells. Taken together, LIN28B might function in controlling cell survival and differentiation in Wilms' tumor. To further study the role of LIN28 in Wilms' tumor formation, we will transplant "normal" and knockdown human Wilms' tumor cell lines under the kidney capsule of immunodeficient mice to compare each other. We will also over-express LIN28A/B during normal mouse kidney development.

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Investigating the contribution of centrosome amplification in tumorigenesis.

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As the major microtubule organizing centers, centrosomes play a central role in facilitating the formation of a bipolar mitotic spindle. Defects in centrosome duplication can induce an abnormal centrosome number and may lead to chromosome missegregation and subsequent aneuploidy. Although aneuploidy is an extremely common feature of tumor cells, its status as a cause or a consequence of cancer is highly controversial. In vertebrates and invertebrates, the conserved protein kinase Polo-like kinase 4 (Plk4) plays a key role in initiating centriole duplication and overexpression of Plk4 promotes the formation of extra centrosomes. Here we will describe the construction of two mouse models in which centrosome amplification can be induced through conditional overexpression of Plk4. We have made use of both a doxycycline-inducible promoter and the Cre-LoxP system to allow reversible and non-reversible expression of Plk4 in a tissue specific manner. These mice are used to study the contribution of centrosome amplification in tumorigenesis. Preliminary results suggest that centrosome amplification cannot be the sole driver mechanism leading to tumor development.

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Discovery of cancer drug targets using RNAi screening with pooled lentiviral shRNA libraries.

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This presentation will provide an overview of the recent innovations in the development of a lentiviral pooled format RNAi screening platform, in bioinformatics target prioritization, and in validation of potential drug targets in xenograft mouse models. We will present results of large-scale viability screens for cell-specific lethal and synthetic-lethal genes in a panel of blood, prostate, and breast cancer cell lines. Subsequent validation using single shRNA-expressing constructs showed that about 80% of the shRNAs identified in each complex library screen did in fact lead to cell death when transduced in cells. Analysis of the identified essential genes for known biological interactions revealed non-random clusters of interacting proteins that provide a useful strategy for prioritization of potential targets. Analysis of the lethal combinations indicates redundant, complementary, and compensatory responses in cancer cells. Genes modulating proliferation and survival in oncogenic cells have been further validated using *ex vivo* genetic screens with sub-pooled lentiviral shRNA libraries in xenograft mouse models. Also, we have recently developed combinatorial RNAi screening technology with bi-specific pooled shRNA libraries for the unbiased discovery and annotation of additive and synergistic synthetically lethal interactions. We will present results of combinatorial RNAi screening of DNA damage and repair genes in a breast cancer cell model.

Tumor Invasion and Metastasis I

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Macrophages induce invadopodium formation in tumor cells during intravasation.

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Most cancer patients die as a result of metastasis. Spread of metastatic cancer cells by blood circulation is responsible for most distant metastases, thus it is important to understand the molecular mechanisms of transendothelial migration. The discovery that a tripartite structure composed of an invasive carcinoma cell, a macrophage and an endothelial cell, termed Tumor MicroEnvironment of Metastasis (TMEM), can be used as a predictor of distant metastasis in patients (Robinson et al., 2009) highlights the importance of the interaction of these three cell types during transendothelial migration. The mechanistic role of each cell type in transendothelial migration, however, is not well understood. Additionally, while much is known about the process of extravasation, far less is known about intravasation. Thus, we studied intravasation using a combination of fixed and live imaging, and found that human breast cancer cells undergo intercellular transendothelial migration *in vitro* and *in vivo*. As tumor cells penetrate through the endothelium, they form invadopodia, actin-rich structures that degrade basement membrane. Using pharmacological inhibitors, we found that tumor cell intravasation is dependent on signaling from macrophages. We further found that macrophages induce the formation of invadopodia in tumor cells. Taken together, our results suggest that signals from macrophages induce the formation of invadopodia in tumor cells to break through endothelial barriers during intravasation, and thereby demonstrates the significance of TMEM.

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Extracellular matrix guides tumor cell invadopodia formation by modulating β 1-integrin signaling.

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Cancer cell invasion and metastasis to distant sites are hallmarks of cancer malignancy and the leading cause of cancer deaths. Invadopodia are specialized microscopic membrane protrusions of invading tumor cells that extend into the extracellular matrix (ECM) to mediate proteolytic digestion of, and invasion through, the ECM. We recently reported that structural cores of invadopodia rich in actin and cortactin are formed first, then the metalloprotease MT1-MMP accumulates and triggers ECM degradation; membranes at these invadopodia are highly dynamic compared to podosomes and focal adhesions. Our current research is focused on: (1) understanding the role of the ECM in induction of invadopodia, and (2) identifying the regulatory roles of integrins and downstream signaling pathways leading to invadopodia formation and function in proteolytic degradation of ECM.

We have developed novel, physiologically relevant fibrillar collagen matrices that are superior for inducing large numbers of invadopodia in several different types of invading tumor cells. We have discovered that integrin α 2 β 1 regulates invadopodia initiation by fibrillar collagen, and that α 2 β 1 signaling does not require co-stimulation from the epidermal growth factor receptor (EGFR) for assembly of numerous mature invadopodia. In contrast, conventional gelatin matrices mimic fibronectin matrices and induce only modest numbers of invadopodia in metastatic cancer cells. We have found that fibronectin matrices engage α 5 β 1 integrin to induce only preinvadopodia, and maturation of invadopodia on these substrates requires co-signaling from EGFR. Analyses of the localization of small GTPases to invadopodia and FRET studies using Rac1 biosensors allowed us to determine that activated Rac1 preferentially localizes to invadopodia induced by collagen matrix. Over-expression of a dominant-negative Rac1 mutant or siRNA-targeted depletion of Rac1 inhibited invadopodia formation in cells invading collagen. Thus, collagen- and fibronectin-rich ECMs regulate invadopodia formation via activation of different β 1-integrin heterodimers that elicit unique regulatory effects on activation of small GTPases and on the efficiency of invadopodia formation. Our findings also identify an important role for matrix itself in regulating tumor cell invadopodia.

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Comparison of cellular invasion through cross-linked and non-cross-linked fibrillar collagen.

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The process of cellular invasion through extracellular matrices is increasingly recognized to be heterogeneous with respect to variations in both the phenotypes of the invading cells and the properties of the extracellular matrix. One aspect of the extracellular matrix that influences invasion is the degree of cross-linking of collagenous matrices. We have compared invasion characteristics of tumor cells through gels composed of acid-extracted (cross-linked) and pepsin-extracted (non-cross-linked) type I collagens in a modified Boyden chamber assay. We find that, in general, tumor cells invade through gels consisting of non-cross-linked collagen at a higher rate than through gels composed of cross-linked collagen. A highly invasive cell type, MDA-MB-231, and a moderately invasive cell type, Hs578T, are capable of invading both forms of collagen, but the rate and magnitude of invasion is higher through non-cross-linked than cross-linked collagen. In addition, the use of non-cross-linked collagen allows lower quantities of invasive cells to be used than are required with cross-linked collagen. Finally, B16F1

melanoma cells were able to invade non-cross-linked collagen but were not observed to invade cross-linked collagen. Although non-cross-linked collagen has been reported to support amoeboid, protease-independent invasion and cross-linked collagen reported to require protease-dependent, mesenchymal invasion, we find that invasion through both cross-linked and non-cross-linked collagen is completely inhibited by a cocktail of protease inhibitors in the modified Boyden chamber assay format. We conclude that use of comparison of tumor cell invasion through cross-linked and non-cross-linked collagen provides extra insight into the invasive potential of the cells, and that the increased sensitivity of non-cross-linked collagen may be useful for characterizing less aggressively invasive cell types.

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Oncogenic K-Ras Promotes Basal Extrusion of Epithelial Cells.

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Epithelia provide a protective barrier for the organs they encase, yet the cells comprising the epithelia are constantly turning over via cell death and cell division. To maintain a functional barrier, cells destined to die are squeezed out of the epithelium by a mechanism that we have termed "cell extrusion". In order to extrude, a cell produces sphingosine 1-phosphate (S1P), which binds to a G-protein coupled receptor (S1P2) in the neighboring cells to trigger contraction of an intercellular actomyosin band. This contraction squeezes the cell out of the epithelial sheet while closing any gaps that may have resulted from the cell's exit. Typically, live cells are extruded and later die by anoikis, a type of cell death caused by the loss of survival signals from cell matrix detachment. Since oncogenic K-Ras cells upregulate survival signals and override anoikis, we tested if extruded oncogenic K-Ras cells survive following extrusion. Surprisingly, we found that oncogenic K-Ras cells not only survive and proliferate after extrusion, but also extrude basally into the tissue the epithelium encases, rather than apically out of the tissue (the normal direction). Moreover, K-Ras cell basal extrusion is cell-autonomous. We found that high levels of autophagy in extruding oncogenic K-Ras cells disrupt the normal S1P production and signaling required for apical extrusion. S1P normally forms puncta at the interface between an apically extruding cell and its neighboring cells and is also required only for apical but not basal extrusion. In extruding oncogenic K-Ras cells, however, S1P is greatly decreased despite the fact that its precursor, sphingosine kinase, is upregulated. We found that markers of autophagy, typically upregulated in oncogenic K-Ras cells, are even more pronounced in extruding K-Ras cells. Blocking autophagy rescues S1P localization and apical extrusion. Thus, we propose that elevated autophagy in oncogenic extruding K-Ras cells induces increased survival and basal extrusion. K-Ras transformation, therefore, could promote basal extrusion and enable cells with higher survival and proliferation potential to exit the epithelia and initiate invasion.

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Versican V2 enhances angiogenesis by regulating endothelial cell activities and fibronectin expression.

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Versican is a proteoglycan expressed in the extracellular matrix, where it regulates a variety of cell activities and affects tumor development. With alternative splicing, there are four versican isoforms, denoted V0, V1, V2 and V3. The V2 isoform is highly expressed in the mature brain but its function in the mature brain has not yet been elucidated. Since brain tumors are among the most angiogenic of human tumors, we investigated whether or not the V2 isoform plays a

role in angiogenesis and found that astrocytoma cell line U87 stably transfected with V2 formed tumors containing extensive vasculature. Although the V2-expressing cells grew slowly, they survived well in serum-free medium. They also displayed high adhesive ability to endothelial cells and facilitated tube-like structure formation. Importantly, fibronectin was up-regulated by V2 and mediated V2 function. To understand the role of endogenous versican function, we expressed the versican 3'UTR fragment in the cancer cells. We hypothesized that expression of versican 3'UTR could promote the function of existing mRNAs by regulating endogenous miRNA activity. Human breast cancer cells, which express high levels of versican V2 mRNA, when stably transfected with the 3'UTR expression construct, were found to increase expression of versican V2 protein. The cell also displayed decreased proliferation but increased survival and tube-like structure formation. Tumors formed by these cells showed decreased rate of growth but increased rate of angiogenesis. We investigated whether or not expression of versican 3'UTR could regulate endogenous microRNA function and found that versican 3'UTR could bind miRNAs miR-133a, miR-199a*, and miR-431, which also interacted with fibronectin. As a consequence, expression of versican and fibronectin was up-regulated by ectopic transfection of versican 3'UTR. Transfection with siRNAs against versican 3'UTR abolished the 3'UTR's effects on regulation of the expression of versican and fibronectin and tumor growth and angiogenesis. Taken together, our results demonstrated that versican can regulate fibronectin expression by the V2 isoform and by expression of the 3'UTR. Our studies suggest that versican V2 and fibronectin could be potential targets for intervention of brain tumor angiogenesis.

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Caveolin-1 expression alters MT1-MMP and cell behavior in human melanoma lines.

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Caveolin-1 is known for its role in cell signaling and trafficking. A recent study suggests that upregulation of caveolin-1 greatly attenuates melanoma metastasis and could be a potential therapeutic target. The mechanism by which caveolin-1 might attenuate metastasis remains unclear and may involve multiple signaling pathways. The metalloproteinase MT1-MMP associates with caveolin-1 in lipid rafts of endothelial cells, and this association could also occur in melanoma and be involved in the anti-metastatic effect of caveolin-1 expression in melanoma. The aim of our study was to examine the consequences of caveolin-1 upregulation in human melanoma cell lines, 1205Lu and BLM, and its possible interaction with MT1-MMP. 1205 and BLM wild-type cells were transfected with a GFP tagged caveolin-1 plasmid allowing examination of caveolin-1 location within the cell. Colocalization of MT1-MMP and caveolin-1 along the plasma membrane was observed in the caveolin-1 expressing cells through the use of immunofluorescent staining. An increase in pro-MMP2 activity, an inactive precursor form of MT1-MMP, was observed in caveolin-1 expressers versus wild-type cells when performing gelatin zymography. An increase in net proliferation of caveolin-1 expresser lines versus wild-type lines was demonstrated by MTT assay. A decrease in migration rate measured by wound healing assay was observed in both caveolin-1 expresser lines as compared to wild type lines, and as compared to wild type lines treated with the metalloproteinase stimulant concanavalin-A. Our collective data suggests that induced caveolin-1 expression in 1205Lu and BLM lines attenuates MT1-MMP activation versus the corresponding wild-type lines while simultaneously slowing migration. The attenuation of these metastatic behaviors may come at the expense of greater melanoma proliferation rates. Given that rates of metastasis are directly related to mortality in melanoma patients, caveolin-1 upregulation remains a reasonable target for further studies directed at increasing patient survival rates in melanoma victims and may exert its action in part by attenuating MT1-MMP activation.

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Cav1 suppresses tumor growth and metastasis in a murine model of cutaneous SCC.

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Caveolin-1 (Cav1) is the main structural protein of specialized lipid rafts called caveolae, and it functions as a scaffolding protein that is able to bind and negatively regulate a multitude of signaling molecules. Because many of those molecules are involved in cellular proliferation and survival, the loss or misregulation of Cav1 has been linked to transformation and tumorigenesis in several cancer types, including breast cancer and melanoma. However, Cav1's role in the development of cutaneous squamous cell carcinoma (cSCC) remains largely unknown. The objective of this study was to clarify the function of Cav1 in cSCC development.

To this end, we stably transduced a murine keratinocyte cell line, PAM212, to over-express or down-regulate Cav1, and assessed the function of this protein in skin tumor biology. We first show that Cav1 over-expression decreases cell proliferation and tumor incidence and growth. In contrast, Cav1 knock-down dramatically increases primary tumor growth. Additionally, Cav1 knock-down increases both invasive ability and the incidence of spontaneous lymph node metastasis. We attribute this phenotype to increased Erk1/2 MAPK/AP-1 pathway activation, as inhibition of MAPK pathways rescues the invasive advantage conferred by Cav1 knock-down. We note that the expression of the AP-1 transcriptional targets K18 and cyclin D1 are inversely correlated to the expression of Cav1 in PAM212. In conclusion, we demonstrate that Cav1 may be an important modulator of both primary tumor development and metastatic dissemination in cSCC. These studies provide further evidence that Cav1 acts as a putative tumor suppressor in the skin

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Regulation of breast tumor dormancy by the perivascular niche.

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It is now well-established that breast cancer cells (BCCs) disseminate early during metastatic progression. However, distant metastases may not emerge until years or decades after treatment. Whether growth of disseminated tumor cells (DTCs) is regulated by the microenvironment of distant organs, and how, are fundamental questions of tumor biology. To address these problems, we adopted a multi-disciplinary approach incorporating murine models of human breast cancer dissemination and novel engineered tumor microenvironments. Two different in vivo assays revealed that dormant (i.e., Ki67-negative) breast DTCs reside upon the microvasculature of lung, bone marrow, and brain. To show whether this association was coincidental or if it reflected functional interactions, we created organotypic models of lung and bone marrow microvascular niches in which sparsely seeded BCCs could be tracked long-term. Using 3 different BCC lines, we confirmed that lung and BoMa stroma each promote rampant outgrowth of sparsely seeded BCCs. As suggested also by our in vivo data, endothelial cells (ECs) restrained this outgrowth (~5-fold) and induced quiescence of up to 90% of resultant tumor clusters. Importantly, these tumor cells remained quiescent long-term-- i.e., they were dormant. Conditioned media experiments suggested that the putative endothelial-derived ('angiocrine') mediators of tumor dormancy were not soluble; therefore, we performed comparative tandem mass spectrometry on decellularized extracellular matrix from microvascular niche cultures and their stromal counterparts. This approach revealed a number of potential angiocrine regulators of DTC dormancy. Gain- and loss-of-function studies were

conducted to validate one such mediator. Importantly our experiments also revealed that suppressive cues were downregulated at the neovascular tips of microvasculature. Time-lapse analysis revealed that neovascular sub-niches actually accelerate BCC growth. We confirmed this surprising result using different approaches to enrich and deplete these sub-niches, and performed further LC-MS/MS analysis to identify neovascular mediators of this tumor-promoting behavior. The localization of a subset of these factors to the neovascular sub-niche was confirmed via immunofluorescence, and gain-of-function studies demonstrated that these factors permit BCCs to overcome the suppressive effects of the stable microvasculature. This study defines distinct endothelial sub-niches that regulate dormancy or expansion of disseminated breast tumor cells depending on the nature of the local microenvironments. The identification of inhibitory- and growth-promoting- factors derived from each of these sub-niches may yield supplementation therapies that enforce dormancy of DTCs and provide novel molecules to target in order to prevent disruption of tumor dormancy.

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Laminin-derived peptide C16 induces invasion and invadopodia activity in oral squamous cell carcinoma and fibrosarcoma cells.

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Our Laboratory has been studying the effects of laminin-derived peptides in tumor biology. Here we addressed whether the peptide C16 (Kafdiyvrkfk, gamma 1 chain) would stimulate invasion activity in cell lines derived from oral squamous cell carcinoma (CAL27) and fibrosarcoma (HT1080), two malignant tumors with different origins and poor prognosis. C16 increased invasion rate in both cell lines compared to scrambled control peptide (C16SX), as shown by invasion assays in Boyden chambers coated by Matrigel. Tumor cells that actively invade surrounding tissues depend on invadopodia to degrade extracellular matrix barriers. Invadopodia are actin-rich membrane protrusions associated with pericellular proteolysis. Therefore, we analyzed the role of C16 on invadopodia activity of CAL27 and HT1080 cells, through a fluorescent substrate degradation assay. Measurements of digestion spots (black areas in fluorescent background) showed that C16 significantly increased invadopodia activity of both neoplastic cell lines. This peptide also increased expression of phospho-cortactin and MT1-MMP in CAL27 and HT1080 cells. These proteins are regarded as important invadopodia markers. Using time-lapse 4D fluorescence microscopy, we explored invadopodia dynamics in C16-treated cells. CAL27 and HT1080 cells were transfected with GFP-cortactin, followed by growth on fluorescent gelatin. Time-lapse videos showed that C16 increased invadopodia activity of both cell lines over time. Furthermore, we analyzed signaling pathways related to C16 effects in CAL27 and HT1080 cells. Immunoblots showed that C16 increased activation of ERK 1/2 and Rac1 pathways in CAL27 and HT1080 cells. Moreover, inhibition of ERK 1/2 signaling pathway by MEK inhibitor U0126 decreased invasion rate and invadopodia activity in both cell lines. We propose that C16 stimulates invasion and invadopodia activity in cells derived from oral squamous cell carcinoma and fibrosarcoma, probably through ERK1/2 and Rac1 signaling pathways. Support: FAPESP (2008/57103-8; 2009/17336-6) and CNPq (304986/2009-7).

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Characterization of Two Subtypes of the SW-13 Human Adenocarcinoma Cell Line.

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The human adenocarcinoma cell line SW13 has been reported to switch between two subtypes, one expressing vimentin (SW13+) and the other not (SW13-) and that this switch is at the post-transcriptional level. Given the importance of epithelial to mesenchymal transition (EMT) in metastasis and the fact that vimentin expression induced during as cells adopt the mesenchymal or metastatic form, we have investigated the properties of the two SW13 subtypes further. We have found that the vimentin expressing SW13+ appear to divide more slowly but have the more spindly morphology characteristic of mesenchymal cells while the vimentin negative SW13- cells appear to divide more quickly and are more rounded. More interesting is that mixed cultures appear to maintain different characteristics than homogeneous cultures of either subtype, suggestive of communication during culture between the two subtypes. As increased cell motility is a characteristic of mesenchymal cells, we tested the ability of each subtype to chemotax in response to serum in a transwell migration assay after serum starvation. Preliminary results suggest that vimentin positive SW13+ subtype may have greater motility than SW13- in our transwell chemotaxis assay. We are also very interested in investigating the unknown factors that might trigger the switch between SW13+ and SW13- subtypes. As Rho and Rac GTPases are associated with regulating the mechanisms of migration involved in metastasis, we investigated the ability of inhibitors and activators of both Rho and Rac to facilitate the switch between SW13+ and SW13- subtypes. Preliminary results suggest that alterations in the activity of these GTPases changes in the percentage of SW13+ cells in co-cultures. These results suggest that the subtype switch may be related to the ability of these cancer cells to metastasize and that the ability of SW13 cells to switch between subtypes may be controlled by altering GTPase activity.

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Role of the endocytic adaptor Epsin in cancer cell migration.

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One of the most common links between cancer and endocytosis is defective internalization of Receptor Tyrosine Kinases, for example, linked to a deficit in endocytic proteins. This scenario leads to hypersignaling and in turn to malignant transformation. Nevertheless, there are also cases in which endocytic proteins, such as the epsin adaptors, are found upregulated in invasive cancers. Indeed, we demonstrated that overexpression of the epsins enhances cancer cell migration and invasion. Furthermore, depletion of epsins (by siRNA) led to impairment of cancer cell invasion. However, the different epsin paralogs varied in their capacity to enhance migration/invasion in the following order: epsin 3 > epsin2 >> epsin1. The epsin paralogs have a highly conserved Epsin N-terminal Homology or the ENTH domain (N-terminus) and a C-terminus where the most divergent regions are housed. While our data shows that both the ENTH domain and the C-terminus are required for enhancing migration, we speculate that the differential ability of the paralogs to migrate/invade lies in their less conserved C-terminus region.

This study aims to uncover the mechanism by which epsin overexpression leads to enhanced migration/invasion and to identify the regions of the epsin C-terminus which gives the paralogs their differential ability to enhance migration/invasion. Here we describe a systematic combinatorial approach for mapping the epsin determinants involved in localizing epsin at

endocytic sites and required for its effect on cancer cell migration/invasion. Our data clearly shows that Clathrin binding motifs (out of the 4 types of endocytic determinants) are the strongest localization determinants of epsin2, however are not sufficient. In fact, 2 types of endocytic determinants are required for proper epsin2 localization suggesting co-operativity and redundancy. We also found that mutation of any 2 types of endocytic determinants can also reduce the ability of epsin2 to enhance migration as compared to the wild-type protein. Expanding the results obtained from epsin2 to epsin3 will reveal the similarities and differences between the paralogs. Taking together our findings and the requirement of these proteins for cancer cell invasion, we envision epsin2 and epsin3 as targets for novel anti-cancer and anti-metastatic approaches.

Cancer Therapy I

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Galectin-3 Plays a Role in Murine NK cell-mediated INF- γ Production.

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Natural killer (NK) cells serve as a crucial first line of defense against tumors and a diverse range of pathogens in the innate immunity. Highly related to receptors recognizing tumor cells, the activation receptors trigger cytotoxicity and cytokine production. IFN- γ , secreted by NK cells, plays a critical role in suppressing pathogen challenge, both to contain the initial infection, and to promote an appropriate adaptive response. Galectin-3, a member of β -galactoside-binding animal lectin, acts as immune modulators implicated in a variety of biological functions such as inflammation, tumor metastasis, and cell apoptosis. However, the role of immune regulation of galectin-3 on NK cells is not known. In our study, galectin-3 induced IFN- γ production in murine NK cells in a dose-dependent manner. IFN- γ production of NK cells by galectin-3 was inhibited by either anti-galectin-3 antibody or NF- κ B inhibitor, but not lactose. These indicated that active site of galectin-3 in IFN- γ generation was in N-terminal but not in lectin domain that has many other physiological function of the molecule. IFN- γ production by galectin-3 was dependent on the activation, translocation and the transcriptional control of NF- κ B. Galectin-3 induced IFN- γ production was enhanced through signaling pathway of phosphorylated Erk1/2. Erk1/2 phosphorylation was synergistically increased in galectin-3 treated NK cells and MEK1/2 inhibitor abrogated the phosphorylation of Erk1/2 by galectin-3. Our results provided new insights into mechanism of NK cell activation and, also, a novel function of galectin-3 in immune reaction.

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Docetaxel and Metformin act synergistically to inhibit growth of prostate cancer cell lines.

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Treatments for prostate cancer, one of the most prevalent cancers among men, are an area of ongoing investigation. While docetaxel, a microtubule-stabilizing drug, is effective against many forms of prostate cancer this drug, while the best available treatment to date, shows little improvement in the duration of patient survival (only a few months) in castration resistant prostate cancers. It has been previously shown that increasing microtubule stability (by multiple mechanisms, including paclitaxel treatment, a natural analog of docetaxel) can lead to a cell cycle delay/mitotic arrest and an increase in cell death. Inhibiting intracellular metabolism has also been proposed as a treatment for multiple cancer types because many cancers favor

glycolysis for most of their ATP production (Warburg effect). It has been shown previously that most glycolytic enzymes are localized to the microtubule cytoskeleton; therefore we hypothesize that disturbing the microtubule network should further interfere with ATP production via glycolysis and limit growth of cancer cells. Normal cells, however, should be resistant to these treatments due to their reliance on oxidative phosphorylation. Here we test the hypothesis that there is a synergy between the microtubule stabilizing drug, docetaxel, and drugs that can block intracellular metabolism, such as metformin (a drug that inhibits metabolism at multiple points). Cell proliferation was measured for each drug alone and in combination using a colorimetric assay (MTT). While both drugs individually significantly inhibited cell proliferation, initial results show a super-additive (synergistic) effect between docetaxel and metformin in multiple prostate cancer cell lines including LNCaP (androgen dependent) and PC-3 (androgen independent) cell lines. This synergy both decreases the concentration of each drug needed to inhibit cell proliferation (therefore decreasing possible toxicity) while also increasing the potency of the combination. Based on our results the data suggests a potential new drug combination to treat castration resistant prostate cancers.

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Sensitivity of Human Acute Myeloid Leukemia Cells to a Recombinant Human Arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000].

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Though combination induction and consolidation chemotherapy induce complete remission in a high proportion of patients with Acute Myeloid Leukemia (AML), most eventually relapse. Hence, alternative approaches with potent, more selective mechanisms for targeting AML are needed. In this study, we attempt to target the potential Arginine auxotrophy of AML cells using a pegylated recombinant human Arginase I cobalt [HuArgI (Co)-PEG5000].

Potency and selectivity of HuArgI (Co)-PEG5000 were tested on a panel of 10 human AML cell lines and on normal human monocytes using a proliferation inhibition assay. Cell cycle analysis was carried out by propidium iodide (PI)-staining and type of cell death was determined by AnnexinV/PI staining and caspase staining using flow cytometry.

Nine AML cell lines were sensitive to HuArgI (Co)-PEG5000 at 48-hour incubation (IC_{50} = 14-550 pM, Percent cell death > 80%) with only one cell line deemed not sensitive to arginine depletion (IC_{50} > 10,000 pM). Human monocytes were not sensitive to HuArgI (Co)-PEG5000 (IC_{50} > 10,000 pM). Incubation times of 48 and 72 hours had similar potency but were 7-fold more potent than 24-hour incubation. Addition of L-citrulline (40 mM) did not salvage AML cells from arginine depletion-induced cytotoxicity, thus demonstrating the complete arginine auxotrophy of these cells. There was no impact of treatment on cell cycle with both the G0/G1 and G2/M peaks decreasing significantly with increased concentrations of HuArgI (Co)-PEG5000, along with a significant increase in the pre-G0/G1 peak (up to 90% of total events). AnnexinV/PI staining showed a dose-dependent increase in cells stained with both annexinV and PI (4.5% and 55% in non-treated vs treated cells) with no increase in cells staining with annexinV alone. In addition, caspase staining revealed the lack of caspase activation following incubation with HuArgI (Co)-PEG5000 demonstrating that arginine depletion, through HuArgI (Co)-PEG5000, induces caspase-independent, non-apoptotic cell death in AML cells.

These findings indicate that AML cell lines are auxotrophic for arginine and demonstrate the selective cytotoxicity of HuArgI (Co)-PEG5000-induced arginine depletion to these cells. Hence,

HuArgI (Co)-PEG5000 is a potent and selective molecule which constitutes a potential targeted therapeutic for AML.

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Cayaponia tayuya and Dihydrocucurbitacin B are Potent Inducers of Apoptosis in Immune Cells.

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A malfunctioning immune system contributes to a number of autoimmune disorders and dysregulated immune cell growth can result in leukemia. Treatments for autoimmune disorders and leukemia often include agents which suppress the immune system by reducing populations of immune cells. Current immunosuppressive treatments can, sometimes, have limited effectiveness or serious side-effects, thus causing the need for new options. *Cayaponia tayuya* (tayuya) is a plant found in South America and has been shown to possess anti-inflammatory properties. Understanding the underlying mechanism of this effect could result in additional therapies for immune disorders. These anti-inflammatory properties may be a result of chemical compounds known as cucurbitacins found in the tayuya plant. This study aims to investigate the reported anti-inflammatory activity of tayuya by determining the effects of tayuya and cucurbitacin on immune cell function and survival. To determine whether tayuya modulated T cell growth and/or survival, Jurkat T cells, a human leukemic cell line, were incubated with or without various concentrations of tayuya extract over time. Culturing cells with 25 μ l/ml of a 40 mg/ml tayuya extract for 24 hours resulted in a 43% decrease in viable cell numbers as compared to controls. In addition, culturing cells with 2.5 μ M dihydrocucurbitacin B resulted in an 83% reduction. Western blot analysis for proteins involved in eliciting apoptosis confirmed that both tayuya and dihydrocucurbitacin B reduce Jurkat cell concentrations by inducing apoptotic cell death. Treated cells showed a dramatic increase in cleaved caspases 7, 9 and PARP. Similar results were observed with the THP-1 monocytic leukemia cell line. These studies show that tayuya significantly reduced immune cell viability and survival by inducing apoptosis. Additionally, dihydrocucurbitacin B demonstrated similar effects when added alone, supporting the suggestion that it is the main active immunosuppressive component in tayuya. These results indicate that tayuya and dihydrocucurbitacin B warrant further investigation as possible therapies in immune cell disorders.

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A Plant Lectin can Target Podoplanin to Inhibit Melanoma Cell Growth and Migration.

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Skin cancer is extremely common, with melanoma accounting for about 80% of skin cancer deaths in many countries. Melanoma cell motility contributes to metastatic invasion that is responsible for the vast majority of skin cancer deaths. Extracellular receptors modified by α 2,3-sialic acids that promote this motility can serve as ideal chemotherapeutic targets. For example, the extracellular domain of the mucin receptor podoplanin (PDPN) is highly O-glycosylated with α 2,3-sialic acid linked to galactose. PDPN is activated by endogenous ligands to induce tumor cell motility and metastasis. Some lectins that target proteins containing α 2,3-sialic acid can inhibit tumor cell growth. However, anti-cancer lectins that have been examined thus far target

receptors that have not been identified. We report here that *Maackia amurensis* seed lectin (MASL), which binds O-linked carbohydrate chains containing sialic acid, targets PDPN to inhibit transformed cell growth and motility at nanomolar concentrations. Interestingly, the biological activity of this lectin survives gastrointestinal proteolysis and enters the cardiovascular system to inhibit melanoma cell growth, migration, and tumorigenesis. These studies demonstrate how lectins may be used to help develop oral drugs that target specific receptors to combat malignant cell growth.

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Inhibition of Phosphatidic Acid Phosphohydrolase activity induces EGFR, EGFvIII and ErbB2 endocytosis and decreases their mitogenic potential.

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A signaling pathway involving phospholipase D (PLD)-generated phosphatidic acid (PA), activation of type 4 phosphodiesterases (PDE4) and subsequent decrease of protein kinase A (PKA) activity (PA/PKA pathway), leads to removal of empty/inactive EGFR from the cell surface by endocytosis and accumulation in recycling endosomes, in a reversible manner (Norambuena et. al Mol Biol Cell-2010). This pathway might provide several potential targets for counteracting the oncogenic action of EGFR and other ErbB receptors in tumoral cells. Signaling PA levels mainly depend on rates of generation by PLD and hydrolysis by phosphatidic acid phosphohydrolase (PAP) activity. The relative contribution of plasma membrane-residing lipid phosphatidate phosphatases (LPPs) or cytosolic lipins to PA degradation, the PKA downstream elements and the consequence of the PA/PKA signaling to oncogenesis remain undefined. The PA/PKA pathway can be easily activated by PAP inhibitors, such as propranolol, resulting in transient elevations of PA levels. Here we show that cells transfected to overexpress LPP-1 or LPP-3 display increased levels of EGFR at the cell surface without changes in total receptor expression levels. LPPs seem to be crucial regulators of the PA/PKA signaling pathway that modulate the relative distribution of EGFR between the cell surface and intracellular compartments. Inhibition of the three MAPKs (ERK, p38, JNK) completely abrogates the internalization of EGFR induced by propranolol. These MAPKs seem to act downstream of PKA. EGFRvIII variant and ErbB2 are also internalized by the effect of propranolol. Strikingly, cell proliferation that depends on overexpression of EGFR, EGFRvIII or ErbB2 is highly sensitive to PAP inhibitors. Therefore, decreased accessibility of EGFR to ligand stimuli, as well as exaggerated tyrosine kinase signaling from intracellular compartments, might be particularly deleterious for cells "addicted" to ErbB mitogenic signals emerging from the plasma membrane. PAP inhibition might be a potential target to counteract malignancy of oncogenic ErbB tyrosine kinase receptors.

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High-Content Screening of Crude Extracts Identifies Inhibitors of Triple-Negative Breast Cancer Subtypes.

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Triple-negative breast cancers (TNBC) remain difficult to treat with current chemotherapy regimens, with no currently approved targeted therapies for metastatic disease. The lack of development of targeted therapies for TNBC is partly due to the lack of clearly identified pharmacological targets. Recent subtyping of TNBC patients and identification of driver pathways has made it possible to begin searching for targeted therapies with efficacy for treating particular TNBC subtypes. New therapeutic strategies targeting these various molecular phenotypes of breast cancer are needed to more effectively treat this heterogeneous disease. Libraries of crude extracts from understudied Texas plants and fungal cultures were screened for cytotoxic and antiproliferative activity against a panel of cell lines representative several breast cancer subtypes; including estrogen receptor positive, HER2-positive and 5 triple-negative subtypes with a fluorescence-based assay using the Operetta High-Content Imaging system and Columbus Image Data Storage and Analysis software. These extracts provide novel sources for drug discovery and a majority of their biological sources have never been studied for potential anticancer effects. In total, 920 different fungal extracts and 764 extracts from 367 different species of Texas plants were screened against 9 breast cancer cell lines. We identified 21 extracts with selective activity against one TNBC cell line. 4 extracts were selective for the MDA-MB-468 cell line, 4 extracts were selective for the MDA-MB-231 cell line, and 13 extracts were selective for the MDA-MB-453 cell line. Additionally, 6 extracts showed selectivity for the HER2+ cell line, SK-BR-3. Many of these extracts demonstrated a dose-dependent ability to selectively inhibit cells possessing each of these phenotypes. Future bioassay-guided fractionation will identify the biologically active compounds from these crude extracts. Our results demonstrate the potential to identify specific inhibitors of molecularly distinct breast cancer subtypes from nature.

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Pharmacological and Genetic Abrogation of Histone Deacetylase 6 (HDAC6) Modulates the Jak/STAT Pathway and Proliferation of Melanoma.

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An understanding of the cellular and molecular processes involved in the progression of melanoma is crucial for the development of more efficacious treatments and improved diagnostics. Acetylation and deacetylation of proteins plays a crucial role in the control of important cellular pathways, such as transcriptional control, proliferation, apoptosis, immunogenicity, etc. Specifically, histone deacetylases (HDACs) have garnered significant interest due to the availability of drugs that selectively inhibits these protein modifications and therefore, control these cellular functions.

In our studies we investigated the effect of HDAC6 selective inhibitor(s) (HDAC6i) in human melanoma cell lines (WM164, WM983A and SKMEL21) over the proliferation and cell cycle profile. In all melanomas analyzed pharmacological inhibition of HDAC6 resulted in a delayed growth characterized by a G1 cell cycle arrest. In addition to the pharmacological inhibition of HDAC6, we explored the effect of knocking-down HDAC6 (HDAC6KD) by the generation of monoclonal melanoma cell lines lacking HDAC6 using specific shRNA. As expected, the proliferation of HDAC6KD melanoma cells was diminished when compared to wild-type and

non-target shRNA control cells. Likewise, we observed a similar G1 arrest in the cell cycle that was observed with the use of HDAC6 specific inhibitors.

Our data has also implicated HDAC6 in the regulation of the STAT1 and STAT3 pathways in melanoma cells. These findings were demonstrated by the stimulation of HDAC6KD melanoma cells with cytokines, such as IL-6, IFN[gamma] or TNF[alpha], we found significant changes in the expression of genes involved in cell cycle control and immune regulation, specifically target genes of STAT3 and STAT1 measure by qRT-PCR. We also observed changes in the phosphorylation of STAT3 and STAT1 proteins in cells lacking HDAC6 and/or treated with HDAC6i by immunoblot. To further validate our findings, we evaluated, by ELISA the effect of HDAC6KD over the production of selected cytokines in melanoma cells. These studies provide critical insights into the molecular pathways and/or partners that are involved in the regulatory role of HDAC6 in cell proliferation survival and immunogenicity of human melanoma cells. Collectively, our data has identified HDAC6 as an attractive therapeutic target in melanoma due to its ability to delay tumor growth and target the STAT1/STAT3 pathways, known to be altered in several melanoma malignancies, providing rationale for the development and use of selective HDAC6i.

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Histone Deacetylase 6 (HDAC6) regulates the Jak/STAT pathway in leukemic cells of myeloid lineage.

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The role of histone deacetylases (HDACs) in cell biology, initially limited to their effects upon histones, now encompasses more complex regulatory functions that are dependent on their tissue expression, cellular compartment distribution, pathophysiological conditions and stage of cellular differentiation. Although major advances have been made in understanding the role of specific HDACs in cell proliferation and the survival of cancer cells, their individual participation in specific intracellular pathways is not completely understood. Here we present evidence for the participation of HDAC6 in the control of the proliferation of leukemic cells through the regulation of the Jak/STAT3 pathway.

We initially evaluated the pharmacological effects of both pan-HDAC inhibitors (LBH589) and selective HDAC6 inhibitors (i.e. Tubastatin A), on several leukemic cell lines of myeloid lineage (MO7e, THP-1, K562, HEL, UKE-1, SET2 and TF-1). On this first approach we observed a pronounced inhibition in proliferation of all cell lines when treated with pan-HDACi. However, when these cells were treated with selective HDAC6 inhibitors, only the cell lines MO7e, THP-1, K562 and TF-1 had decreased proliferation, whereas the leukemic cell lines that have an activating mutation in the pseudokinase domain of Jak2 (Jak2V617F), including HEL, UKE-1 and SET2, did not. MO7e, a cytokine dependent cell line, displayed the highest degree of sensitivity to HDAC6 inhibition. These results were mirrored in the HDAC6 shRNA knockdown (HDAC6KD) cell lines. Additionally, cell lines that demonstrated a slower rate of proliferation had a diminished expression of STAT3 target genes by qRT-PCR and a decrease in the amount of phosphorylated STAT3 in both the wild type cell lines treated with HDACi and in the HDAC6KD cell lines where the Jak2 mutations were absent.

In summary, these results demonstrate a decrease in the proliferation of leukemic cells using selective HDAC6i that is comparable to the results obtained from pan-HDACi. However, when the leukemic cell lines contain a Jak2 mutation, both the pharmacological intervention and

the shRNA knockdown of HDAC6 had little to no effect on the proliferation of the cells. Furthermore, cell lines that are particularly dependent on the activation of the Jak/STAT pathway had increased sensitivity to both the inhibition of HDAC6 pharmacologically and by knocking down HDAC6, as was the case in particular with the cell line MO7e. Our findings have demonstrated that HDAC6 may play a role in the regulation of the Jak/STAT pathway.

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Direct inhibition of the Glut1 hexose transporter by resveratrol.

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Interest in the biological actions of resveratrol (3,5,4'-trihydroxystilbene) arises from its identification as a chemo preventive agent for cancer, its cardio protective effects, and as a potential anti-obesity and anti-diabetic compound, since it seems to mimic the effects of energy restriction, thus leading to reduced body fat and improved glucose homeostasis. The exact mechanisms involved in improving hyperglycemic state are not known, but most of glucose uptake into mammalian cells is facilitated by the GLUT hexose transporters. Resveratrol is structurally similar to isoflavones such as genistein, which has been reported to inhibit the glucose uptake facilitated by the GLUT1 hexose transporter.

In the present study we examined the direct effects of resveratrol on glucose uptake in human red cells and in HL-60 and U937 leukemic cell lines, which express mainly GLUT1, and investigated the mechanism involved. We show here that resveratrol inhibits glucose exit in human red cells, and the displacement of previously bound cytochalasin B revealed the direct interaction of resveratrol with GLUT1. Resveratrol behaves as a competitive blocker of glucose uptake under zero-trans exit and exchange kinetic assays, but it becomes a mixed noncompetitive blocker when zero-trans entry transport was assayed, suggesting that the binding site for resveratrol lies on the endofacial face of the transporter. The effect of resveratrol over hexose transport was also tested in human HL-60 and U937 cells under conditions that discriminate transport from the intracellular substrate phosphorylation/ accumulation. Resveratrol also blocks GLUT1-mediated hexose uptake and independently affects cellular substrate accumulation on these cells.

We propose that resveratrol interacts directly with the human GLUT1 hexose transporter by binding to an endofacial site and that this interaction overrides the transport of hexoses across the plasma membrane.

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Analysis of drug resistance in HER-2 positive breast cancer.

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Breast cancer is a deadly disease, and approximately one in four women with breast cancer show overexpression of the HER-2 protein in their tumors. There are a number of effective treatments for women with HER-2 positive breast cancer, including the combination of chemotherapy with HER-2 targeting drugs such as lapatinib, trastuzumab or the recently approved pertuzumab. However, in a number of cases, tumors that initially respond to therapy eventually develop drug resistance, leading to the relapse of the disease. By studying the genes of different drug resistant variants, we have observed two mechanisms by which drug resistance

may develop. Thus, some HER-2 human breast cancer cells (e.g., 231-H2N) treated with the clinically used trastuzumab agent, can escape therapy by shedding, or losing, the target HER-2 gene. We can better understand the biology of drug resistant cancers and devise new therapies to treat cancers that no longer respond to currently available treatments by analyzing the RNA in drug sensitive and drug resistant cell lines. This way, we can identify genes whose copy number or expression level correlates with drug resistance. Previous data shows that tumors relapsing to anti-HER-2 regimens overexpressed a gene termed VEGF. Anti-VEGF treatment was then shown to delay the growth of such drug resistant tumors.

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USP7 and Daxx regulate mitosis progression and taxane sensitivity by affecting stability of Aurora A kinase.

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A large number of patients are resistant to taxane-based chemotherapy. Functional mitotic checkpoints are essential for taxane sensitivity. Thus, mitotic regulators are potential markers for therapy response and could be targeted for anticancer therapy. In this study we identified a novel function of Ubiquitin Specific processing Protease-7 (USP7) that interacts and cooperates with protein Daxx in regulation of mitosis and taxane resistance. Depletion of USP7 impairs mitotic progression, stabilizes cyclin B and reduces stability of the mitotic E3 ubiquitin ligase, Checkpoint with Forkhead and RING finger (CHFR). Consequently, cells with depleted of USP7 accumulate Aurora A kinase, a CHFR substrate, thus elevating multipolar mitoses. We further show that these effects are independent from the USP7 substrate p53. Thus, USP7 and Daxx are necessary to regulate proper execution of mitosis, partially via regulation of CHFR and Aurora A kinase stability.

Results from colony formation assay, in silico analysis across the NCI60 platform and in breast cancer patients suggest that USP7 levels inversely correlate with response to taxanes, pointing at the USP7 protein as a potential predictive factor for taxane response in cancer patients. In addition, we demonstrated that inhibition of Aurora A attenuates USP7-mediated taxane resistance, suggesting that combinatorial drug regimens of Taxol and Aurora A inhibitors may improve the outcome of chemotherapy response in cancer patients resistant to taxane treatment. Finally our study offers novel insights on USP7 inhibition as cancer therapy.

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Sub-lethal High Intensity Focused Ultrasound Exposure Results in Altered Mechanosensitive Gene Expression in Mammary Epithelial Cells.

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High Intensity Focused Ultrasound (HIFU) is a non-invasive therapeutic modality that allows the ablation of unwanted tissues inside the body. In recent years, HIFU gained much attention for its potential applications in the treatment of multiple disorders including breast cancer. At the focal spot, intensified energy results in immediate cell death due to instantaneous temperature rise and/or cavitation effects in the target tissue. The surrounding tissue, though routinely receives residual energy, is believed to be left undamaged. Considering that HIFU exposure results in pressure/tension waves which can potentially cause cellular deformations in exposed tissue due

to altered mechanotransduction, and that a number of mechanosensitive genes have been shown to be implicated in tumorigenesis, we hypothesize that sub-lethal HIFU treatment would result in mechanotransduction alterations that may induce tumorigenesis of mammary epithelial cells. The objective of this study is to investigate the *in vitro* effects of residual HIFU exposure on cellular viability, proliferation, and mechanosensitive gene expression in mammary epithelial cells. An experimental setup was custom-designed and manufactured to permit utilization of a 2.158 MHz HIFU transducer for *in vitro* exposure of MCF-10A immortalized human mammary epithelial cells and MDA-MB-231 invasive human breast cancer cells. Cellular viability and proliferation were assessed days 1-to-4 post HIFU treatment using: i) trypan blue vital stain exclusion assay, ii) WST-1 assay, and iii) standard phase-contrast microscopy. Our results show significant decrease in cellular viability and proliferation of MCF-10A and MDA-MB-231 cells exposed at the focal spot in comparison to mock controls ($P < 0.01$ for both). Post exposure to 2.5% and 22% residual ultrasound intensity, we find no significant difference in cellular viability and proliferation in comparison to the mock controls in both cell lines. Real-Time PCR quantification of *CAV-1 α* (encodes for caveolin-1 α) and *PXN* (encodes for paxillin) gene expression in MCF-10A cells shows 1.65- and 2.39- fold increase in *CAV-1 α* ; 1.54- and 2.40- fold increase in *PXN*, when assessed at 1 hour following 2.5% and 22% HIFU application respectively (mean fold change, $P < 0.01$). Our findings indicate that while a single exposure to residual HIFU has no significant effect on cellular viability and proliferation in MCF-10A and MDA-MB-231 cells, it does result in a significant immediate-early increase in the transcriptional levels of two mechanosensitive genes known to be implicated in mammary tumorigenesis. Future directions will address the bio-functional relevance of these modulations by evaluating their effects on cell motility, migration, invasiveness, and cytotoxic response to anti-neoplastic agents, hence providing insights into the potential role of sub-lethal HIFU exposure in breast cancer pathogenesis.

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Cytotoxic Effects of Novel Compounds on Human Breast Cancer Cells.

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A deeper understanding of the biochemistry of cancer has resulted in the generation of a large number of novel compounds that could be used as anticancer agents that may improve anticancer therapy. The main goal of this project was to determine if a novel set of compounds had selective cytotoxicity on a panel of human breast cancer cell lines, but with lower toxicity on non-cancer cells. Novel compounds were characterized for their cytotoxicity on a panel of cancer and non-cancer cells, measured by the Differential Nuclear Staining (DNS) assay validated for screening by our group. This assay was used to determine the percentage of cytotoxicity after compound treatment. The DNS assay utilizes hoechst to label all the cell nuclei and propidium iodide to stain dead cell nuclei. Selected compounds were tested in secondary assays for the mechanism of cell death (apoptosis/necrosis). Our results confirmed that novel compounds have significant anticancer activity and they induce cell death via apoptosis. One of the most significant findings of our drug screens was the finding that one breast cancer cell line derived from an African American (AA) woman was more sensitive to the treatments than controls and other breast cancer cell lines. Further studies are underway to determine if other AA-derived breast cancer lines are also sensitive to the same compounds. In addition, the specific pathway(s) that induce cell death will be characterized. The present results could provide valuable information for future research applicable in the anti-cancer chemotherapy

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Hormonal Regulation of HB-EGF and SMAD4 in an Endometrial Cancer Cell Line, RL95-2 Cells.

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Endometrial cancer has been linked to deregulation of cell signaling pathways, especially apoptotic pathways. Identifying molecular biomarkers and their role in affecting the proliferation of endometrial cancer cells will be paramount in ensuring early prognosis and overall survival of patients. Unregulated secretion of HB-EGF exerts acute effects on endometrial cells resulting in proliferation and progression of endometrial cancer. SMAD4 on the other hand is one of the molecules involved in TGF signaling pathway and it has been implicated in playing a role in the development of several carcinomas. Ovarian steroid hormones, Progesterone (P4) and estrogen (E2), affect normal endometrial functions as well as their associated abnormalities. The aim of this project is to evaluate the role of P4 and E2 on the expression and secretion of HB-EGF and SMAD4 in an endometrial cancer cell line, RL95-2 cells. We hypothesized that P4 and E2, would down-regulate the expression of HB-EGF and SMAD4 in RL95-2 cells, in vitro. To test this hypothesis, RL95-2 cells were cultured and treated for four days as follows: (a) 10^{-8} M E2, (b) 10^{-6} M P4, (c) 10^{-8} M E2 + 10^{-6} M P4 (d) 10^{-8} M E2 in two days followed by 10^{-6} M P4 for another two days. Immunocytochemistry (ICC) and western blot analysis revealed E2 treated cells followed by P4 treatment resulted in reduced expression and secretion levels of HB-EGF whereas SMAD4 expression levels increased in E2-treated cells, but decreased in P4-treated cells. Our results indicate that ovarian steroid hormones may modulate the expression of HB-EGF and SMAD4, and may impede the progression of endometrial cancer.

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Chitosan and Docosahexanoic Acid Exert Synergistic Effects on NF- κ B Expression in an Ovarian Cancer Cell Line, SKOV-3

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The current treatments for the deadliest gynecological disease, ovarian cancer, have a significantly high level of reoccurrence. Hence, it is imperative that alternative treatments, and more specifically a natural therapeutic product be adopted. Recent studies have shown that natural products, such as chitosan and docosahexanoic acid (DHA), an omega-3 fatty acid, inhibit the proliferation of cancer cells. Nuclear factor kappa B (NF- κ B) is a latent gene regulatory protein transcription factor that lies at the heart of most inflammatory responses. It has been shown to be upregulated in ovarian cancer patients. Therefore, the objective of this study was to determine the effect of chitosan and DHA on NF- κ B expression levels in an ovarian cancer cell line, SKOV-3 cells. We hypothesized that chitosan and DHA will decrease NF- κ B expression in a dose-dependent manner. To test our hypothesis, SKOV-3 cells were treated with (a) 0 ng/mL, (b) 1% Acetic acid; (c) 250 ng/mL of chitosan, (d) 100 μ M of DHA, (e) 250 ng/mL of chitosan + 100 μ M of DHA for 48 hours. Western Blot analysis and immunocytochemistry were performed to observe the expression and localization of NF- κ B, while an ELISA was used to determine NF- κ B DNA binding activity. Quantitative RT-PCR was also performed to determine NF- κ B mRNA expression in the treated cells. Nuclear factor kappa

B protein was localized within the nucleus of SKOV-3 cells and its DNA binding activity decreased when treated with chitosan and DHA individually. Furthermore in cells treated with both chitosan and DHA, there was an even greater decrease in NF- κ B expression. The synergistic effects of chitosan and DHA suggest that these natural products may serve as potential natural treatment for ovarian cancer.

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Expression of Kallikrein 6 in a Chitosan-Treated Ovarian Cancer Cell Line, SKOV-3

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Epithelial ovarian cancer is the deadliest gynecological malignancy and despite current treatments, such as chemotherapy and radiotherapy, reoccurrence of this deadly malignancy is high. Early detection of ovarian cancer is imperative, and furthermore, novel treatments with fewer adverse effects than current treatments of chemotherapy and radiation, are currently being investigated. Kallikreins (KLK) are serine proteases that may play a role in ovarian carcinogenesis. The expression of one member of this family, KLK 6, has been reported to significantly increase in ovarian carcinogenesis. Chitosan, a natural polysaccharide that is biodegradable and biocompatible, may serve as a potential alternative treatment for ovarian cancer, and may function by regulating the expression of KLK 6. Therefore, the objective of this study was to determine the effects of chitosan on the expression and localization of KLK 6 in SKOV-3 cells. It was hypothesized that the expression of KLK 6 protein will be down-regulated in a dose-dependent manner. To test this hypothesis, SKOV-3 cells were treated with 0, 1% Acetic Acid, 50, 100, 250, 500, and 1000ng/mL of chitosan for 48 hours. Cells were also harvested, total protein extracted and the expression of KLK 6 was determined by Western blot analysis. Protein localization and expression of KLK 6 were also determined by immunocytochemistry. Western blotting and immunochemical studies revealed that chitosan treatment resulted in a decreased expression of KLK 6 protein in the cytoplasm of SKOV-3 cells, in a dose-dependent manner. These data indicate that chitosan may inhibit SKOV-3 cell proliferation by decreasing KLK 6 expression.

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Activation of tyrosine phosphatase SHP-1 determines the apoptotic effect of sorafenib on HCC cells.

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Human hepatocellular carcinoma (HCC) is the fifth most prevalent solid tumor in the world and the fourth main inducer of cancer-related death. In 2007, sorafenib (NexavarR), a multiple kinase inhibitor, has shown survival benefits in patients with advanced HCC and become the first clinically approved drug for HCC. Unfortunately, the response rate of sorafenib is not satisfied in clinical. Recently, we have reported that sorafenib sensitized hepatocellular carcinoma (HCC) cells to TRAIL via Src homology-2 containing protein tyrosine phosphatase-1 (SHP-1) dependent inhibition of signaling transducer and activator of transcription 3 (STAT3) (Clinical Cancer Res, 2010). In addition, we identified the effect of sorafenib on STAT3 was

independent to kinases and discovered a series of sorafenib derivatives as SHP-1 enhancers (J Hepatol, 2011; Euro J Med Chem, 2011). Here, we report the role of SHP-1 in sorafenib's determinant for anti-HCC effect. We found that sorafenib increases SHP-1 activity in vitro and in vivo, indicating that sorafenib affects phosphatase activity directly. Based on a series of deletion mutants of SHP-1, we observed that N-terminal SH2 domains (N1) strongly involved in sorafenib-induced STAT3 inhibition and apoptotic effect. Moreover, D61, a critical residue in N1 responsible to form inhibitory salt bridge with catalytic domain, also abrogates the biological effect of sorafenib. In co-IP experiments, sorafenib impairs the interaction between N1 and PTP directly. Furthermore, the role of elevated SHP-1 served as tumor suppressor of HCC was confirmed in cells expressed constitutively activate mutant (dN1 and D61A). Together, we propose a conformational change model for sorafenib-induced SHP-1 activity. Sorafenib potentially opened the inhibited structure of SHP-1 through impairing the linkage between N-SH2 and PTP domains to increase phosphatase activity and further inhibit p-STAT3-related proliferative pathway.

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a5e induces apoptosis via activation of both extrinsic and intrinsic pathways and inhibition of pi3k/akt survival signaling pathways in lung cancer cells.

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it has been reported about the preventive and therapeutic effects of asian traditional medical plants extracts on the cancers, metabolic diseases, inflammatory diseases, and any other intractable diseases. in these studies, it has been assessed the molecular mechanism of the anticancer effect of korean medicinal plants extracts a5e. we examined the cytotoxic and apoptotic pathway for the cancer chemopreventive activity of a5e in non-small-cell lung cancer (nslc) cell lines nci-h460 and nci-h1299. a5e inhibited more efficiently proliferation of nci-h460 than nci-h1299 (p53 -/-) cells. a5e induced apoptosis via sub-g1 cell-cycle arrest in nci-h460 and cells. apoptosis was detected by nuclear condensation, annexin v-fitc/pi staining, cell cycle analysis, western blot, rt-pcr and mitochondria membrane potential analysis. a5e induced cell morphological changes and nuclear condensation at 24h in dose dependent manner. a5e also perturbed cell cycle progression at sub-g1 and altered cell cycle regulatory factors. furthermore, a5e activated apoptotic intrinsic and extrinsic pathways. each expression levels of the extrinsic death receptor complex fasl, fas, and fadd were increased by a5e. also, a5e treatment cleaved caspase-8, caspase-9, caspase-3, parp and downregulated bcl-2 and bcl-xl expression. a5e induced mitochondrial membrane potential collapse and cytochrome c release. otherwise, a5e diminished levels of cell survival factors, pi3k, akt and p-akt dose dependently. taken all these results, our results suggest that the a5e induces apoptosis via activation of both extrinsic and intrinsic pathways and inhibition of pi3k/akt survival signaling pathways in nci-h460. in conclusion, these data demonstrate that a5e can be used as a novel chemotherapeutic agent in nslc.

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Regulation of CoCl₂-induced HIF1 α expression by a natural compound

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Hypoxia induces HIF1 α expression, leading to the malignant cell transformation. In screening of inhibitors against HIF1 α expression using a reporter gene assay system, MO was found to strongly reduce the level of HIF1 in HeLa cells treated with a hypoxia-mimetic, CoCl₂. Identification of binding proteins using agarose-bead conjugated MO (AC-685) combined with subsequent MS data revealed several proteins affected by MO. AC-685 colocalized with a nuclear protein (Np-K) in the nucleus of CoCl₂ treated HeLa cells. Amongst several cytoplasmic or nuclear proteins, Np-K was only found to be responsible for CoCl₂-induced Hif-1 α expression as supported by Np-K siRNA. In this study, detailed regulatory mechanism of HIF1 α expression by MO in association with some nuclear and cytoplasmic proteins will be presented, which might be a clue to treat cancer. This is the first report showing the interaction of Np-K and Hif-1 α .

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Inhibition of Ovarian Cancer Cell Proliferation by Oleoyl Ethanolamide and Its Metabolically Stable Analog AM3102.

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The acylethanolamide oleoylethanolamide (OEA), a well-known appetite suppressant (Nature 414:209, 2001; 425:90, 2003) also induces apoptosis of human neuroepithelioma (BBRC 255:456,1999), glioma (Cell Death Differ 11:853, 2004), and prostate (Int J Urol 13:148, 2006) cancer cells. Although OEA has also been found in the human ovary (Chem Phys Lipids 121:211, 2002), its effect on ovarian cells has not been studied. In the present study, we have investigated the role of OEA and its metabolically stable analog AM3102 (N-[(1R)-2-hydroxy-1-methylethyl-9Z-octadecenamide) on human ovarian cancer cell line OV2008. In serum-free medium, both OEA and AM3102 inhibited the proliferation of subconfluent OV2008 cells. Numerous apoptotic cells were observed floating in the culture medium in the presence of these acylethanolamides. In the presence of charcoal stripped serum, the inhibitory effect of OEA and AM3102 on the cells was markedly reduced. However, inclusion of 20 μ M of palmitoyl trifluoromethyl ketone (PTK), an inhibitor of phospholipase A₂, to the culture medium enhanced the toxicity of the cells to both OEA and AM3102. No toxicity was observed in the presence of an inactive analog of PTK (PACOH3). Moreover, PTK by itself did not inhibit the proliferation of the cells. The inhibitory effect of OEA and AM3102 in serum-free medium or in the presence of PTK in serum-containing medium was significantly reversed if the cells were incubated with 100-200 μ M of the antioxidant α -tocopherol but not by its water-soluble analog Trolox. Our results suggest that both OEA and AM3102 may generate reactive oxygen species, which leads to the inhibition of proliferation in OV2008 cells. Further, phospholipase A₂ inhibition may play a role in the generation of reactive oxygen species in response to OEA and AM3102 (This undergraduate student research was supported by funds from the University Research Council).

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Green barley extract possesses selective antiproliferative and cytotoxic activity on human cancer cells.

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The use of plants for medical purposes is an ancient practice and this knowledge has been transferred by oral communication and/or by imitation among various cultures for generations. Green barley (GB) has been proposed to contain an anticancer curative activity and has been actively promoted by producers of natural herbal supplements with little, if any, evidence for the validity of these proclamations. In this study, we have attempted to confirm some of the proposed activities ascribed to GB and have investigated if such extracts possesses antiproliferative and cytotoxic effect. Treatment of three human lymphoma cell lines with GB extracts resulted in decreased cell numbers after 96h of incubation as compared to untreated cells. However, treatment of non-cancer cells (Hs27 human fibroblasts) did not result in a significant negative effect. Cell cycle analysis of treated lymphoma cells (BJAB) revealed that GB induced slight, but statistically significant, apoptosis after 96h, as evidenced by an increase in the sub G0/G1 population. This result was confirmed by using annexin V-FITC and propidium iodide assay, which detected a subpopulation of treated cells undergoing apoptosis. In addition, GB altered the cell cycle by arresting BJAB cells at the G2/M phase. On the other hand, GB inflicted strong cytotoxicity on another lymphoma cell line (NALM-6) after 48h of exposure and this was mediated by caspase-3 activation. In conclusion, GB exhibited selective antiproliferative and cytotoxicity activity on lymphoma cells. This study suggests that GB extract could possibly be used in a combinatorial therapy with well-known anti-cancer drugs.

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The anti-tumor effects of ethanolic extract of *Antrodia cinnamomea* in human lung cancer-derived A549 cell.

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Aim of the study: *Antrodia cinnamomea* (AC) is a popular Taiwanese folk medicine with numerous beneficial biological effects. The purpose of the present studies is to explore the potential activities of ethanolic extract of *Antrodia cinnamomea* (EEAC) in inhibiting cell growth and migration of human lung adenocarcinoma epithelial cell line (A549).

Materials and Methods: A549 cells were treated with various concentration of EEAC. Anti-proliferative effects of EEAC were analyzed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay and flow cytometry; wound healing assay was used to examine anti-migration property; antioxidation activity was measured by 1-diphenyl-2-picrylhydrazyl (DPPH) and malondialdehyde (MDA) assay; Western blot was employed for examining the molecular mechanisms of EEAC in anti-tumor. HPLC separation was applied to verify the potential bioactive compounds within EEAC.

Results: EEAC has been found to decrease cell viability of A549 cells in a dose- and time-dependent manner, which might be caused through cell cycle arrest at G0/G1 and G2/M phase. In the regulation of the proliferation-associated proteins, EEAC could increase and activate AMPK protein but downregulate the proteins expression of p-Akt, Akt, p-ERK1/2 and ERK1/2. EEAC also suppressed cells migration of A549 cells, and the expressions of migration-related proteins (MMP2 and MMP9) and its upstream regulator, p-ERK1/2, have been evidenced to be

downregulated in A549 cells after EEAC treatment. Additionally, EEAC has more potential activity in scavenging free radicals and significantly reduce free radicals-induced MDA production during lipid peroxidation. The result of HPLC showed that both adenosine and cordycepin have been detected within EEAC.

Conclusions: These results suggested that EEAC could be developed as potential clinical chemotherapy drug for lung cancer or nutraceutical supplements for preventing free radical-associated diseased conditions.

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Zingiber Officinale Extracts Prevent Proliferation and Promote Apoptosis of Human Glioblastoma Cells.

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Glioblastoma multiforme (GBM) are aggressive malignant tumors that develop in the brain. GBMs demonstrate increased rates of cell proliferation and reduced rates of apoptosis. The clinical management of glioblastoma consists of surgical resection followed by radiotherapy and chemotherapy. This treatment regimen is not very effective and more effective therapies need to be developed. Ginger and ginger-based extracts have been widely used as natural remedies. *Zingiber officinale* plant extracts have been shown to prevent chemical induced skin tumors in a mouse model as well as prevent general inflammation. We hypothesize that bioactive ginger compounds will inhibit cell proliferation and promote apoptosis. Specifically, we tested the anti-proliferative effects of [6]-gingerol and [6]-shogaol, two chemically-defined extracts from the *Zingiber officinale* plant, on human GBM cell lines (A-172, U-251 MG and U-1242 MG). Treatment of all the cell lines with the [6]-gingerol or [6]-shogaol (100µg/µl – 10µg/µl) decreased the amount of serum-induced cell proliferation. Additionally, an increased amount of apoptosis was associated with the tested cell lines following exposure to the ginger extracts. More importantly, apoptosis was confirmed using PARP cleavage as a mechanistic measure. These data suggest that [6]-gingerol and [6]-shogaol, are capable of preventing GBM cell proliferation and promoting apoptosis in the GBM cell lines tested. More importantly, *Zingiber officinale* plant extracts may serve as an alternative or supplement to current therapeutic regimens for GBMs.

Cell Biology of Viruses

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The role of Bst-2/Tetherin in HIV transmission from primary human macrophages.

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Bst-2/Tetherin is a cellular protein that restricts HIV release and is antagonized by the viral protein U (Vpu). The restriction factor is thought to physically link budded virions to the host cell surface, preventing their release and cell-free transmission. Although Tetherin has been extensively studied in T cells and cell lines, little is known about its localization and functions in macrophages, and its role in the direct cell-cell transmission of HIV remains controversial.

Here we show that upon interferon treatment of primary monocyte-derived macrophages (MDM), Tetherin is highly up-regulated on both the mRNA and protein levels. Immunostaining shows that in IFN-treated MDM, Tetherin localizes to the cell surface as well as to an intracellular compartment positive for a marker of the trans-Golgi network. MDM infected with

Vpu-deficient HIV show increased overall and cell surface levels of Tetherin, higher levels of cell-associated virus and decreased HIV release into the culture supernatant, suggesting that Tetherin restricts the release and cell-free transmission of HIV in these cells.

Direct cell-cell transmission of HIV is thought to be more efficient than cell-free propagation. One of the key structures mediating cell-cell transmission is the so-called virological synapse, which is characterised by the accumulation of viral proteins and cellular receptors at the interface of the infected and the target cell. To investigate the role of Tetherin in cell-cell transmission of HIV from macrophages to T cells, we co-cultured infected MDM with autologous CD4⁺ T cells. We find that T cells associate with MDM within minutes, while the formation of virological synapses takes at least half an hour. qPCR experiments show that in our system cell-cell transmission of HIV from MDM to T cells is about ten fold more efficient than cell-free infection of the T cells. Using Western Blot analysis to detect HIV protein in the T cells early (24 h) after the co-culture with infected MDM, we find that T cell infection is greatly diminished in the absence of the Tetherin antagonist Vpu. Depleting MDM of Tetherin by RNAi rescues the infection of the T cells.

In conclusion we find that Tetherin efficiently inhibits the release of cell-free HIV from primary macrophages and also inhibits cell-cell transmission of HIV from macrophages to autologous CD4⁺ T cells. These results support the notion of high evolutionary pressure on HIV and related viruses to antagonize Tetherin.

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Annexin A1 is involved in virus-induced cell-cell fusion and syncytiogenesis.

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Cell-to-cell fusion and syncytium formation is involved in numerous biological processes including cell differentiation, development, and organogenesis. Syncytium formation is also associated with pathological conditions such as inflammation, virus cytopathic effects and possibly cancer. Despite the ubiquitous nature of this reaction, little is known about the cellular protein machinery participating in syncytiogenesis. The fusion-associated small transmembrane (FAST) proteins encoded by the non-enveloped, fusogenic reoviruses are the smallest known membrane fusion proteins and the only viral fusogens dedicated to inducing cell-to-cell fusion and syncytium formation. The measles F protein is a canonical class I enveloped virus fusogen whose primary function is to promote virus-cell fusion but also induces cell-cell fusion in virus-infected or transfected cells. We have used these two different classes of viral fusogens to explore the role of cellular proteins and pathways in the process of syncytium formation. A yeast two-hybrid screen identified annexin A1 as a genetic interaction partner of the cytosolic domain of the p14 FAST protein. Annexin A1 is a calcium- and phospholipid-binding protein involved in multiple cellular events including anti-inflammatory signalling, differentiation, cytoskeleton rearrangement, and apoptosis. Co-immunoprecipitation results using HT-1080 cells co-transfected with p14 and FLAG-tagged annexin A1 indicated a calcium-dependent interaction between p14 and annexin A1. Furthermore, siRNA knockdown of annexin A1 dramatically impaired cell-cell fusion and syncytiogenesis mediated by both p14 and measles virus F protein. Since the p14 FAST protein and measles F protein differ in their predicted mechanisms of membrane fusion, our findings suggest that annexin A1 may participate in a ubiquitous cellular response to a membrane fusion event that promotes syncytiogenesis.

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Cell-type specific requirements for ER exit during BK Polyomavirus entry.S. M. Bennett¹, M. Jiang¹, M. J. Imperiale¹; ¹University of Michigan, Ann Arbor, MI

BK Polyomavirus (BKPyV) is a non-enveloped DNA virus that establishes a lifelong subclinical infection in the majority of the population and can cause severe disease in immunocompromised individuals. In particular, BKPyV reactivation in kidney transplant patients can lead to polyomavirus-associated nephropathy (PVAN) and subsequent loss of the graft. In PVAN biopsies, evidence of BKPyV lytic infection can be detected in proximal tubule epithelial cells; therefore, to study BKPyV in a natural host cell, our laboratory has established a cell culture model of lytic infection using primary renal proximal tubule epithelial (RPTE) cells.

The productive entry pathway of BKPyV includes a microtubule-dependent endocytic trafficking route to the endoplasmic reticulum (ER), followed by entry into the nucleus. The trafficking of polyomaviruses from the ER to the nucleus is not well understood, and whether the virus enters the nucleus directly by crossing the inner nuclear membrane or first enters the cytosol and subsequently is imported into the nucleus, remains controversial. Previous studies have suggested that polyomaviruses hijack host ER quality control mechanisms including ER-associated degradation (ERAD) for capsid disassembly and crossing of the ER membrane.

Our study is the first to address BKPyV trafficking in a natural host cell, and our data indicate involvement of both the proteasome and ERAD during entry of BKPyV. Viral capsid proteins colocalize with the ERAD substrate CD3 δ , and inhibition of the proteasome with epoximycin leads to an accumulation of BKPyV in an ER subcompartment, as visualized by fluorescence in situ hybridization of the viral genome and immunofluorescence staining for viral capsid proteins. Employing a retro-translocation assay, we have evidence that BKPyV enters the cytosol during infection, beginning at a timepoint soon after ER trafficking. However, epoximycin treatment does not inhibit ER exit of BKPyV. Interestingly, under epoximycin treatment, the related polyomavirus SV40 was able to enter the cytosol as well in RPTE cells, and furthermore we saw an increase in cytosolic virus for both BKPyV and SV40. This finding differs from what has been shown for SV40 in CV-1 cells. Together these data suggest that the ERAD machinery plays a role during BKPyV entry and cytosolic ERAD factors such as the proteasome play a role after the virion crosses the ER membrane, before nuclear entry.

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Nuclear receptor interaction protein enhances HPV gene expression via interaction with either glucocorticoid receptor or E2.S-L. Chen¹, S-W. Chang¹; ¹Microbiology, National Taiwan University, College of Medicine, Taipei, Taiwan

We previously identified a gene, nuclear receptor-interaction protein (NRIP), which functions as a transcription cofactor in glucocorticoid receptor (GR) and human papillomavirus E2 (HPV E2)-driven gene expression (JBC, 2005; JV 2011). Here, we comprehensively evaluated the role of NRIP in HPV-16 gene expression. NRIP acts as a transcription cofactor to enhance GR-regulated HPV-16 gene expression in the presence of hormone. NRIP also can form complex with E2 that caused NRIP-induced HPV gene expression via E2-binding sites in a hormone-independent manner. Furthermore, NRIP can associate with GR and E2 to form tri-protein complex to activate HPV gene expression via GRE, not the E2-binding site, in a hormone-dependent manner. These results indicate that NRIP and GR are viral E2-binding proteins and that NRIP regulates HPV gene expression via GRE and/or E2 binding site in the HPV promoter in a hormone-dependent or independent manner, respectively. Due to NRIP has a function to dephosphorylate E2, therefore the complex formation with the status of E2 phosphorylation are currently investigating.

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Fighting HIV Infection by Defining Mechanisms to Disaggregate SEVI Fibrils.

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Human immunodeficiency virus (HIV) is a major public health threat worldwide, with 80% of infections acquired through sexual transmission. Naturally occurring fragments of prostatic acid phosphatase (PAP), a protein abundant in human seminal fluid, form amyloid fibrils that promote HIV infection. These amyloid fibrils, termed SEVI (semen derived enhancer of viral infection), augment HIV infectivity by up to $\sim 10^5$ -fold by facilitating virion attachment and fusion to target cells. Despite the extremely stable structure of amyloid fibrils, certain agents can disrupt and reverse amyloid formation. Hsp104, a AAA+ ATPase found in yeast, uses energy from ATP hydrolysis to disaggregate amyloids by disrupting their generic cross- β structure. In addition, an Hsp104 variant termed HAP has been generated that interacts with the chambered bacterial protease ClpP to degrade disaggregated substrates. Both Hsp104 and the HAP/ClpP proteolytic system were found to remodel SEVI amyloid fibrils resulting in a significant reduction in SEVI-mediated enhancement of HIV infection. In addition, a small molecule “molecular tweezer” is able to disassemble SEVI fibrils through interactions with lysine residues and completely abolishes SEVI-mediated enhancement of HIV transmission. In the future, it will be important to determine whether these agents can eradicate the enhancing effects of SEVI in the more complex milieu of seminal fluid.

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ZASC1 Knockout Mice Exhibit an Early Bone Marrow-specific Defect in Murine Leukemia Virus Replication.

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ZASC1 is a zinc finger-containing transcription factor that was previously shown to bind to specific DNA binding sites in the Moloney murine leukemia virus (Mo-MuLV) promoter and is required for efficient viral mRNA transcription (1). To determine whether this cellular factor influences Mo-MuLV replication and virus disease pathogenesis in vivo, we generated a ZASC1 knockout mouse model. Mice lacking ZASC1 were born at the expected Mendelian ratio and showed no obvious physical or behavioral defects. Analysis of bone marrow samples revealed a specific increase in a common myeloid progenitor cell population in ZASC1-deficient mice, a result that is of considerable interest because osteoclasts derived from the myeloid lineage are amongst the first bone marrow cells infected by Mo-MuLV (2). Indeed, Mo-MuLV infection of neonatal mice revealed that ZASC1 is required for efficient early virus replication in the bone marrow, but not in the thymus or spleen. The absence of ZASC1 however did not influence the timing of subsequent tumor progression or the types of tumors resulting from virus infection. These studies have revealed that ZASC1 is important for myeloid cell differentiation in the bone

marrow compartment and that this cellular factor is required for efficient Mo-MuLV replication in this tissue at an early time point post-infection.

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Visualization of Murine Norovirus proteins and its genome RNA.

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The study on Human Norwalk virus (HuNoV), a member of the family Caliciviridae, is hampered because there is no cell culture system or small animal model. However murine norovirus (MNV) that belongs to the same genus as HuNoV is replicated in RAW264.7 cells.

To understand MNV life cycle in cells, we used immunofluorescence microscopy to visualize MNV proteins (VPg, RNA dependent RNA polymerase: RdRp, NTPase, and structural proteins VP1), double stranded viral RNA (dsRNA), and newly synthesized viral RNA (nsRNA) during replication in RAW264.7 cells. We also detected the host proteins as markers of organelle (Calnexin as ER, GM130 as Golgi, EEA1 as endosome, and LAMP1 as lysosome markers) to address the localization of the viral proteins, and of the viral dsRNA and nsRNA in the cells.

At 13 hours post infection, the dsRNA was observed at the perinuclear region. The small amount of nsRNA was also localized at the perinuclear region, however most of the nsRNA were spread diffusely throughout the cytoplasm. The RdRp, NTPase and VPg were co-localized at the perinuclear region with viral dsRNA, indicating that MNV replicates at perinuclear region, and that the replication complex is at least composed of dsRNA, RdRp, NTPase, VPg and a small amount of nsRNA. The RdRp is partially co-localized with Calnexin, indicating that the virus replicates on ER. The structural protein, VP1 was detected in the cytoplasm and appeared to co-localize with nsRNA, suggesting that virion assembly, which involved at least nsRNA and VP1. VP1 was co-localized with EEA1 used as endosome markers indicating that endosome is involved in the virion assembly, which is the different site from the replication on ER.

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HCV infects and impairs CNS function.

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Hepatitis C virus (HCV) is the major agent that causes chronic liver disease and according to the OMS approximately 170 million are infected with this virus. In addition, to being a dangerous infection, HCV also is associated with co infections in HIV, drug abuse and other infectious diseases. HCV infects hepatic cells, however, recent evidence indicates that HCV also results in CNS compromise, but the mechanism of this brain dysfunction is unknown. Our proposal is that HCV can directly infect the brain resulting in CNS dysfunction and damage. Thus, we examined whether human brain primary cells express the receptors required for HCV entry, such as claudin-1, occludin and ephrin B2. Our results indicated that neurons, astrocytes, pericytes, macrophages and brain microvascular endothelial cells may be susceptible to HCV infection, resulting in CNS dysfunction and compromise. Our studies indicate that HCV directly attack the

brain causing CNS dysfunction and cognitive impairment in millions of people infected with this virus.

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Baculoviruses use actin-based motility for nuclear egress and transit to the cell surface.

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Many viruses utilize the microtubule cytoskeleton for intracellular transport. In contrast, the baculovirus *Autographa californica M nucleopolyhedrovirus* (AcMNPV) utilizes the actin cytoskeleton at several points in its replication cycle. Early in infection, following viral uptake by endocytosis, AcMNPV undergoes actin-based motility, a process that accelerates transit to the cell nucleus where replication takes place. Following nuclear translocation, one of the most striking features of AcMNPV late stage infection is the polymerization of actin within the nucleus, concomitant with viral replication. However, beyond an essential role in progeny virus production, a specific function of this nuclear filamentous actin was previously unknown. We have found, using live cell imaging of virus, actin and the nuclear envelope marker lamin B2, labeled with fluorescent protein tags, that AcMNPV nucleocapsids undergo actin-based motility within the nucleus. Moving nucleocapsids collide with and form protrusions in the nuclear membrane, apparently in the process of exiting the nucleus. Using electron microscopy we observed that these protrusions are absent and nucleocapsids fail to exit the nucleus in the presence of drugs that interfere with actin polymerization, indicating that actin is essential for nuclear egress. Following nuclear exit, nucleocapsids again use actin-based motility in the cytoplasm, suggesting that actin is also important for viral translocation to the plasma membrane prior to budding out of the cell. These results indicate that manipulation of the actin cytoskeleton is essential for numerous stages of the process of baculovirus egress, and reveal new roles for actin in pathogen transport within host cells.

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Evidence of Co-purification of the Human T-cell Leukemia Virus Type 1 and Heat Shock Protein-90 (HSP90).

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Retroviruses cause many human diseases, such as, immunodeficiency syndromes, autoimmune, neurological disorders, and cancer. Human T-cell leukemia virus 1 (HTLV-1) is a retrovirus that causes adult T-cell Leukemia (ATL). How HTLV-1 infection progresses to ATL is not well understood. However, identification of cellular proteins that interact with the virus will provide much needed insight into HTLV-1 pathogenesis. Evidence suggests that cellular proteins contained within the virus particle are important for the infection process. We have hypothesized that heat shock protein 90 (HSP90) is a cellular factor packaged within HTLV-1 virion. We have conducted tripartite transfection to produce HTLV-1 virus-like particles (VLPs). This procedure involved using three plasmids, which contain all the necessary viral genes. Western blots analyses demonstrated that the HTLV-1 VLPs were produced and are structurally correct. Next, we purified the HTLV-1 VLPs by spinning the virus through a 20% and 30% sucrose cushion. To further purify the HTLV-1 VLPs, samples were placed onto a 10% - 50% sucrose gradient. Western blot analyses revealed that HTLV-1 and HSP-90 are localized in the same sucrose gradient fractions. Collectively, our data supports the notion that HSP90 is packaged in the HTLV-1 virion. Currently, we are planning to use immunogold-labeling technique to investigate if HSP90 is contained within the HTLV-1 virion.

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Elucidating the Molecular Mechanism of Bacteriophage P22 Genome Packaging.

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The Salmonella-infecting bacteriophage P22 packages its genome from concatemers of dsDNA. This packaging machinery consists of a large (L-) and small (S-) terminase complex. The small terminase functions to properly position the large terminase for packaging initiation via the pac site. The large terminase uses ATP hydrolysis to translocate a single copy of the genome into the bacteriophage procapsid. The motor activity of L-terminase results in dsDNA packaging at a rate of up to 2000 bp/sec. Although poorly understood, termination and cleavage are induced upon reaching a filled procapsid. Upon cleavage, the L-/S-terminase complex disassociates from the capsid enabling tail and associated proteins to bind, sealing the genome inside the procapsid. There are structures of procapsids and mature phage, but no high-resolution structures with terminase proteins bound to the particle. Swift packaging, followed by rapid disassociation, has complicated procapsid-terminase complex isolation and structure elucidation.

We report here preliminary results of L-terminase mixed with procapsids visualized with negatively stained EM images that display density corresponding to the procapsid-terminase complex. Data collection of images of the complex are underway with the goal of producing a 3D reconstruction of the complex. The structure will provide insights for other viruses with related packaging mechanisms, such as Herpes virus.

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Purification of Human T-cell Leukemia Virus Type 1 for Mass Spectrometry Analysis.

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Human T-cell leukemia virus 1 (HTLV-1) is an enveloped retrovirus that causes adult T-cell leukemia. How HTLV-1 infection progresses to T-cell malignancy is not well understood. However, identification of cellular proteins that interact with the virus will provide much needed insight into HTLV-1 pathogenesis. It has been reported that enveloped viruses contain proteins derived from the host cell. **Our hypothesis is that HTLV-1 contains host cell proteins essential for the virus to infect naïve cells.** To identify cellular proteins, we will perform proteomic analysis. Initially, we transfected HEK 293T cells with three different plasmids (pCMVRU5-GFP_{Luc}, pCMVHT1 delta X-M, and pCMV-VSV-G) to produce HTLV-1 virus-like particles (VLPs). Western blot analyses of HTLV-1 VLPs revealed that the viral protein, called capsid, is produced and structurally correct. Next, HTLV-1 VLPs were purified by sucrose gradient centrifugation. Immunoblot studies of sucrose gradient fractions revealed that HTLV-1 VLPs are purified and sediment at the 30% – 40% sucrose region. Silver stained polyacrylamide gels of the purified HTLV-1 VLPs revealed the presence of half the known viral proteins. Currently, we are further purifying the HTLV-1 VLPs to obtain samples enriched with all known viral proteins. Samples containing all known HTLV-1 viral proteins will be prepared for mass spectrometry analysis by digestion with trypsin. These studies will advance our understanding of host-pathogen interactions and should facilitate the development of novel therapeutic targets for HTLV-1 infections.

Protists and Parasites

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Epigallocatechin-3-gallate (EGCG) blocks development of *Dictyostelium discoideum*

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Green tea catechins have received significant attention due to their potential health benefits, but the precise mechanisms by which these compounds act are not known. Here, we assess whether the model organism *Dictyostelium discoideum* is a useful tool with which to characterize the effects of these natural products. Epigallocatechin-3-gallate (EGCG), the most abundant and potent catechin in green tea, affects *Dictyostelium* aggregation and morphogenesis; aggregating amoebae do not stream and development is stalled at the loose aggregate stage. These developmental effects are accompanied by defects in cell-cell signaling and delayed expression of developmentally-regulated genes. These data suggest that catechins and their derivatives may be useful tools with which to better understand cell movement and morphogenesis in *Dictyostelium*. Furthermore, because the methods used here are relatively simple, quick, and easily adaptable for high-throughput screening, *Dictyostelium* is likely to be a useful model with which to identify and characterize other bioactive natural products.

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New insights into Golgi complex of *Tritrichomonas foetus*: production of a specific marker and identification of the adenosine triphosphatase and beta-tubulin.

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Tritrichomonas foetus is the causative agent of bovine trichomoniasis, and is among the earliest diverging eukaryotes. This protist presents a well-developed Golgi complex and, although current information shows that the Golgi complex plays an important role in protein glycosylation, sorting and lysosome biogenesis, there are very few studies in the Golgi of trichomonads. The absence of reactivity of commercial antibodies to the Golgi complex of trichomonads contributes to the lack of new data. In this work, several approaches such as cell fractionation, Golgi isolation and monoclonal antibodies were raised as tools on Golgi studies of *T. foetus*. In addition, complementary techniques, such as immunolabelling, immunoblotting, mass spectrometry analysis and database searches were performed to identify which proteins were recognised by this antibody. The Golgi complex of *T. foetus* was purified and among the antibodies, one was named mAb anti-Golgi 20.3, which exhibited intense reactivity by light and electron microscopy. This antibody recognised proteins of 60 kDa and 66 kDa. Using proteomic approaches, these proteins were identified as putative beta-tubulin and adenosine triphosphatase, respectively. However, antigenic peptides analysis indicated a cross-reactivity

with beta-tubulin which could carry this protein during immunoprecipitation. The mAb anti-Golgi 20.3 also recognised the Golgi of *Trichomonas vaginalis* which presents an adenosine triphosphatase with an identity rate higher than 80%. The nucleotides sequences that codify these proteins were identified and included on *T. foetus* database, and the 3D structure of the proteins was also obtained. This study is the first to demonstrate the presence of ATPase and beta-tubulin in the Golgi complex from *T. foetus* using mass spectrometry and genomic data. These data indicated (1) the presence of adenosine triphosphatase in the Golgi, (2) it is conserved in *T. foetus* and *T. vaginalis*, (3) new information concerning nucleic acid sequences and proteins structures of adenosine triphosphatase and beta-tubulin from *T. foetus* and (4) the mAb anti-Golgi 20.3 is a good marker for this organelle that can be used in future studies. This work was supported by AUSU, CNPq, FAPERJ, INMETRO and Pronex.

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Experimental Verification of microRNAs in *Leishmania braziliensis*.

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miRNAs have been described in most organisms from worms to human and more recently in protozoans. miRNAs are a group of small RNAs that regulate gene expression post-transcriptionally in a complex process of binding to mRNA in a perfect complement or almost perfect complement that cleave mRNAs or inhibit their translation. Gene expression in *Leishmania* is not well understood, however, it is known to be post-transcriptionally regulated. Argonaute-like and Dicer-like protein, the machinery needed for the processing of miRNAs have been shown to exist computationally in *Leishmania braziliensis*. Our hypothesis is that miRNAs regulate gene expression in *L. braziliensis*. We have shown by computational data analysis that there are miRNAs in *L. braziliensis*. Here we show the continuation of our study and plans for experimental verification of miRNAs in *L. braziliensis*.

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Identification of differentially expressed genes among pathogenic and nonpathogenic *Leishmania* species.

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There are 31 species of *Leishmania* that are known to infect humans. The disease spectrum of leishmaniasis ranges from mild self-limiting cutaneous ulcers to a potentially fatal visceral infection, each dependant on the species of parasite with which one is infected. It is poorly understood which genes and how many proteins are involved in the survival and virulence of the pathogenesis of *Leishmania*. We hypothesize that differences in the expression of genes among pathogenic species and between pathogenic and nonpathogenic species should identify some of the proteins that are critical to the survival and important to the virulence of these parasites. Thus, in the current study we identified differentially expressed genes between pathogenic and nonpathogenic species of *Leishmania* (*L. donovani*, *L. mexicana*, *L. major* and *L. tarentolae*) by cDNA-AFLP (Amplified Fragment Length Polymorphism) and subsequently characterized these genes by DNA sequence analyses. AFLP analysis with 32 different primer sets resulted in 8 AFLP primer sets that gave data in the 50-400 bp range and therefore were considered for further analysis. We have identified 296 polymorphic fragments and 148 unique fragments to date. Interestingly, the calculated polymorphism is 25-35% across the species; this corresponds to the high degree of similarity that is seen at the genome sequence level. Analysis of some of these fragments has evolved 3 general categories of proteins. 1) hypothetical proteins in the

Leishmania genome database with no known function or cellular location 2) 18s and 24s rRNA and most likely result from using total RNA in the AFLP reactions and 3) protein coding sequences including calreticulin in *L. donovani*, superoxide dismutase, peptidase and ascorbate peroxidase in *L. mexicana*. Characterization of the remaining unique AFLP fragments is underway. Once characterized these cDNA-AFLP fragments may be used as probes to obtain their corresponding full length genes from genomic DNA and used to blast search against known *Leishmania* genome sequences. As toxic antimony compounds are often used unsuccessfully for its treatment, the characterization of genes differentially expressed between pathogenic and nonpathogenic species is a first step in identifying potential novel targets for the development of nontoxic drugs to be used in the treatment of leishmaniasis, a devastating and potentially fatal collection of human diseases.

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Hepatic metalloproteases activity in mice infected with malaria parasite *P. chabaudi*.

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Malaria, the disease caused by *Plasmodium* infection, remains one of the most prevalent and deadly maladies. Matrix metalloproteinases (MMPs) are a family of secreted and membrane-bound zinc-dependent endopeptidases that have the combined capacity to degrade all the components of the extracellular matrix, and to shed, activate, or inactivate cytokines, chemokines, and other MMPs through cleavage from their precursors. Among them are ACE and ACE2, critical regulators of the renin–angiotensin system. The liver is affected in two stages during the life cycle of the malarial parasite: initially in the pre-erythrocytic cycle and then in the erythrocytic phase. The latter is responsible for the clinical manifestations of malaria. The involvement of the metalloproteinases activity in the liver injury mediated by malaria remains unknown. Herein we report the evaluation of metalloproteases catalytic activity in liver homogenates (40µg / assay) infected by *Plasmodium chabaudi* with blood parasitemia of 80%. For this purpose, selective fluorogenic substrates were used as follows: MCA-PLGL-Dpa-AR-NH₂ (collagenase and gelatinase enzymes), Abz- GPQGLAGQ-EDDnp (MMP-9), Abz-FRK(Dnp)P-OH (ACE) and Abz-APK(Dnp)-OH (ACE2). Our data show a significant decrease (38,1 UAF/min ±3 to 11,8 UAF/min ±0,5 n=5) of hydrolysis of MCA-PLGL-Dpa-AR-NH₂ (general substrate for metalloproteases) in the infected liver compared to non-infected. MMP-9 activity presented a slight increase (33,2 UAF/min ± 1 to 35,8 UAF/min ±2 n=5), which is reflection of the liver inflammation by the infection. However, ACE hydrolytic activity of liver tissue upon the selective substrate Abz-FRK(Dnp)P-OH did not changed after *Plasmodium* infection and was 50% inhibited by captopril (1mM) (0,97 UAF/min ±2 to 0,45 UAF/min ± 0,7 n=5). Interestingly, we distinguished the ACE2 activity with selective substrate Abz-APK(Dnp)-OH in infected liver homogenates (70 UAF/min ± 7 n=3) and noninfected (97 UAF/min ± 12 n=3). In both situations we observed a specific inhibition of the ACE2 with addition of inhibitor DX600 (20 µM): infected liver (inhibition of 70%±3) and noninfected liver (inhibition of 80%±2). This data provide new insights for renin-angiotensin modulation in infected liver by *Plasmodium*. Our data suggest that the metalloproteases analyzed are directly involved in inflammation process and liver hemodynamics, which are important for malaria physiopathology. Supported by FAPESP.

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The unique insert in the ribosomal stalk protein, phosphoprotein P0, shared by members of the Ciliophora.

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Phosphoprotein P0 is a eukaryotic 60S ribosomal protein which is a member of the L10 protein family. It functions as a scaffold for the P protein stalk complex, located at the GTPase-associated center required for protein elongation. P0 has been implicated in systemic lupus erythematosus. Recent evidence suggests that P0 may locate to the cell surface of apicomplexan parasites such as *Plasmodium* and *Toxoplasma*, and that it may play an important role in host invasion. The apicomplexans and ciliophorans are sister clades, within the protistan alveolates. Therefore, we are investigating the potential of the ciliophoran *Tetrahymena thermophila* as a model organism for vaccine research and drug development applicable to apicomplexan diseases. Our comparison of P0 orthologs from *Tetrahymena* with those of prokaryotes and other eukaryotes has revealed a 15-17 amino acid-long insert exclusive to members of the protist phylum, Ciliophora. Using several bioinformatics methods, including sequence alignments, homology modeling and motif prediction tools, we have begun to characterize the insert in *T. thermophila* and other ciliates. With the exception of *Paramecium tetraurelia*, the nine ciliophoran species examined shared a region within the insert, with the consensus sequence (D/E)XX(Y/F)(D/E). Within this region, there is a predicted casein kinase II phosphorylation site in *T. thermophila* and some species of *Euplotes* and *Eufolliculina*, with the consensus sequence (S/T)XX(D/E). Homology modeling of the *T. thermophila* P0 (TtP0) shows that the insert is located within the N terminal region, and is situated near the surface of the ribosome, near the base of the stalk. The predicted conformation of the insert is highly variable, suggesting that it could interact with the ribosomal RNA, another part of P0 or with one of the P protein dimers that bind to P0. We have also found that this insert is absent in several apicomplexan species, so the insert may have arisen after the divergence of the ciliate and apicomplexan lineages. Based on our evidence, we are continuing to investigate the hypothesis that the insert may regulate ribosomal function, via its phosphorylation site and putative interactions with stalk proteins or rRNA, and extra-ribosomal factors.

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Architecture and assembly of TbBILBO1, a protein that is essential for the survival of *Trypanosoma brucei*

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Protozoan Trypanosomatidae including *Trypanosoma brucei*, *T. cruzi* and *Leishmania spp.* are parasites to human and animals and cause significant mortality each year. The flagellar pocket (FP) is a unique compartment that resides at the base of the flagellum of these protists. It is the sole site for endo- and exocytosis and plays crucial roles in the cell's defense against the host immune system. TbBilbo1 is the first characterized FP cytoskeletal protein and is an integral component of the flagellar pocket collar (FPC). This protein is essential for both FP biogenesis and the viability of the parasites. TbBilbo1 consists of a globular N-terminal domain (NTD), two predicted central EF-hand motifs and a long coiled-coil domain followed by a C-terminal leucine zipper motif. In order to illuminate the molecular basis for TbBilbo1 in FP biogenesis and cell survival, we aim to determine the structure of the protein and to understand the structure-

function relationship by means of x-ray crystallography, NMR spectroscopy, electron microscopy, and in vivo immunofluorescence studies.

Our localization analysis of various truncated constructs of TbBilbo1 in the procyclic form of *T. brucei* 427 showed that the C-terminal leucine zipper is responsible for the protein's localization to the FPC. Overexpression of TbBilbo1- Δ NTD, but not of TbBilbo1-FL, with 20 ng/ml tetracycline was lethal to the cells after 4 days of induction. Our determined NMR structure of the TbBilbo1-NTD reveals that it adopts a ubiquitin-like fold with a surface patch consisting of eight absolutely conserved residues (5 aromatic and 3 positively charged). We have further generated two sets of mutations in the NTD for in vivo functional analysis. The first set, namely TbBilbo1-M3M0, contains F12A, K15A, K60A and K62A, and the second set, namely TbBilbo1-M1b2b, contains W71A, Y87A and F89A. Overexpression of both mutated TbBilbo1 had similar effect to that of the TbBilbo1- Δ NTD, indicating that these residues play an important role for cell viability. Based on our rotary shadowing EM studies, we conclude that TbBilbo1 dimerizes in an anti-parallel manner via its coiled-coil domain. This dimer further assembles into a long filament-like oligomer via its C-terminal leucine zipper. We are currently investigating how the EF-hand motifs might control the gating function of the FPC.

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Evaluation of recombinant filarial Troponin and Paramyosin as Diagnostic tools in lymphatic filariasis.

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Lymphatic filariasis is a mosquito vector borne disease caused by the filarial nematodes *Wuchereria bancrofti* and *Brugia malayi*. This extremely painful, debilitating and disfiguring disease is a cause for major public health problem in the tropical and subtropical world. World Health Organisation estimates that at least 120 million people are affected in more than 80 countries and another 1.5 billion people are at risk. The development in molecular biology research and the sequence mapping data provided by the Filarial Genome Project is being utilised by the nematode research community to develop a better understanding of the biology of filarial parasites and to identify new diagnostic candidates for the detection of human filariasis. The production of diagnostically important recombinant antigens can now be produced in variety of expression vectors which represents the key success to the preparation of commercial diagnostic kits. Hence our focus on to detect antigens by immunoscreening from cDNA libraries and to evaluate its diagnostic efficacy. The DNA inserts from the purified plaques were amplified by PCR using T3 and T7 universal primers and sequenced.

Epitope mapping of rWbPmy and rWbTN by Kolaskar and Tongaonkar antigenicity method were able to predict the presence of potential antigenic determinant sites based on the antigenic propensity values. This analysis has shown several antigenic sites in both rWbPmy and rWbTN antigens. The filarial IgG4 antibody detection assays with individual recombinant antigens rWbL2, rWbPMY and rWbTN showed 45%, 28.88% and 22.22 % sensitivity respectively for the detection of the microfilaremics and clinical filariasis samples and the specificity was 98-100% for all the three antigens. The Filarial IgG4 antibody detection assays with different

combinations of the individual recombinant antigens (rWbL2 & rWbMY/rWbL2 & rWbTN and rWbL2,rWbPMY & rWbTN showed sensitivity of 81.81%,74.54% and 85.45% respectively. Based on the IgG4 ELISA assay results we have selected cocktail of three recombinant antigens to develop sensitive, specific, rapid and user-friendly Dot-blot assay for the detection of filarial IgG4 antibodies in different clinical groups of filariasis. The Dot-Blot assay using the cocktail of three recombinant antigens yielded a sensitivity and specificity of 100% to detect microfilaremic and clinical filariasis cases

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Ultrastructural alterations induced by SQS-inhibitor and metronidazole on *Trichomonas vaginalis*.

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Trichomonas vaginalis is a human parasite that causes trichomoniasis, a cosmopolitan sexually transmitted disease. In women, this disease causes adverse pregnancy outcomes, as pre-terms rupture of membranes, pre-term delivery, cervical cancer and also increases the transmission of HIV. Currently, the treatment of choice for trichomoniasis is the drug metronidazole. However, this compound presents undesirable side effects and has been observed an increase in metronidazole-resistant strains. In addition, during pregnancy, the use of metronidazole is not advised. Therefore, there is a search for alternative chemotherapy, a top priority for the management of this disease. 3-(biphenyl-4-yl)-3-hydroxyquinuclidine (BPQ-OH) is an inhibitor of the squalene synthase, an enzyme that catalyses sterol biosynthesis. The effects of BPQ-OH have already been described in *Trypanosoma cruzi*, *Leishmania mexicana* and *Candida tropicalis*. Thus, the aim of this study is to analyze the effect of this compound on *T. vaginalis* in comparison to metronidazole effects. Parasites were grown in TYM medium and different concentrations of compounds were added after 24h. The ultrastructural changes of treated cells were observed by scanning and transmission electron microscopy. The cytotoxicity of BPQ-OH on mammalian cells (HeLa, MDCK and Caco-2) was analyzed by MTT procedure. Several antiproliferative experiments demonstrated a dose-dependent effect of BPQ-OH and metronidazole on the growth of *T. vaginalis* with an IC₅₀ of 46µM and 1,8µM (0,3 µg/ml) after 24h treatment, respectively. Morphological analyses showed that both compounds induced changes in the ultrastructure of the parasite. The most significant alterations observed were (1) membrane blebbing, (2) cell wrinkling, (3) cell clusters and (4) pseudocyst formation. Cytotoxicity assays using mammalian cells showed no effect of the BPQ-OH on the viability of these cells at higher concentrations than IC₅₀. The opposite was observed when metronidazole was used at lower concentrations. Ultrastructural analyses demonstrated that both compounds provoked alterations, such as intense vacuolization. In conclusion, the results suggest that BPQ-OH could be, after more tests, an important compound in the development of a novel chemotherapeutic agent against *T. vaginalis*.

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Live cell measurement of cysteine protease activity in malaria parasites – evaluation of calcium dependent proteolysis (calpain) and modulation by cell signaling messengers.

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Malaria is caused by *Plasmodium* parasites and remains one of the most prevalent and persistent diseases. Due to increased resistance to the available antimalarial drugs, a better understanding of the cellular and biochemical pathways of the parasite are fundamental to achieve better strategies to control the disease. Proteolytic activity is an essential process in parasite metabolism during intraerythrocytic cycle. The regulation of *Plasmodium* proteases through cellular signaling mechanisms, such as calcium and phosphorylation, are poorly understood. Using a fluorogenic peptide as substrate, we performed a confocal microscopy analysis to investigate the intracellular proteolysis of *P. falciparum* and *P. chabaudi* (human and rodent malaria). We investigated either the role of calcium and phosphorylation in the modulation of parasites cysteine protease activity. Our data shows a rapid increase in intracellular proteolysis by calcium in the cytosol after release from the ER (89,5% ± 16 n=6) with THG (10µM) addition, which was greatly inhibited (82% ± 1 n=5) by the cysteine protease inhibitor E-64 (10µM) and by the specific calpain I inhibitor (ALLN, 0.5µM) (80,5% ± 4 n=6). *Plasmodium falciparum* calpain is believed to be one of the central mediators of parasite cell cycle and previous work shows a disruption of *Plasmodium* culture by ALLN. Interestingly, inhibition of calmodulin with calmidazolium (10µM), which is a key factor in CaMK activation and calcium homeostasis, resulted a rise in cytosolic calcium followed by a significant increase (107% ± 15 n=5) in the *P. falciparum* intracellular hydrolysis of the Z-Phe-Arg-AMC substrate. However, phosphorylation modulators for PKA (cAMP analogs: 8Br-cAMP, N6-cAMP and PKA inhibitor: H89) and PKC (PKC activator phorbol 12,13-dibutyrate and PKC inhibitor Gö6976) did not promote directly inhibition of cysteine proteases activity however could modulate the Ca²⁺ signaling crosstalk like observed with 8Br-cAMP (20µM) and N6-cAMP (20µM) treatments with interruption of Ca²⁺-calmodulin proteolysis modulation. Our results establish a selective protocol for measurement of calcium dependent proteases activity (i.e. calpain) in *Plasmodium* and also verified if phosphorylation pathways (PKA and PKC) have an important modulatory effect in proteolysis. Supported by FAPESP.

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The formation of the cyst wall in *Giardia lamblia*: a missing piece of the puzzle.

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Giardia lamblia is a parasite protist considered a living fossil because it is placed in the earliest divergence and deepest branch in eukaryote evolution. *Giardia* is a cosmopolitan parasite and the causative agent of giardiasis, which can leads an enormous burden of human diarrheal diseases. Nonetheless, the basic biology of this protist is incompletely understood. The parasite cycle includes two developmental and morphological forms, the trophozoite and the cyst. Trophozoites colonize in the upper small intestine by attaching to the epithelial cells in which they can cause disease. While the trophozoites pass through the small intestine, they produce a rigid extracellular matrix or cyst wall (CW) that protects them from the external environment. The

CW is composed by proteins and carbohydrates. The formation of the resistant extracellular CW begins with intestinal signals, where the cyst wall proteins (CWP) 1, 2 and 3 are synthesized. All of the CWPs are transported via encystation-specific secretory vesicles (ESVs) and are released at the site of CW assembly. Although the presence of carbohydrates in cyst wall has been reported, the detection and origin of the cyst wall carbohydrate-rich moieties is the missing piece of the puzzle. The lack of specialized vesicles that contain large amounts of this carbohydrate raises the question concerning how this material is exported to the CW. In this study, the distribution of the different sugar residues and the origin of the carbohydrate components of the cyst wall were studied using transmission electron microscopy, cytochemistry for carbohydrates and immunocytochemistry. Immunofluorescence microscopy using anti-cyst wall protein 1 (CWP1) and gold- and fluorescent-conjugated lectins, such as WGA and DBA, were also used. Interestingly, encystation carbohydrate-containing vesicles (ECVs), positive for N-acetyl-galactosamine, were found in the encysting cells. The ECVs are distinct from the encystation specific vesicles (ESVs) because: (1) they are electron lucent whereas ESVs are electron dense; (2) they do not react with antibodies against cyst wall proteins; (3) their contents are positive for carbohydrates, whereas ESVs display a negative reaction; and (4) this cell compartment exhibits a positive labeling for DBA lectin indicating the presence of N-acetyl-galactosamine, whereas ESVs are negative. To evaluate if ECVs could be vesicles involved in the endocytic pathway, endocytic markers, such as Lucifer Yellow and Acridine Orange, were used. No co-localization of these markers with ECVs was observed. We suggest that the ECVs may represent a new structure involved in the cyst wall formation. This research was supported by AUSU, CNPq, FAPERJ and PRONEX.

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Acetyl-CoA Carboxylase Links Lipid Metabolism and Immune Evasion in African Trypanosomes.

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Trypanosoma brucei, a lethal unicellular parasite transmitted by the tsetse fly, infects the bloodstream and cerebrospinal fluid of humans, causing African sleeping sickness. In addition to antigenic variation of its surface glycoprotein coat, *T. brucei* employs a secondary immune evasion tactic: the upregulation of endocytosis to clear lytic cell surface immune complexes, a strategy that likely involves membrane turnover and fatty acid synthesis (FAS). Acetyl-CoA carboxylase (ACC) catalyzes the first committed step of FAS and is a control point for regulating FAS. Previous work showed that a *T. brucei* bloodstream form (BF) ACC RNA interference (RNAi) strain exhibited reduced virulence in mice, but normal growth in culture. We hypothesized that ACC is required for immune evasion via endocytosis upregulation. To test our hypothesis, we examined endocytosis in BF ACC RNAi parasites using fluorescent dextran and transferrin as markers for fluid-phase and receptor-mediated endocytosis, respectively. After 9 days of ACC RNAi, we observed a significant reduction in fluid phase (88%) and receptor-mediated endocytosis (87%). Next, we tested the clearance of surface bound antibodies and found that ACC RNAi caused a significant 26% delay in surface antibody clearance. Lastly, we tested avoidance of complement-mediated lysis. ACC RNAi resulted in a significant 42% increase in complement-mediated lysis. Because upregulation of endocytosis might increase demand for FAS, we examined if ACC is regulated in response to changes in lipid demand, which we modulated by growing the parasites in normal, low, or high lipid media. In BFs, we found no change in ACC phosphorylation, expression, or activity upon growth in low or high lipid media. Also, under these growth conditions, ACC RNAi had similar effects on endocytosis, antibody clearance, and complement-mediated lysis. In contrast, insect form parasites exhibited

a 2-fold increase in ACC protein and enzymatic activity in low lipid media. ACC phosphorylation in insect forms increased 3-fold in high lipid media and decreased by 80% in low lipid media. Based on these data we propose a model whereby ACC is up-regulated in *T. brucei* within the mammalian host to support increased endocytosis and immune evasion, while within the insect host, ACC is regulated in *T. brucei* in response to the environmental lipid supply.

New Technologies for Cell Biology I

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Encapsulation of cytoplasmic extracts in lipid bilayer vesicles for spatially and temporally controlled cellular reconstitutions.

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Cytoplasmic extracts and purified proteins offer a powerful tool for understanding a broad range of cellular behaviors at the molecular level. However, dissecting how these behaviors are coordinated over space and time requires an environment that closely mimics that of the cell, including interactions with and confinement within lipid bilayer membranes. The ability to encapsulate proteins and cytoplasmic extracts within lipid membranes has traditionally presented a significant barrier for cellular reconstitution. Although giant unilamellar vesicles (GUVs) offer a promising platform for addressing this challenge, traditional techniques for forming vesicles by hydration, sonication and electroformation provide limited flexibility for encapsulating protein solutions and may produce highly polydisperse or multilamellar vesicles. To circumvent these challenges, we developed microfluidic jetting, a technique capable of loading functional cytoplasmic extracts and purified protein solutions into GUVs of controlled size, similar to the inverse emulsion technique. Unlike inverse emulsions, however, microfluidic jetting uses liquid impulses expelled from a small (~10 μm) nozzle to deform a planar lipid bilayer into spherical vesicles. Because the solution expelled from the nozzle mixes with the surrounding liquid immediately before being encapsulated inside the GUV, jetting offers excellent control over both the internal composition of vesicles as well as the timing of biochemical reactions. Previously, we demonstrated encapsulation of whole-cell extracts with viscosities up to fourfold higher than water. Here, we extend this technique to encapsulate *E. coli* extracts that are competent for the transcription and translation of plasmid DNA, and show that proteins can be expressed and targeted to the membrane or vesicle lumen depending on the presence or absence of a hydrophobic helix. Because we are able to control the size of the vesicles and the timing of transcription initiation, this technique offers a step towards the reconstitution of dynamic cellular processes and an understanding of how the self-organization of the cell is influenced by spatial constraints.

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A Novel Pulse-Chase Paradigm to Visualize the Trafficking of Transport Vesicles in Neurons.

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The polarized structure and function of neurons is established and maintained by the differential trafficking of axonal and dendritic proteins. Transmembrane proteins are targeted to dendrites in vesicles that traffic solely within the somatodendritic compartment. How these vesicles are

retained within the somatodendritic domain is unknown. Here, we use a novel pulse-chase system, which allows synchronous release of exogenous transmembrane proteins from the endoplasmic reticulum to follow movements of post-Golgi transport vesicles. Surprisingly, we found that post-Golgi vesicles carrying dendritic proteins were equally likely to enter axons and dendrites. However, once such vesicles entered the axon, they very rarely moved beyond the axon initial segment but instead either halted or reversed direction in an actin and Myosin Va-dependent manner. In contrast, vesicles carrying either an axonal or a nonspecifically localized protein only rarely halted or reversed and instead generally proceeded to the distal axon. Thus, our results are consistent with the axon initial segment behaving as a vesicle filter that mediates the differential trafficking of transport vesicles.

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Spontaneous membrane translocating peptides for drug delivery.

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Using orthogonal high-throughput screening and rational combinatorial peptide libraries, we have identified a family of peptides that can spontaneously translocate across membranes without toxicity or membrane permeabilization. The conserved 9-residue translocation motif is mostly hydrophobic, composed of leucine, isoleucine and proline, but also has several conserved arginine or lysine residues and an open terminal amino group. Using confocal fluorescence microscopy, we tested the ability of the selected peptides to transport large polar cargoes across the plasma membranes of living cells. They translocate rapidly, filling the cytoplasm with a uniform fluorescence. Translocation occurs without endocytosis and does not require a cellular energy source. These peptides could be used as delivery vehicles for a variety of polar drugs.

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Magnosphere™-based Rapid Exosome Isolation for Biomarker Detection.

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Exosomes play an increasingly important role in autoimmune diseases, cancer, stress response, and metabolic disorders. Exosomal cargo may include several biomarkers, such as membrane and soluble proteins, mRNAs, long non-coding RNAs and miRNAs. Current exosomal isolation methods are tedious and laborious. Additionally, some of these methods can indiscriminately precipitate exosomes along with other biological moieties including free circulating proteins or nucleic acids. To address the challenges of exosomal isolation, we developed a magnetic bead based isolation method. Magnosphere™ magnetic beads, exclusively manufactured by JSR Corporation, are functionalized for immunoaffinity isolation of exosomes using a proprietary combination of antibodies. Confirmation of exosomal isolation from cell culture is visualized by western blot of the soluble exosomal biomarker, HSC70 along with other exosomal proteins. Additionally, a selected few miRNAs (hsa-Let 7a, hsa-miR-21, hsa-miR-222 and hsa-miR-223) are analyzed for further verification. To evaluate non-specific binding, we introduced either a random synthetic small RNA or non-exosomal peptides into the samples. Our studies reveal successful exosomal isolation by immunoaffinity with functionalized Magnosphere beads. Furthermore, functionalized Magnosphere beads facilitate

rapid exosomal isolation from cell culture or biological fluids with high affinity and low non-specificity.

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Endophilin B is associated with caveolin containing vesicles in living cells.

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Endophilins A and B comprise a family of proteins that are involved in membrane vesiculation. Both forms of endophilin contain two well-characterized domains, an amino-terminal N-BAR (Bin-Amphiphysin-Rvs) domain and a carboxy-terminal SH3 (Src-homology 3) domain. The N-BAR domain is required for binding to the lipid bilayer and the subsequent induction and stabilization of membrane curvature, while the SH3 domain interacts with proteins that contain proline-rich (PXXP) motifs. Oligomerization of endophilin A has been characterized in cells and shown to be a very tight dimer by fluorescence fluctuation spectroscopy. Here, we examine the oligomerization/multimerization of endophilin B. To our surprise, endophilin B displays much higher multimerization than endophilin A. We demonstrate that the multimerization of endophilin B is primarily controlled by its BAR domain and by its association with vesicular membranes. The endophilin BAR domain alone constitutively associates with vesicles, whereas vesicle binding of full-length endophilin B is very sensitive to dynamin 2 activity, as it is strongly inhibited by expression of a dominant negative form of the enzyme, dynamin 2-K44A. Furthermore, we directly detect that endophilin B associates with caveolin1- but not clathrin-containing vesicles using dual-color heterospecies partition analysis. This analysis allows us to unequivocally demonstrate that endophilin B engages in caveolin-dependent but clathrin-independent endocytic pathways. This work is supported by the National Institutes of Health (R01 GM64589), and the National Science Foundation (PHY-0346782).

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Characterization of Foodborne Escherichia coli Profiles.

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The cellular fatty acid profiles of eight strains of Bacillus, Staphylococcus, and Enterobacteriaceae (Escherichia coli, and Salmonella) were analyzed by comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GCxGC/TOF MS). A novel template method was developed to standardize the raw GCxGC retention data through the use of a chemical indexing mixture. Analyte retention coordinates were normalized in the primary dimension with respect to a series of n-alkanes (Kovats index) and in the secondary dimension with respect to a series of aromatic hydrocarbons (Lee index). Fatty acid profiles extracted from the templates were compared by multidimensional scaling (MDS) and principal component analysis (PCA). Differences in the profiles of Gram positive and Gram negative bacteria were observed, and a series of heterogeneous mixtures comprised of different fractions (containing one Gram positive and one Gram negative bacteria strains) were also distinguished from their homogeneous constituents.

923B1481

In situ direct live single-cell exhaustive molecular analysis by video-mass spectrometry.

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Direct and quick exhaustive molecular detection and its site or stage specific molecular picking have been succeeded for a live single cell which is under observation of a video-microscope.

Method: We called this method as "Live single-cell video mass spectrometry" in which a live cell was directly sucked its contents by a nano-spray tip under the scope and the content was introduced into mass spec. directly after adding a ionization solven to the tip. It takes less than 10 minutes. Mammalian cell lines of RBL-2H3 and Hep G2 were used for single organelle level molecular tracing and for single cell drug metabolism, respectively. For plant cells, radish sprouts was used for detecting light responding molecular extraction.

Results: We have succeeded to detect hundreds to thousand molecular peaks from a single cell and site specific or stage specific molecular peaks have been extracted by t-test or principal component analysis. In a granule of an RBL-2H3, not only Histamine but also many granule specific molecules were found. Using stable isotope labeled Histidine, the kinetic aspect of this biosynthesis or metabolism in a granule and cytosol in a single cell were monitored directly to check the dynamic metabolic pathways. Drug metabolism can be monitored only using a single Hep G2 cells and found the drug metabolites can only found in cytosol. Incorporations of the drug molecules into a vesicle or nucleus were also monitored directly within 10 minutes.

Conclusion: Since this method is so direct, speedy and versatile, it seems to open new era of bioanalysis for cell biology.

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Making cell arrays out of suspended cells by open channel nanoprinting.

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When working with cell suspensions it is frequently required that individual cells are observed under a microscope in standard conditions. This permits making meaningful comparisons of cellular morphology and intensity of labeling. For that, the cells are often centrifuged onto the slides from suspensions. However this tends to produce poor images, can damage the cells, and affects their morphology. These drawbacks are more pronounced in cell suspensions composed of several cell types, such as those obtained in blood tests.

To overcome these limitations, we have developed a new approach which permits making orderly and uniform cell arrays out of suspended cells in a matter of seconds. The approach is an application of nanotechnology to cell biology. It does not require centrifugation. Instead, it uses microscope slides patterned by the open channel Nano eNabler system, so that they can trap cells directly from cell suspensions. The approach creates arrays of cells firmly positioned on a microscope slide. The cells are undamaged and are presented in a uniform, orderly fashion required for their quantitative evaluations.

The approach is useful in comparing effects of various interventions and has general applicability in cell biology and in cell imaging.

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Applications of the microfluidic cell culture system with on-chip CO₂ incubation for cell biology.

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We have developed a microfluidic cell culture system in which we can cultivate and monitor several mammalian cells continuously for a long-term period. Our culture system does not require conventional huge cell culture equipment such as CO₂ incubator, carbon dioxide bottle and gas-regulators. The system is a microscope-slide-sized chip which is capable to diffuse CO₂ through a gas-permeable poly (dimethylsiloxane) (PDMS) wall of nested medium reservoir which is surrounded a tiny water-jacket reservoir containing sodium bicarbonate and sodium carbonate buffer. This developed CO₂ incubation system, consequently, could maintain physiological conditions of cell culture medium without any external gas chamber or gas supply. The entire microfluidic cell culture system has been set up to pocket sized equipment with a temperature and pumping control device [1]. The proposed chip consists of a PDMS-microfluidic chip and flip-mounted on a microscope slide that contains a nested pair of cell culture medium reservoir and water-jacket insulated by a gas-permeable PDMS wall. By using 0.8 M sodium bicarbonate with 65mM sodium carbonate as the water-jacket and placing on a 37°C surface, the chip maintained osmolality shift and the partial CO₂ pressure (pCO₂) in the medium reservoir stabilized at least few weeks. Since microfluidics is effective approach to manipulate small amount of fluid, it could minimize the consumption of required cells, reagents, amount of cell culture medium. Therefore, microfluidics provides many advantages for the experimental approach of cell biology, especially single cell analysis, such as patch clamp analysis, axon guidance and signal patch on the cell surface. On the other hand, our long-term cell culture systems available for cell-based biosensor. In fact, we integrate the microfluidic cell culture system that has an inline culture well with a shear horizontal surface acoustic wave (SH-SAW) as for cell-based biosensor could be used to assay cell-cell and/or cell-surface interaction. In addition to the biosensor, we are trying to improve the cell culture system to promote the differentiation of embryonic stem cells (ES cells).

[1] A. Takano, M. Tanaka, and N. Futai, On-chip CO₂ incubation for pocket-sized microfluidic cell culture, *Microfluidics and Nanofluidics*, **12**, 907-915 (2012)

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Non-invasive/destructive single cell purification method for re-cultivation of functionally identified specific cells using spot digestion of double alginate sol layers on a multi-electrode array chip.

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1. Objective

We have studied the meaning of community effect of cell networks using re-constructive approach from single cells such as creating artificial cell networks using individual cells set on a micropatterned biochip as a minimum functional unit. Hence, we have developed several techniques for studying above cell-to-cell communications, such as a non-destructive cell sorting method using digestive DNA aptamer equipped with an imaging flow cell sorting system, and a constructive cell network patterning method using stepwise spot heating with focused infrared laser on a thin agarose layer set on a multi-electrode action potential recording system for creating desired microchamber patterns even during cultivation. Adding to the developed

non-invasive/destructive purification of target cells using DNA aptamer, which identified target cells using the expressed surface proteins, we need to identify and purify the target cells from the viewpoint of their functions. To overcome this problem, a non-destructive method of collecting cultured cells after identifying their in situ functional characteristics has been developed using thin alginate sheet on a culture dish and multi-electrode array chip.

2. Methods

Primary hippocampal neurons, or beating human embryonic stem (hES) cell-derived cardiomyocytes are cultivated on an alginate layer in a culture dish or multi-electrode array dish, following released cells at the single-cell level by spot application of a calcium chelate buffer that locally melts the alginate layer.

3. Results

Primary hippocampal neurons, and beating human embryonic stem (hES) cell-derived cardiomyocytes cultivated on an alginate layer were first measured their field potential waveforms, and the target cells having particular field potential profile were successfully released from the substrate from single cell level, and collected using a micropipette. Then the collected target cells were re-cultivated on the other biochip from single cell level successfully maintaining their physiological function and their spatial patterns such as neurite patterns.

4. Discussion

We have developed a new approach of the non-invasive/destructive functional cell identification and purification method. This method can be expanded for further applications for phenotypic identification and re-cultivation combining with imaging technologies such as cell network pattern, and cell cycle state, which are not possible with commercialized fluorescence-activated cell sorting (FACS) methods.

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Microfluidic deformability cytometry is a high-throughput screening tool for stem cell pluripotency.

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Introduction: Due to high risks of genetic and phenotypic instability associated with cell culture, quality control strategies to ensure proper and consistent human embryonic stem cells (hESC) functionality for potential use in regenerative medicine are vitally important. The expression of certain markers usually assessed by flow cytometry and the ability to form embryoid bodies and teratomas in mice are two main categories of assays used for quality control. In addition to variability of these tests between different labs and their limited robustness, these assays are highly costly and labor-intensive. Recent studies using atomic force microscopy (AFM) and micropipette aspiration have shown that cell mechanical properties could be a promising biomarker of pluripotency. However, AFM and other recent techniques are manual approaches severely limited in throughput and are not suitable for cell screening. Here we introduce microfluidic deformability cytometry as a screening tool for stem cell pluripotency that addresses the need for high throughput.

Methods: The microfluidic device was designed to apply uniform stretching forces to cells using an extensional flow. The positioned cells arrive one at a time at the extensional flow while they are continuously imaged using high-speed microscopic imaging and cell size and deformability are extracted by image analysis. Individual cells are plotted based on these parameters as points on color density plots.

Results: The deformability of three hESC cell lines was analyzed before and after induction to differentiation for up to 12 days. The density plots captured from the deformability cytometer

show that while the pluripotent cells exhibit a deformable, small diameter profile, they switch to a larger, less deformable state as they lose their pluripotency. We assessed pluripotency of live cells using conventional pluripotency markers, which consistently show down regulation of these markers following differentiation as expected. The high correlation between our mechanical measure and pluripotency markers (≥ 0.9 , for the expression of Oct4 and Nanog) indicates the potential of deformability cytometry as a label-free assay of pluripotency.

Conclusions: The high information content (multiparameter mechanophenotype), low cost (elimination of antibody use with reduced sample preparation), and high throughput (> 1000 cells/sec) of deformability cytometry technology provides further evidence for the feasibility of using deformability as a biomarker for routine screening of ESC quality and differentiation for research and clinical use.

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Light-induced inhibition of Src via LOV domain insertion into a conserved portion of the catalytic domain.

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Src is a non-receptor tyrosine kinase that couples with cellular receptors to participate in a diverse spectrum of signaling pathways, and has been implicated in numerous types of cancer. Src activation leads to increased invasiveness, increased motility and decreased cell adhesion in tumor cells. The interactions of Src with upstream activators and downstream targets is dependent on the subcellular localization and kinetics of activation, necessitating the use of live cell techniques to understand Src signaling. We demonstrate here a new genetically encoded approach, potentially broadly applicable to kinases, for light-induced inhibition of Src. The light oxygen voltage (LOV) domain of *Avena Sativa* phototropin undergoes light-induced conformational changes upon irradiation at wavelengths between 400 and 500 nm. We have identified a site in Src, highly conserved in kinases, where insertion of the LOV domain minimally perturbs Src in the dark, but inhibits catalytic activity upon irradiation. Molecular dynamics studies indicate a mechanism in which the unwinding of the LOV α helix results in narrowing of the ATP binding site. The activation loop and beta-sheet move closer to each other, emulating the structure of the inactive state. Inhibiting Src activity through irradiation decreases migration rates in wound healing assays and alters protrusion behaviors. Cells transformed by overexpression of LOV-Src revert to a normal phenotype upon irradiation. Structural studies and insertion of other engineered folds into the Src site we have identified indicates that the same strategy can be applied to other kinases.

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Precise control of signal transduction in living cells by light.

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With a limited set of signaling pathways, cells exploit time control in order to ensure proper conversion of specific environmental stimuli into distinct cellular output. However, there are very limited means to control the temporal dimension of intracellular pathways with high accuracy. Here, we report a light-gated protein-protein interaction system that precisely regulates the activation and inactivation of the mitogen-activated protein kinase (MAPK) signaling pathway. We show that sustained MAPK activation through continuous light stimulation is sufficient to

induce significant neurite outgrowth in PC12 cells in the absence of nerve growth factor. Light-gated activation leads to an interesting discovery that MAPK alone is sufficient to account for neurite elongation, it only partially contributes to the full development of sodium channels in PC12 cells. The strategy of using light-gated protein interaction shows a great promise in dissecting detailed mechanisms of signal transduction in cells.

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Real-time light-driven temporal control of gene expression and protein concentration in *S. cerevisiae*.

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Studies of signaling and transcriptional networks are limited by the tools currently available to systematically perturb and interrogate such networks. Standard genetic tools such as deletion, overexpression, and mutation are effective for identifying network components but less suitable for understanding kinetics and identifying feedback interactions. Here we describe the development of a system for real-time light-inducible control of gene expression in *S. cerevisiae*. Controlling transcription enables fine-tuned temporal modulation of protein levels useful for interrogating genetic networks.

Light-induction is achieved through fusion of the *Arabidopsis thaliana* proteins cryptochrome 2 (CRY2) and its interaction partner (CIB1) to appropriate DNA-binding and activation domains (1). CRY2 and CIB1 naturally dimerize on blue-light exposure, bringing the DNA-binding domain and the activation domain together to drive expression of the desired gene. We characterize the kinetics, off-target effects, and dose-response of this induction system and develop improved fusion proteins to allow for specific and controllable induction.

To implement real-time control we developed an integrated robotics system. Cells from a steady-state chemostat culture are continuously imaged for fluorescence. Information from this imaging is used to adjust the inducing light levels to vary the concentration of the fluorescently-tagged protein. By controlling the light-intensity, pulse frequency, and pulse duration we are able to maintain a steady-state protein concentration of our choosing. By modulating the inducing light we can produce dynamic protein level perturbations such as pulses and oscillations. This tool allows us to flexibly perturb protein levels to interrogate the kinetics and structure of genetic networks.

(1) Kennedy, MJ et al 2010 Nature Methods 7: 973-975

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Selective Differentiation of Human Neural Stem Cells via Spatial Exposure Control.

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Modeling *in vitro* neuronal culture system has been a challenging task because the model requires multiple cell types working together in order to approximate physiologic microenvironment. Co-culture has provided a method to look into various cell-cell interactions; however, the inability to precisely control the distribution of different cell types as well as the difficulty in spatially controlling the cellular placement significantly reduce the robustness and repeatability of co-culture experiments. Here we developed a method to create spatially distinctive populations of the neuronal cells, in this case, neurons and astrocytes, for studying neuronal cell-cell communications *in vitro*. Using a commercially available microfluidic platform, two extracellular matrices were selectively patterned on the top and bottom half of a circular

microchamber (3mm in diameter and 0.1mm in height) at the same time using the hydrodynamic focusing effect at micro-scale. We also used fluorescent green dye FITC and red dye Texas red with similar viscosity to validate and optimize the dual ECM coating profiles in the chamber. H9-derived human neural stem cells were then seeded into the chamber. After cells stabilized in the microchamber for 24 hours, the same hydrodynamic principle was applied again to allow two differentiation reagents to continuously perfuse through the top and the bottom half of the chamber, respectively.

Before the differentiation reagents were introduced, the cells were immunostained for the pluripotency markers Nestin and Sox2, as well as the astrocyte marker GFAP and neuron marker MAP2. After the differentiation reagents were applied to the neural stem cells, we continuously monitored the cellular morphology at the top and the bottom part of the microchamber for two weeks. At the end, the cells were again stained with the same set of the neural markers. Using Cell Profiler software for image analysis, we were able to obtain information on both the proliferation and the differentiation of the two spatially distinctive populations. In this study, we have created a novel system to distinctly differentiate the human hippocampal stem cells into two different types of neural cells in one culture chamber. This novel method has the potential to create co-culture methods for the multiple neural cell types through selective ECM patterning as well as spatially controlled differentiation reagent introduction.

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Rapid Feeder-Free Xeno-Free iPSC Generation using mRNA.

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“Footprint-free” methods for reprogramming somatic cells to pluripotency are essential if iPSC technology is to advance to the clinic. Currently, protocols based on non-integrating episomal DNA, Sendai virus, and messenger RNA vectors can meet the footprint-free requirement while still producing iPSCs with practical levels of efficiency. Of these methods, only the mRNA approach obviates screening and cleanup of derived iPSCs to eliminate risks associated with residual traces of the reprogramming vector. The main drawback to the mRNA method is the increased hands-on time needed to perform reprogramming, which arises from the need to resupply the short-lived mRNA vector every day for ~2 weeks in order to fully activate endogenous pluripotency circuitry. We sought to address this problem by increasing the potency of the reprogramming cocktail, and were able to show that use of a six-factor cocktail incorporating M3O – an engineered variant of Oct4 – along with Sox2, Klf4, c-Myc, Lin28 and Nanog significantly accelerates reprogramming relative to the established OSKM+Lin28 mRNA regimen. We leveraged these kinetic gains to define a xeno-free, feeder-free protocol for rapid reprogramming of human fibroblasts to pluripotency. The new protocol achieves efficient reprogramming with 9 days of transfection, and the transfection process itself has been streamlined so that RNA/vehicle cocktails can be stored for days and applied by simple supplementation of culture media prior to routine media changes. This novel methodology is readily applicable to high-throughput iPSC derivation and circumvents the inconvenience and uncertainty attending the main alternative approaches to footprint-free reprogramming.

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Combinatorial Application of Multiple MicroRNAs to Induce Non-Integrating Cell Fate Conversion on Microfluidic Chips.

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Since *in vivo* cell fate determination and cell-cell signaling involve multiple genes, extracellular factors, and intracellular signaling pathways, finding the right combinations for *in vitro* cell culture conditions is invaluable for studying fundamental cell biology questions and for cell-based therapies. However, using traditional cell culture systems, multi-factorial experiments are often laborious and difficult to reproduce. Microfluidic technologies allow for precise control of microenvironment of cells, facilitate studies of multi-factorial combinations, and enable development of robust, reproducible and chemically defined cell culture systems. We have designed and fabricated a prototype microfluidic chip and an automated instrument that can culture cells on chip for extended period of time and deliver multiple combinations of different factors to cells. Each chip includes 32 cell culture microchambers and 16 reagent inlets. Reagents can be automatically multiplexed to desired combinations and ratios at various pre-defined time points. Cells can also be harvested from the chip for continued off-chip culturing, single-cell genomic analysis, and/or functional assays. Culturing human BJ fibroblasts on chip, we have developed a safe non-integrating method for direct reprogramming of fibroblasts to neurons by dosing cells with combinations of synthetic microRNA mimics. The identities of cells were confirmed with immunostaining and gene expression profiling. This non-integrating approach of miRNA administration generated neurons with high efficiency and cell viability. The results were consistent with published reports and were confirmed in large well-dish format. In summary, the prototype automated microfluidic system reported here provides the potential to study and screen the precise combinatorial effects of multiple factors on cell culture maintenance, reprogramming and differentiation, hence could be a valuable tool for the cell biology research community.

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Transdifferentiation of Cells into Brown Adipose Tissue.

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Objective. Recently, investigators have focused on brown fat as a tool to combat obesity, which according to the Centers for Disease Control, more than one-third of Americans are obese and projections speculate 42% of Americans will be considered obese by the year 2030. The goal of this study is to transdifferentiate cells into a brown adipose tissue (BAT) phenotype by co-expression of heparin-binding EGF-like growth factor (HB-EGF) and a disintegrin and metalloprotease (ADAM) 12S. **Methods.** HB-EGF and ADAM 12S cDNAs were cloned, sequenced, and stably expressed in mammalian cells including mouse fibroblasts and human epidermoid carcinoma cells (A431) resulting in formation of oil droplets as evidenced by positive Oil Red O staining. In order determine whether the Oil Red O positive cells are BAT or white adipose tissue (WAT), we employed a quantitative real-time RT-PCR approach for known genes involved in the WAT pathway including peroxisome proliferator-activated receptor gamma (PPAR γ), CCAAT/enhancer binding protein alpha (C/EBP α), and serine-threonine protein kinase (AKT-1) as well as genes involved in the BAT pathway including PR domain 16 (PRDM16) transcriptional coregulator, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α) and uncoupling protein 1 (UCP-1). No significant differences in PPAR γ , C/EBP α , or AKT-1 mRNA expression levels were present in all stable cell lines

examined, but were up-regulated in WAT 13-, 400- and 3.5-fold, respectively. PRDM16 mRNA levels were significantly up-regulated 12-fold in HB-EGF/ADAM 12S co-expressing cells and 138-fold in BAT compared to mouse fibroblasts, mock-transfected, HB-EGF, and ADAM 12S cell lines. PGC-1 α mRNA was significantly up-regulated 38-fold in ADAM 12S/HB-EGF co-expressing cells and 100-fold in BAT compared to other cell lines. Finally, UCP-1 mRNA was significantly up-regulated 5-fold and 7,322-fold in ADAM 12S/ HB-EGF co-expressing cells and BAT, respectively. In order to support these results, human adipogenesis PCR arrays were performed using RNA from A431 cells stably transfected with ADAM 12S and HB-EGF and resulted in increased expression of PGC-1 α (30-fold), Kruppel like factor 3 (Klf3) (5-fold), Klf4 (2-fold), and fibroblast growth factor (FGF-2) (4-fold) compared to mock transfected A431 cells. Further supportive of BAT transdifferentiation was down-regulation of the WAT marker genes C\EBP α , Lamin A/C (LMNA), and solute carrier family 2 (GLUT4) (-5, -2, and -2.7 -fold, respectively). In order to demonstrate BAT transdifferentiation in vivo, recombinant adenovirus vectors directing the expression of ADAM 12S and HB-EGF were engineered and used to co-infect A431 cells resulting in positive Oil Red O staining and upregulation of PGC-1 α (15.6-fold) and FGF-2 (4-fold) and down-regulation of C\EBP α and LMNA (-10.5 and -7.6 -fold respectively). **Conclusions.** Co-expression of ADAM 12S and HB-EGF stimulate adipogenesis utilizing the canonical BAT pathway while suppressing genes involved in the WAT pathway. The adenoviral vectors directing ADAM 12S and HB-EGF will be useful tools to demonstrate BAT synthesis in vivo.

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Induced pluripotent cells derived from peripheral lymphocytes can be differentiated into functional neural cells.

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Human induced pluripotent stem cells (hiPSCs) derived from somatic cells of patients, are recognized as promising source for regenerative medicine and useful tool for disease modeling. Currently, most of hiPS clones are generated from dermal skin fibroblasts by inducing reprogramming factors. However, there are undeniable invasive risks of bleeding, infection, and scarring in skin biopsies. Although T-cell derived iPSCs (TiPS) (Seki et al., Cell Stem Cell, 2010) are perhaps the ideal cell source for patient-specific iPSCs due to the ease of obtaining patient samples, recent studies have indicated that iPSCs retain an epigenetic memory relating to their cell of origin that restricts their differentiation potential.

To investigate whether TiPS have ability to generate functional neural cells as well as fibroblast-derived iPSCs and can be used for the study of neural diseases, we established both TiPS and fibroblast-derived iPSCs from the same patient by various method including Sendai virus (SeV), retrovirus and episomal vectors. Although microarray and principal component analysis revealed that global gene expression of TiPS was significantly distinct from that of fibroblast-derived iPSCs from the same donor, all TiPS clones were able to differentiate three germ layers as well as fibroblast-derived iPSCs. While the efficiency of differentiation into neural lineage is significantly lower in TiPS, neural cells induced from TiPS were functionally similar to that from fibroblast-derived iPSCs. Therefore, we concluded that TiPS can be used as tools to study neural diseases.

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Profiles of genomic instability in high-carcinogenicity genetic disease cell lines collection by high-resolution array-based comparative genomic hybridization (aCGH).

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Introduction: Genomic instability has been proposed to play an important role in cancer by accelerating the accumulation of genetic changes responsible for cancer cell evolution. Genomic aberrations in the form of subchromosomal DNA copy number changes including c-myc amplification are a hallmark of cell line. The goal of the present study was to analyze such aberrations in high-carcinogenicity genetic disease cell lines for scientific research at high resolution.

Methods: The patient origin cell lines of xeroderma pigmentosum (XP) and fanconi anemia (FA) was used for analysis. We employed high-resolution array comparative genomic hybridization with Affymetrix GeneChip SNP6.0 to analyze genomic aberrations in genetic disease cell lines. And also numerical chromosomal aberrations and rearrangements were analyzed by multicolor fluorescence in situ hybridization (mFISH) and G-banding.

Results: The detailed chromosome analysis result of XP group and FA group became clear by aCGH. The chromosomal region which changes in common was found out by comparing XP cell lines with FA cell lines mutually. The partial loss of chromosome 12 and 17 were non-random and was detected by analysis of microsatellites and single-nucleotide polymorphism (SNP)-based loss of heterozygosity (LOH). Also, DNA copy number aberrations identified in XP and FA cell lines. The relation of these aberration and clinical information is just going to be interesting very much.

Conclusion: High resolution CGH analysis of high-carcinogenicity genetic disease cell lines reveals powerful tool for detection of chromosomal aberrations and gene amplification. In the quality control and characterization of cell lines, the chromosome aberration is an important problem, and CGH might be useful a convenient and efficient screening method. Then, precise gene amplification analysis is important to elucidate a mechanism for oncogene amplifications for better understanding of tumor initiation or progression.

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Multiplexed High Content Hepatotoxicity Assays using Induced Pluripotent Stem Cell Derived Hepatocytes.

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Hepatotoxicity is one of the main safety concerns in drug development. Highly predictive in vitro assays suitable for safety and efficacy testing are extremely important for improving the drug development process and reducing drug attrition. Accordingly, there is great interest in using stem cells as tools for screening compounds during early drug development. Human hepatocytes derived from stem cells can greatly accelerate the development of new chemical entities and improve drug safety by offering more clinically relevant cell-based models than those presently available. Such cells can express appropriate hepatocyte markers and demonstrate intrinsic hepatocyte functions similar to primary cells. We demonstrate several models for assessing general and specific hepatotoxicity that are well-suited for automated assays. First, multi-parametric image analysis using an automated microscope is used to monitor changes in cell viability (Calcein AM), nuclear shape (Hoechst), and mitochondria potential (Mitotracker Orange) associated with different types of toxicity. A number of known hepatotoxic compounds were evaluated using these assays and IC50 values determined. Other assay models targeting specific mechanisms of hepatotoxicity will also be presented.

Mitochondrial depolarization is an early signal for hypoxic damage or oxidative stress. Mitochondria membrane potential was monitored with the mitochondria active dye JC-10 on the ImageXpress Micro system. This assay can be used either as an end-point or live-cell real time assay. We evaluated the effect of apigenin, CCCP, and several other compounds and determined IC50 values with this assay. Phospholipidosis is a lysosomal storage disorder characterized by the excess accumulation of phospholipids in tissues. The increase in undigested materials results in the abnormal accumulation of multi-lamellar bodies (myeloid bodies) in tissues. Phospholipidolysis was detected using LipidTOX reagent with an automated microscope system. A dramatic increase in phospholipid levels was observed in response to treatment with Amiodarone, Cyclosporin A, Propranolol, and Chloroquine and IC50 values were determined. We have also monitored modulation of the autophagy pathway in hepatocytes by high content imaging using Cyto-ID dye. In disease, autophagy may function as a survival mechanism by removing damaged organelles and toxic metabolites to maintain viability during periods of stress. We have characterized several compounds that affect autophagy in hepatocytes, including chloroquine verapamil, and rapamycin. The results demonstrate that method has potentially significant value for compound screening and early safety evaluation in drug development process.

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Predictive Assays for High Throughput Assessment of Cardiac Toxicity and Drug Safety.*O. Sirenko¹, C. Crittenden¹, E. Cromwell¹; ¹Molecular Devices, Sunnyvale, CA*

Assessing cardiotoxicity is important in the early stages of drug discovery to enable elimination of potentially toxic compounds from further development. There is a need for cardiotoxicity assays that use more biologically relevant cell-based models to aid development of new chemical entities and ensure drug safety. Accordingly, there is great interest in using stem cells as tools for screening compounds during early drug development. Stem cell derived cardiomyocytes are especially attractive for this because they express ion channels, and demonstrate spontaneous mechanical and electrical activity similar to native cardiac cells. We have developed methods for measuring the impact of pharmacological compounds on the beating rate of stem cell derived cardiomyocytes using imaging methods and FLIPR® Tetra cellular analysis system. Cardiomyocyte contractions rate and patterns characterized by monitoring changes in intracellular Ca²⁺ measured using calcium sensitive dyes. Automated peak detection and analysis processes the data concurrently with acquisition and provides user selectable outputs. The assay allows characterization of deviations from normal contractions that include changes in beating rate and other characteristics such as spacing between peaks, peak width or pattern irregularities. Multi-parameter characterization of beating profiles provides additional insights into mechanisms of action indicating distinct characteristics of different types of receptor antagonists or ion channel blockers. We have characterized more than 100 known pharmacological compounds and detected concentration-dependent modulation of beating rate as well as atypical patterns caused by alpha and beta blockers, hERG inhibitors, ion channel blockers, kinase inhibitors, anti-cancer drugs, and other cardiotoxic compounds. Identifying these parameters early in the drug discovery process has the potential to identify a compound that should be failed as a pre-clinical candidate. For example, this assay may allow prediction of adverse cardiac effects such as elongated QT syndrome. We demonstrate that the IC50 values determined by FLIPR cardiac beating assay had significant rank correlation with published values determined by other cardiac models and also good concordance with reported human plasma C_{max} values. Finally, we have estimated assay predictivity by testing the Screen-Well™ Cardiotoxicity library that consists of 131 compound representing different classes of cardiotoxicity. We found that estimated predictive value of the assay was greater than

80%, and therefore method could be very useful for compound screening and early safety evaluation in drug development process.

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Image-Based Functional Assay to Screen Therapies for Inherited Heart Disease.

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Many single cell drug-screening technologies test changes in electrochemical properties of contractile heart cells (cardiomyocytes - CMs). However, as researchers and clinicians alike turn to stem cell-derived CMs for applications in pharmacology and tissue engineering, there is a need for rapid, easy-to-use mechanical functional assays of immature cells such as neonatal and stem cell-derived CMs. This paper reports a new image-based minimally invasive functional assay that quickly and rapidly quantifies cell contractility and detects changes in CM mechanical function in standard culture conditions.

While commercial image-based functional assays (IFAs) exist for adult CMs, their algorithms rely on mature sarcomeric structures and are thus unsuitable for immature cells like neonatal and stem cell-derived CMs. Researchers have created IFAs to study neonatal cardiomyocytes, but these algorithms only detect contraction frequency or effective mechanical contractility, not both. Our IFA routine utilizes three unique components to enable simultaneous frequency and mechanical contractility analysis: soft hydrogel substrates for cell culture that enable large cellular displacements, edge-finding algorithm for each frame of high-speed brightfield videos that facilitates velocity and contractility calculations, and Fourier transforms of the raw data to identify a characteristic frequency.

The poly(acrylamide-co-acrylic acid) hydrogels were functionalized with SulfoSANPAH followed by laminin. Neonatal cells were harvested from mouse pups and plated on gels the same day. Brightfield videos of single cells were captured six days later, before and after treatment with 10 μ M isoprenaline. Videos were then cropped and converted to AVI files using ImageJ. AVI files were read into MATLAB, and for a given video, each frame was processed to trace the outer border of the cell and the major axis recorded. Contraction and relaxation velocities were also calculated for the cell. Calculations of change in length, scaled by original length, yield an average contractility. Contractility has physiological correlation to the ability of the heart to pump blood. Discrete Fourier transforms of the major axis data identifies the dominant spontaneous beat frequency.

Using this method, statistically significant increases in contractility ($p = 0.030$) and frequency ($p = 0.002$) are detected in the presence of isoprenaline. Other techniques have not been able to generate this combined data with such robust statistical significance. We are currently using our IFA to identify changes in contractility due to inherited mutations in contractile proteins like Myosin Regulatory Light Chain (MYL2-N47K) and Myosin Heavy Chain (MYH7-R403Q) that cause hypertrophic cardiomyopathy.

Our mathematical and experimental approaches showed that our image-based functional technology was able to capture the fastest kinetic properties of the cells. The outcome of these studies shows a reliable new technology that can be used for single cell functional studies and drug screening for cardiovascular diseases.

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Cord blood platelet gel applications in thoracic surgery.

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Objective: Prolonged air leak is the major cause of morbidity after pulmonary resection. In this study we tested an innovative approach based on the use of human umbilical cord blood platelet gel (CBPG) in repairing pleural damage through in vitro and in vivo experimental approaches.

Methods: In vitro, scratch assay was performed to test the tissue repair capability mediated by CBPG compared to the standard culture conditions using human primary mesothelial cells.

In vivo, a iatrogenic injury on the left lung of 54 Wistar rats was performed. CBPG was placed on the injured area only in treated animals and at different times histological changes and presence of pleural adhesions were evaluated. In addition, changes in the soluble inflammatory factor pattern were tested using a multiplex proteome array.

Results: In vitro, CBPG repaired the damage of mesothelial cells in a shorter time in comparison with the control (24 vs 35 hours, respectively). In vivo, formation of new mesothelial tissue and complete tissue recovery was observed at 45+1 and 75+1 hours in treated animals vs 130+2.5 and 160+6 hours in controls, respectively. Pleural adhesions were evident in 43% of treated animals compared to 17% of controls. No complication was observed.

Interestingly, some crucial soluble factors involved in inflammation were significantly reduced in treated animals.

Conclusions: CBPG accelerates the repair of pleural damage and stimulates the development of pleural adhesions. Both properties could be particularly useful in the management of prolonged air leak, and to reduce inflammation.

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Analysis of Neurotransmitters and Behavioral Responses in 835 MHz Radiofrequency Exposed Mice.

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Neurotransmitters (NTs) are signaling molecules, which play critical roles in neuronal communication of nervous system. The changes of NTs concentration in several brain regions have been known to involve many psychiatric diseases developments and neurodegenerative diseases progresses. Our lab has developed a simple and rapid liquid chromatography tandem mass spectrometry (LC-MS/MS) method for analyzing the level of major NTs in brain regions. By using this newly developed method, we measured various NTs of mouse brain regions following chronic exposure to 835 MHz radiofrequency radiation (RFR). We investigated the effect of chronic RF exposure on mice at 835 MHz with high energy (specific absorption rate: SAR=4.0 W/kg) for 4 weeks (10 hrs/day) by using determining NTs. We found that the levels of dopamine at striatum and midbrain were continuously decreased in parallel with the duration of radiofrequency exposures. The levels of serotonin and epinephrine in brain following radiofrequency exposure did not change at all brain regions in mice. Norepinephrine levels significantly decreased at brain stem region but increased at pituitary glands (PT) region in mice. Glutamate levels significantly decreased at PT and cerebellum (CB) regions and GABA levels increased at CB region in mice. The moving distances of RFR and rearing frequencies in

RFR exposed mice were significantly increased at the 2 or 3 weeks after exposure. These results suggest that RFR-exposed mice show the hyperactivities. The underlying mechanisms affecting RFR exposed mice behavior are under investigation. In summary, newly developed analytical method for NTs significantly improves assay performance characteristics and will be well-suited for many relevant studies to quantify the concentration of various NTs.

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Possible involvement of protein citrullination in the production of brain-reactive autoantibodies related to Alzheimer's disease pathology.

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Brain-reactive autoantibodies (BAs) in human sera, which are immunoreactive to antigens in the brain, may contribute to AD pathology. Some BAs have shown promise as biomarkers that can be used to diagnose AD. However, mechanisms involved in their generation are unknown. Here, we used immunohistochemistry and immunoblotting to investigate whether protein citrullination (PC) plays a role in this process. PC is a post-translational protein modification whereby arginine, a basic amino acid, is converted to citrulline, a neutral amino-acid. This is mediated by peptidyl arginine deiminases (PADs). Loss of basic charges can modify protein structure which can enhance antigenicity. PC leading to the generation of disease-specific autoantibodies is well established in rheumatoid arthritis. In the present study, we found selective expression of PAD4 and PAD2 in neurons and astrocytes, respectively, in human brain. In the cerebral cortex and hippocampus PAD4 was expressed selectively in pyramidal neurons. Although comparable levels of PAD4 expression were observed in AD and control brains, cortical expression of PAD2 was higher in AD brains compared to controls. PAD4 was localized to the neuronal perikaryon, but was also present in axons, dendrites and synapses. Similarly, PAD2 was localized in the cell body and processes of astrocytes. Localization of citrullinated proteins closely matched that of PADs in both cell types in AD and control brains. Co-localization of PAD4, citrullinated proteins and amyloid beta1-42 was consistently observed in pyramidal neurons, suggesting a link between PAD expression, protein citrullination and accumulation of amyloid during AD pathogenesis. Lastly, pentatricopeptide repeat domain 2, a potent diagnostic marker of AD, was citrullinated in human brain. We propose that protein citrullination has a vital function that remains unidentified in the brain. However, our results suggest that selective neuronal degeneration in AD brains releases citrullinated proteins into the blood that elicits an immune response and the appearance of BAs. Under conditions of blood-brain barrier compromise, extravasation of these BAs into brain could exacerbate AD pathology.

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Computational, Physical, and Synthetic Biology

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Single Molecule Study of Thrombospondin-1 Receptor, CD36, In The Endothelial Cell Plasma Membrane.

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Thrombospondin-1 (TSP-1), an endogenous extracellular matrix anti-angiogenic factor, is down-regulated in many tumors. CD36 is a key receptor transmitting the anti-angiogenic activity of TSP-1 through induction of endothelial cell apoptosis. The binding of multivalent ligand to CD36 (anti-CD36 immunoglobulin M (IgM)), similarly induces endothelial cells apoptosis, while divalent anti-CD36 immunoglobulin G (IgG) does not, suggesting that CD36 clustering is critical for this apoptotic signal. Our objectives are to examine the dynamic organization of CD36 into multimeric complexes upon TSP-1 binding, and determine the factors that regulate its reorganization and clustering upon TSP1 binding. To achieve this, we used single molecule and super-resolution imaging to assess the dynamics and spatial organization of CD36 in the plasma membrane of human dermal microvascular endothelial cells. We compared CD36 dynamics and organization in unstimulated and TSP-1 stimulated cells. Treatment with 10 nM TSP-1, which induced endothelial cell apoptosis, resulted in a significant increase in CD36 clustering and actin-guided organization and mobility. TSP-1 also induced phosphorylation of the Src family kinase Fyn in a CD36-dependent manner at focal adhesions along actin filaments. Based on our data, we propose that TSP-1 binding results in a change in CD36 dynamics, potentially mediated by actin, which facilitates CD36 clustering and subsequent downstream signaling.

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Dynamain torque and membrane elasticity sets location and duration of the endocytic fission reaction.

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The GTPase Dynamain polymerizes into a helical coat that constricts membrane necks of Clathrin-coated pits to promote their fission. However minimal requirements for fission are still debated as Dynamain constriction is necessary but not sufficient for fission. Here we show that fission occurs at the interface between the Dynamain coat and the uncoated membrane (Morlot et al., in press). At this location, the considerable change in membrane curvature increases

locally membrane elastic energy, reducing the energy barrier for fission. Fission kinetics depends on tension, bending rigidity and the Dynamin constriction torque. Indeed, we experimentally find that the time it takes for fission depends on membrane tension *in vitro* and during Clathrin-mediated endocytosis *in vivo*. By i) estimating the energy barrier from the increased elastic energy at the edge of the Dynamin coat, and ii) measuring the Dynamin torque, we show that the mechanical energy spent in Dynamin constriction can reduce sufficiently the energy barrier for fission to promote spontaneous fission.

These results allow us to quantitatively validate a membrane fission scenario in which Dynamin constricts fast (<few 100 ms), and waits a few seconds for spontaneous fission to occur.

Ref :

Morlot, S., Galli, V., Klein, M., Manzi, J., Humbert, F., Chiaruttini, N., Dinis, L., Lenz, M., Cappello, G., and Roux, A. (2012). Membrane shape of the edge of the dynamin helix sets location and duration of the fission reaction. *Cell*. in press

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Investigating the spatial correlation and dynamics of protein nanoclusters in live cells, using spatial statistics.

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Quantitative analysis of live fluorescence microscopy images is a key tool in the study of the intracellular localization and dynamics of proteins, and of the cellular components they are part of. However, significant advances in our understanding of cell biological processes can only be attained by linking that information with functional and/or biophysical models.

Here we present a mathematical framework based on spatial statistics to quantitatively assess punctate protein aggregate co-localisation and dynamics in cells, and relate them to biophysical models.

Punctate objects abound in cellular fluorescence microscopy (vesicles, protein clusters, exo- or endocytosis sites, etc) and the main question they raise concerns their spatio-temporal (co-)localisation, that is how given populations of points are distributed, either alone or with respect to one another, and how that changes with time. Co-localisation is often studied using a variety of ad-hoc techniques, and dynamics using Single Particle Tracking (SPT) methods. We use spatial statistics as a tool to tackle both problems in a unified way and ask: are the distributions of two proteins correlated in space? how do the spatial correlations between different pairs of proteins compare with one another? what biophysical or biological model(s) can best explain an observed correlation? In particular we are able, without particle tracking and using noisy data, to distinguish free diffusion from sub- or constrained diffusion and directed motion and compare experimentally observed spatio-temporal behaviour with the output of complex simulated systems.

Focusing on the protein machineries that regulate cell polarity in fission yeast (*S. pombe*) cells, we recently discovered that *S. pombe* polarity factors are not smoothly distributed at the cortex of cells but rather are organized in nanoscopic protein clusters [Dodgson, Chessel and al., under review]. We illustrate how using spatial statistics we have been able to shed light on the relative spatio-temporal distribution of polarity factor clusters in *S. pombe*, revealing the existence of several distinct but interacting (possibly cell-cycle regulated) polarity complexes as well as investigating their dynamics with the help of biophysical models.

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Multiscale Modeling of Lipid Droplet Formation.

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Lipid droplets (LD) are intracellular organelles containing neutral lipid esters enclosed in a lipid monolayer. They have long been thought of as passive globules for intracellular lipid storage. However, recent studies have shown that they are dynamic structures involved in fat metabolism and may have important roles in obesity, diabetes and atherosclerosis. Furthermore, viruses such as the hepatitis C virus are known to hijack LDs for their function. Despite the importance of LDs in many of these processes, very little is known about their structure, formation and regulation. Understanding the biogenesis of these organelles will provide new avenues in therapies for these diseases.

The canonical model of LD formation hypothesizes that neutral lipids accumulate between the leaflets of the endoplasmic reticulum membrane and subsequently bud off into vesicles. We are studying this problem using two different approaches—mesoscopic modeling and continuum mechanics. We use a coarse-grained lipid model, and hybrid Monte Carlo–dissipative particle dynamics simulations to study properties of individual lipids surrounding the droplet and the mechanism by which they facilitate budding of the LD. We model the contents of the LD as ‘super-hydrophobic’ particles secreted within the membrane.

Alongside the coarse-grained model, we are also developing continuum models of the membrane. We use lipid tilt as a key degree of freedom in our continuum model to capture not just the change in the shape of the membrane but also the change in the orientation of the lipids in response to the deformation caused by the LD. Our results show that the early stages of LD formation are ellipsoidal in shape. Furthermore, there is a change in the thickness of the lipid monolayer along the LD, indicating that the lipids in the membrane monolayer are adjusting their orientation to accommodate the LD. Ongoing studies are focused on understanding the different stages in the formation of the droplet and scission.

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Membrane curvature-induced sorting of transmembrane proteins.

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The targeting of transmembrane proteins to specific cellular regions is essential for proper cell function. It depends upon both enrichment in trafficking vesicles, and lateral redistribution upon delivery to the plasma membrane or organelle of interest. Extensive work has been done to identify specific protein/protein interactions, active processes governing protein targeting as well as the contribution of hydrophobic mismatch. However, membranes such as transport intermediates, the dendrites of neurons or primary cilia can be highly curved, with curvature radii of tens of nanometers. Thus, the matching of protein shape to the underlying membrane curvature may also provide a targeting mechanism that was not demonstrated yet.

We have used a quantitative in-vitro assay to demonstrate that membrane curvature can strongly affect the lateral distribution of transmembrane proteins. We consider two proteins: a voltage-gated potassium channel, KvAP, and Aquaporin AQP0. The redistribution of these two proteins between flat and highly curved membranes is strikingly different. Our model system consisted of purified, fluorescently-labeled, proteins reconstituted into Giant Unilamellar Vesicles (GUV) with a typical diameter of ~10 μm. Using micromanipulation techniques, we

pulled membrane nanotubes from GUVs and measured the protein concentration via quantitative confocal fluorescence microscopy while varying the nanotube radii from 100 nm to 7nm.

We observe strong curvature-induced sorting of KvAP; up to a 10-fold enrichment of KvAP is measured on the tube compared to that of the GUV. This sorting is measured as a function of tube radius and protein density on the GUV. In addition, FRAP measurements on the nanotube confirm that proteins can freely diffuse between the quasi-flat GUV membrane and the highly curved membrane tube. Thus, the membrane nanotube neck does not act as a diffusion barrier. In contrast, no curvature-induced sorting of AQP0 has been observed.

The markedly different sorting of these two transmembrane proteins can be understood in terms of a theoretical model of the protein-imposed spontaneous membrane curvature. Thus, these measurements of curvature-induced sorting provide supra-molecular scale information about the shape of membrane-embedded proteins, which is usually not accessible using crystallographic methods.

In conclusion, our work shows a new sorting mechanism based on the shape of transmembrane proteins. Consequently, cells can sort proteins based on matching of their shape and the local membrane curvature; otherwise, active means or interactions with other cell constituents are necessary to efficiently create and maintain protein spatial inhomogeneities.

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Bayesian imaging fluorescence correlation spectroscopy to resolve heterogeneity in cell membranes.

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Fluorescence Correlation Spectroscopy (FCS) is a powerful tool to measure molecular dynamics with single molecule sensitivity, including transport mechanisms, local concentrations, and aggregation states. Due to its high spatial resolution and non-invasive nature, FCS has been widely used to probe the dynamics of various cellular processes in live cells. In contrast to single point detection in traditional confocal FCS, advances in EM-CCD camera technology and Total Internal Reflection Fluorescence Microscopy (TIRFM) now enable parallel measurements at hundreds to thousands of locations on the cell surface. However, inferring models from FCS measurements on biological samples is challenging due to noise from sampling limitations, heterogeneity present in the sample, and the often transient nature of biological processes. In addition, imaging FCS measurements contain up to thousands of temporal autocorrelation functions (TACFs) in a single acquisition, rendering manual data analysis and model selection impractical. Thus, automated, unbiased analysis procedures are required for proper data analysis and interpretation. Here, we present and apply a systematic Bayesian approach to multiple hypothesis testing of competing models for FCS datasets of multiple TACF curves or single intensity traces. By testing the approach with simulations and TIRF data of supported lipid bilayers, we demonstrate that this Bayesian approach selects the simplest model that can describe the observed data, thereby capturing heterogeneity in the sample while avoiding overfitting. Further, model probabilities provide a reliability test for the downstream interpretation of measured parameter values. Application of our approach to Epidermal Growth Factor Receptor (EGFR) indicates that diffusion of EGFR on the cell surface is more heterogeneous than the membrane marker Dil and requires at least two species to describe the data. These results demonstrate that Bayesian analysis offers a quantitative and unbiased approach for FCS data analysis and has broad applications in resolving heterogeneous dynamics in various cellular processes.

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Understanding heterogeneity of cellular responses in tumors by computational and systems biology.

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Heterogeneity in the cellular make up and response to molecular signals and chemical challenges in micro and macro-domains of tumors has fundamental implications in understanding the dynamics of pathways and signaling, the development of predictive diagnostic and prognostic tests, and the optimal delivery of personalized therapeutics. The objective of this study is to relate heterogeneity in cellular activity, to variations in specific cellular pathways, using computational models. Our approach is based on large-scale cell biological analyses using multiplexed fluorescence with High Content Analysis (HCA). The present study pushes the limits of quantitative HCA by calibrating the "systems response" of the imaging platform using fluorescent dye solutions and bead standards for stability, sensitivity, dynamic range, and linearity, so that variations in the biological responses can be optimally interpreted. We have validated this approach by correlating the same measurements in both flow and image (HCA) cytometry. In addition, we are building a continuum of biological models including 2D arrays of relevant cancer cells, 3D arrays of tumor microenvironments and cohorts of patient tumor sections. We have demonstrated that the calibrated intensities of 2 μm fluorescent beads, measured by imaging, exhibit a coefficient of variation (CV) of ca. 4%, which is comparable to the best CV's obtained by flow cytometry of ca. 3%. We used the cell cycle distribution as a key comparative parameter in validating measurements of heterogeneity. When a head and neck cancer cell line (Cal33), labeled with a fluorescent DNA probe, was analyzed, we demonstrated that with the optimal systems calibration a CV of ca. 8% for the G0/G1 peak and a fraction of S-phase of ca. 28% were obtained. This result compares well to a CV of ca. 6% and a ca. 31% fraction for the S-phase in the same cell samples measured by flow cytometry. The initial model system is focused on the Cal33 cells plated over a range of densities and relating key components of STAT3, STAT1 and NF κ B pathways to the stage of cell cycle in normally cycling cells and in response to stimulation by IL-6, INF- α , TNF α and selected test compounds. Even in the simple 2D cell model, we have characterized significant heterogeneity of responses, for which we are developing network models to reveal finer details of sub-populations.

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Relating cellular heterogeneity to drug sensitivity.

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Cancer is a disease associated with a high degree of cellular heterogeneity. Here, we show that quantification of pre-existing heterogeneity in H460 lung cancer clones can be used to predict drug response. Cellular heterogeneity within clones was described by mixtures of similarity of signaling states (i.e. colocalization patterns of signaling molecules from microscopy fluorescence images). Clones with similar ensembles appeared to have similar drug sensitivities, and relative similarity among the clones was largely independent of signaling readout. Our work suggests that measures of cellular heterogeneity can contain useful information for the diagnosis and treatment of cancer.

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Modeling the Cell Biology of the Heat Shock Response of Barley Aleurone Cells.

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When heat shocked, the secretory cells of barley aleurone layers reallocate cellular resources by combining heat shock and ER stress responses. Cells of the barley aleurone layer secrete endosperm-degrading hydrolytic enzymes in response to the phytohormone gibberellic acid which is sent by the germinating embryo. The hydrolases, chief among them α -amylase, mobilize nutrients stored in the endosperm to support the growth of the embryo through the soil until it is capable of sustaining photosynthesis. Upon heat shock, secretory protein synthesis is suppressed by rapid and targeted destabilization of secretory protein mRNAs. The ER upon which these secretory protein mRNAs are translated into proteins undergoes major structural and compositional changes. In addition, signal recognition particles that ferry secretory protein-translating polysomes to translocons at the ER fail to release from the ER upon heat shock. We present a mathematical model to analyze how these elements interact to regulate α -amylase synthesis in heat-shocked aleurone cells. Our model was created using the mass-action principle to transform biological reactions into differential equations. We then fit the model with our experimental data which measure protein concentrations at three different temperature schemes (plunge [heat shock], slow and fast ramps [heat stresses]). The computational simulations show good qualitative agreement with the literature and experimental data. α -Amylase is most severely affected by the plunge temperature scheme but is not as drastically decreased in the heat stresses, since the ramping periods afford cells some time to adapt to the temperature stimulus. Continuing work is aimed at refining the model with deeper experimental data and introducing membrane fluidity into the model. We will test predictions about the ability of aleurone layers to maintain normal cellular function under heat stress conditions. This will provide important insights about the limits of plants to adapt to temperature extremes, insights important to addressing challenges imposed by global climate change. Supported by the NSF grant 0926702 and Trinity University.

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The temperature dependence of cell cycle timing.

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The timing of cell division and therefore the developmental timing of cold-blooded organisms is temperature (T) dependent. It is known that the rates (R) of many biological processes increase exponentially with the inverse of T, following the empirical Arrhenius equation. But consideration of the full viable T range often reveals more complex T dependence above a breakpoint temperature T*, where R decreases with temperature, giving rise to asymmetric humped shapes in R vs T plots. We are interested in the origin of this non-monotonous T dependence of the speed of biological processes. Are cellular rates controlled by a master regulator that fails at higher temperatures, or can the high temperature behavior be explained by the network structure of a set of regulatory proteins?

One of the best-understood regulatory networks in cell biology is the CDK-1/APC feedback system that is believed to give rise to the stable oscillations underlying cell cycle progression. Based on models of the CDK-1/APC oscillatory network, we developed a protein network model that assumes Arrhenius-like T dependence of rates on the protein level and therefore accounts for the T dependence of cell cycle timing. We found that a three-node model that allows for

dynamic looping trajectories in the graph of network states shows the non-monotonous T dependence typical for physiological rates. To test our model we measured the timing of early embryonic cell cycles of the nematodes *C. elegans*, *C. briggsae* and *P. pacificus* at different temperatures and could show that the model successfully captures both aspects of the biological data, the asymptotic Arrhenius tail at low T, as well as the non-monotonous behavior at high T. This suggests that the T dependence of cell cycle rates is due to the network structure of regulatory proteins.

The formula obtained from the dynamics of the model, explains the temperature rate relationship observed for developmental processes. Besides adding temperature dependence to the current cell cycle models, our analysis gives accurate fits to the nematode data that allows us to address the question of how organisms adapt their cell cycle machinery to evolve to a different temperature niche.

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Wanted: a calibration standard for anomalous subdiffusion.

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Much work is being done on anomalous subdiffusion in the plasma membrane, cytoplasm, and nucleus of cells, and in model systems. The main question: Is diffusion anomalous, transient anomalous, or normal? If it is anomalous, what are the exponent and the mechanism? If it is transient anomalous, what is the crossover time from anomalous to normal? The type of diffusion is of interest as a factor in reaction kinetics and as a probe of submicroscopic organization, unfortunately not uniquely invertible. The area is controversial, especially the hypothesis that crowding causes anomalous subdiffusion. There is strong experimental evidence supporting the hypothesis in model systems, but the problem is not yet well enough understood in terms of theory, simulation, and experiment to settle the question. Resolving the experimental question requires a calibration standard for anomalous subdiffusion, preferably one able to crosscalibrate the various optical (FRAP, FCS, single-particle tracking) and NMR measurements. A standard would provide a positive control to experimentalists who do not observe anomalous subdiffusion in cells, and a calibration point to those who do. The main requirements for a calibration standard are as follows. Diffusion must be anomalous over the length and time scales of the measurements, several μm and s for the optical measurements. The length scale is fundamental; the time scale can be tuned via the viscosity of the medium. The standard must be theoretically well understood, with a known anomalous subdiffusion exponent, ideally readily tunable. The standard must be simple, reproducible, and independently characterizable, say by electron microscopy for nanostructures. If subdiffusion is transient, the anomalous regime must extend over at least two or three orders of magnitude. Among the candidate experimental systems discussed are (1) obstructed motion in a supported lipid bilayer; (2) computer-driven physical trajectories in which a stable fluorophore is moved by a piezo stage; (3) continuum diffusion in a randomly ablated lattice of nanoscale obstacles, a novel percolation problem chosen to facilitate nanofabrication; and (4) transient anomalous diffusion in which the tracer is trapped by a hierarchy of binding sites, for example a transcription factor in a random DNA array. (Supported by NIH grant GM038133.)

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A Single-Molecule Hershey-Chase Experiment.

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Ever since Hershey and Chase used phages to establish DNA as the carrier of genetic information in 1952, the precise mechanisms of phage DNA translocation have been a mystery. Although bulk measurements have set a timescale for in vivo DNA translocation during bacteriophage infection, measurements of DNA ejection by single bacteriophages have only been made in vitro. Here, we present direct visualization of single bacteriophages infecting individual *Escherichia coli* cells. For bacteriophage λ , we establish a mean ejection time of roughly 5 min with significant cell-to-cell variability, including pausing events. In contrast, corresponding in vitro single-molecule ejections are more uniform and finish within 10 s. Our data reveal that when plotted against the amount of DNA ejected, the velocity of ejection for two different genome lengths collapses onto a single curve. This suggests that in vivo ejections are controlled by the amount of DNA ejected. In contrast, in vitro DNA ejections are governed by the amount of DNA left inside the capsid. This analysis provides evidence against a purely intrastrand repulsion-based mechanism and suggests that cell-internal processes dominate. This provides a picture of the early stages of phage infection and sheds light on the problem of polymer translocation.

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Predicting rates of cell state change due to stochastic fluctuations using a data-driven landscape model.

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We develop a potential landscape approach to quantitatively describe experimental data from a fibroblast cell line that exhibits a wide range of green fluorescent protein (GFP) expression levels under the control of the promoter for tenascin-C. Time lapse live cell microscopy provides data about short term fluctuations in promoter activity, and flow cytometry measurements provide data about the long term kinetics as isolated subpopulations of cells relax from a relatively narrow distribution of GFP expression back to the original broad distribution of responses. The landscape is obtained from the steady state distribution of GFP expression and is connected to a potential-like function using a stochastic differential equation description (Langevin/Fokker-Planck). The range of cell states is constrained by a “force” that is proportional to the gradient of the potential, and biochemical noise causes movement of cells within the landscape. Analyzing the mean square displacement of GFP intensity changes in live cells indicates that these fluctuations are described by a single diffusion constant in log GFP space. This allows application of the Kramers’ model to calculate rates of switching between two attractor states, and enables an accurate simulation of the dynamics of relaxation back to the steady state with no adjustable parameters. With this approach, it is possible to use the steady state distribution of phenotypes and a quantitative description of the short term fluctuations in individual cells to accurately predict the rates at which different phenotypes will arise from an isolated subpopulation of cells.

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The Thermodynamics of Prediction.

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Biological systems (such as organisms, cells or molecules) inhabit constantly-fluctuating environments. They learn and exploit environmental statistics. What governs their adaptation? Here we make concrete the connection between two important perspectives: the view that biological systems have evolved (in part) to predict their environment, and the view that biological systems have evolved to use energy efficiently. A system responding to a stochastically fluctuating environment can be interpreted as computing, by means of its dynamics, an implicit model of the environment. The system's state retains information about past environmental fluctuations, and a fraction of this information is predictive of future ones. The remaining nonpredictive information reflects model complexity that does not improve predictive power. We expose the fundamental equivalence between this model inefficiency and thermodynamic inefficiency, measured by the energy dissipated during the interaction between system and environment. Our results hold arbitrarily far from thermodynamic equilibrium and are applicable to a wide range of biomolecular systems. They highlight a profound connection between the effective use of information and efficient thermodynamic operation: any system constructed to keep memory about its environment and to operate with maximal energetic efficiency has to be predictive.

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Thermodynamic Metrics and Optimal Perturbation Paths.

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A fundamental question in biology is how a molecular-scale machine performs useful work in a cellular milieu, while operating away from thermal equilibrium without excessive dissipation. In particular, how can control be exerted to rapidly manipulate a microscopic substrate so as to minimize the work required and hence the metabolic burden? For example, how should a linear motor step through its mechanical cycle so as to translate its cargo with minimal energy wasted? To address this general question of optimal control, we derive a friction tensor that induces a Riemannian manifold on the space of thermodynamic states. Within the linear-response regime, this metric structure governs the dissipation of rapid perturbations, and bestows optimal perturbation protocols with many useful properties. We demonstrate the utility of this metric by solving for optimal control parameter protocols in a simple microscopic nonequilibrium model.

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Is transcriptional noise universal? Single cell readout of mRNA copy numbers in systematically designed promoters.*R. C. Brewster¹, D. Jones¹, R. Phillips¹; ¹Applied physics, California Institute of Technology, Pasadena, CA*

Recent efforts have demonstrated the diverse and widespread importance of noise to biological function. Theoretical efforts to quantify transcription often uses the language of rates to describe how the relevant molecular players will interact with the various DNA target binding sites which constitute the promoter. The output of such a theory is a distinct prediction for the mean and noise in expression as a function of the promoter architecture and of the concentrations of those molecular players. An inescapable prediction from this theory is that for any transcriptional architecture one can model, the predicted dependence of both the mean and noise in expression on these parameters is unique to that architecture. On the other hand, recent experiments measuring the mRNA copy number from a collection of unrelated promoters suggests that the noise in expression is universal and fits a single phenomenological model which depends only on the mean. In an attempt to understand this discrepancy, we have used single cell mRNA fish measurements in *E. coli* to study the expression profile from constitutive promoters where we have systematically altered the affinity of RNAP binding to the promoter; a quantity that is directly relateable to a parameter in our theory. As a result we have constructed over a dozen constitutive promoters with mean expression levels varying over 3 decades from an identical architecture where the systematic tuning of the RNAP binding affinity acts as the sole parameter determining the expression.

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Comparison of the theoretical and real world evolutionary potential of a genetic circuit.*M. Razo-Mejia^{1,2}, J. Boedicker¹, R. Phillips¹; ¹Applied Physics and Biology, California Institute of Technology, Pasadena, CA, ²Biotechnological Engineering, National Polytechnic Institute, Silao de la Victoria, Mexico*

Many large scale genomic efforts aim to map genotypic variability among individuals. However, a simple model system where the link between genetic variability, gene regulation, and function can be studied in detail is missing. Here we implement a thermodynamic model of gene regulation in the wild type *lac* operon as the theoretical framework to explore the potential evolutionary pathways for this gene circuit. We used the model parameters controlling gene regulation (such as the number of *Lac* repressor molecules per cell, the binding energies of transcription factors encoded in the DNA, the propensity for the formation of secondary structures involved in gene regulation, and the RBS binding sites of the genes) to gain insight into the possible sources and patterns of variability among individuals. We then analyzed how this operon actually evolved in a collection of ≈ 1000 *E. coli* natural isolates from all over the world, measuring both the genetic and phenotypic diversity of the *lac* operon in each isolate in order to inquire into the paths of evolutionary change. Thus far we have shown a wide range of phenotypic potential within the natural isolates and have begun to correlate these changes with the evolutionary story of the *lac* operon in each individual strain.

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Heritable changes to network architecture in genetically rewired yeast

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Genome rewiring events (recruiting genes to foreign regulatory systems) alter the network architecture of a cell resulting in major physiological changes that ultimately lead to the emergence of new phenotypes. Deciphering the mapping between genotype and phenotype is essential for many branches of biology, e.g. development and cancer and is at the basis of our understanding of evolution. We have developed an experimental system to study the ability of a cell to recover from a genome rewiring event by measuring the dynamics of various cell phenotypes (division rate, cell size, protein expression levels) over hundreds of generations using genetically rewired strains of the yeast *S. cerevisiae*. These cells have been modified to remove the essential gene HIS3 from its native context in the genome and place it under sole regulation of a promoter in the galactose metabolic system, which is isolated from the histidine pathway in wild type cells. Glucose-containing medium strongly represses the GAL genes and HIS3 resulting in a severe challenge to the rewired cells in this medium. Greater than 50% of the population overcome the challenge within 20 generations and the adapted state can propagate indefinitely. In our experiments, adaptation was due to a reorganization of the system architecture within many individual cells as a response to the environmental challenge and is not a result of selection of rare advantageous phenotypes. An induced response that resolves an environmental challenge, accessible to many cells but is nevertheless inherited extends the common evolutionary theory and attests to the adaptive potential of regulatory circuits. [Stolovicki et al 2006, David et al 2010] Here I will present new results that show that the adaptation process involves complex dynamics manifested both at the molecular and cellular levels. Using time-lapse microscopy, we show the surprising result that the adapting population is composed of cells spanning a broad distribution of growth rates and that the population dynamics are not simply driven by fixation of the fastest growing cells. The build-up of a stable adapted population thus relies on the ability of cells to inherit a stable growth phenotype and to transmit it for generations. These results suggest the existence of a wide variety of stable adaptation mechanisms, resulting in a new, robust regulatory system for the rewired genome in the initially challenging environment. It calls for a new framework for population dynamics based on epigenetic mechanisms.

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Bistable Switch in Glycolysis as a Control of Metabolic State.

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Glycolysis may operate at two different states: a high flux state in which the majority of glucose consumed is converted to lactate and a low flux state in which most glucose consumed results in complete oxidation through the citric acid cycle. Different cells may operate at different flux states; for example quiescent cells are often at a low flux state, whereas upon oncogenic transformation cells metabolize glucose through the high flux state to produce lactate, known as the Warburg effect. In liver and muscle cells the metabolism may switch between a high and a low flux state depending on the hormonal state. To understand the regulation of the switch of the metabolic state, we developed a mathematical model based on mechanistic enzyme kinetics. We showed that the glycolytic pathway exhibits multiple steady states and that the steady state behavior is dependent on the make-up of glycolytic isozymes. With the isozymes

seen in quiescent tissues, glycolysis resides only at the low flux state in the physiological range of glucose concentration. Whereas in rapidly growing cells such as cancerous cells and embryonic stem cells, glycolysis resides at a high flux state. A bistable switch allows the change of flux state only at the “switch” concentration. Our modeling results show that in the physiological range of glucose concentration, the switch from a particular flux state is prevented in both quiescent cells and in fast growing cells. In contrast, in liver cells, the bistable behavior is determined by the ratio of kinase/phosphatase activity of PFKFB which is modulated by the hormones insulin/glucagon. Thus the change in the “switch concentration” under different K/P ratios allows the flux state to switch from a high flux state to a low flux state (gluconeogenesis) in the presence of glucagon.

A better understanding of the bistable behavior will enable a better control of metabolism under different disease conditions. We evaluated a number glycolysis inhibitors which have illicited interest in clinical applications. Some inhibitors shift the “switch concentration” while others change the flux level without affecting the bistable behavior fundamentally, thus potentially presenting different strategies and even efficacies in metabolic control. Insights from the switch behavior of glycolysis may reveal new means of metabolic intervention in the treatment of cancer and other metabolic disorders through suppression of glycolysis.

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The Response of Bacterial Growth Rate to Changes in Osmotic Pressure.

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Several theoretical studies (e.g. Koch 1983, Huang 2011) have proffered osmotic pressure as an essential factor of bacterial cell growth, proposing that it is required to drive mechanical expansion of the stiff cell wall, but this idea has yet to be studied experimentally. Here, we combine microfluidics with high-resolution, time-lapse microscopy to measure the response of single-cell growth rate to repeated, rapid changes in osmolarity of the growth media. We find that while lowering the osmotic pressure of the Gram-negative bacterium *Escherichia coli* transiently lowers its growth rate, that these cells are capable of making up for this by exhibiting particularly rapid bursts of growth upon re-pressurization, thereby attaining the same size that they would have attained had they never been slowed. This suggests that it is actually wall synthesis, not osmotic pressure, that ultimately determines cell size in this species. On the other hand, the same phenomenon is not observed in the Gram-positive *Bacillus subtilis*. In *B. subtilis*, the retardation of growth in response to osmotic shock is longer-lived. Furthermore, *B. subtilis* cells that have experienced successive osmotic up- and downshocks are smaller than they would have been had they never been shocked. Thus, it appears that in Gram-positive species, growth depends critically on both wall synthesis and osmotic pressure and suggests a model where the cell wall behaves as a viscoelastic solid for which the yielding properties are dependent on synthesis and hydrolysis of wall cross-links. I will discuss these observations in the context of the co-evolution of cell wall ultrastructure, osmoregulation, and the proposed fundamental growth strategies associated with Gram-negative and Gram-positive organisms.

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Spatial gradients in bacteria.

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Establishment of spatial asymmetry is critical to development. One mechanism for generating asymmetry involves the localized synthesis of a regulatory protein that diffuses away from its

source, forming a spatial gradient. While gradients are prevalent in eukaryotes at both the tissue and intracellular levels, it has only recently been shown that gradients of freely diffusible proteins can form within bacterial cells despite their small size and the speed of diffusion.

We use mathematical modeling to investigate the biochemical and physical constraints on the generation of intracellular gradients by the asymmetric localization of a source and a sink [1]. Our results suggest that gradients are a robust and potentially common mechanism for providing intracellular spatial cues. We present two examples illustrating the importance of spatial asymmetry in bacteria. First, employing a combination of mathematical modeling, single-cell microscopy and genetic manipulation, we demonstrate that a gradient of the master cell-cycle regulator CtrA in *Caulobacter crescentus* robustly establishes the asymmetric fates of daughter cells prior to the compartmentalization resulting from cell division [2]. Second, we present a systematic computational analysis of the effects of changes in protein localization and abundance to determine whether localization of the histidine kinase PleC is required for correct developmental timing of the flagellar pole in *Caulobacter* [3].

Taken together, our results suggest that bacteria are able to create functional spatial gradients that are robust to perturbations in localization phenotypes, regulatory mechanisms and cellular morphology.

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Quantitative characterization assisted design of novel pattern forming E coli.

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Biological pattern formation is a process involving cell motion, growth and spatial gene expression regulation. A number of mechanisms has been identified using traditional reverse genetic method. We have taken a build-to-understand approach using synthetic biology to engineer E coli motion control. We have engineered a novel cell density suppression cell motility mechanism, and it leads to form a robust sequential and periodic stripe pattern. A key element to this area of study is to develop quantitative cell motion measurement with sufficient spatial and temporal resolution. We have developed a continuous fluorescent photobleaching method to measure the diffusion and drift coefficients of the engineered E coli strain. It enable us to identify an additional pattern forming motion regulation of engineered strains.

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Configurable tool for automated exocytotic events quantification.

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Background

Exocytosis is one of the fundamental cellular processes. Many key molecules involved in exocytosis have been identified. However, its underlying mechanisms are complex and are still largely unknown. Total internal reflection fluorescence (TIRF) microscopy allows the direct imaging of the exocytosis dynamics. Using TIRF movies to quantify exocytosis event statistics, such as the types, occurrence counts and timings for different assay scenarios could provide a powerful tool in basic research and drug development for new discoveries in exocytosis processes.

Challenges

Exocytosis is generally a rare event among many vesicles in cells. In our study of 20 movies with a variety of assay scenarios, the exocytosis rate is < 1%. Therefore it is impractical (tedious, irreproducible) to quantify exocytosis statistics manually. There is a strong demand for automated exocytotic quantification. However, the kinetic profiles of exocytosis events vary significantly in TIRF images depending on factors such as cell types, probes, imaging systems, assays, etc. It is challenging to create a universal classifier for automated exocytosis event detection.

Solutions

We developed a tool that can be configured to automatically detect a variety of exocytotic events in TIRF images such as rapid emerging events, slow emerging events and gross events. The configuration parameters include event size, contrast and lasting durations. The tool is based on a spatiotemporal structure guided processing method. The method uniquely performs multi-duration event energy integration, event energy contrast detection, spatiotemporal event qualification and resulting event quantification.

Objective

The objective of this study is to assess the effectiveness of our tool for broad exocytosis detection.

Studies We performed a verification study to assess the exocytotic event detection accuracy using TIRF movies containing a variety of exocytotic events.

Study data: The study set includes 15 movies of endocrine cells (MIN6 and GLUTag) that were provoked with various secretagogues, resulting in rapid emerging, slow emerging, and gross exocytotic events.

Accuracy metrics: sensitivity and positive predictive value are used to assess the accuracy of the tool detection results. The exocytosis events were manually detected and independently confirmed as truth for the study.

Results The study results show high sensitivity: $88.44 \pm 4.76\%$ (153/173) and high positive predictive values: $91.07 \pm 4.31\%$ (153/168) for all exocytotic event types .

Conclusion Our configurable tool could automatically provide accurate statistics for the quantification of broad exocytosis events.

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The Conserved Domain Database (CDD) at NCBI.

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The Conserved Domain Database (CDD) at the National Center for Biotechnology Information (NCBI) is a collection of structure based multiple sequence alignments that represent ancient conserved domains. CDD provides annotation and tools for the rapid annotation of functional domains on protein and coding nucleotide sequences. In addition protein BLAST searches by default display the results of the CD-Search giving users a quick overview of the protein domains present. CDD includes high quality curated NCBI protein domain models as well as imported models from Pfam, SMART, TIGRFAM, and COG, combining data from several disjoint resources. DELTA-BLAST, the latest in an arsenal of BLAST tools relies heavily on PSSMs generated from CDD and offers the most sensitive protein search to date, outperforming BLASTP, PSI-BLAST, and CS-BLAST. Currently, over 92% of proteins with known 3D structure and over 76% of proteins with defined source organism are annotated by CDD in NCBI's Entrez database, and about 13,000 functional sites have been recorded on close to 5000 domain models, almost half of which are supported by direct evidence observed in 3D structures of protein complexes.

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The pSeg Library: A toolset for simplifying live-cell image analysis.

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A thorough understanding of biochemical pathways requires knowledge of how pathway components change in time. Investigators have become increasingly aware of the importance of measuring pathway dynamics at the single cell level, as population-level approaches may average away interesting behaviors. Additionally, the single cell variability seen in pathway dynamics may itself be an important aspect of the underlying biology. Detailed cell-level studies require careful data collection of hundreds to thousands (or more) of cells, and so fluorescence microscopy is often used due to the wealth of information available in images (position, texture, intensity distribution, and hundreds more) and because single cells can be monitored over time. Automatic identification of cellular regions in an image, termed *segmentation*, is a difficult problem, requiring some level of expertise. Segmentation depends on fluorescence signals that separately identify a cell's nucleus and cytoplasm. Such signals are not readily available for live-cell studies. To simplify live-cell image analysis, we have developed a toolset consisting of two parts: (1) a library of *segmentation plasmids* (pSegs) for differentially labeling the cytosol and nucleus of live cells, and (2) software for intensity-based segmentation that is simple to use. Each pSeg includes both a cytosolic and a nuclear fluorescent protein expressed together for a total of over 20 color localization combinations. Finally, our software identifies the nuclei in fluorescence images based on a single parameter and outputs measurements of each nucleus along with the perinuclear pixels. We hope that this toolset will simplify single-cell studies by removing the requirement for image analysis expertise and by providing an efficient experimental foundation.

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Simple Physics and the Possible Origin of Life between Mica Sheets.*H. G. Hansma¹; ¹Department of Physics, University of California, Santa Barbara, CA*

The origins of life require reliable energy sources. One feasible energy source has not been considered until recently. This is mechanical energy - work (1, 2). The spaces between moving muscovite mica sheets are the environment in which mechanical energy is hypothesized to have been involved in the origins of life. Mechanical energy from moving mica sheets has two main sources: 1. The open-and-shut motions of mica sheets in response to water movements in and out between the sheets, and 2. Thermal cycles of day and night acting on bubble 'defects' between mica sheets. This mechanical energy is hypothesized to have been involved in the formation (and breaking) of covalent bonds, the rearrangement of polymers and molecular aggregates, and the budding off of protocells, in the earliest form of cell division. Furthermore, it is hypothesized that: The mechanical energy from mica sheets moving open-and-shut is the source of the common open-and-shut motions of enzymes, originating from a protobiotic era when mechanical energy was plentiful and chemical energy was not yet available.

(1) Hansma, H. G. (2010). Possible origin of life between mica sheets. *Journal of Theoretical Biology*, 266(1), 175-188.

(2) Hansma, H. G. (2012). Possible Origin of Life between Mica Sheets: How Life Imitates Mica. *J. Biol. Struct. Dynamics*, in press.

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unPAK: Developing an Undergraduate Research Network to Analyze Fitness Phenotypes of *Arabidopsis thaliana* Single Gene Knockout Lines.

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Providing an authentic research experience for undergraduates that reflects the collaborative nature of modern biology research is a consistent challenge for faculty. A solution to this challenge is the formation of research collaborative networks in which undergraduates directly work with one another in classroom or independent research settings and take the lead in the gathering and analysis of biological data. unPAK (**u**ndergraduates **P**henotyping **A**rabidopsis **K**nockouts) is a collaborative research network between the College of Charleston, Barnard College, Hampden-Sydney College, and the University of Georgia in which undergraduates phenotype and genotype single gene knockouts (SALK insertion lines) of the genetic model plant *Arabidopsis thaliana*. unPAK seeks to serve the *A. thaliana* research community by developing a comprehensive database with specific phenotypic and genotypic information for the SALK insertion lines. Several previous targeted investigations have not uncovered phenotypes in these mutant lines. The information gathered by unPAK researchers is expected to uncover a wide variety of phenotypes in these lines that are only revealed under specific environmental conditions that mimic natural ecological variation. Additionally, mutant phenotypes may be more likely to be found by screening complex fitness traits (i.e. germination, fruit production, plant size). Molecular analysis via conventional and quantitative PCR is expected to reveal which SALK lines contain multiple T-DNA insertions that have the potential to influence and complicate total phenotypic assessment. Undergraduates have, to date, grown more than 8000 plant lines encompassing over 1000 distinct SALK T-DNA lines for the development of seed stocks and tissue samples for genotyping. Fitness experiments are ongoing to phenotypically characterize each line. Undergraduate genotypic assessment of ~1000 distinct tissue samples have suggested that approximately half of these lines possess multiple T-DNA inserts across their genomes. As unPAK continues to develop and expand, it will serve as a model for the development of cross-campus undergraduate research collaboration, provide undergraduate research experiences both in and out of the classroom, and provide an invaluable resource for the study of plant genetics.

971

Investigation of Benefits and Challenges of Mentoring Undergraduates from Underrepresented Groups.

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Mentors are critical in the development of an individual's career, and most mentors in the biological sciences have had little formal training in managing undergraduates. In order to understand the benefits and challenges associated with mentoring undergraduates, we conducted semi-structured interviews with 15 undergraduate mentors at the University of Minnesota Duluth. All of the mentors we contacted agreed to participate in the interview and the interviewed mentors represented a broad array of perspectives including an equal

representation of male and female mentors, mentors from underrepresented groups, mentors at different levels of their careers, and mentors from undergraduate and professional school departments. In addition, the mentors in our study had all served as mentors for the Pathways program, which is designed to increase the number of students from underrepresented groups in science careers. Themes we explored with the mentors included: What evidence suggests that the mentor-mentee relationship is working well? How does diversity contribute to your relationship with the mentee? How does mentorship of undergraduates affect professional development? Our interview data help to identify issues relevant to designing programs and courses focused on undergraduate student research.

IRB Study Number: 1202S09842

972

Using HeLa as an Organizing Theme for an Advanced Cell Biology Course.

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HeLa cells have had an enormous impact on the field of cell biology. The story behind the first immortalized human cell line also provides insights into the role of science in society. I have developed an upper division, undergraduate, cell biology course that uses HeLa as an organizing theme. Students explore a range of topics in cell biology through review of primary literature utilizing HeLa cells as a research model. In addition, students read and discussed *The Immortal Life of Henrietta Lacks* by R. Skloot. Discussions of the sociological and philosophical aspects of the HeLa story were supplemented through the inclusion of guest lectures by faculty from the College of Humanities and Social Science. Learning outcomes for the course included **scientific literacy** as demonstrated by the ability to access, read and evaluate primary literature as well as explain methodologies and derive independent interpretation of data; **application and integration of knowledge of cell biology** as evidenced by the ability to relate specific research problems to larger question in the field of cell biology, evaluate how methodology influences interpretation, and recognize science as a human endeavor; and **scientific communication** measured by the ability of students to present scientific literature and lead an effective discussion. Students also completed several writing assignments, including a 'mini-review' on a topic of their choosing. Student response to the structure and outcomes of the course was very positive. The involvement of colleagues from outside the College of Natural Sciences and Mathematics also provided an exciting opportunity for trans-disciplinary collaboration.

973

The Genomics Education Partnership: An undergraduate team research experience.

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Research into how students learn science has confirmed that inquiry-driven research experiences that address important biological questions are more successful at producing scientifically literate students with good critical thinking skills than traditional content-driven

laboratory classes. The Genomics Education Partnership (GEP), a consortium of faculty from over 90 colleges and universities with different characteristics and missions, uses comparative genomics to engage students in research within the regular academic year. GEP curriculum has been adapted to many different settings, from a short module in a genetics course to the core of a laboratory course. The current GEP research project focuses on the evolution of the Muller F element (a largely heterochromatic domain) in five *Drosophila* species (*D. melanogaster*, *D. erecta*, *D. ananassae*, *D. grimshawi* and *D. mojavensis*). GEP undergraduates work to improve the draft genomic sequences and/or annotate these improved sequences. More than 4.2 million bases of draft sequence have been improved and hundreds of gene models produced using evidence-based manual annotation, leading to student poster presentations and collaborative publications in the scientific literature. The flexibility in the way GEP materials can be used means that each faculty member can adapt the materials in a manner that best suits his or her unique pedagogical goals. Anonymous surveys of GEP faculty and students show a high level of satisfaction with this active-learning approach. GEP students show similar self-reported gains in scientific problem solving and data assessment when compared to students who have participated in traditional summer research, as well as comprehension gains on a quiz about genes and genomes. The GEP pedagogical model offers an exciting opportunity for institutions with limited genomics resources and/or expertise to engage undergraduates in a research experience that will more fully prepare them for future careers in the life sciences. We invite additional faculty to join us (no previous training in bioinformatics required; see <http://gеп.wustl.edu>; training workshops will be held summer 2013). Supported by HHMI grant # 52005780 to SCRE.

974

Characterization of *C. elegans* capture by a nematophagous fungus in an undergraduate laboratory course.

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Incorporation of discovery driven experiments into the undergraduate laboratory curriculum brings students one step closer to a true research experience. The purpose of this study is to assess whether a new undergraduate lab course exercise allows students to conduct novel scientific research while achieving the learning objectives established for the module. This experiment examines the regulation of *Caenorhabditis elegans* capture by *Arthrobotrys oligospora*, a nematophagous fungus. The protocol was generated by students in an advanced molecular biology lab course using the published literature as a guide. It has been adapted for a large TA instructed microbiology lab course consisting of eight sections (160 students total) per quarter. The students in this class are presented the background information regarding the experiment. Groups of four then conduct their own literature search to identify a variable, which may affect the efficiency of *C. elegans* capture by *A. oligospora*. Briefly, the experiment involves the addition of a known number of *C. elegans* to a plate of growing fungus. At two time points following *C. elegans* incubation (6 hours, 24 hours), students count the number of surviving worms and compare to a control plate lacking fungus. The percentage of surviving worms is then compared to the chosen variable. Following data collection, students write a lab report in the format of a primary research article. From this experimental module, students were able to produce results that agree with published data as well as add to the existing literature. A number of the established learning goals were achieved, with the students demonstrating a greater understanding of the subject material and an improved ability to apply the scientific method. From self-assessments, students expressed positive gains as well. Overall, these benefits demonstrate the utility of discovery driven experiments compared to traditional “cookbook” labs.

975

A simple microscopy assay to teach the processes of phagocytosis and exocytosis.

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Phagocytosis and exocytosis are two cellular processes involving membrane dynamics. While it is easy to understand the purpose of these processes, it can be extremely difficult for students to comprehend the actual mechanisms. As membrane dynamics play a significant role in many cellular processes ranging from cell signaling, to division, to organelle renewal and maintenance, we felt that we needed to do a better job of teaching these types of processes. Thus, we developed a classroom-based protocol to simultaneously study phagocytosis and exocytosis in *Tetrahymena pyriformis*. Using simple compound microscopes and a pulse-chase set-up where the cells are supplied India Ink as the pulse and carmine dye as the chase, we quantified black and red vesicles over time in the presence and absence of cytoskeletal inhibitors. Here we present the results demonstrating that our undergraduate classroom experiment delivers results comparable to that acquired in a professional research lab. The students performing the experiment did learn the mechanisms of phagocytosis and exocytosis and finally, we demonstrate a mathematical exercise to help the students apply their data to the cell. Ultimately, this assay sets the stage for future inquiry-based experiments where the students develop their own experimental questions and delve deeper into the mechanisms of phagocytosis and exocytosis.

976

Preparing Science & Math Undergraduate Majors to Teach in K-12 Classrooms.

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Recruiting, preparing, and retaining high quality K-12 teachers are critical issues for promoting scientific literacy for our nation's youth, particularly those from socially and economically disadvantaged backgrounds. Education leaders have advocated for teacher education programs to provide stronger preparation and support for beginning teachers. Proponents of innovation in teacher education pathways argue that in subject areas with severe teacher shortage problems, such as science and math, there should be a deliberate effort to open channels for attracting strong candidates such as science and math undergraduate majors into teaching. Cal Teach Berkeley is a new interdisciplinary teacher education program that allows undergraduate students to complete their degrees in life sciences, physical sciences, engineering and math while simultaneously earning a single-subject teaching credential with a focus on teaching in urban schools. We hypothesize that the integration of science and math disciplinary coursework with education coursework and simultaneous opportunities for apprentice teaching through multiple field placements in local K-12 classrooms will produce highly qualified science and math teachers. We present data that address the following research questions: (1) How does the integration of disciplinary coursework, education coursework, and field placements in urban classrooms the development of pedagogical content knowledge (PCK)? (2) How does this integration influence the development of productive beliefs about subject matter knowledge, teaching, and learning? (3) How are Cal Teach graduates able to translate their beliefs and PCK into classroom practices that promote pupil learning? In particular, we highlight a research methods course that provide research experiences for Cal Teach students so that they may gain an understanding of and comfort level with the process of research by engaging in in-depth, authentic research projects and subsequently engage their K-12 students in meaningful inquiry and problem-solving. We describe the Cal Teach Summer Research Institute and instruments we developed for measuring outcomes of this program. A

Knowledge Integration scoring rubric specific for the research process effectively measures conceptual understanding of research by analyzing open-ended responses from pre/post surveys. We show that the opportunity to engage in scientific research experiences provides participants with a deeper understanding of the process of experimentation and the nature of science, as well as strategies for teaching these concepts and skills to K-12 students.

977

The flip-side of integrating research and teaching: the research laboratory as a classroom.

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Undergraduate independent research projects provide opportunities to aid student growth towards the independence required for a professional career. In addition to producing publishable work, the goal of an independent study project is to help students transition from viewing knowledge as something that is provided to realizing that they can participate in the analysis, interpretation, and creation of new knowledge. Just like any other skill, the expertise required to become an effective scientist is learned. However, students accustomed to traditional courses can struggle when faced with less structured experiences like independent study. Even if a student is receiving course credit for an independent project, traditional coursework can seem more important because of the internal checkpoints (exams and assignments) by which a student can easily gauge success. Additionally, since the emphasis for independent research projects is often placed on the actual doing of experiments, students can be left without a proper understanding of the preparation, effort, and “skills away from the bench” required of an effective researcher. Here we describe student-centered approaches designed to aid students in transitioning from traditional coursework to independent research by overlaying a structure onto independent study. Projects are built around a formal syllabus. They also contain specific mechanisms to assess student progress, generating feedback useful in improving the experience for all involved. From the onset of the project, student involvement is key to developing an individualized plan that is tailored to the goals of both mentor and student. Written materials relevant to working as a scientist (such as searching the scientific literature, presenting one’s own research, and keeping a lab notebook) are provided to supplement oral instruction. To introduce students to the idea of external review and accountability, a portion of their final grade comes from an external reviewer. Assessments in the middle and at the end of the experience allow for the students and mentors to revise and improve the project for the next iteration. Student feedback indicates that participants have a realistic understanding of the complexity of the scientific endeavor, beyond what is necessary to complete their specific experiments. Those that choose to enter graduate programs are well prepared--those that have chosen other careers have developed a skill set that will help them to be successful professionals.

978

Integrating research and teaching: The classroom as a research laboratory.

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While new active pedagogies that expand student engagement and investment are common in the classroom environment, use of similar active pedagogies is not common in laboratory sections of many undergraduate courses. In addition, more students seek research experiences than are available, and faculty at primarily undergraduate institutions often have difficulty balancing large teaching loads with their desire to answer exciting research questions. I have addressed these issues by creating a research-and inquiry-based undergraduate

laboratory section of an advanced genetics course. The course focuses on chromosomes; their inheritance, their structure, their evolution, and chromosome-based sex determination. The reading material for the course consists of reviews and articles from the primary literature. The laboratory component of the course is a semester-long research project that establishes connections between course material and my own research. The research project evolves from year to year as the research question progresses. Topics have included meiosis in holocentric chromosomes, chromosome behavior on the spindle, and evolution of chromosome number, structure, and sex determination. At the beginning of each semester, students are instructed in the basic methods they will need to study their topic, and provided with all background literature. Lab groups of 2-4 students are then formed, I discuss with each group the approach they will take to studying their topic, and the students then design and analyze their own experiments. To assess whether the lab meets its learning goals, I used both course evaluations and the CURE (Classroom Undergraduate Research Experience) survey. Students reported gains in understanding the research process, in confidence that they could create hypotheses and design experiments to test those hypotheses successfully, and in critically interpreting and reporting research results. Students also reported learning about the struggles associated with science, e.g. when experiments fail to test their hypothesis, or when organisms die or are difficult to work with. As a faculty member and researcher, I have been impressed with the quality of student work, and have obtained publishable results each year I have taught this course, with the most recent version significantly enhancing my research program. The research projects designed for this laboratory could easily be altered and used as a laboratory for a non-majors biology class, or for an introductory genetics or cell biology class, and the critical thinking and analysis gains should carry into any situation.

979

Effectiveness of Supplementing Traditional Lecture with Animation.

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Many students are intimidated by courses in cell and molecular biology because these courses require them to conceptualize intangible processes. Illustrating these abstract concepts with textbook figures is helpful, but a mounting body of evidence indicates that presenting simple animations may improve learning outcomes, because it allows students to see a topic as a continuous process. In this study, student learning outcomes were compared following cell biology lessons taught with three different instructive strategies. One group was instructed with traditional written material and figures; another group was instructed with narrated animations; and a third group was instructed with both written material and with animations. Learning outcomes were measured using a pre-test/post-test analysis. Mean post-test score improvements for the three methods of teaching were compared pairwise using independent t-tests with 2 tails. No significant difference in learning outcomes was observed between students who learned from traditional written lessons as compared to those who learned from narrated animation lessons. In the post-study survey, many students reported anecdotally that they learned better from one method or the other, indicating that the best teaching strategy might be to incorporate both written lessons and animations into lessons, in order to accommodate multiple learning styles of students. Surprisingly, for three of the four topics taught in the study there was no significant improvement in outcomes for students who learned from both methods of instruction prior to taking the post-test as compared to outcomes for students who learned from only written lessons or from only narrated animation lessons. These results show that more studies are needed to determine if students who learn via multiple instructive strategies can achieve better learning outcomes than students who learn from only one method of teaching.

980

Integrating Inquiry-Based Laboratory Exercises throughout an Undergraduate Biology Curriculum.

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It is nationally recognized that undergraduate students profit from exposure to research activities. Large undergraduate institutions that cannot accommodate all students in independent research projects due to financial and personnel constraints need to search for alternatives that effectively expose undergraduates to research endeavors. The Biology Department at the University of Puerto Rico Mayagüez Campus has developed an initiative (funded by HHMI) in which research has been successfully integrated into the curriculum involving all departmental undergraduate students (approximately 1,300 per year). In this approach, faculty researchers designed inquiry-based laboratory modules that have been integrated into undergraduate biology courses (General Biology, Microbiology, Genetics, Botany, Zoology, Immunology, Cell Biology, Microbial Ecology, Entomology and Plant Physiology). Each laboratory module comprises of 3-12 contact hours and focuses on molecular and/or structural research in the given course field. The lab module mimics as much as possible an authentic research environment within the constraints of the large undergraduate and teaching assistant populations. Students take a minimum of four inquiry-based lab modules as they progress through their undergraduate curriculum thus being exposed to multiple research projects in different disciplines prior to graduation. With this approach, our large undergraduate science population acquires research awareness and appreciation as well as research skills. Assessment conducted revealed that students, including those with independent research experiences, have learned novel research techniques and skills in the teaching labs of their biology courses. Our approach of impacting students early in their undergraduate careers, and doing so multiple times, also enhanced student's interest in pursuing other research activities and graduate / professional degrees. Each inquiry-based lab module was pilot tested, thoroughly assessed and modified accordingly, before permanent integration into the course lab schedule. Challenges encountered, especially at the beginning, included resistance-to-change attitude by lab technicians and coordinators, research faculties' general lack of experience in conducting assessment in education, as well as the intense coordination required for graduate teaching assistants training and for gathering of assessment data. We will describe in detail the process followed to design and integrate the modules in the courses, challenges encountered, assessment tools developed and the impact of this endeavor on our undergraduate population. Other science departments with large undergraduate student population as ours will find this initiative useful to further enrich their undergraduate student's research experiences.

981

Improving student performance in a first year biology course through an emphasis on learning strategies.

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Objective: Introductory courses are one of the major reasons for student attrition in the STEM majors. The U.S. Department of Education strongly recommends the development of stronger programs and customized approaches to increase student retention in the STEM majors. Undergraduate Research and Mentoring (URM) program at Miami University is funded by the NSF. The chief objective of the program is to increase diversity and retention in the biological sciences. . Introductory biology course, BMZ 115 is a large enrollment class aimed at teaching

basic biology concepts and is a gateway course designed primarily for biology majors. BMZ 115 is perceived by many students to be one of the weed-out courses at Miami University. The attrition rate for the course over the past 3 years is ~40%. The URM program designed and implemented a supplemental sprint course for BMZ 115 in fall 2011, emphasizing learning strategies to improve student performance and thereby retention in the biological sciences.

Description: The sprint course was offered for seven weeks during the second half of the semester. Students who had a C or lower grade in the first exam in BMZ 115 were encouraged to enroll in the sprint course. Sixty students enrolled in the course. The students in the sprint course focused on learning strategies based on Merlin C. Wittrock's generative learning theory which was derived from David Ausubel's assimilation theory. David Ausubel defined assimilation as a fundamental process involved in meaningful learning, where new concepts and propositions are hierarchically arranged and incorporated in to already existing cognitive structures. The strategies practiced in the sprint course were the following:

- Making outline before every lecture focusing on the significance of the material to be taught in relation to previously learned material
- Reorganizing lecture notes using concept maps and flow charts
- Developing the habit of asking questions while studying, to evaluate oneself on the learned material

Results: (1) Study habits of the students in the sprint course improved during the course (2) An achievement gap observed between the control and the sprint group in the first exam in BMZ 115 during the beginning of the sprint course, gradually decreased in the subsequent BMZ 115 exams and closed in the final exam.

982

Connecting the Core: Comprehensive assessment model of basic concepts and research skills in a four-year undergraduate biology curriculum.

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In 2004, the Department of Biology at Texas Wesleyan adopted a research-based curriculum for its undergraduate biology major in response to the call for reform by several noted educational groups (National Academy of Sciences, AAAS, etc). Based on these recommendations, the department designed a curriculum focused on developing analytical skills and emphasizing exposure to biological research techniques. Curricular changes included the introduction of inquiry-based laboratory explorations in all courses, and the implementation of a two-semester capstone course sequence for all biology majors to develop skills in independent research. We developed a comprehensive assessment approach that evaluated student performance in both concept acquisition and hands-on research skills, utilizing both tools designed specifically by the department and nationally standardized assessment tools. Our strategy is unique and notable because of its breadth (preliminary evaluations starting in the freshman year, with both mid-program and end-of program evaluations) and depth (specific tools developed to assess laboratory-based skills, analytical skills, and basic concept acquisition). We submit our curricular design and assessment efforts as a model of how to incorporate sustained evaluation throughout the curriculum, and present a novel method for assessment of laboratory skills and analytical abilities for students in the biological sciences.

983

Integrating teaching and research in a research-based introductory biology laboratory curriculum: results of a three-year comparison evaluation.

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National reports including Bio2010 and Vision and Change have emphasized the positive impact that a research-based curriculum can have on undergraduate biology laboratories. However, few research-based curricula have been implemented in large introductory laboratory courses at research-intensive institutions due to logistical challenges and few incentives for faculty to dedicate time to teaching rather than research, with teaching and research often perceived as competing demands.

We present an introductory ecology-based lab course implemented at a research-intensive institution that has many of the hallmarks of authentic research – a single longitudinal question, research questions with unknown answers, the use of modern ecological and molecular techniques in the field and in the laboratory, experimental designs and protocols that are not pre-determined, and collaboration among lab peers.

As part of the assessment of this lab course, we compared students enrolled in the research-based ecology course to students enrolled in a traditional “cookbook” lab course using pre- and post-course Likert-scale surveys. The first year assessment using a matched-pair design comparing volunteers in the new course with matched students in the traditional course revealed that the new course had a significant positive effect on student attitudes regarding authentic research practices, student perceptions of their ability to do lab-related tasks, and student interest in pursuing future research. The second year assessment using a randomized study design showed that the new course compared to the cookbook course had a significant positive effect on student attitudes regarding authentic research practices, but there were no differences in either student perceptions of their ability to do lab-related tasks or student interest in pursuing future research. The third year assessment using a randomized study design that scaled the number of students up to over 100 showed similar results to the second year assessment, specifically gains in student attitudes towards authentic research practices.

Notably, this curriculum goes beyond its pedagogical functions to provide a source of novel data that can have an epistemic function. For example, student-collected data are being used to answer research questions that are the subject of research-based manuscripts from the professor’s research lab.

The success of this curriculum serves as a case study showing that, by merging research and teaching, a research-based curriculum can provide a mutual benefit to undergraduates and faculty. Based on our experience and formal assessment, we provide general ideas and specific protocols that can be easily applied to implement similar curricula elsewhere, in particular at research-intensive institutions where instruction has often taken a backseat to research.

984

Improvisation: Building and Understanding Environments for Creative Learning.

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Recent research in learning suggests that people learn and develop expertise through social interaction, and it is the qualitative aspects of those interactions that affords learning (Vygotsky,

1976; Bransford, Brown, Cocking, 1999; Holzman, 2009) It is also increasingly recognized that an integral component of collaborative learning environments is improvisational conversation (Sawyer, 2007).

Yet, scientists and science educators are rarely invited to learn and practice the social-cultural skills involved in building environments of cooperative communication and interdependent, collaborative work. In contrast, actors in improvisational (improv) theatre explicitly learn how to develop such environments, to experiment with different performances and interactions, and to take risks. Both groups are challenged to collectively create an ensemble of individuals from diverse disciplinary, ethnic and socio-economic backgrounds to work together.

We created improv theater workshops to 1) teach the principles of improv, 2) engage scientists in developing their abilities for group creativity and 3) discover if and how improv theater training affects learning and creativity within computational biology. The 90 minute workshops used improv theater exercises that focus on the development of the ensemble, accepting and giving “offers”, and active listening.

The evaluation of this effort is accomplished through socio-cultural research questions focused on workshop participants’ perspectives of creativity in science, production of creative ideas, opportunities to practice creative thought in collaboration, and abilities to project their creativity in their future research. Our findings suggest improvisational theater training can help participants to create a social environment and a perspective on interaction practices that provide new ways of building community within working groups and a new view of the discursive practices that may be common to science professions. Specifically, participants: 1) realized interpersonal relationships affect learning and creativity within interdisciplinary research teams, 2) learned about their own and others’ perceptions of their field through discussions with individuals from various disciplines, 3) reexamined their notions of learning, and 4) identified having a supportive, collaborative environment as most valuable experience of improvisational training for computational biology.

985

Jumpstarting STEM Careers.

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A successful career in science, technology, engineering and mathematics requires not only a great education and research experiences but also extensive training and preparation with dedicated and enthusiastic mentors and role models. To help promote this preparation, initiatives by the National Science Foundation, National Institutes of Health and other funding institutions now require that post-doctoral mentoring plans be incorporated into funded research proposals to maintain a pipeline of future professionals. Women and minorities are especially affected by low rates of career advancement. Although a similar number of women obtain high-level degrees as compared to men, it appears that women do not continue within academia in proportion to their male contemporaries; such that, significantly fewer women occupy higher ranked, tenured and tenure-track positions. For example, recent statistics show that women received over 40 percent of all BA/BS degrees awarded by U.S. 4-year colleges and universities in the life sciences and that the proportion of women with doctorates has exceeded 40 percent for the last two decades with some reports as high as 60 percent in the biomedical fields. However, in the basic science departments of most medical schools and universities, the

proportion of women associate professors is still below 30 percent, and the proportion with rank of full professor is only 20 percent. In an effort to address these concerns, the Central Arizona Chapter of the Association for Women in Science, based at Arizona State University, and in collaboration with colleagues from George Washington, Gallaudet and Ottawa Universities, has developed a program to help prepare graduate students, post docs and early faculty for a career in stem. This nsf advance funded program tackles the problem of low career advancement of women and minorities by hosting a series of career development seminars and workshops to provide training, mentoring and networking opportunities to graduate students and post docs as they progress through their program of study. Such career training programs that include mentoring and networking will help address the complex problem encountered by women and minorities and thus will aid in restoring a pipeline of diverse stem professionals.

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Abstract art can enhance student learning about quantitative measurements of cells in biological tissues.

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To enhance the histology and embryology curriculum for first year medical students at Touro University California, paintings of the abstract expressionist artist Sam Francis that resemble, and may have been inspired by, biological images, were also presented to the students. We then developed a research project in which a student performed a mathematical comparison between shapes of cell-like forms in the paintings and shapes of cells in these biological images. Our analyses showed that the shapes of cells in Sam Francis paintings are more varied than those of biological cells. Cell like forms in the Sam Francis painting 'Red in Red' were more diverse in elongation (measured as Length to Width (LW) ratio) than red blood cells in a Blood Smear slide. In addition, cells in the Sam Francis painting 'Abstraction' varied more in their skewness (measured as Kurtosis) than cells from an image of embryonic tissue undergoing morphogenesis. This underscores the unconstrained aspect of Sam Francis paintings compared to nature, as well as the variability of cell shapes between different paintings of Sam Francis. This also shows that art can be used as an effective tool to enhance medical student learning about histology and embryology, and quantitative morphometric techniques common in contemporary bio-informatics research in cell and developmental biology.

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The SUN Project Significantly Improves High School Biology Students' Knowledge of Biological Energy Transfer Relative to Cluster-Randomized Controls.

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The SUN (Students Understanding eNergy) Project has developed a new approach to learning about biological energy transfer. During the SUN teacher workshop, a series of experiences emphasizes the role of moving electrons in both abiotic and biotic instances of energy release

and capture. The common terminology of ultimate electron donor and electron acceptor allows one to highlight this physical basis for energy transfer in both a hydrogen fuel cell and in the mitochondria and chloroplasts of living things. This terminology also allows one to conceive of life forms that do not require oxygen (that have a different ultimate electron acceptor) or that use electrons from an atypical source (not a carbon compound) as the ultimate electron donor. Our physical manipulatives, now with some digital support, are designed to help learners understand how electrons move in the chloroplast and mitochondrion, what gets them moving, and how some of the energy released can be stored in a usable form as a result. Previously we reported significant effects of this approach on teacher knowledge and self-efficacy demonstrated both in a traditional exam and in a drawing with explanation assessment. Here we report significant effects upon regular biology students from a cluster-randomized controlled trial. Importantly, students were assayed at the beginning and end of the course, not at the beginning and end of instruction. We found that when pre-scores, SES (free lunch eligibility), GPA and gender were controlled, the SUN participants demonstrated significantly higher scores with a medium effect size of .44. Interestingly ANOVA analysis demonstrated no significant gain in knowledge by control students, which supports a plethora of data regarding student difficulty learning this topic. We will discuss additional data that explore the affordances of these manipulatives, and also propose a learning progression that highlights fundamental laws and applies them to biological energy transfer.

988

Turning Grandparents on to Content Based Cell Biology.

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The Cheshire Academy for Lifelong Learning (CALL) program at Keene State College is organized around classes that are 90 minutes long and run for eight weeks. Having 12 hours of class time over an extended period allows one to teach content based science courses with time to allow the "students", or in this case 55+ year old grandparents, to build an understanding of the early parts of the course before progressing to the more complicated content near the end of the course. The courses I have taught to this group have been highly successful to the extent that I have developed a following of about 20 students who take every new course I offer. Another 20 to 30 additional students have taken one or two of my courses. Courses I have taught for the CALL program include: The A, B, C's of Cell Biology, Cancer Cell Biology, Understanding Genetics and How Animals Work. Toward the end of each class I have, when possible, tied the course to relevant information in the news. Feedback from "students" indicates that they better understand biological topics they read about in the news, can talk with their grandchildren regarding their new understanding of cell biology, and have a better understanding of their own bodies and health.

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From lab to classroom: Science with mobile phone microscopes.

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Microscopy is a critical tool for biological and clinical research, but high-quality microscopes are typically confined to well-equipped laboratories. We recently developed a mobile phone-based microscope for disease diagnostics in developing countries that enables high-resolution imaging outside traditional laboratory environments. After a chance encounter with a middle school

science teacher in San Francisco, we introduced these mobile phone microscopes, called CellScopes, to students at the San Francisco Friends School and found that they could provide new educational opportunities in the classroom and motivate middle school students to pursue discovery-based learning. Over the course of a year, students used a set of CellScopes to carry out a 'Micro : Macro' project in which they took the microscopes outside the classroom to take macroscopic and microscopic pictures of objects in their homes, gardens, parks and playgrounds. The images were displayed in real time on the touchscreen of the phone and could be viewed by multiple students simultaneously, which allowed for interactive discussions among students and teachers. Image modifications and annotations were performed directly on the smartphone screen and results were subsequently posted to social media platforms for further discussions. This project required both students and academic researchers to be actively involved, take initiative, and think creatively about the microscopic world around them. We are now extending the use of CellScope to educational outreach activities with researchers from the University of Hawaii where the microscopes are taken to the beach and students participate in plankton diversity monitoring.

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Foldscope: Origami based print and fold paper microscopes.

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Here we describe Foldscope, a fully functional microscope printed and folded out of paper. Foldscope utilizes the principles of origami to implement bright field, multi-fluorescence, polarization, dark-field and projection microscopy. Foldscopes are manufactured in a roll-to-roll process where all the optical components including lenses, apertures and illumination electronics are directly printed on paper. Novel optical components including spherical GRIN lenses and integrated micro-apertures are also introduced. Panning and focus stage are implemented as a flexure mechanism in paper. The entire microscope can be assembled in minutes, includes no mechanical moving parts, is extremely light (paper), packs in a completely flat configuration in a very small volume, operates with no external power, can be dropped from a 5-story building or stomped upon by a person and can be incinerated if required. We describe several potential applications of foldscope in ultra low cost "use and throw" microscopy for field diagnostics of diseases including malaria, chagas, giardiasis. Costing less than a dollar, Foldscopes can bring microscopy out of the lab and into the hands of citizen scientists enabling large-scale hands-on life science education opportunities.

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Teaching Cellular and Molecular Biology with a Video Game, Evaluation Results and Game Development.

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Immune Attack is a third person shooter that is designed for Advanced Placement (AP) biology students in high school to learn about the immune system, cell to cell communication, and how proteins are required to mediate the signals that allow the immune system to function. We have developed an assessment of cellular immunology knowledge gained as well as confidence with the graphical presentation of the subject matter, and correlated these scores with the amount of time players report spending on video games, how much of the game students finished in the time allotted, whether the students understood the game mechanism, and whether they found the game difficult to play. Our short, 3 day, protocol allowed us to collect a large data sample,

N=180 for each group: middle school, Immune Attack, middle school control, high school Immune Attack and high school control. Classes were each divided randomly into Immune Attack or control groups to ensure that the teacher quality, subjects covered, socio economic status were controlled. Additionally, parental consent was obtained, as well as student assent. Our results show that students gain confidence with the material regardless of how much of the game they play through or how much they play games in general. Additionally, we found that middle school students also enjoyed the game, learned cellular immunology concepts and gained confidence with the graphical representations of cellular immunology, despite the fact that the material covered in the game was at the AP level. Using the results from our evaluation and play testing, we built a sequel game called Immune Defense. Immune Defense is a two dimensional game that can be downloaded and played on Macintosh and PC computers and in browser windows online. The game is a real time strategy style game, in which players must strategically deploy cells of the innate and adaptive immune system to destroy ever more difficult Pathogens. Our evaluation of how well Immune Defense teaches cellular immunology is ongoing. It will include a comparison of the game versus a video on the same topics. We will also investigate whether students learn more when they watch the video before or after playing the game.

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Rising to the challenge of 'Vision and Change in Undergraduate Biology Education'

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In 2009 AAAS published a call for reform in undergraduate biology education based on several guiding recommendations: Integrate Core Concepts and Competencies throughout the Curriculum, Focus on Student-Centered Learning, Promote a Campuswide Commitment to Change, and Engage the Biology Community in the Implementation of Change. The College of Biological Sciences at the University of Minnesota-Twin Cities has established courses and programs to meet several aspects of the recommendations found in the Vision and Change report. We provide coursework that emphasizes student-centered learning, problem solving, team responsibilities, and core concepts and competencies both at a foundational biology and in some upper division courses, including cell biology. Other instructors are noticing the increased preparation and critical thinking skills of students who have participated in student-centered courses, and graduates of these courses report they are better prepared for upper division work, lab research and graduate school than students in traditional lecture courses. We established a series of Conversations on Teaching and Learning as one element of a commitment to creating a community of biology scholars dedicated to improving undergraduate courses and contributing to the teaching and learning literature. We also have begun a Scientific Teaching Program for advanced graduate students and postdocs in biology as a way to introduce them to principles of effective teaching that include classroom diversity, active learning and assessment, as informed by current literature. All participants practice what they have learned by working on a team to create a small instructional module in their sub-discipline. As current or former teaching assistants, they typically recognize the power of this education reform and are eager to put its principles into action as the next generation of biology educators.

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An In-class Activity that Promotes Understanding of Experimental Design for Undergraduates.

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Understanding experimental design is essential to all fields of science. This fundamental skill is taught beginning in elementary school and continuing through to the college classroom. Although students quickly grasp the general idea of creating an experiment to test a hypothesis, they are frequently unable to put these ideas into practice. Our aim was to develop a twenty minute in-class group activity suitable for the large lecture hall which would promote student understanding of experimental design. We designed two alternative activities to test the hypothesis that analysis of well-designed and confounded experiments would be more effective at teaching experimental design than creating a new experiment. Students in an introductory biology course were randomly assigned one of the two activities which they completed in groups of two to four during the same class period. To assess the effectiveness of these alternative interventions we measured student performance using the open-ended experimental design ability test (EDAT) administered before and after the in-class activity. The EDAT and scoring rubric were expanded in order to capture students' ability to justify their proposed experimental design. Comparison of change in pre/post EDAT scores using a general linear model to control for student academic ability and ethnicity revealed no significant differences between the two interventions. However, both activities increased student understanding of experimental design relative to a control group which received a traditional lecture in place of the activities (effect size = 0.25; $p = 0.047$). Moreover, the median performance of students who received either activity exceeds the median performance of graduate students on the same test ($p = 0.01$). Thus, creating a well-controlled experiment and justifying the design elements remains a challenge for entry-level Ph.D. students in biology.

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Promoting Understanding of Scientific Literature among College Students.

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The current system of undergraduate education in science fields often includes a limited amount of training in understanding professional research publications. The pursuit of a higher educational degree, however, requires this training. We conclude that there can be an academic gap between undergraduate training and postgraduate expectations in biomedical professional schools.

With this in mind, we undergraduates have created the Health and Biological Research (HBR) News Foundation, which serves to increase undergraduate students' understanding of scientific publications. Our goals include education of young scientists, especially college students, by encouraging them to read contemporary biological research and write and report summaries of their applications. We aim to create working professional relationships within a team of motivated individuals through interactive groups and peer-to-peer review. We also want to further worldwide dissemination of biological knowledge by publishing our works and translations of them on our website. To ensure scientific quality of our articles, our members use primary scientific literature from journals such as Nature and Science, and send their works to authors of the original publications for revision and approval. Another goal is to create an online

database where other college students and the general public can receive updates on the latest biological and medical discoveries.

To accomplish our goals, the Foundation established its first chapter at the University of Minnesota. This chapter also serves as a model as we strive to establish more at other universities. So far our colleagues at Tufts and Pennsylvania State University have agreed to establish a chapter at those campuses.

After eight months, the results show that students who participated in our chapter at the University of Minnesota have succeeded in their general biology courses, developed a better understanding of biology, sharpened their scientific reading and writing skills, and actively engaged in scientific discussions. Members report that they have discovered more biological areas of interest through reading and writing about various research articles. In addition, members enjoy the scientific, peer-reviewed, and professional environment within the chapter. Our website consistently has more than 60 unique viewers every day, 200 every week, and 700 every month.

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Strategies to improve time management and content retention in undergraduate Biology majors.

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Current undergraduate students expect immediate access to information on any topic, due in large part to advances and accessibility of technology. As a consequence, this generation struggles with tasks that require a consistent commitment over a long period of time. These tasks can include acquiring and retaining a set of information over a semester or completing a multi-week long project. To help students improve their time management skills in Cell Techniques, a laboratory-based course, a large project was broken down into small tasks with intermediate deadlines. Students kept a log of the time they spent completing all laboratory assignments over the course of the semester. On the six-week project with intermediate deadlines, students spent on average 2.8 hours per week in addition to the scheduled lab time and 3.5 hours the week before the final due date. In contrast, on a four-week single deadline project, students spent 2.7 hours per week for the first three weeks of the project and 5.0 hours of outside time the week before the project was due. Students were more consistent in the amount of time they spent each week completing a project with multiple deadlines than they were in completing a single deadline project. To help students improve their retention of information and time management skills in Genetics, a content-centered course, reading worksheets were designed and evaluated for effectiveness. These reading worksheets forced students to read the assigned material on time, produce a tangible product that could be used for studying for the exams, focus on information the instructor deemed important, and engage in higher order thinking. Student learning was evaluated using pre and post unit assessments. For example, on the pre-unit I assessment, only 22% of students could demonstrate they knew how many chromatids would be in a cell at prophase I of meiosis. On the unit I exam, 68% of students demonstrated knowing this concept and 93% of students answered the question correctly on the final exam. Student feedback was solicited using an anonymous survey. Ninety-three percent of the students who completed the survey found the reading worksheets helpful and ninety-seven percent recommended that I continue to use reading worksheets in the future offerings of the course. Improved student learning, content retention, and satisfaction support the continued use of instructor-designed reading worksheets.

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How Does Active Learning Influence Student Learning in Large-Enrollment Introductory Biology Courses?*C. A. Saunders¹, S. Kazmi¹, M. D. Withers¹; ¹Biology, West Virginia University, Morgantown, WV*

The goal of this study was to begin to understand which aspects of an active learning classroom, level of engagement or level of alignment between the assessments used for the course and for evaluation, contribute most to improvements in student learning. To do this, we calculated learning gains for freshmen science majors in three large introductory biology classes using pre-/post-tests composed of validated concept inventory questions. We used timing analysis and the Reformed Teaching Observation Protocol (RTOP) to quantify the level of interactivity and student-centeredness, respectively, of each class. We used the Blooming Biology Tool to determine the Bloom's level of cognitive processing for both the course exams and the pre-/post assessments. We then compared the average Bloom's cognitive levels of each topic to determine the level of alignment between the course exams and the course evaluation, i.e. pre-/post-tests. Finally, we compared the level of interactivity and level of alignment with the magnitude of student learning gains for each topic to determine which had the biggest impact. Preliminary findings suggest that alignment may be more important for student learning than level of interactivity.

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Science, Biotechnology and Society: A Novel Introductory Course in which Non-STEM Undergraduates Engage in Active Learning Performing Molecular Biology Laboratory Experiences.*G. Arroyo-Cruzado¹, J. Rodríguez-Echegaray¹, C. I. Ayarza-Real¹; ¹Biological Sciences Department, General Studies College, UPR-Río Piedras Campus, San Juan, Puerto Rico*

Since August 2010, the Department of Biological Sciences (DBS) of the University of Puerto Rico, Río Piedras Campus (UPR-RP), is offering a novel course to introduce non-STEM undergraduates to biotechnology and the new trends in this scientific field. The course was designed with the 7E model strategy described by Eisenkraft (The Science Teacher, 2003), as the strategy for "the transfer of learning". Scientific Methodology, Chemistry of Life, Cell Physiology, Molecular Biology and Biotechnology are the main topics presented using an interdisciplinary approach, through the discussion of technical papers related to the biotechnological evolution. The students are engaged in active learning, introducing themselves in the discussion of current socioeconomic, environmental and bioethical issues related to biotech. In the laboratory, students execute a sequence of experiences in basic microbiology and DNA technology resembling a research simulation. These experiences provide to the students the opportunity to learn how scientists do science while develop research skills. Pre-post tests show students significant outcomes in knowledge and comprehension about DNA structure, gene expression, recombinant DNA, gene cloning, stem cell resources and DNA technology fundamentals. The next objective in the evolution of this course is to integrate non-science majors in authentic research activities.

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Closing the Loop—Assessing a Mandatory Research Course in an Undergraduate Biology Curriculum.*H. G. Kuruville¹; ¹Science and Mathematics, Cedarville University, Cedarville, OH*

Scientists learn much of what they know about methods of science by practicing those methods in a research setting. However, getting undergraduates to add research experiences to an already busy schedule can be challenging. In order to ensure that our students receive firsthand exposure to scientific research, Cedarville University currently requires that all of our B.S. majors (biology, environmental science, and cellular and molecular biology) complete a research requirement. Students may choose to pursue either a one-semester on-campus research experience, or may elect to do a 10-week summer internship. The research program has four stated learning goals: 1. students will investigate a research problem and state an appropriate hypothesis, 2. students will read and critically evaluate published research and write a review of the literature related to the research question, 3. students will participate in research design, data collection, and hypothesis testing using appropriate instruments and tests, and 4. students will perform appropriate data analyses, interpretations, and representations. Using Survey Monkey, I surveyed all 39 of our senior students to determine whether these learning goals had been met. Twenty four students answered the survey. The results indicated that most learning goals have been met; however, students are not receiving consistent exposure to the literature in terms of writing a literature review. In response to this data, the biology faculty are working to further standardize our expectations for this research experience. I also found that more than half of our students were asked about their research during an interview, and that of these, the vast majority (87%) felt that they could communicate effectively about their research during the interview. We plan to continue making research a mandatory part of our biology curriculum at Cedarville University.

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Quantitative Biology Laboratory for Transfer Students.*A. K. Wilson¹, R. Rylaarsdam¹, K. Kelly²; ¹Biological Sciences, Benedictine University, Lisle, IL, ²Psychology, Benedictine University, Lisle, IL*

Transfer students often cite issues with the process of transferring, especially from a two year institution to a four year university. Benedictine University provides articulation and advising services through our New Student Advising Center but does not offer a formal orientation for transfer students as it does for incoming freshmen. Because optional orientation activities rarely work, we now offer a “transfer’s lab” to these students. This lab offers students the chance to 1) learn/brush up on lab skills required for upper level labs, 2) a formal orientation to key persons, offerings and expectations in the College of Science and more specifically, the Department of Biological Sciences and 3) discussions of University policies and academic advising concerns. This course counts as a 200 level biology elective towards the major. Assessment has confirmed the need for addressing lab skills such as micropipetting, calculating and making solutions and dilutions, proper methodology to collect observational, quantitative, and qualitative data along with statistical analyses and interpretation of that data and the use of equipment such as electronic balances, compound light microscopy, spectrophotometers, and gel electrophoresis. Students have responded favorably to the orientation and advising components of the course.

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Biology Undergraduate Mentoring Program (BUMP) at Central Michigan University.*C. K. Damer¹, D. L. Linton¹, A. K. Monfils¹; ¹Biology, Central Michigan University, Mount Pleasant, MI*

We have created an undergraduate research opportunity funded by the National Science Foundation at Central Michigan University (CMU) called Biology Undergraduate Mentoring Program (BUMP). The goal of BUMP is to increase the diversity of individuals pursuing graduate studies in biological research. BUMP recruits students from historically underrepresented groups, engages them in high-quality research projects mentored by our faculty, and prepares them for graduate school. Students in the program receive financial support for two years and begin the program in their first or second year. Starting in 2009, BUMP has recruited three cohorts of ~ five students. In addition to being closely mentored by a faculty member in research, students in the program take several courses to introduce them to careers in biological research, to develop their scientific communication skills, and to prepare them for the GRE. BUMP students are supported by and integrated into the University, scientific, and local communities through collaborations and partnerships. These include collaborations with the Biology Department, the Multicultural Education Center, GEAR-UP, and the McNair Program on campus. Outside of CMU, BUMP students interact with scientific professionals in the community, volunteer at the Chippewa Nature Center, and mentor students from Holmes Middle School. Although it is too soon to determine the overall impact of the program, the self-efficacy surveys completed by students in the new courses suggest that students had a significant increase in self-efficacy, a clear perspective on career opportunities in biological research, and a realistic plan for their career pathway into biological research.

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The Open Learning Initiative: development and evaluation of free online content delivery system for introductory and advanced biology courses.*A. L. Goodman¹, A. M. Barra², W. Riggs³, M. Brown⁴, G. Rule⁴; ¹California Polytechnic State University, San Luis Obispo, CA, ²National University, Costa Mesa, CA, ³College of the Redwoods, Eureka, CA, ⁴Carnegie Mellon University, Pittsburgh, PA*

The Open Learning Initiative (OLI; <https://oli.web.cmu.edu/openlearning/>) provides a robust content delivery platform used with a variety of instructional approaches, including traditional and inverted classrooms, and hybrid and online instruction. OLI offers three levels of biology courses. (1) Introductory Biology, developed specifically to meet the needs of students at community colleges, has minimal prerequisite knowledge and covers wide range of topics from molecular and cellular biology to evolution and ecology. (2) Modern Biology, a more advanced course, emphasizes cell and molecular biology. (3) Biochemistry is designed for advanced students studying biochemistry and biotechnology. All courses are available as open-and-free versions that contain all course materials, with the exception of graded activities. Instructors who use the courses can customize the content to specifically meet the needs of their students.

The OLI environment offers a wide range of educational materials including text with imbedded assessments, static drawings, Flash-based animations, molecular simulators, three-dimensional images of biological structures using Jmol, and quizzes aligned to learning objectives. The rich assessment environment in OLI provides feedback to the student and reports student progress to the instructor. Student performance on the interpretation of 3D models was improved using Jmol-based ungraded quizzes and mini-tutors. In a student survey, the majority of respondents (68%) recommended using a combination of OLI materials and a traditional textbook. When asked to evaluate individual components of the OLI learning

environment, students found the following aspects helpful: simulations and dynamic figures (71%), ungraded quizzes (68%) and mini-tutors (61%). All of these features offer immediate feedback to students. Graded quizzes, in the same format as ungraded quizzes, were endorsed as helpful by only 32% of the students. Written comments revealed that students valued immediate feedback from the ungraded quizzes as a learning tool, but considered graded quizzes too stressful.

We are looking for partner institutions and instructors to further evaluate and improve the OLI materials, as well as use common assessment tools to investigate effectiveness of pedagogical approaches like the inverted classroom (contact email: oli-participate@andrew.cmu.edu)

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Identifying insect species using taxonomy and DNA Barcoding: a research experience that bridges two undergraduate courses.

J. Bassong¹, A. D'Costa¹; ¹Biology, Georgia Gwinnett College, Lawrenceville, GA

As part of GGC's 4-year undergraduate research experience (4-yr URE), students are exposed to research in all 4 years of their undergraduate career. These course-embedded research experiences provide students the research skills needed for independent research projects in their senior year and later. The goal of this project is to measure insect biodiversity on our campus, and has students in multiple sections of Introductory Biology and Cell biology courses collaborating over a semester. Students in Introductory Biology set up insect traps at three locations on campus. In lab, they sort and classify the insects, usually down to "order", using traditional taxonomic techniques. Each student picks a specimen and researches its distinguishing morphological features, life cycle, and other important characteristics. The insects are then handed over to students in Cell biology to DNA barcode (www.barcodeoflife.org). DNA barcoding involves first extracting DNA and then amplifying a region of the cytochrome oxidase subunit I gene (CO1) using PCR. Apart from learning the PCR technique, we cast our own agarose gels, and learn to trouble-shoot to obtain a PCR product. The PCR products are sent off campus for sequencing. We learn basic bioinformatics, and aligned our sequences in the NCBI and Barcode of Life databases to identify our insect down to the "species" level. Information from each specimen (morphological characteristics, taxonomic and barcode) is catalogued in an online biodiversity database. Pre and post content assessments and surveys to determine students' attitudes towards science are administered, and assessment data will be presented, along with my experiences and as a student and a peer mentor.

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Figure Facts: Encouraging undergraduates to take a data-centered approach to reading primary literature.

J. E. Round¹, A. M. Campbell¹; ¹Biology, Davidson College, Davidson, NC

The ability to comprehend and interpret experimental data is essential to understanding and participating in the process of scientific discovery. Reading primary research articles can be a frustrating experience for many undergraduate biology students because they have very little experience interpreting data. To enhance their analytical skills, students used a template called "Figure Facts" to assist them with primary literature-based reading assignments in an advanced cellular neuroscience course. The Figure Facts template encourages students to adopt a data-centric approach, rather than a text-based approach, to understand research articles. Specifically, Figure Facts requires students to focus on the experimental data presented in each figure and identify specific conclusions that may be drawn from those results. Students who used Figure Facts for one semester increased the amount of time they spent analyzing figures in a primary research article, and regular examination of primary literature was associated with

improved student performance on a data analysis skills test. Students reported decreased frustration associated with interpreting data figures, and their opinions of Figure Facts were overwhelmingly positive. Here, we present Figure Facts for others to adopt and adapt, with reflection on its implementation and effectiveness to improve undergraduate science education.

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Genome Solver: Genome Analysis for 21st Century Faculty.

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The face of biology education is changing rapidly, becoming more interdisciplinary and using larger datasets, in keeping with the recommendations of influential documents such as Vision and Change in Undergraduate Biology. However, faculty trained in the mid-20th century may not be equipped to teach courses involving 21st century techniques, such as genomics and bioinformatics. To address this gap, we, in collaboration with bioinformatics specialists at the J. Craig Venter Institute, have developed workshops to teach faculty to mine the rich data sources being created by the Human Microbiome Project (HMP). The aims of the faculty workshops are several-fold: (1) To teach faculty to use open-source bioinformatics tools, (2) to teach faculty where to obtain HMP data, and (3) to develop a faculty community to discuss curriculum ideas and best practices. The workshops in turn help support the networking possible through our parallel on-line community at www.genomesolver.org. Here we report on the outcomes of our first two workshops, held in the summer of 2012.

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Are There Cell-Cycle Dependent Variations in Cadherin-Mediated Cell Adhesion?

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Cell adhesion makes possible the development and maintenance of tissues and organs. It has thus been an important topic in biomedical research, cancer research in particular. One method used to quantify cell adhesion, the dual pipette assay, has uncovered considerable variability in the force required to separate pairs of adherent cells. This variability, persistent despite strict protocols and assay automation, has necessitated high numbers of replicate trials to obtain statistically valid data. We are using FUCCI (fluorescence ubiquitination cell cycle indicator) to determine if cell-cycle dependent changes might account for the observed variability in separation force. The FUCCI technique involves antiphase oscillating proteins that mark the cell-cycle transitions with corresponding fluorescent probes. Initial comparison of fluorescence distributions in aggregated and unaggregated FUCCI E cad cells showed no preferential accumulation in aggregates of cells in a particular stage of the cell cycle. Work continues with recently acquired equipment of higher sensitivity to improve the resolution and reproducibility of the tests. Positive results could lead to enhanced efficiency of the dual pipette assay.

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New tools that enable blind students to tactilely visualize image data.

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In the fields of microscopy and astronomy items that are either too small or distant are visualized through optics and this image data is captured through pictures, but not everyone is capable of seeing pictorial information. In the United States there are approximately 21.5 million people that are either totally blind or vision impaired. Recent studies have shown that by the age of 16 years old, the average vision impaired student is 3 years behind in subjects such as mathematics in comparison to their peers. From the population of individuals who have STEM (science, technology, engineering and mathematics) careers, less than 5% will be held by those who have some form of vision disability. There is no evidence that individuals who are blind lack the necessary analytical abilities to pursue a STEM career but the lack of opportunities to develop and use those abilities impedes both their educational and employment advancement in these disciplines. Since almost all aspects of life are vision based this creates an unintentional bias because the mechanisms for doing most anything rely on sight. STEM fields are image based, and image rich content must be converted into a tactile format to enable the visually impaired student to have the same access to course materials as their peers. Within the 3D IMAGINE project, we are addressing the issue of low participation in STEM courses by creating 3D tactile prints and models that will help the blind “visualize” images that are critical in understanding key concepts or interpreting data. We are incorporating these tactile displays into entry level STEM classes to assist blind students with learning image rich material. Various technologies are used to create projected tactile surfaces such as printing using raised ink and swell paper or changing 2D images into height field 3D surface geometries that are used to create HDPE (high-density polyethylene) boards or rapid 3D prototype models. Through the development of these pilot courses which are enriched with tactile image displays, we are exploring the limitations encountered by those who are visually impaired and are working to develop new technologies that will make it possible for these students to overcome image based obstacles and have the opportunity to learn and find their place in the STEM community.

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Group Assignment for a 200-level cell biology class that integrates the arts to express and explore basic concepts.

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Students in a 200-level Cell biology course were involved in a semester-long assignment on “Cells and Disease” to allow for an opportunity to apply the basic concepts in an out-of-class activity. The assignment had 3 parts- (1) using popular science articles to learn about an assigned cell and a diseased condition, (2) using research articles and reviews to develop disciplinary knowledge about the cell and its disease, (3) in keeping with the “year of the arts” at the institution, a final assignment was to develop two formats to communicate basic concepts to a middle school audience. The objective of the final assignment was for students to express themselves creatively in a science classroom and to target it to a middle school audience. Students developed two formats- choice of comic strip/poem/artwork/trading cards/other, and choice of Short story/Learning unit/video or animation/Other. The assignment had the following additional components- (i) a narrative that discussed why particular formats were chosen (ii) reflective statements that commented on the role that art/visuals play in understanding science concepts, how the assignment engaged students with the scientific content they read/learned about, and an examination of the importance of communicating science to the non-scientist/middle school audience. Students were polled to determine which assignment engaged

them the most. Greater than 75% of students indicated it was the creative assignment. Qualitative comments were solicited about the learning experiences of the creative assignment. Greater than 40% of responses indicated an awareness of/the ability to communicate science to a lay audience. Examples of student work will be presented. Future directions of this project include the use of student generated material in teacher professional development workshops.

Kinesins: Regulation and Activity

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Tuning Multiple Motor Travel via Single Motor Velocity.

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Microtubule-based molecular motors often work in small groups to transport cargos in cells. A key question in understanding transport (and its regulation in vivo) is to identify the sensitivity of multiple-motor-based motion to various single molecule properties. Whereas both single motor travel distance and microtubule binding rate have been demonstrated to contribute to cargo travel, the role of single-motor velocity is yet to be explored. Here, we recast a previous theoretical study, and make explicit a potential contribution of velocity to cargo travel. We test this possibility experimentally, and demonstrate a strong negative correlation between single-motor velocity and cargo travel for transport driven by two motors. Our study thus discovers a previously unappreciated role of single-motor velocity in regulating multiple-motor transport.

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Quantitative optical trapping on single cellular organelles in cell extract.

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Molecular motors transport many cargos such as Endosomes, Lysosomes and Lipid Droplets inside cells. How motor-function on an organelle is modulated in response to metabolic or cellular signaling is under intense investigation. One way to address these questions is by utilizing optical trapping to measure the force exerted by motors on cellular organelles in response to different metabolic stimuli. However, cellular organelles vary in size from hundreds of nanometers to few microns. Since the force exerted by an optical trap depends on the size of trapped object, this makes calibration of the trap difficult for cellular organelles.

Here we present and validate a simple method that uses motion generated by the motors to precisely calibrate the optical trap for force measurements on cargos of unknown size in a cell extract. This permits the extension of quantitative optical trapping to cellular organelles in controlled assays to understand how motors and motor-associated proteins function. We first validate this method using kinesin-coated beads to confirm a force of 5.7 ± 1.1 pN for kinesin-1. We then use this method to measure kinesin-1 forces driving lipid droplets isolated from rat liver. A histogram of stall forces shows a single kinesin force peak at 7.1 ± 1.4 pN and a second peak at 13.2 ± 1.5 pN. Using the same methodology we further show that kinesin-1 activity reduces on lipid droplets isolated from 16hr fasted rat liver. This apparent change in kinesin-1 activity as a function of metabolic state of the liver will be a subject of future investigation.

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Traffic control: Serine 176 phosphorylation attenuates kinesin's stall force and biases bidirectional transport.

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In neurons, microtubule motor driven transport is crucial for communication between processes and the cell body. Disruptions in transport are associated with a variety of neurodegenerative diseases. Recent studies implicate phosphorylation of serine 176, a conserved residue found in all three isoforms of kinesin-1 in the impaired axonal transport associated with Huntington's disease. In isolated squid axoplasm, introduction of pathogenic huntingtin protein activates the kinase, JNK3, which specifically phosphorylates kinesin at S176[1]. The mechanism by which S176 modification leads to impaired transport is not very well understood and it is not known whether phosphorylation of kinesin alone is sufficient to cause impaired cargo transport. To investigate the isolated effect of residue 176 on kinesin transport, we use optical trapping and single-molecule fluorescence imaging to study purified kinesin. We employ two constructs, S176A and S176D, truncated at residue 888 to remove the autoinhibition domain, resulting in constitutively active motors. There is no significant difference in the processivity or ATPase activity of the phosphomimetic S176D construct or the non-phosphorylatable S176A construct. However, we do find that S176D has an attenuated stall force (5pN) compared to S176A (7 pN). Furthermore, polystyrene bead cargos coated with dynein and S176D are transported preferentially in the minus direction in comparison with cargos coated with equivalent concentrations of dynein and S176A. We also perform phosphorylation assays using JNK3 on both 888-truncated and full-length wild-type kinesin in which radiolabeling is used to quantify the percentage of protein phosphorylated in the assay. The pattern of stall force attenuation observed for the S176D mutant is also observed in JNK3 phosphorylated samples in which 60-80% of the wild type protein has been phosphorylated by the kinase. These results show that modification of serine 176 alone is sufficient to alter the behavior of kinesin. Efforts are currently underway to investigate the effect of S176 phosphorylation in PC-12 cells as model for neuronal traffic via tracking of up-conversion nanoparticles (UCNP) in neurite growth factor induced processes. UCNP are excitable by two photon processes and do not photobleach, making them ideal for tracking on long timescales. Understanding how modification of this kinesin residue affects transport will allow us to better model the molecular events associated with the development of Huntington's disease.

[1] Morfini, G. A. et al., Nat. Neuroscience 12, 864-871 (2009).

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In Vivo Optical Trapping Reveals Kinesin Drags Dynein During Intracellular Transport.

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Kinesin and dynein are fundamental components of intracellular transport, but their interactions when simultaneously present on cargos are unknown. We have built an optical trap that can be calibrated in vivo to measure forces in living cells. By comparing directional stall forces in vivo and in vitro, we found that cytoplasmic dynein is active during minus- and plus-end directed

motion, while kinesin(s) is only active in the plus-end direction. In vivo, we found that outward (\approx plus-end) stall forces range from 2-7 pN, significantly less than the 5-7 pN stall force measured in vitro for single kinesin molecules. In vitro measurements on beads with both kinesin-1 and dynein bound revealed a similar distribution, implying that an interaction between these opposite polarity motors causes the reduction in kinesin stall force. Finally, inward (\approx minus-end) stalls in vivo were 2-3 pN, higher than the 1.1 pN stall force of a single dynein, implying multiple active dynein.

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Investigating properties of kinesin-2 motors optimized for bidirectional transport.

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Kinesin-2 and dynein motors transport intracellular cargo bidirectionally along both axonemal and cytoplasmic microtubule. These motor activities underlie intraflagellar transport, melanosome dynamics and other vital transport functions in cells, but the mechanism by which the activities of these oppositely-directed motors are coordinated is not well understood. One important question is whether the properties of kinesin-2 motors are specifically tuned for bidirectional transport rather than long-distance plus-ended transport. Consistent with this, kinesin-2 motors were found to detach much more readily than kinesin-1 under hindering loads, and also to rapidly rebind and continue stepping following detachment (slipping behavior). To test the hypothesis that kinesin-2 motors are specifically tuned for bidirectional transport, we carried out stopped-flow and steady-state biochemical studies of monomeric and dimeric kinesin-1 and kinesin-2. In solution, kinesin-2 motors were found to have a 30-fold higher affinity for mantADP than unmodified ADP, presumably due to the hydrophobic nature of the mant moiety. While this feature complicates experimental interpretations, it also offers a new tool to study the role of ADP dissociation in the kinesin-2 chemomechanical cycle. Extrapolated ADP off-rates in the presence of microtubules indicated that for unlabeled nucleotide, ADP dissociation is not rate limiting for either kinesin-1 or kinesin-2. Microtubule pelleting experiments indicated that in the ADP state, monomeric kinesin-2 motor domains have a 10-fold higher microtubule affinity than kinesin-1 motor domains. However, this increased microtubule affinity does not translate into enhanced processivity of dimeric kinesin-2 when compared to kinesin-1. This result suggests that kinesin-2 spends a larger fraction of its hydrolysis cycle in the ADP state and thus is more prone to detaching under hindering loads, but following detachment it readily rebinds to the microtubule. This behavior results in a more dynamic competition with dynein that avoids the cargo coming to a complete standstill due to motor stalling. Supported by NIH.

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The kinesin catalytic motor domains possess a plus-end directionality.

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Drosophila kinesin-14, Ncd, moves toward the minus-end of microtubules. Ncd has a neck region between the C-terminal motor domain, the catalytic core of ATPase, and the N-terminal stalk domain, the dimerizing region. The neck is composed of a rigid coiled-coil structure. The widely accepted model of its force-producing mechanism is the power-stroke model in which Ncd strokes its N-terminal neck region as a rigid lever-arm around the motor domain as a pivot in the direction to the minus-end of microtubules. (Thomas et al., EMBO, 2002) Here, to test the power-stroke model, we have engineered Ncd that is fused to a gelsolin protein or a biotinylated tag sequence (Avi-tag) at the C-terminus. These C-linked Ncds are able to be fixed to a glass

surface or a biotin-coated quantum dot via their C-terminus respectively, leaving the N-terminal coiled-coil structure hanging free. We found that these C-linked Ncd molecules slid microtubules or moved along immobilized microtubules with robust processivity at similar rates to N-linked Ncds (wild type), thereby ruling out models in which the rigid coiled-coil structure is the only element able to exert or transmit force. Instead, our findings indicate another force-generating mechanism that shifts both the N and C terminus of the head, so that force can almost equally well be exerted by either the N or C terminus of the head. We also found that these C-linked Ncds are the plus-directed motors as is Kinesin-1. It was previously reported that a chimera Ncd whose C-terminus was replaced with kinesin-1 neck slid microtubules with the minus-end leading when it was attached to the glass surface at its C-terminus (Henningsson and Schliwa, *Nature*, 1997), showing the relative movement of these Ncds toward the plus-end of microtubules. This implied that the kinesin-1 C-terminal neck was a critical part in determination of the directionality. But our result of the plus-ended directionality of C-linked Ncds, leaving the N-terminal coiled-coil structure hanging free, indicates that the Kinesin-1 neck is not needed for the plus-ended directionality. It means that the kinesin motor domains would possess a 'default' plus-ended directionality.

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Exaggerating the switch - a chimeric Kinesin-tetramer with fulsome motile features.

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Mitosis is driven by a fundamental rearrangement of cytoskeletal structures and the concomitant activity of a wealth of motor-proteins. The tetrameric Kinesin-5 Eg5 has been shown to cross-link antiparallel microtubules and drive long-range uni-directional and plus-end directed relative sliding (on-state). This activity is required in the spindle mid-zone during spindle elongation. When interacting with a single microtubule, however, Eg5 displays a less effective mode of activity (off-state), most likely consisting of a ATP-independent diffusive component (type-1) and a ATP-dependent directional component (type-2), as shown by MSD-analysis. To understand the switch between on and off-state as well as the factors influencing transitions between type 1 and type-2-interactions, it is desirable to be able to directly observe transitions between and quantify the contributions of both interaction types to Eg5-motility. Given the inherently slow speed and short processive run-length of Eg5 quantitative information is very difficult to obtain. To accentuate the transition between type-1- and type-2-interactions, we exchanged the motor-domain and neck-linker of Eg5 with corresponding parts of Kinesin-1. Single molecule fluorescence assays of this chimeric model system, DK4mer, directly confirm the alternation of type-1 and type 2 interactions. Using a truncated chimera we find that characteristics of type 2 interactions are predominantly determined by the motor domain and the initial, dimeric coiled coil of the neck. Thoroughly investigating the influence of ionic strength on the motility of DK4mer on single microtubules and in relative sliding assays, we find that type 2 interactions dominate over type 1 interactions, but also that Type 1 interactions are likely to be required for the initiation of type 2 interactions. This study of our chimeric model system lays the base for a more detailed understanding of motility and regulation of Kinesin-5 motors, representing important players in Mitosis, but also development of cellular polarity.

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Force transduction by the kinesin motors.

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Kinesin motors use ATP to do work by coupling steps of nucleotide hydrolysis to microtubule binding to produce force. Large conformational changes, detected in previous structural studies, have led to the proposal that the central beta-sheet of the motor plays an essential role in force transduction by the motor. We now find that an invariant beta-sheet residue has large, unexpected effects on force transduction by a kinesin motor. Amino acid changes in this residue greatly affect the ability of the motor to hydrolyze ATP and bind to microtubules – force production by the motor is increased, as indicated by motility assays and effects on spindles in oocytes. The spindle effects can be reproduced by simulations of assembly, indicating that they arise because of the altered motor properties. The findings show that the residue is involved in mechanotransduction by the motor. Movements by the residue in different states of the ATP hydrolysis cycle could trigger conformational changes in the motor that store and release free energy, producing strain that leads to force generation.

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Characterization of Hereditary Spastic Paraplegia-Causing Mutations in Kinesin Kif5A.

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Hereditary Spastic Paraplegias (HSPs) are a group of neurodegenerative disorders that arise from the progressive degeneration of corticospinal tract axons, causing lower limb spasticity and weakness. An autosomal dominant form of HSP (AD-HSP) is caused by mutations in Kif5A, a neuronally enriched form of the kinesin-1 family of cellular transport motors. While nineteen separate AD-HSP-causing missense mutations in Kif5A have been mapped, eighteen are in the motor domain, suggesting a deficit in catalytic activity as the mechanistic cause of the disease. We have mutated the wild type Kif5A gene with each of the separate AD-HSP-causing mutations and have begun to test the mechanical properties of these recombinantly-expressed mutant motors in a series of *in vitro* biochemical and biophysical assays. Here we report the results of four separate mutations in the ATP binding and hydrolysis pocket (S202N, S203C, R204W, and V231L) and one mutation in the L11 loop predicted to be at the microtubule interface (E251K). Performing microtubule pelleting assays in the presence of saturating levels of ATP, we found that S202N and S203C had a significantly higher microtubule affinity than wild type Kif5A or the other mutants. Conversely, in the presence of the non-hydrolyzable ATP analog AMPPNP, R204W, V231L and E251K had a significantly lower microtubule affinity than wild type and the other mutants. In microtubule gliding assays, wild type Kif5A moved microtubules at a rate of 0.47 ± 0.02 μm per second. The S203C mutant moved microtubules at a 60-fold slower rate of 0.0074 ± 0.0028 μm per second, while the S202N, R204W, and E251K mutants bound microtubules to the glass surface in rigor and the V231L mutant was unable to recruit microtubules from solution. These contrasting results suggest that AD-HSP may be caused by a variety of different mechanical deficits in the kinesin catalytic core. We will report the results of our ongoing studies to parse the deficits involved in each of the AD-HSP-causing mutations in Kif5A.

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The ubiquitous kinesin KIF5B binds light chain heterodimers in human cells.

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Conventional kinesin (Kinesin-1a) is a ubiquitously expressed molecular motor transporting a variety of cargoes towards microtubule plus-ends. However, the molecular details of cargo discrimination and destination choice are poorly understood. The Kinesin 1a holoenzyme consists of two heavy chains (KIF5B) and two regulatory light chains encoded by one of four kinesin light chains (KLC) genes, each encoding multiple splice variants. Whether the cellular ensemble of Kinesin-1a is associated only with KLC homodimers, or also heterodimers, impacts the diversity of motors in the cell and how they might be regulated. To study this question in detail, we combined the sensitivity of reciprocal affinity-purification mass spectrometry (rAP-MS) with the advantages of recombinant bacterial artificial chromosome (BAC) transgenes because these vectors contain native non-coding cis-regulatory information to express proteins at wild-type levels. Western blot analysis showed that stable HeLa lines express native levels of KIF5B and the correct KLC isoforms. Recombinant KIF5B and KLC1 also bind light membranes by density-gradient centrifugation, indicating the tagged proteins bind cargo. As a final demonstration of biochemical function, all 4 KLCs are captured by recombinant KIF5B and all recombinant KLC transgenes capture endogenous KIF5B by AP-MS. We found that recombinant KLC1 selectively captures endogenous KIF5B and a short, endogenous KLC1 isoform. Systematic AP-MS using all tagged KLCs further revealed that only KLC3, the most divergent member of this protein family, is uniquely homodimeric in the context of the Kinesin-1a holoenzyme. This study demonstrates that asymmetric Kinesin-1a motors, containing different light chains, exist in cells expressing multiple light chain genes. Although not all species contain multiple KLC genes, we propose that Kinesin-1a light chain asymmetry is a general phenomenon in mammals and highlights a previously unexplored regulatory mechanism in cells.

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Molecular mechanisms of kinesin-8 regulation in budding yeast.

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Accurate segregation of the duplicated genetic material during mitosis not only requires the essential load-bearing bridge between kinetochores and microtubules, but also elements that fine-tune microtubule dynamics and spindle behavior. While none of the four nuclear kinesins in yeast is individually required for viability, nevertheless several important mitotic functions are performed by these motors. The family of kinesin-8 motors combines plus-end directed motility with depolymerization activity to provide length-dependent regulation of microtubules (Varga et al., 2006). Recent studies have indicated important roles for the non-motor carboxyterminal domain of the yeast kinesin-8 Kip3 in allowing full motor activity (Su et al., 2011). How the cell spatially and temporally coordinates the activities of microtubule polymerases and depolymerases to allow spindle morphogenesis remains an important open question.

We show here that mutations within the carboxyterminal tail of the yeast kinesin-8 Kip3 have differential effects on motor activity. While large truncations of the c-terminal domain interfere with dimerization and behave similar to Kip3 deletion alleles, truncations of the extreme carboxyterminus have the opposite effect and lead to increased benomyl-sensitivity and shortened anaphase spindles in vivo. This suggests that this domain has an important role in

restricting Kip3 activity. Interestingly, several cell-cycle dependent phosphorylation sites map to this region, suggesting a possible mode of regulation. We will present biochemical and cell biological experiments to gain further insights into the regulation of a major microtubule-depolymerizing enzyme in the cell.

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Huntington-associated phosphorylation of kinesin-1 enhances autoinhibition in a phosphomimic.

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One of the consequences of the triplet expansion in Huntington's disease is inhibition of fast axonal transport (FAT). Phosphorylation of Ser176 in human kinesin-1 by JNK3 has been implicated in this inhibition (Morfini, et al., *Nature Neuro*. 12, 866 (2009)). To investigate the molecular basis for the inhibition of FAT, we have generated the S182E phosphomimic of the homologous residue in *Drosophila* kinesin-1. The S182E mutation produces only a modest 30% decrease in the maximum rate of microtubule-stimulated ATPase rate for a short dimer of motor domains, indicating that direct inhibition of MT-stimulated ATPase activity is not likely to be the cause of the pronounced inhibition of FAT. However, free kinesin is known to be autoinhibited through the binding of a tail domain to a dimer of motor domains (heads) and the Ser182 phosphorylation site is near the tail binding site on the heads (Kaan, et al., *Science* 33, 883 (2011)) where it could influence autoinhibition. One possibility is that the increased negative charge on the heads due to phosphorylation of Ser182 could produce a stronger interaction with the positively charge tail domain that would strengthen autoinhibition and inhibit FAT. To test the effect of the phosphomimic on autoinhibition, the kinetics of binding for a monomeric tail domain to a dimer of motor domains was determined using a FRET assay. In 100 mM KCl, the binding rates were 61 and 117 $\mu\text{M}^{-1}\text{s}^{-1}$ and the dissociation rates were 65 and 35 s^{-1} for the wild type and phosphomimic respectively. These rates predict K_d values for the tail-motor domain interaction of 1.1 and 0.3 μM for the wild type and mutant respectively, indicating that the phosphomimic motor domains bind tail domains 3-fold more tightly. The increased affinity of tails for motor domains in the phosphomimic suggests that reversal of autoinhibition is more difficult for phosphorylated kinesin and that this may be a significant factor in inhibition of FAT.

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KIF17 activity is regulated by its C-terminal tail domain and by EB1.

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KIF17 is a kinesin-2 family, plus-end directed microtubule (MT) motor that interacts with EB1 and APC and contributes to MT stabilization and polarization in epithelial cells. To gain insight into the mechanism by which KIF17 participates in MT stabilization, and to elucidate the functional significance of its interaction with EB1, we analyzed KIF17 activities in the absence or presence of EB1 *in vitro*. Purified, full-length EB1 enhanced the steady-state MT-stimulated ATPase activity of KIF17 motor domain 2.0 fold and increased the number of motility events as observed in MT gliding assays. This EB1-mediated increase in ATPase and MT gliding activity was abrogated by addition of purified KIF17 Tail domain, consistent with a role of head-tail interactions in regulating kinesin activity. Interestingly, we found EB1 and the KIF17 tail domain compete for binding to the KIF17 catalytic motor domain. In two *in vitro* assays, the KIF17 catalytic domain was sufficient to protect MTs from depolymerization. This MT stabilizing activity was also enhanced by EB1 but was unaffected by the KIF17 tail domain. Consistent with *in vitro* MT stabilization assays, we also found that overexpressed KIF17 motor domain was sufficient to stabilize MTs in MDCK epithelial cells. As expected, the KIF17 motor domain co-sedimented with MTs. Unexpectedly however, we also found that the KIF17 tail domain co-sediments with MTs and indeed, bound with higher affinity to MTs than the motor domain. This suggests that the KIF17 tail, in addition to regulating the ATPase activity of the motor, may also contribute to binding of the holoenzyme to MTs. Furthermore, the KIF17 tail domain also interacts with soluble tubulin heterodimers, suggesting this kinesin could potentially regulate MT dynamics by dual mechanisms in cells. In support of this, we also found that expression of the KIF17 tail domain in cells transiently attenuated MT regrowth after washout of MT depolymerizing drugs, possibly by sequestering tubulin dimers. The emerging role of various kinesins in disease raises the possibility of using them as targets for treatment and therapy but very little is known about the intrinsic biochemical properties of each kinesin contribute to determining patterns of motility and patterns of microtubule dynamics within eukaryotic cells. Our result suggested some unique control of KIF17 motor and tail domain over MT growth and stabilization needs further research to explore its novel regulation in microtubule dynamics.

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Src kinase regulates the human kinesin-5, Eg5, by phosphorylating tyrosines in the motor domain of Eg5.

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The human kinesin-5, Eg5, is required to establish and maintain the mitotic spindle. While Eg5 localization is regulated by phosphorylation and protein-protein interactions, it is unclear if any endogenous cell mechanisms directly regulate the motor's activity. Using *in silico* and *in vitro* methods, we show that the Eg5 motor domain contains a previously unidentified SH3-binding motif that allows the Src kinase to phosphorylate tyrosine residues near the nucleotide pocket of Eg5. Characterization of phosphomimetic and non-phosphorylatable Eg5 mutant proteins reveals that two of these phosphorylation events slow ATPase activity and microtubule sliding. Src phosphorylation of Eg5 represents a novel regulatory mechanism for a human kinesin, which may be of particular interest as both Src and Eg5 are antimitotic drug targets.

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Protein kinase Darkener of Apricot and microtubule-binding protein EF1- γ are global regulators of transport along microtubules.A. S. Serpinskaya¹, L. Rabinow², V. I. Gelfand¹;¹Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, IL,²Centre de Neurosciences de Paris Sud, Université Paris Sud 11, Orsay Cedex, France

Regulation of organelle transport along microtubules is important for proper distribution of membrane cargo in the cytoplasm. We used RNAi-mediated knock-down in cultured *Drosophila* S2 cells to study the role of microtubule-binding proteins in this regulation. Our results demonstrate that two microtubule-binding proteins, a unique isoform of Darkener of apricot (DOA) protein kinase and its substrate, translational elongation factor-1 γ (EF1 γ) negatively regulate transport of several classes of membrane organelles along microtubules. Inhibition of transport by EF1 γ requires its phosphorylation by DOA on Serine 294. Together, our results indicate a new role for two proteins that have not been previously implicated in regulation of the cytoskeleton and suggest that the biological role of some of the proteins that bind to microtubule track is to mediate cargo transport along these tracks.

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The microtubule-binding protein ensconsin is an essential cofactor of kinesin-1.K. Barlan¹, V. I. Gelfand¹; ¹Cell and Molecular Biology, Northwestern University, Chicago, IL

Kinesin-1 is a major microtubule motor that drives transport of a multitude of cellular cargoes toward the plus-ends of microtubules. In the cell, kinesin-1 exists primarily in an inactive, autoinhibited state, and motor activation is thought to occur upon binding to cargo or adaptors through the C-terminus. Using RNAi-mediated depletion in *Drosophila* S2 cells, we demonstrate that kinesin-1 requires ensconsin (MAP7, E-MAP-115), a ubiquitous microtubule-associated protein, for its primary function of organelle transport. A “hingeless” mutant of kinesin-1, which mimics the active conformation of kinesin-1, does not require ensconsin, suggesting that ensconsin acts to promote unfolding of the autoinhibited motor on the microtubule. Surprisingly, an ensconsin truncation that cannot bind microtubules is sufficient to activate organelle transport by kinesin-1, indicating that this activating domain functions independently of microtubule binding. Our study demonstrates that cargo-binding alone is insufficient to activate kinesin-1 for transport and indicates that ensconsin is an essential cofactor for kinesin-1 in organelle transport.

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Kinesin as a Small G-protein, with CK2 as an Exchange Factor.M. K. Mattson¹, Y. Jun¹, S. K. Tripathy¹, B. J. Reddy¹, A. Strawn¹, S. P. Gross¹; ¹Developmental and Cell Biology, University of California, Irvine, Irvine, CA

The kinesin motor protein is required for proper function and organization of the cell; loss of function leads to neurodegeneration and death. Currently, the dominant model for how kinesin is regulated involves head-tail auto-inhibition, which is believed to be relieved upon cargo binding. However, cellular cargos can change motion (and force production) without a change in the amount of cargo-bound kinesins, suggesting the existence of additional kinesin regulatory mechanisms. Recently, Xu et al. discovered that kinesin inactivates over time and activity can be rescued with treatment of the signaling kinase casein kinase 2 (CK2), but the mechanism was unknown. Here, we provide a mechanistic understanding of kinesin's inactivation. Kinesin

shares structural homology with small G-proteins, and we find that when inactive, kinesin is in a previously unknown stable ADP-bound state, and unable to release the ADP. Methods used to characterize GEFs were modified for use with kinesin, and nucleotide movement was monitored. We find that CK2 promotes release of ADP, essentially acting as a nucleotide exchange factor for kinesin. Because this process requires only the kinesin head domain, with many family members share structural homology, this mechanism will likely turn out to be quite general, with potentially different exchange factors contributing to regulation of different motors.

Actin and Actin-Associated Proteins II

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The role of the actin-bundling protein fascin in cell motility.

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Fascin is an actin binding protein that cross-links filamentous actin into tightly packed parallel bundles. In normal mammalian tissues, it is the most highly expressed in neuronal and glial cells, microcapillary endothelial cells, and antigen-presenting dendritic cells but is largely absent or present in very low levels in epithelial cells. After the initial observation that GFP-fascin is very highly localized to filopodia, fascin has been the most intensely studied within the framework of filopodia dynamics.

More recently, it has been established that high fascin expression is correlated with poor prognoses and high rates of metastases in many cancers. Because fascin is highly expressed in cancer cells but not in normal epithelia, it is emerging as an attractive novel therapeutic target. Although it has been shown that drugs targeting fascin are able to suppress metastasis, very little is known about the exact nature of the relationship between fascin and cell motility.

To study the role of fascin in cell motility, we use PTK-1 as well as HCT116 and HT29 colon cancer cell lines. HCT116 expresses fascin at high levels while HT29 expresses very little fascin. In a wound healing assay, the migration rate of HCT116 was fourfold larger than that of HT29. In a transwell migration assay, the migration rate of HCT116 was also higher, by a factor of 2.5, than the one of HT29. This difference could be partially reduced by knocking down fascin in HCT116 with shRNA, confirming fascin's central role as a positive regulator of cell motility.

We then employed high-resolution imaging of PTK-1 and HCT116. From movies of cells expressing fluorescent proteins or fluorescently-tagged cells, we are able to segment a cell, define probing windows that dynamically adapt to the changing shape of a migrating cell, and thus follow local variations in protein recruitment relative to the local cell motion. Such analyses allowed us to positively correlate fascin localization with protruding lamellipodia, suggesting that fascin may play essential roles in regulating actin dynamics outside filopodia. Further experiments showed that increased fascin expression may be correlated with increased maximum protrusion velocity and decreased total retraction distance in both cell lines. This could be interpreted as fascin bundling increasing the mechanical persistence of the entire protrusive actin network structures. We are currently consolidating these initial data and continue to systematically probe non-filopodia related functions of fascin.

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Actin crosslinking by *V. cholera* ACD toxin: kinetic parameters and the role of high-energy glutamyl-phosphate intermediate.*E. Kudryashova*¹, *C. Kalda*¹, *D. S. Kudryashov*¹; ¹*Chemistry and Biochemistry, Ohio State University, Columbus, OH*

Actin Crosslinking Domain (ACD) is produced by several life-threatening Gram-negative pathogenic bacteria (*V. cholerae*, *V. vulnificus*, and *A. hydrophila*) as an effector domain in larger MARTX and VgrG toxins. ACD together with other effector toxins contributes to the inactivation of macrophage cells, intestinal inflammation, and bacterial colonization of intestinal epithelium. Upon intracellular delivery, ACD disrupts the actin cytoskeleton by irreversible covalent crosslinking of cytoplasmic actin. At the molecular level, ACD-catalyzed formation of an intermolecular amide bond between E270 and K50 residues of actin (Kudryashov, D.S. (2008) Proc. Natl. Acad. Sci. USA 105, 18537-18542) leads to the accumulation of polymerization-deficient actin oligomers, ultimately resulting in structural and functional failure of the actin cytoskeleton in affected cells.

In the present study, we determined the pH optimum for the reaction catalyzed by MARTX ACD, measured the kinetic parameters of the enzyme for both of its substrates (actin and ATP), and deciphered the role of the nucleotide in the ACD catalyzed actin crosslinking.

The optimal pH for ACD activity was in the range of 7.0-9.0. We established that similar to ATP, GTP also supports the crosslinking reaction by ACD, but the kinetic parameters ($K_M=8\mu\text{M}$ and $50\mu\text{M}$ for ATP and GTP, respectively) suggest that ATP is the primary substrate of ACD *in vivo*. ACD showed sigmoidal, non-Michaelis-Menten kinetics for actin ($K_{0.5}=30\mu\text{M}$) reflecting involvement of two actin molecules in a single crosslinking event. Furthermore, we found that the actin crosslinking reaction proceeds via formation of a high-energy acyl-phosphate intermediate. First, the γ -phosphoryl group of ATP is transferred to the E270 residue of actin resulting in the formation of an activated glutamyl phosphate derivative. Subsequently, this high-energy intermediate is hydrolyzed and the energy of hydrolysis is utilized for the formation of the amide bond between actin subunits. The elucidated kinetic mechanism of ACD toxicity adds to understanding of complex mechanisms of host-pathogen interactions.

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Electron microscopy and 3D reconstruction reveals Filamin Ig-domain binding to F-actin.*W. Suphamungmee*¹, *F. Nakamura*², *J. Hartwig*², *W. Lehman*¹; ¹*Department of Physiology & Biophysics, Boston University School of Medicine, Boston, MA*, ²*Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, Boston, MA*

Filamin A (FLNa) is an actin-binding protein that cross-links F-actin into networks of orthogonally branched filaments. FLNa also directs the networks to integrins while responding to mechanochemical signaling pathways. Elongated, 80 nm long FLNa molecules are subunits of a dimer and contain an N-terminal calponin-homology/actin-binding domain connected by a series of 24 immunoglobulin (Ig) repeats to a dimerization site at their C-terminal end. Whereas the CH-homology domains' contribution to F-actin affinity is weak ($K_a \sim 10^5$), the binding of the intact protein to F-actin is strong ($K_a \sim 10^7$), suggesting involvement of additional parts of the molecule in this association. Indeed, previous results indicate that Ig-repeats along FLNa contribute significantly to the strength of the actin filament interaction. In the current study, we have used electron microscopy and three-dimensional reconstruction to elucidate the structural basis of the Ig-repeat – F-actin binding. We find that FLNa density is clearly delineated in reconstructions of F-actin complexed with either a 4 Ig-repeat segment of FLNa containing Ig-repeat 10 or with IgFLNa10 alone. The mass attributable to IgFLNa10 lies peripherally along the actin-helix over the N-terminus of actin subdomain 1. The interaction appears to be specific,

since no other fragment of the FLNa molecule or individual Ig-repeats examined, besides ones with CH-domains or IgFLNa10, decorated F-actin filaments or were detected in reconstructions. We conclude that the combined interactions of CH-domains and the IgFLNa10 repeat provide the binding strength displayed by the whole FLNa molecule and propose a model for the association of filamin on actin filaments.

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New Structural Units in the Actin Cross linking Protein Filamin C.

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Dynamic nature of actin cytoskeleton is essential for cell division, adhesion, migration, signal transduction and various other cellular processes. The nucleation, polymerization and organization of the actin filament network is mediated by a number of proteins. Filamins are actin cross-linking proteins composed of two 290 kDa subunits. Each subunit is composed of N terminal actin binding domain followed by a flexible rod region containing 24 Immunoglobulin-like domains. We have earlier found that in the C-terminal rod-2 part of filamin there are three closely interacting pairs of immunoglobulin-like domains. Two of these domains pairs can serve as mechanically regulated binding sites for Integrins, other transmembrane receptors and for cytoplasmic adaptor proteins. This provides a way how mechanical contraction of actomyosin cytoskeleton can regulate cell adhesion and signaling. To find out whether other areas of Filamins have similar structures we expressed all possible two immunoglobulin-like domain fragments of the rod-1 region from human Filamin C. We found three compact two-domain pairs. Of these, we have crystallized the fragment consisting of immunoglobulin domains 4 and 5. This fragment shows a novel type of domain-domain interaction, where the two domains form a loop-like structure with large interaction surface. While the previously characterized domain pairs of the Filamin rod 2 region interact through the edge of a beta-sheet, the domains 4-5 interact face-to-back along their beta-sheets. This kind of structure could be easily opened by mechanical force and we are currently investigating if this provides a new functional site in Filamins.

1030

Visualizing the exposure of filamin A's cryptic binding sites in living cells.

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Filamin A (FLNA) is a ubiquitous actin-crosslinking and scaffold protein consisting of 24 Ig-like repeats that bind and modulate multiple intracellular signaling effectors. Structural studies of repeats 19-21 of FLNA indicate that the integrin-binding site on repeat 21 is sterically masked by strand A of repeat 20, and that mechanical/chemical stimuli are required to unmask this cryptic site. We have previously shown that mechanical straining of actin-FLNA networks *in vitro* induces a conformational change in FLNA that successfully exposes its integrin binding site. Here we have engineered a novel photoquenching fluorescence resonance energy transfer (PQ-FRET)-based system (sensor cassette) that reports on this conformational change in cells. Fluorescence emission of the monomeric enhanced green fluorescent protein (mEGFP) is diminished by ~ 73% when the quencher, sREACH, is attached to its C-terminus. Insertion of the two tandem FLNA repeats 1-2 (and even only one repeat) between the FRET pair obliterates this quenching. However, insertion of the FLNA repeats 20-21, generates a structure that quenches mEGFP fluorescence intensity by ~50%, confirming the repeats to posit the probes on the two molecule ends near one another. We then expressed recombinant FLNA containing RFP as an internal control and the FRET pair sensor cassettes in HEK-293 and COS-7 cells. We tested sensor cassettes containing either the "stretchable" FLNA repeats (20-

21) or the control repeats (1-2) between the FRET pairs. Ratio imaging (mEGFP vs RFP) of FLNA molecules in cells having the stretch-detecting repeat 20-21 cassette report that the delivery of FLNA to F-actin enriched regions of cells and the mechanical opening of FLNA molecules can occur in separate steps. By contrast, no changes in fluorescence intensity were observed in FLNA molecules carrying the control sensor cassette (containing repeats 1-2). Time-lapse cell imaging further demonstrates that opening the cryptic binding site is associated with motile activity at the leading edge of cells. These data confirm that FLNA-mediated mechanotransduction occurs in motile cells and that the stretch-detecting PQ-FRET probe is capable of revealing important conformational changes.

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Catch bond behavior of alpha-actinin in vivo.

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Our cells are subjected to an incredible variety of static and dynamic forces; the correct chemical and mechanical responses of cells to these stimuli are essential, and when incorrect can cause medical disorders. The actin cytoskeleton's ubiquitous nature and connectivity enables it to sustain & transmit forces rapidly over large distances, implicating it in these physiological and pathological responses. At the core of actin mesh dynamics are actin-binding proteins, which can crosslink the network and set its mechanical properties. Here we find that the actin-binding protein, alpha-actinin (ACTN4), an essential crosslinker displays unique behavior under stress in vivo; under stress, ACTN4 binds more tightly, which is in stark contrast to the behavior of most binding interactions. To study this behavior, we transfect GFP tagged ACTN4 into cells cultured on deformable substrates with Young's moduli of 1-30 kPa. Cell spreading and internal stresses increase on stiffer materials, and we use this to stress the cells as a function of substrate stiffness. Using Fluorescence Recovery After Photobleaching (FRAP), we demonstrate that ACTN4 binds an order of magnitude longer to actin under greater stress. Additionally, we impose an external strain on the substrate, which stretches the cells a precise amount, confirming the strain-mediated increase in binding affinity. We hypothesize that this strain-dependent binding is mediated by the CH1 & CH2 domains opening and either exposing an additional actin binding site, or by removing a steric hindrance. We verified the specificity of the strain-dependent binding with a series of ACTN4 point mutations which constitutively open or close the CH1-CH2 conformations, and disrupt the putative additional actin binding site. These results may have immediate bearing on renal medical disorders; we find that in kidney disease (Focal segmental glomerulosclerosis) an ACTN4 variant (K255E), is a severe disease causing mutation. In vivo K255E binds substantially longer than the wildtype, suggesting that interrupting or increasing ACTN4's strain dependent binding may underlie certain diseases.

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CAS-2, a second cyclase-associated protein isoform in *C. elegans*, regulates ADF/cofilin-dependent actin filament dynamics in an ATP-dependent manner.

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Cyclase-associated protein (CAP) is a conserved actin-monomer binding protein that regulates actin dynamics. In particular, CAP enhances actin filament turnover in the presence of ADF/cofilin. In multicellular organisms, multiple CAP isoforms are expressed in a tissue-specific manner, but their functional differences are not clearly understood. Mammals have CAP1 (a non-muscle isoform) and CAP2 (a muscle isoform). Recently, we have reported that CAS-1 is a muscle isoform of CAP in *C. elegans* and is essential for sarcomeric actin organization in

striated muscle (Nomura et al., 2012). In addition, *C. elegans* has CAS-2, a second isoform of CAP, which remains uncharacterized. CAS-2 is 41 % identical to CAS-1. *cas-2*-null worms were homozygous viable and showed disorganized actin cytoskeleton in the distal gonad. Although we have been unsuccessful in localizing the CAS-2 protein in worms, gonadal expression of *cas-2* mRNA has been demonstrated in the NextDB *in situ* hybridization database (National Institute of Genetics, Mishima, Japan) and is consistent with the observed phenotypes. These results strongly suggest that CAS-2 is a non-muscle CAP isoform in *C. elegans*. To understand the actin-regulatory function of CAS-2, we produced recombinant CAS-2 protein and characterized its biochemical properties *in vitro*. CAS-2 bound to G-actin without strong preference to either ATP-actin or ADP-actin and sequestered it from polymerization when CAS-2 was present at high concentrations. CAS-2 strongly enhanced the rate of exchange of actin-bound ATP/ADP. Although UNC-60A, a non-muscle ADF/cofilin isoform, inhibited exchange of actin-bound nucleotides, substoichiometric concentrations of CAS-2 was able to enhance nucleotide exchange in the presence of UNC-60A. The C-terminal half of CAS-2 was necessary and sufficient for G-actin binding and enhancement of nucleotide exchange and had nearly as strong activities as the full-length CAS-2 protein. UNC-60A promoted depolymerization of F-actin and inhibited polymerization. However, substoichiometric concentrations of CAS-2 antagonized UNC-60A and promoted actin polymerization in the presence of ATP but not in the presence of ADP or in the absence of nucleotides. These results suggest a role of CAS-2 to load ATP onto UNC-60A-bound ADP-actin to promote their dissociation and a new round of actin polymerization and depolymerization.

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Swiprosin-1 modulates membrane dynamics by regulating cofilin accessibility to F-actin.

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Membrane protrusions, like lamellipodia, and cell movement are dependent on actin dynamics, which are regulated by a variety of actin binding proteins acting cooperatively to reorganize actin filaments. Here we provide evidence that Swiprosin-1, a recently identified actin bundling protein, modulates lamellipodia formation and cell motility by regulating the accessibility of F-actin to cofilin. Overexpression of Swiprosin-1 increased lamellipodia formation and cell motility in B16F10 melanoma cells, whereas knockdown of Swiprosin-1 inhibited EGF-induced lamellipodia formation and cell motility, and led to a loss of actin stress fibers at the leading edges of cells but not in the cell cortex. Swiprosin-1 strongly facilitated the formation of F-actin that was closely packed into parallel or entangled bundles, which made the F-actin inaccessible of cofilin. Taken together, these findings suggest that Swiprosin-1 is a novel molecule that regulates actin dynamics by modulating the pattern of cofilin activity at the leading edges of cells.

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Cofilin uses intrinsic zones of instability within F-actin to disrupt the actin filament.

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Actin plays a major role in many cellular processes including cell motility, cell division, endocytosis, and exocytosis. Recently, we showed that frozen-hydrated actin filaments contain a multiplicity of different structural states. We also reported an unconventional structural state of actin (tilted actin or T-actin) where the interaction between the actin protomers weakens

compared to “canonical” F-actin. To evaluate the role of T-actin in the stability of the actin filament we analyzed the structural composition of individual frozen hydrated filaments. We show that within regions of actin filaments enriched with T-actin the majority of neighboring actin protomers have their subdomain 2 completely disordered. The coupling in the spatial distributions of the two structural modes that possess less connectivity between subunits suggests an existence of intrinsic zones of instability within the actin filament. Cofilin/ADF proteins depolymerize actin filaments to maintain the pool of monomeric actin. To evaluate the role of the proposed intrinsic zones of instability within the actin filaments in presence of ADF/cofilin proteins we analyzed individual frozen hydrated actin filaments partially decorated with cofilin-2. We show that the bare zones between the regions of F-actin occupied by cofilin are structurally similar to the intrinsic zones of instability found in pure F-actin. The weakening of the contacts between actin subunits in those zones reaches its maximum at 50%-75% occupancy by cofilin-2. Our data show that at substoichiometric concentrations ADF/cofilin proteins boost the frequency of intrinsic zones of instability within the actin filament. We provide a structural explanation for the maximal depolymerization activity of ADF/cofilin proteins when bound at low stoichiometry to actin filaments.

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Mammalian CAP1 (adenylyl Cyclase-Associated Protein 1) Regulates Cofilin function, the Actin Cytoskeleton and Cell Adhesion.

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Background: CAP (adenylyl Cyclase-Associated Protein) was first identified as a yeast protein that participates in the regulation of both the actin cytoskeleton and the Ras regulation of cAMP. While the role in Ras signaling does not extend beyond yeast, CAP regulates actin in all eukaryotes. *In vitro* biochemical assays and knockdown experiments have shown that mammalian CAP1 and its yeast homologue facilitate cofilin-driven actin filament turnover. However, it remains unclear whether mammalian CAP participates in other cellular functions.

Methods and Results: We efficiently knocked down CAP1 in HeLa cells using stable knockdown with vector-based shRNA constructs. Imaging and fractionation assays show that depletion of CAP1 led to larger cell size, cell protrusions and accumulation of filamentous (F) actin. We suggest that loss of CAP1 function in sequestering actin monomers is responsible for the actin filament phenotype. Moreover, we unexpectedly found that CAP1 knockdown led to activation of FAK (Focal Adhesion Kinase) and enhanced cell spreading. We further demonstrated that CAP1 forms complex with adhesion molecules FAK and Talin, which may explain the increases in adhesion and spreading of the CAP1 knockdown cells. Knockdown of CAP1 in HeLa cells also led to substantially elevated cell motility and invasion through Matrigel. Finally, our studies showed biochemical and functional interactions between CAP1 and cofilin as well as the underlying mechanisms; CAP1 depletion in cells caused cofilin dephosphorylation and accumulation into aggregates, and CAP1 facilitates cofilin phosphorylation by LIM kinase in *in vitro* biochemical assays. We also mapped cofilin binding to the N-terminus of CAP1 by GST-cofilin pull-down. In summary, in addition to further establishing the role of mammalian CAP1 in actin dynamics, we identified a novel cellular function for CAP1 in regulating cell adhesion signaling.

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Cortactin directly targets nascent actin filament branch junctions to synergize with N-WASp and activate branching nucleation by Arp2/3 complex.*L. Helgeson¹, A. Wagner¹, B. Nolen¹; ¹Institute of Molecular Biology, University of Oregon, Eugene, OR*

Arp2/3 complex is a critical actin regulatory protein that nucleates branched actin filaments in response to cellular signals. Cortactin has been shown to regulate Arp2/3 and is required for assembly of actin filaments in podosomes and invasive actin structures in cancer cells called invadopodia. Cortactin acts synergistically with WASp/Scar family proteins, the prototypical Arp2/3 complex activators, to dramatically increase branching nucleation by the complex. Cortactin has an actin filament binding domain and no actin monomer binding region, suggesting it uses a distinct mechanism of activation compared to WASp/Scar proteins. Despite its critical role in regulating actin, how cortactin synergistically activates the Arp2/3 complex is unknown. Here we show that neither the previously proposed actin filament recruitment model nor the WASp displacement/cycling model can account for cortactin-mediated synergy. Instead, our data supports an obligatory displacement model in which cortactin actively displaces N-WASp from nascent branch junctions as a prerequisite for branching nucleation. Our data show that the oligomerization state of N-WASp, the number of actin monomer binding regions it harbors, and its affinity for actin monomers (and Arp2/3 complex) are all critical determinants in synergy. We find that the N-terminal acidic (NtA) region of cortactin alone is sufficient for synergy. By mapping regions of NtA required for synergy we show that it is functionally distinct from the acidic region of N-WASp. In contrast to previously published data, we demonstrate that high concentrations of cortactin do not inhibit branching. Using single-molecule TIRF microscopy we show that cortactin has a 150-fold preference for binding branches over filament sides, explaining how nascent branches are directly targeted by cortactin, a prediction of the obligatory displacement model. Using kinetic rate constants derived from our single molecule studies, we built mathematical models which show that an obligatory displacement model but not the cortactin-mediated WASp cycling or filament recruitment models can account for synergy observed in our bulk actin polymerization assays. These results have important implications for understanding how cortactin cooperates with other Arp2/3 complex regulators to control branching nucleation.

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Arg/Abl2 modulates the affinity and stoichiometry of cortactin binding to F-actin.*S. M. MacGrath¹, A. J. Koleske²; ¹Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, ²Molecular Biophysics and Biochemistry, Interdepartmental Neuroscience Program, Department of Neurobiology, Yale University, New Haven, CT*

The Abl family nonreceptor tyrosine kinase Arg/Abl2 interacts with cortactin, an Arp2/3 complex activator, to promote actin-driven cell edge protrusion. Both Arg and cortactin bind directly to filamentous (F-) actin. While protein:protein interactions between Arg and cortactin have well-characterized downstream effects on the actin cytoskeleton, it is unclear whether and how Arg and cortactin affect each other's actin binding properties. We employ actin cosedimentation assays to show that Arg increases the stoichiometry of cortactin binding to F-actin at saturation. Using a series of Arg deletion mutants and fragments, we demonstrate that the Arg C-terminal calponin homology (CH) domain is necessary and sufficient to increase the stoichiometry of cortactin binding to F-actin. We also show that interactions between Arg and cortactin are required for optimal affinity between cortactin and the actin filament. In summary, our data suggest a mechanism for Arg-dependent stimulation of cortactin binding to F-actin, which may facilitate cortactin recruitment to sites of local actin network assembly.

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Effects of actin binding proteins on polarizing cortical flow mechanics.

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The establishment of cell polarity is crucial for many developmental processes. In *C. elegans* one-cell embryos, cellular polarization and anteroposterior axis establishment is facilitated by actomyosin cortical flows (Mayer M *et al.*, 2010, Goehring N *et al.*, 2011). Flows proceed on scales that are comparable to the size of the cell. Describing flows requires the identification of mesoscale physical properties of the actomyosin cytoskeleton, such as active stresses, active torques, 2D viscosity of the cortex and friction with the cell membrane and the intracellular cytosol (Mayer M *et al.*, 2010). However, how large-scale actomyosin flow and associated physical properties depends on molecular functions of actin binding proteins (ABPs) and other cortex-associated proteins remains unknown. Here, we devised a candidate RNAi screen of ABPs and inferred the molecular regulation of large scale flow by performing a systematic comparison to a mesoscale hydrodynamic description of the cortex. Interestingly, our candidate-based RNAi screen yielded only a reduced spectrum of flow phenotypes. This points towards a type of mechanical redundancy, with diverse molecular mechanisms affecting a limited number of mesoscale physical properties. In summary, by applying the hydrodynamic model on molecular perturbation experiments performed on a developing *C. elegans* embryo, we reveal the molecular links that give rise to emergent flow properties of the actomyosin cortex.

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Slings enable leukocyte rolling during inflammation.

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Leukocyte recruitment to the sites of inflammation involves leukocyte rolling along the inflamed venules. Rolling is mediated by P-selectin on the endothelium binding to P-selectin glycoprotein ligand-1 (PSGL-1) constitutively expressed on leukocytes. Most leukocytes can roll along the walls of venules at low shear stress (1 dyn/cm²), but neutrophils have the ability to roll at 10-fold higher shear stress in microvessels in vivo. Although the shear-resistant neutrophil rolling is known to be facilitated by cell flattening and pulling of long membrane tethers, the mechanisms that enable neutrophil rolling during inflammation are poorly understood. Isolated mouse bone marrow neutrophils stained with an intercalating membrane dye (Dil or DiO) were allowed to roll on a P-selectin (20 molecules/μm²) coated cover slip in a microfluidic device at a shear stress of 6 to 10 dyn/cm² and footprints recorded using dual-color quantitative dynamic footprinting (DqDF). Epifluorescence intravital microscopy was used to observe rolling of neutrophils in the mice cremaster venules. As anticipated, rolling neutrophils formed long membrane tethers (> 10 μm) at the rear. However, following detachment these long tethers did not retract as postulated, but instead persisted and appeared as 'slings' at the front of rolling cells. Rolling neutrophils formed slings in a model of acute inflammation in vivo and on P-selectin in vitro, where PSGL-1 was presented as discrete sticky patches (1.6 μm apart) while LFA-1 was expressed over the entire length on slings. As neutrophils rolled forward, slings wrapped around the rolling cells and underwent a step-wise peeling from the P-selectin substrate. As each PSGL-1 patch failed, the rolling neutrophil was unable to accelerate because a new downstream patch on the same sling was already lined up and became load-bearing. LFA-1 on the sling interacted with inter-cellular-adhesion-molecule (ICAM)-2 on the neutrophil surface in trans to facilitate wrapping of slings and blocking this interaction resulted in less efficient wrapping and thus, reduced rolling velocity

on P-selectin substrate. The discovery of slings provides a mechanism by which neutrophils rolling at high shear stress pave their own way for enhanced rolling. Besides stabilizing rolling, slings are unique structures that also enable rolling neutrophils to present LFA-1 to its ligand ICAM-2 in trans. This study was supported by the NCRP-Scientist Development Grant 11SDG7340005 from the American Heart Association (P.S.) and NIH EB02185 (K.L.).

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Degradation of E-cadherin by aspartyl proteases secreted by *Candida albicans* causes changes in F-actin dynamics and disruption of epithelial adherens junctions.

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Pathogenic *Candida albicans* invade human epithelia by a complex mechanism that allows them to penetrate the protective outer layer. E-cadherin has been identified as one of the epithelial cell receptors for the host-cell association with *C. albicans*. In a previous study, we showed that secreted aspartyl proteases (Saps) that contribute to the virulence of *C. albicans* affected E-cadherin-mediated cell-cell adhesions in cultures of human oral keratinocytes and decreased E-cadherin levels in a time-dependent manner during coculture with *C. albicans*. The mechanism by which *C. albicans* induces epithelial cell damage is incompletely understood but Saps most likely contribute to this process. To determine the effects on epithelial adherens junctions by individual Sap4-6, keratinocytes were treated with single *SAP4-6* and *SAP4-6*-deficient mutants. We used OKF6/TERT-2 cells, an immortalized cell line of human oral keratinocytes cocultured with hyphae-forming *C. albicans*. The cellular dynamics of proteins that form cell-cell adhesions was determined at defined time intervals by multi-mode high-resolution microscopy and quantitative image analysis.

OKF6/TERT-2 cells exposed to wild-type *C. albicans* showed that E-cadherin mediated cell-cell adhesions were compromised compared to untreated cultures. Coculture with *C. albicans* initially stimulated formation of cell-cell adhesions at early time intervals (0 – 6 hours). At longer intervals of coculture (12 – 24 hours) cell-cell adhesions began to disintegrate and were disrupted completely after 24 hours. Fluorescence intensity profiles illustrated both the dynamics of cell-cell adhesion proteins and their colocalization with the actin cytoskeleton. Exposure of OKF6/TERT-2 cells to individual tetracycline-inducible *SAP4-6* promoter strains and triple *SAP4-6* mutants were found to elicit attenuated changes in keratinocyte responses compared to those that were cocultured with wild-type *C. albicans*. Disruption of cell-cell adhesions by *C. albicans* was blocked by the anti-fungal drug fluconazole. In addition, affinity binding studies using GST-E-cadherin and purified Saps showed binding between these proteins, which resulted in the degradation of E-cadherin. We, therefore, conclude that the binding of Sap4-6 to epithelial E-cadherin caused its degradation and led to the simultaneous reorganization of the actin cytoskeleton. The degradation of E-cadherin by Sap4-6 may be important for *C. albicans* to invade epithelial surfaces by active penetration between epithelial cells.

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F-actin during mitosis: how spindle-associated F-actin contributes to mitotic spindle structure and function.

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Mitotic spindles are microtubule-based structures responsible for partitioning genetic information into each new daughter cell. The role of microtubules and microtubule-associated proteins in

this process has been well characterized, however whether or not F-actin is required—or is even present—within the spindle has long been controversial. In this work we examine spindle-associated F-actin in the intact *Xenopus laevis* epithelium. Imaging of fluorescently labeled phalloidin revealed F-actin at spindle poles, F-actin cables extending from poles towards the cortex, and F-actin cables spanning between poles within the spindle. Similar localization patterns are detected with gamma-actin antibodies. Live imaging experiments corroborated these results and highlighted the dynamic nature of these actin cables as they move within and around the spindle throughout mitosis. In addition, treatment with the formin inhibitor SMIFH2 resulted in a loss of spindle-associated F-actin and mitotic spindle defects, while cortical F-actin remained intact. Collectively, these results show that a) mitotic spindles in intact vertebrate epithelia contain a substantial amount of F-actin structures; b) these structures form coincident with spindle assembly, and; c) spindle F-actin, rather than cortical F-actin, may be essential for proper mitotic spindle structure and function.

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Drosophila dorsal closure as a model for actin regulation.

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From bundled myofibrils in muscle cells to dynamic filopodia and lamellipodia in migratory axons, proper development requires cells in different tissues, or cells within the same tissue at different times, to build distinct actin-based structures. Each structure presumably arises from different underlying actin geometries, created by the action of different suites and levels of actin regulators. We are using *Drosophila* morphogenesis, and in particular dorsal closure, as a model for understanding in-vivo mechanisms of actin regulation both within the same and across different tissue types. Dorsal closure is a highly dynamic process that results in enclosing the embryo in epidermis, through both the dorsalward migration of contralateral epidermal sheets, and apical constriction of central amnioserosa cells. These two cell types produce morphologically and dynamically distinct suites of actin-based protrusions. We are comparing the structure and dynamics of these protrusions, and identifying their underlying suite of actin regulators. We started by exploring the contribution and relationship of the actin elongation and filopodia promoting factors, Enabled (Ena) and Diaphanous (Dia), in formation and persistence of filopodia within and between these two cell types. Our preliminary data suggest that while both Ena and Dia can promote filopodia, the nature and dynamics of these filopodia differ significantly. Further, our data suggest that Ena binds and inhibits Dia in vitro, in cultured cells, and in vivo in the epidermis. While elongation factors are required to promote filopodia formation at the tip, the shaft is comprised of bundled actin. We thus are extending our assessment of the role of actin regulators by determining the requirement of actin bundling for morphology and persistence of filopodia, by removing zygotic fascin. Taken together these experiments will help us understand the mechanistic differences that drive these two very distinct sets of actin-based structures contributing to the same developmental process.

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Cell Adhesion Assemblies and the Origins of Multicellularity.

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The emergence of multicellular eukaryotes is one of the major transitions in the evolution of life. Multicellularity presented eukaryotic organisms with novel challenges. Principal among these are: (1) Cell Adhesion: How do individual cells in the multicellular organism interact with one another and their environment within the organism, and (2) Cell Signaling: How do cells

communicate with each other and sense their extracellular environment within the organism? Despite its fundamental importance in eukaryote evolution, relatively little is known about the origins of multicellular animals (Metazoa) at the cellular level. Completion of the genome sequences of several unconventional model organisms (e.g., Choanoflagellates, Basal Fungi, Apusozoa, and Filasterea) has provided the foundation for addressing this question. These model genomes have revealed that several adhesion/signaling complex proteins are ubiquitous, or nearly so, in close unicellular relatives of animals. These include talin, which is found in all unicellular taxa closely related to animals, and is essential for cell adhesion and cell motility in these organisms. Vinculin and integrin, which are physiological partners of talin in metazoans, are also present in these organisms. Given that talin, integrin, and vinculin have essential roles in cell adhesion and signaling in multicellular organisms, the identification of the molecular and cellular roles of these conserved proteins in unicellular relatives of animals may reveal fundamental information about the origin of multicellularity in the Metazoa. We have performed extensive phylogenetic and bioinformatic analyses of talin, vinculin, and beta-integrin from the choanoflagellates *Monosiga brevicollis* and *Salpingoeca rosetta*, the apusozoan *Thecamonas trahens*, the filasterean *Capsaspora owczarzaki*, and the basidiomycete *Spizellomyces punctatus* and *Allomyces macrogynus* and have identified the putative functional domains of these conserved, modular proteins. The phylogenetic affinities of these proteins are congruent with the conventional phylogenies of these taxa. Molecular and cellular analyses of these protein domains have revealed functional conservation, e.g., actin binding, between the proteins from these largely unicellular organisms thought to be representative of the immediate ancestor(s) of multicellular organisms and those from model metazoan organisms. While it is not possible to study the last common ancestor of unicellular organisms and animals and directly characterize the transition to multicellularity, the comparative approach described here should allow us to define the structure and function of the putative simple ancestral states of multicomponent adhesion assemblies such as the focal adhesion complex and the adherens junction. These results may then provide a foundation for understanding how the subsequent evolution of the extant complex derived state of these adhesion assemblies contributed to the emergence of multicellular organisms.

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Enabled antagonizes Actin Capping Protein function to regulate growth of motor nerves in the *Drosophila* embryo.

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During neural development, axons migrate to their targets by responding to guidance cues in the environment. In response to transmembrane receptor activation of signaling cascades, the growth cone of the axon builds actin-based cell protrusions including filopodia and lamellipodia that help determine the speed and direction of axon growth. Enabled (Ena), a member of the Ena/VASP/EVL family of proteins, promotes formation of filopodia *in vitro* and is essential for many aspects of neuron migration and axon guidance *in vitro* and *in vivo*. Ena has several biochemical activities including promoting actin polymerization, preventing capping of actin filaments, bundling actin filaments and anchoring actin to the cell cortex. In the *Drosophila* embryo, Ena is required for pathfinding of the Intersegmental Nerve b (ISNb) motor nerve. In *ena* mutants, the axons of this nerve fail to defasciculate from the Intersegmental Nerve after exiting the CNS, causing them to “bypass” their target muscles. Based on experiments in cell culture it has been hypothesized that the anti-capping function of Ena is particularly important

for motility, but this has not been tested in axon guidance *in vivo*. We manipulated levels of Ena and Actin Capping Protein (CP) to test this hypothesis. Our data suggest that suppressing CP activity is an essential aspect of Ena function in ISNb motor nerves. Using Fasciclin II as an axonal marker, we compared the nervous system defects in *ena* and *cp* single mutants with those in mutants deficient in Ena and with reduced Capping Protein. Strikingly, reducing the dose of *cp β* by half in *ena* mutants produces substantial rescue of the ISNb “bypass” defect. This supports the importance of the anti-capping function of Ena in promoting ISNb motor axon guidance. In our future experiments we will selectively inactivate functional domains of Ena required for its other biochemical activities to assess their contributions to Ena-dependent axon guidance.

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Capturing the Action: Visualizing *in vivo* Actin Dynamics in the *Drosophila* Ovary.

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Drosophila oogenesis is comprised of 14 morphologically defined stages of egg or follicle maturation. Late in this maturation process, the 15 germline-derived support cells, termed nurse cells, rapidly transfer their cytoplasmic contents to the oocyte in a process termed nurse cell dumping. Nurse cell dumping requires the nurse cells to undergo dramatic actin cytoskeletal remodeling. Specifically, the nurse cells must increase their cortical actin to provide the contractile force necessary for dumping while forming a series of parallel actin filament bundles extending from the nurse cell membranes inward towards the nuclei. This developmental model has been used to elucidate the function of numerous actin-binding proteins and their interacting partners. Despite decades of study, *in vivo* actin dynamics in the *Drosophila* germline have not been examined. In order to visualize actin dynamics during nurse cell dumping, we have recently generated transgenic flies expressing either Lifeact::mEGFP or Ftractin::mEGFP/tdTomato under the control of the UASp/Gal4 system. Additionally, we have obtained Utrophin-GFP transgenic flies (UASp and *sqh* promoter driven). Here we compare the utility of these actin labeling tools by assessing colocalization with phalloidin, live imaging capability, and affects on nurse cell dumping and female fertility.

Higher-Order Actin-Based Structures

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Functions of Nonmuscle Myosin IIA and IIB in Assembly of the Cellular Contractile System.

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The contractile system of nonmuscle cells consists of interconnected actomyosin networks and bundles anchored to focal adhesions. The initiation of the contractile system assembly is poorly understood structurally and mechanistically, whereas system’s maturation heavily depends on nonmuscle myosin II (NMII). Using platinum replica electron microscopy in combination with fluorescence microscopy, we characterized the structural mechanisms of the contractile system assembly during spreading or recovery from blebbistatin treatment and investigated roles of NMII in general, as well as specific roles of NMIIA and NMIIIB isoforms, at early stages of this process. We show that inhibition of NMII by blebbistatin, in addition to known effects, such as disassembly of stress fibers and mature focal adhesions, also causes transformation of

lamellipodia into unattached ruffles, loss of immature focal complexes, loss of cytoskeleton-associated NMII filaments and peripheral accumulation of activated, but unpolymerized NMIIA and NMIIIB. After blebbistatin washout, assembly of the contractile system begins with quick and coordinated recovery of lamellipodia and focal complexes that occurs before reappearance of NMII bipolar filaments. The initial formation of focal complexes and subsequent assembly of NMII filaments, preferentially NMIIA, occurred in association with filopodial bundles and concave actin bundles formed by filopodial roots at the lamellipodial base. Over time, accumulating NMII filaments composed of both NMIIA and NMIIIB help to transform the precursor structures, focal complexes and associated thin bundles, into stress fibers and mature focal adhesions. However, semi-sarcomeric organization of stress fibers develops at much slower rate with parallel accumulation of alpha-actinin. Together, our data suggest that activation of NMII motor activity by light chain phosphorylation occurs at the cell edge and is uncoupled from NMII assembly into bipolar filaments. We propose that activated, but unpolymerized NMIIA initiates focal complexes, thus providing traction for lamellipodial protrusion. Subsequently, the mechanical resistance of focal complexes activates a load-dependent mechanism of NMIIA and NMIIIB polymerization in association with attached bundles, leading to assembly of stress fibers and maturation of focal adhesions.

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Live cell, 3-D SIM reveals a myosin II-based, actin arc contractile system operating on the dorsal surface of migrating cells.

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The actin cytoskeleton provides a dynamic structural scaffold that frames the cell and propels its motion. To gain better insight into how this system operates in migrating cells, we employed live-cell structured illumination microscopy (SIM), which doubles both the lateral (x,y) and axial (z) resolution compared with conventional fluorescence techniques. Using 3-D SIM, we observed a novel myosin II-based, contractile system comprised of actin arcs operating on the top surface of migrating cells. The actin arcs glided along the dorsal cell surface, shortening as they moved toward the top of the cell (near the perinuclear region), where they recycled their components. Arranged parallel to the plasma membrane and perpendicular to dorsal actin stress fibers, the arcs contained most of the myosin IIA within cells (up to 80%), with dorsal stress fibers (contacting focal adhesions) having virtually no myosin IIA. Using 3-D SIM we were able to distinguish the myosin IIA motor domain from the coiled-coil domain of individual molecules tagged with mEmerald (N terminus) and mApple (C terminus). This resulted in a distinctive green-red-green (i.e., head-tail-head) pattern that could be used to reveal myosin IIA-filament orientation. We found that myosin IIA-filaments had their long axis oriented parallel to the leading edge. Notably, the myosin II filaments were separated from each other by alpha-actinin, suggesting a sarcomeric arrangement reminiscent of the arrangement in muscle myofibrils. This pattern was acquired by specific recycling of alpha-actinin along the lengths of newly formed actin arcs at sites of myosin IIA filament formation. By revealing a myosin IIA-based, actin arc contractile system at the dorsal surface of cells, our data suggest a model in which a contractile force generated from the top of the cell is used to move the cell forward. This scenario may be particularly relevant for understanding how a cell moves in a more complex 3-D environment, where large ventral stress fibers are not apparent and focal adhesions are small.

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3D PALM shows distinct distributions of Z-disc proteins with the Z-discs in cardiomyocytes.

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Z-discs are important structural and signalling structures that form the boundaries of each muscle sarcomere in striated muscle. Within the Z-disc, electron microscopy indicates that actin filaments, arranged in an anti-parallel organization, are cross-linked by α -actinin dimers arranged at ~20 nm intervals. At least 30 different proteins reside in the Z-disc, and their organisation is not well understood. Conventional light microscopy cannot resolve the localisation of proteins within it as it is too narrow, ~100 nm wide.

We have developed the use of 3-D Photoactivated Localisation Microscopy (PALM [1,2]) to image two specific proteins within the Z-disc; α -actinin2, and Lasp-2 (LIM and SH3 containing protein 2), which binds to α -actinin2. α -actinin2 and LASP fused to mEos2 were expressed in cultured embryonic mouse or isolated adult rat cardiomyocytes. Fixed cells were imaged using PALM, in which a weak cylindrical lens in the light path between the specimen and the camera was used to obtain 3D information from a single 2D plane.

The resulting images show individual molecules of mEos2- α -actinin2 and mEos2-LASP within the Z-disc, using light microscopy, for the first time. The localization precision was 20nm (X,Y) and 50nm in Z. The density of mEos2- α -actinin2 molecules was higher than that for mEos2-LASP, and the densities of both molecules was non-uniform throughout the Z-disc structures. A quantitative analysis of these molecules provides new insight into the organisation of these molecules within the Z-disc structure. These results demonstrate that PALM can be used to localise specific proteins within the narrow Z-disc and thus it has great potential for investigating the organisation of component proteins within this structure.

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Reconstitution of lamellipodium-like structure using micropatterning.

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Cells move, change shape, divide by a dynamic control of their architecture maintained by the actin cytoskeleton and orchestrated by regulatory proteins. The identification of biochemical, biophysical and physical laws that govern this dynamics in cells is rendered difficult by the complexity of life. To reduce this complexity, we have developed new biomimetic experimental systems that reproduce, in simplified terms, the behavior of the cellular actin cytoskeleton. Using this approach, we reconstituted an actin network crosslinked structure similar to that present in the lamellipodium of motile cells. By varying the geometry and the density of the actin nucleation zones, we were able to show that the actin network size, but not its density, affects the lamellipodium-like growth rate. We showed that denser lamellipodia are more rigid and density gradients of actin network orient lamellipodia growth. This work unveils how kinetic and

mechanical properties of growing actin networks can control the overall properties of lamellipodium-like growth and governs force production and cell motility.

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Embryonic Wound Healing Mechanisms Investigated by Experiment and Computer Modeling.

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The healing of embryonic wounds can yield insight into the mechanical forces which shape a developing organism. Here we characterize the healing of wounds in the epithelia of early stage chick embryos, and use a newly developed computational model to investigate the underlying cellular-level mechanisms.

Early stage (HH4) chick embryos were harvested and cultured *ex ovo*. At this stage the embryo consists of planar epithelial sheets two to three cell layers thick. Circular and slash wounds (~100µm in size) were made in the tissue and allowed to heal. The healing process was captured with time lapse microscopy, and wound area and shape were characterized as functions of time. We found that the closure rate of embryonic wounds displayed a two-phase behavior, with rapid constriction lasting about a minute, followed by a period of more gradual contraction until the wound closed. Fluorescent staining revealed that about ten seconds after wounding a broad, faint ring of contractile actin and myosin-II encircles the wound. By one minute post wounding this structure gives way to a narrow actomyosin band at the wound border, consistent with a “purse string” healing mechanism observed in other embryonic systems. We hypothesize that contraction of the broad ring is responsible for the initial, rapid phase of wound closure, and that the narrow purse string drives the later slower phase.

To test the proposed wound healing mechanisms, we implemented both types of contractile response in a finite element computational model. Considering both circular and elliptical wounds, we found that the rapid initial phase of wound closure is consistent only with an isotropic contraction of the broad ring surrounding the wound, and that the slower phase can be accounted for by the formation and circumferential contraction of actomyosin-like fibers at the wound margin. Together, these two mechanisms can quantitatively reproduce the observed wound healing dynamics.

The results of this integrated experimental and computational investigation suggest that a new mechanism, the isotropic contraction of cells in a broad ring around the wound, works together with an actomyosin contractile ring to close an embryonic wound.

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Actin filament cross-linking proteins Daam1 and Fascin share an integrated and essential role in filopodia formation.

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Filopodia are slender, finger like projections that dynamically form at the leading edge of motile cells with important roles in cell migration and environment sensing. Each filopodium contains a dense bundle of parallel actin filaments that grow rapidly from their tips. The molecular basis for the formation of these structures is still not well understood, but in part involves the coordinated actin-assembly activities of Ena/VASP proteins, formins, and Arp2/3 complex. In addition, the

rigid architecture of filopodia depends on the filament bundling protein fascin, which localizes all along the filopodium shaft. Here, we report that the formin Daam1 (Disheveled associated activator of morphogenesis 1) plays a critical role in filopodia formation. Unlike other formins, which localize to the tips of filopodia, endogenous Daam1 localized along the entire filopodial shaft in B16F1 cells, similar to fascin. RNAi silencing of Daam1 caused reduced numbers of filopodia and loss of their structural integrity, similar to fascin silencing. Co-silencing of fascin and Daam1 virtually abolished filopodia formation, suggesting that fascin and Daam1 have related roles in establishing and maintaining filopodial structure. Consistent with this view, purified Daam1 strongly bundled actin filaments *in vitro*, and dual-color TIRF microscopy revealed that fluorescently labeled Daam1 molecules are recruited to the sides of fascin-bundled filaments. Further, fascin-silencing disrupted Daam1 localization to filopodial shafts but not to tips. Taken together, these results demonstrate a closely intertwined and essential role for the actin crosslinking proteins fascin and Daam1 in filopodia formation.

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Drosophila septins bundle and curve actin filaments.

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During cell division, actin filaments and myosin motor proteins form a membrane- anchored contractile ring that constricts the cell midzone to give rise to two daughter cells. A conserved core of about 20 proteins is involved in animal cytokinesis. However, the structural organization and assembly of the actomyosin network remain outstanding questions. Here we focus on septins, one of the conserved core components of cytokinesis. Although septins bind independently membranes, Anillin and nonmuscle Myosin-II in different model systems, the role of septins in the organization of actomyosin is not understood, and their precise biochemical function remains unknown. During *Drosophila* embryo cellularization actomyosin assembles into a cytokinesis-like ring at the tips of invaginating membranes. Pnut/hSep7 depleted embryos fail to accumulate and stabilize actomyosin during cellularization, whereas actomyosin fails to assemble into ring- like structures and instead forms a loose coat beneath the cell membrane, suggesting that septins are involved in compacting or/and curving actomyosin. *In vitro* reconstituted actin filaments in the presence of purified *Drosophila* septin complexes are bundled and organize in rings and curved bundle clusters. Septins decorate specifically actin filament bundles as opposed to single actin filaments, and appear as stitches along curved actin filament bundles. We provide *in vivo* and *in vitro* evidence that *Drosophila* septins constitute a novel actin crosslinker that bundles and curves actin filaments.

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Roles of mammalian profilin1 in cell migration.

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Biochemical analyses have revealed that profilin, one of G-actin binding proteins, regulates actin dynamics in several different manners, namely blocking polymerization at the minus ends of actin filaments, inhibiting spontaneous actin nucleation and acceleration of ATP/ADP exchange of G-actin. We applied retrovirus-mediated RNA interference (RNAi) to profilin1 (Pfn1), the major isoform of mammalian profilin, to better understand cellular roles of profilin. RNAi of Pfn1 in mouse neuroblastoma N2A cells caused significant reduction of filopodia number and length at the leading edges. On the other hand, immunofluorescence with several

lamellipodial markers revealed that lamellipodia formation was enhanced by RNAi of Pfn1. Live cell imaging using mCherry-actin indicated that actin filaments polymerized and depolymerized normally in lamellipodia of the Pfn1 knockdown cells. Though Pfn1 knockdown cells could migrate similarly to the control cells, they were frequently rounded up on the substrate, suggesting that cell-substrate adhesion was affected. We also analyzed RNAi of Pfn1 in Rat2 cells and rat embryonic fibroblasts (REFs), which showed polarized cell migration. By RNAi of Pfn1, lamellipodia became bigger and thicker and contained less filopodia compared to the control cells. Normally these fibroblasts formed the long tails containing F-actin bundle at the rear region during cell migration. RNAi of Pfn1 shortened the rear tails in both Rat2 and REF, resulting that their cell shapes became more round and unpolarized. Interestingly, we could not observe obvious defects caused by RNAi of Pfn1 in cytokinesis or stressfiber formation in either of three tested cell lines. Another mammalian profilin isoform, profilin2, was expressed in these cell lines in that the actin machinery other than lamellipodia and filopodia might not be so severely affected by RNAi of Pfn1. In summary, our results indicate Pfn1 is primarily responsible for balance between lamellipodia versus filopodia formation, probably by cooperating with formin proteins. We also hypothesize that filopodia formation is likely important for subsequent cell-substrate adhesion to establish the cell polarity during cell migration.

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CP and N-WASP cooperate to organize barbed end orientation at the leading edge.

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Motile cells orient actin filament at the leading edge such that fast-growing barbed ends face the membrane. We used *in vitro* motility assays with N-WASP WWCA coated nanofibers and total internal reflection fluorescence (TIRF) microscopy to determine how WWCA domains, capping protein (CP), and arp2/3 generate this barbed end orientation. In the absence of CP, arp2/3 nucleates barbed ends that grow away from the nanofiber surface and branches remain stably attached to their WWCA tethers. CP addition causes barbed ends to pivot toward the nanofiber. CP-mediated reorientation is accompanied by both the shedding of short branches from the nanofiber and barbed end capture by the nanofiber. On drifting nanofibers, reoriented branches pivoted at their barbed end, showing specific barbed end tethering rather than branch-point attachment. Barbed end retention by nanofibers correlated to capping, and barbed end rapidly detached from the nanofiber upon the resumption of growth. These observations indicate that WWCA and CP bind simultaneously to barbed ends. We used filament pull-down assays and fluorescence anisotropy to confirm cooperative binding of WWCA and CP to barbed ends in the absence of arp2/3. In pull-down assays, GST-WWCA coated beads captured an average of 8-9 barbed ends as counted by TIRF microscopy. CP addition lead to a biphasic response with a peak of 13 filaments captured per bead at 5 nM CP and a reduction to ~4 ends per bead at 200 nM CP – half the number captured by WWCA alone. We used bulk WWCA binding assays to measure WWCA affinity for mechanically sheared filament barbed ends. End concentration was estimated from seeded pyrene-actin polymerization assays and WWCA binding was measured in parallel by fluorescence anisotropy. We found that labeled WWCA bound to barbed ends with an affinity of 14 pM and unlabeled WWCA with an affinity of 75 pM, similar to the known 80 – 100 pM affinity of CP for barbed ends. CP addition increased WWCA binding slightly at low CP concentrations and decreased WWCA binding to 50% at high CP concentrations. Reduction of WWCA binding by half in both assays shows that CP competes with the terminal WWCA binding site at the barbed end but does not preclude WWCA binding to the penultimate actin subunit. Molecular models of CP and WH2 domains bound respectively to the terminal and penultimate actin subunit showed no overlap between the two molecules and that CP

orientation might blocks WWCA dissociation from the penultimate subunit. We propose that simultaneous binding of CP and WWCA to barbed ends is essential to the establishment of filament orientation at the leading edge. Rapid CP association limits growth of arp2/3 nucleated barbed ends away from the leading edge. Subsequent N-WASP binding to capped barbed ends causes arp2/3 at the nearby branch junction to detach from N-WASP, pivoting the branch to point in the direction of productive motility. Cooperative CP/N-WASP binding thus presents a new mechanism that refines our understanding of dendritic nucleation at the leading edge.

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Correlation of cross-linked actin network formation and elastic moduli of human trabecular meshwork cells.

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Objective: Cross-linked actin networks (CLANs) are α -actinin rich polygonal actin networks observed in human trabecular meshwork (HTM) cells *in vivo* and *in vitro*. They have been observed with increased incidence in glaucomatous tissue and have been associated with glaucomatous phenotypes *in vivo*. We have recently reported glaucomatous HTM exhibit increased elastic moduli (E) and that Latrunculin B (Lat B), a potential glaucoma therapeutic, can induce transiently increased E in HTM cells *in vitro*. We wished to investigate the role CLANs play in the increased E of Lat B treated HTM cells and to determine if this finding was unique to Lat B, or more broadly a consequence cytoskeletal disrupting agents.

Methods: We treated HTM cells with either 2 μ M Lat B or 100 μ M Y-27632 (a Rho-associated protein kinase inhibitor) and their appropriate vehicle controls for 30 min. After 30 min, the cells were allowed to recover for 0-270 min and their compliance (E) was measured via atomic force microscopy (AFM) or they were fixed and assayed for CLANs.

Results: Both Lat B and Y-27632 treatment resulted in complete ablation of actin stress fibers after 30 min. During recovery, we observed the formation of circumferential cross-linked actin structures after Lat B treatment. We verified that these structures were rich in α -actinin, and identified these as a non-canonical presentation of CLANs. These structures were present in 32.48% \pm 6.29% (mean \pm SEM; n=9) of cells at 90 min and declined to 11.03% \pm 5.18% of cells by 270 min, in close agreement to the previously published timeline of transient increases in E following Lat B treatment. Y-27632 treated cells did not exhibit these structures, nor did they exhibit transient increases in E.

Conclusions: These results suggest this non-canonical presentation of CLANs following Lat B treatment may increase the stiffness of HTM cells, and this effect seems specific to Lat B. Given the recent correlation between stiffness and glaucoma, this behavior is an important consideration when testing therapeutics that target the cytoskeleton of HTM cells.

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Monitoring Actin Cortex Thickness In Live Cells.

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Animal cell shape is determined primarily by the actomyosin cortex, a thin layer of proteins that directly underlies the plasma membrane. The cortex regulates cell shape by controlling the physical properties of the cell, including contractile tension and elasticity, which arise from interactions between actin filaments, myosin motors and other actin binding proteins. The thickness of the cortical network is a key determinant of a cell's physical properties, and modulation of cortex thickness over space and time can drive cell shape changes. Thus, understanding how thickness is controlled is essential to understanding cellular morphogenesis. However, because cortex thickness is on the order of the resolution limit of the light microscope, it is too thin to be measured directly by conventional light microscopy. To address this, we developed an assay to measure cortex thickness based on dual-color sub-resolution localization. We measured cortex thickness by determining the distance between the center of the actin cortex and the plasma membrane, which we labeled with different colored fluorophores. Using a simple theoretical description of cortex geometry, we established a functional definition of cortex thickness that we could use to compare cortices across different experimental conditions. Furthermore, we used this model to account for artificial shifts in the apparent position of the cortex inherent to microscopic imaging. We tested the validity of this model and of our image analysis tools using synthetic images of the actin cortex generated in silico. Applying our technique to living cells, we measured an average cortex thickness of ~130 nm in mitotic HeLa cells and observed an increase in cortex thickness following treatments that prevent F-actin disassembly. We could also follow physiological changes in cortex thickness in real time by monitoring actin cortex regrowth in cellular blebs. Our data demonstrate that our method is sufficiently precise to detect differences in cortex thickness across experimental treatments and during shape changes in living cells. We are currently investigating how changes in cortex thickness contribute to cell deformations during cell division.

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Compliance and Elasticity of Actin Networks from Motile Fish Keratocytes.

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Cells undergo actin-based crawling motility by continuously building, reorganizing, and disassembling an actin network. These processes are driven and coordinated by a series of biochemical reactions and mechanical forces. Because the actin cytoskeleton is a large structure permeating the cell, knowledge of the mechanical properties of this network is crucial in understanding how the cell coordinates a large-scale process like crawling motility. Because the cytoskeleton of a live cell is highly specifically (albeit stochastically) organized, it is possible that their mechanical properties differ from reconstituted protein gels or even among different regions of a cell.

We have used detergent-extracted actin-rich cytoskeletons from fish epithelial keratocytes to directly measure the response to forced deformation by a micromanipulator. When stretched by a microneedle, the thin (approximately 200 nm) lamellipodium stretched extensively, up to several-fold its original dimensions. Deformation propagated through the lamellipodial network, indicating that the cytoskeleton can behave as a coherent sheet and can transduce forces over long distances. At moderate strains, the deformation was almost completely reversible, indicating that the network is highly elastic, at least over the measured timescale (0.2 s to 30 s).

These observations suggest that there is strong mechanical coupling within the cytoskeleton over distance scales close to the whole cell, and may serve as a constraint on physical models of crawling motility.

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Protocadherin-24 and mucin-like protocadherin interact to form inter-microvillar adhesion links that are required for normal enterocyte brush border assembly.

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Intestinal epithelial cells (IECs) possess a highly ordered, densely packed apical array of actin-based, membrane protrusions known as microvilli. Collectively, these microvilli form the brush border, which is known to play a key role in both nutrient absorption and host defense. Despite being critical for proper gut function, little is known about the molecular mechanisms underlying brush border assembly during IEC differentiation. Using the CACO-2_{BBE} cell culture model system, we observed that microvilli cluster together during IEC differentiation, interacting with each other at their distal tips to form 'tepee'-shaped structures. High magnification images of these clusters revealed that microvilli are physically connected to one another by thread-like links. Similarly, electron microscopy of native intestinal tissue revealed an extensive network of inter-microvillar links that exists within a mature brush border. Towards the goal of identifying the constituent(s) of these links, we have identified and characterized the functional role and binding partners of a cadherin superfamily member, protocadherin-24 (PCDH24), which exhibits striking enrichment at the tips of microvilli from IECs. Over-expression of PCDH24 in CACO-2_{BBE} cells correlated with tight microvillar clustering, while knockdown impaired brush border assembly and resulted in structures with loosely packed microvilli of variable length. Deletion of the cytoplasmic tail of PCDH24 resulted in degradation of the protein suggesting that cytoplasmic binding partners are required for stability. Pull-down experiments using a tagged PCDH24 cytoplasmic domain demonstrated interactions with the scaffold protein harmonin-a and the molecular motor myosin-7b (Myo7b). Both harmonin-a and Myo7b colocalized with PCDH24 at the tips of microvilli of CACO-2_{BBE} cells and native tissue. Furthermore, we found that harmonin-a and Myo7b interact directly and knockdown of PCDH24 resulted in loss of both harmonin-a and Myo7b from the brush borders of CACO-2_{BBE} cells. These data indicate that PCDH24, harmonin-a and Myo7b form a tripartite complex at the tips of IEC microvilli. The physiological significance of this complex is highlighted by ultrastructural analysis of harmonin KO mice intestinal tissues, which exhibit severe defects in brush border formation and villus morphology. Analysis of the adhesion capacity of PCDH24 using bead aggregation and protein pull-down assays revealed relatively weak *trans*-homophilic bond formation. Strikingly, these assays demonstrated strong *trans*-heterophilic interaction between PCDH24 and mucin-like protocadherin (MLPCDH), another cadherin that localizes specifically to the tips of IEC microvilli. We propose that PCDH24 is targeted to the tips of IEC microvilli through complex formation with harmonin-a and Myo7b. At the tips, PCDH24 interacts in *trans* with MLPCDH to form inter-microvillar adhesion links that promote the tight packing and uniform length of microvilli during brush border assembly.

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Palladin regulates actin stress fiber assembly via VASP-dependent mechanismG. Gateva¹, S. Tojkander¹, S. Koho², O. Carpen³, P. Lappalainen¹;¹Institute of Biotechnology, University of Helsinki, Helsinki, Finland, ²Institute of Biomedicine and Medicity research laboratories, University of Turku, Turku, Finland, ³Department of Pathology, University of Turku and Turku University Central Hospital, Turku, Finland

Cell motility, adhesion and cytokinesis are key cell features critical for major developmental processes and pathological conditions like metastasis. The ability of cells to move or divide is strictly dependent on a dynamic cytoskeleton characterized by a complex protein composition. A unique protein, actin, is one of the major building blocks of the cytoskeleton. Actin is an extraordinary protein, because it can polymerize into highly dynamic filaments, characterized by rapid assembly and disassembly cycles regulated by physiological stimuli. Actin filaments build up stress fibers which constitute the contractile acto-myosin machinery of non-muscle cells. The assembly of these actin structures is regulated by various actin-binding proteins, but the mechanisms controlling stress fiber stability, organization and contractility are largely unknown. Palladin, like α -actinin, is an abundant stress fiber-associated actin-binding protein, but its exact function in stress fibers remains elusive.

We demonstrated that palladin regulates stress fiber architecture likely via the recruitment of VASP (vasodilator-stimulated phosphoprotein) to stress fiber structures. FRAP (Fluorescent Recovery After Photobleaching) experiments revealed that palladin and VASP display similar highly dynamic rates of association with stress fibers, distinct of the dynamics of classical crosslinking proteins like α -actinin. Depletion of palladin by siRNA caused abnormal actin stress fiber network and over-expression of palladin in 2D and 3D cell cultures resulted in formation of very thick and stable actin stress fibers. Interestingly, the thick actin bundles induced by palladin over-expression resembled the phenotype induced by over-expression of proteins involved in actin filament nucleation and elongation like mDia1 and VASP. Furthermore, we showed by site-directed mutagenesis that the interaction between palladin and VASP was crucial for VASP recruitment to stress fibers and stress fiber stabilization, demonstrated by the formation of thick stress fibers. All our data indicate that palladin acts as a scaffolding protein to promote actin filament assembly in stress fibers at least partially through VASP-dependent actin filament polymerization.

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Effect of Palladin on the mechanical force responses of tumor-associated fibroblasts.M. Azatov¹, B. J. Grooman¹, S. Zhang¹, S. Goicoechea², C. A. Otey², R. F. Hwang³, A.Upadhyaya¹; ¹University of Maryland, College Park, MD, ²UNC School of Medicine, Chapel Hill, NC, ³University of Texas M.D. Anderson Cancer Center, Houston, TX

Pancreatic tumor-associated fibroblasts (TAFs) have been shown to promote the progression of pancreatic tumors. Mechanisms by which these cells stimulate invasiveness and metastasis of cancer cells are not well understood. It is known that the actin-crosslinking protein, palladin, is upregulated in TAFs, but the precise role of palladin in these cells is unknown. Several lines of evidence suggest that the misregulation of actin reorganization resulting from enhanced palladin levels may contribute to aberrant cellular behavior. In this study we use human pancreatic TAFs to investigate the role of palladin in regulating the plasticity of the actin cytoskeleton in response to alterations in substrate stiffness and externally applied local forces. To investigate the cellular response to external forces, we used a magnetic trap device to apply point forces on the cell surface. We detected an increase in stress fiber contraction and displacement after the application of force. We have developed software to track points on the stress fibers to

measure local strain rates within the stress fibers. We found an increase in cellular activity, usually three minutes after the initial force application, and that the force pulses applied must be at least two minutes long to exert this effect. Traction force microscopy revealed that TAFs are sensitive to substrate stiffness, exerting stronger forces on stiffer substrates. Cells knocked down (KD) in palladin by shRNA displayed a similar sensitivity to substrate stiffness in terms of exerted traction forces. However, KD cells exerted higher forces on stiff substrates (20 kPa gels) as compared to wild type or GFP-palladin cells. Finally, we found that palladin KD cells are more sensitive to inhibition of myosin II – treatment with Blebbistatin leads to a greater disruption of force compared to WT cells. However, removal of blebbistatin leads to a greater degree of force recovery in KD cells. Biophysical characterization of the interactions between TAFs and the external mechanics of the surroundings is likely to yield important new insights into the mechanisms that underlie the metastasis-promoting activity of these cells.

Actin-membrane Interactions

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Analysis of synergies between Aip1p and capping protein during clathrin-mediated endocytosis by quantitative microscopy, new alignment methods and mathematical modeling.

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Actin filament dynamics play a major role in reshaping the plasma membrane into vesicles during clathrin-mediated endocytosis. Elongation of actin filament barbed ends in endocytic patches is regulated by at least two classes of capping proteins, heterodimeric Acp1p/Acp2p capping protein and Aip1p. We used quantitative microscopy of live fission yeast cells to count the absolute numbers of protein molecules that assemble to and disassemble from sites of endocytosis. These temporal quantitative data have great potential for improving our understanding of the precise molecular mechanisms of clathrin-mediated endocytosis. However, their collection, analysis and interpretation require advanced theoretical and computational methods to take full advantage of the data.

First, we developed a new, generally applicable method to increase the time resolution of any dataset below the acquisition rate. This new alignment method allowed us to limit many artifacts that appear when averaging datasets such as errors in estimating average values, standard deviations and assembly and disassembly profiles. This method also allowed us to measure more accurately endocytic patch movement and show that actin is not directly involved in propelling the endocytic vesicle but only in its formation. In addition, we built a mathematical model to infer the biochemical mechanisms of clathrin-mediated endocytosis. Reconciliation of simulations of the model with observations showed that dendritic nucleation by the Arp2/3 complex is crucial for a fast assembly and severing filaments from the network by ADF/cofilin is crucial for fast disassembly. This model also predicted quantitative biochemical properties of the actin cytoskeleton in vivo that were later proven experimentally.

Now we have applied our computational and theoretical tools to study synergies between capping protein and Aip1p during endocytosis. The experiments and mathematical models showed that in cells lacking Aip1p capping protein can cap the filaments normally capped by Aip1p. However, Aip1p cannot compensate for the absence of one or both capping protein subunits. In addition, the experiments showed that Acp2p alone has another previously

unknown function, independent from its ability to partner with Acp1p to cap actin filament barbed ends. Indeed Acp2p and Aip1p are individually involved in setting proper polarization of endocytic sites in interphase cells. Strikingly, Acp1p, Acp2 and Aip1p are not involved in the polarization during cytokinesis. Therefore, our data suggest that independent polarization pathways exist during interphase and cytokinesis.

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Severing of actin filaments by cofilin contributes to both assembly and disassembly of endocytic actin patches in fission yeast.

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We used a mutant cofilin with defects in severing actin filaments to test the proposal that cofilin severs actin filaments during endocytosis in fission yeast cells. We used quantitative fluorescence microscopy to track GFP-tagged proteins, including early endocytic adaptor proteins, activators of Arp2/3 complex and actin filaments. Consistent with the hypothesis, actin patches disassembled far slower in cells depending on severing-deficient cofilin than wild type cells. Even more interesting, actin patches assembled slowly in these cofilin mutant cells. Adaptor proteins End4p and Pan1p accumulated and persisted at the endocytic sites more than 10 times longer than in wild type cells, followed by slow put persistent recruitment of activators of Arp2/3 complex, including WASP and myosin-I. Given the selective severing defect of these cofilin mutants, the slow assembly of actin patches may be due to a lack of short actin filaments normally produced by severing that diffuse out of actin patches to serve as mother filaments to stimulate Arp2/3 complex in adjacent patches to nucleate actin filaments. An investigation of actin patch proteins that might recruit these mother filaments revealed that actin filament binding sites on both Pan1p and End4p plays a role in initiating actin polymerization in actin patches.

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Native dynamics of dynamin, actin and cortactin during clathrin-mediated endocytosis revealed by real-time imaging in genome-edited cells.

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Clathrin-mediated endocytosis (CME) is the best-studied endocytic pathway. Numerous molecular actors have been identified, and live-cell imaging studies have revealed the sequence and timing of their recruitment during cargo uptake. Recent application of high precision microscopy and quantitative analysis of clathrin-coated structure (CCS) dynamics during endocytosis revealed high heterogeneity. While the origin of this heterogeneity remains unclear, it also complicates faithful analysis of endocytic protein functions. Recently, we have overcome problems caused by protein overexpression by using zinc finger nuclease (ZFN) technology to express RFP- and GFP-tagged fusions of clathrin and dynamin, respectively, preserving their endogenous levels and stoichiometry. Here, we extend our analysis to explore the function and dependency of the actin machinery relative to dynamin during CME.

Our measurements revealed that dynamin dynamics are highly regular during the last step of CME. We observed that dynamin's arrival is strongly dependent on CCS maturation. Moreover, dynamin lifetime, kinetic accumulation and disappearance are highly predictable despite CCS heterogeneity. Together, these data suggest that early steps of endocytosis are the source of heterogeneity in CCS dynamics, and that the terminal step is highly regular. Interestingly, the

actin cytoskeleton has been proposed to play multiple roles at multiple stages of CME. However, direct imaging of the actin machinery during CME is notoriously challenging. Therefore, understanding the dependency on actin and coordination of its assembly with dynamin recruitment are far from complete. Here, we generated genome-edited cell lines expressing fluorescent protein fusions of actin and cortactin and combined these with fusions of clathrin or dynamin. Strikingly, we observed that actin is consistently recruited before dynamin during CME and is essential for normal dynamin recruitment and function.

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Investigating the function of class-I myosin motors during endocytosis in *Saccharomyces cerevisiae*.

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Many aspects of the clathrin-mediated endocytosis (CME) pathway are conserved among eukaryotes, but a prominent difference between species and cell types is the requirement for class-I myosin activity. The class-I myosins, Myo 3 and 5 (Myo3/5), are essential for CME in the yeast *S. cerevisiae*, while the requirement for a class-I myosin in other eukaryotes less absolute and varies depending on the cell type. Learning the role of myosins in CME would provide important insights into this process overall and may reveal important differences between species. In this study, we use *S. cerevisiae* as a model for studying myosin-dependent endocytosis. Myo3/5 are known to have at least two discrete functions. The CA domain at the carboxy terminus stimulates Arp2/3-mediated actin filament branching. At the N-terminus is a motor domain with high homology to other myosin motors. It has been previously shown that while the CA domain contributes to endocytosis, it is partially redundant with other activators of the Arp2/3 complex, and endocytosis still occurs when the CA domain is deleted. In contrast, complete deletion of Myo3/5 or introduction of a point mutation that blocks ATP binding and prevents actin filament release (rigor mutation) causes a substantial growth defect and abolishes endocytosis. Here we report that deletion of the entire motor domain was actually less deleterious to growth than the rigor point mutation, although CME was still significantly impaired. Furthermore, motor mutants that were engineered to eliminate actin translocation while preserving actin-dependent ATPase activity had growth and CME phenotypes similar to motor domain deletion. We next determined whether these mutants localized properly by GFP-tagging each myosin variant. We found that complete deletion of the motor domain greatly diminished localization at endocytic sites, suggesting that the motor domain contributes to myosin localization. In contrast, both the rigor mutant and actin translocation mutants were recruited to endocytic sites. Taken together, these findings suggest that the motor domains of Myo3/5 are important for at least two roles. First, the motor domain helps recruit myosin to endocytic patches. Second, translocation of actin by the motor domain appears to be required for CME. Moreover, this study suggests that actin polymerization alone is not sufficient for one or more steps of CME, but that forces generated by the Myo3/5 motor domains are also needed.

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Modulation of Ena/VASP processivity by a membrane tethered actin binding protein.

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Actin polymerases of the Ena/VASP protein family promote actin filament barbed end elongation. They are dynamically localized to leading edge membranes where they play an important role in determining the architecture of the underlying actin network. Although Ena/VASP proteins are intrinsically processive actin barbed end polymerases in vitro, this activity is not sufficient for membrane targeting in vivo. Instead, the combined presence of

growing actin filament barbed ends together with a membrane tethering protein are required for persistent membrane association of Ena/VASP proteins in vivo. The molecular mechanisms by which growing barbed ends and membrane tethering proteins synergistically contribute to Ena/VASP protein localization, lifetime, and/or activity at the plasma membrane, however, remain unclear. We present evidence that Lamellipodin, a membrane-associated Ena/VASP tethering protein, can autonomously bind to and organize actin filaments in vitro. In addition, Lamellipodin can enhance Ena/VASP barbed end processivity in vitro by a clustering independent mechanism that requires the interaction with filamentous actin. We propose that the filament binding activity of Lamellipodin acts as part of a “barbed end sensor” that rapidly and efficiently delivers Ena/VASP proteins to growing filament ends. To test this hypothesis, we characterized interactions between Lamellipodin, VASP, and actin filaments in vivo and in vitro, using biomimetic assays of varying degrees of complexity. This work provides insight about how membrane tethered actin barbed end polymerases control the assembly of the actin cytoskeleton during cell migration and morphogenesis.

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Active membrane organization by dynamic actin filaments.

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The plasma membrane (PM) of a living cell is more than just a selective barrier from the outside environment. The PM harbours platforms for signaling, maintains ion and nutrient balance and undergoes diverse structural changes associated with activities like endocytosis, secretion, cell polarization, locomotion and cytokinesis. All these activities require regulated segregation of both membrane proteins and lipids, creating structural and functional platforms which are often highly dynamic, and below optical resolution. For example, several lipid anchored proteins such as glycosylphosphatidylinositol-anchored proteins (GPI-APs) and Ras molecules form dynamic nanocluster assemblies which are of functional consequence to the cell. We have previously shown that the spatio-temporal dynamics of nanoclusters of GPI-APs are actively generated¹. They are sensitive to cortical acto-myosin dynamics. To explain these observations we have recently put forward a theoretical framework², involving the active hydrodynamics of short, dynamic actin filaments coupled to the PM to explain the experimentally observed properties of GPI-APs and other membrane components. Using single molecule imaging and FCS based diffusion measurements we find highly dynamic, and short lived (< 1s) actin filaments near the PM which associate with stable GPI-AP nanocluster enriched domains. To further probe into the molecular basis of the generation of these actin filaments, we undertook specific molecular perturbations of the actin nucleation and polymerization machinery and the cofilin based actin depolymerization machinery. We found that perturbing these two arms of actin turnover significantly affected the GPI-AP nanoclustering and the formation of the dynamic actin filaments. These results therefore strongly support the idea that PM components are locally organized by active processes driven by a regulatable actomyosin dynamics, which potentially can be regulated by the cell in response to specific stimuli and functional needs.

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A Septin-Containing Barrier Differentially Restricts Inheritance of a Yeast Prion and Mitochondria.A. M. Tartakoff¹; ¹Case Western Reserve University, Cleveland, OH

Fusion of haploid cells of *S. cerevisiae* generates zygotes. Their initial terminal buds inherit only a single mitochondrial genome and therefore provide an example of asymmetric cell division. We observe that the zygote midzone includes a septin annulus that restricts both mitochondria and supramolecular complexes (polysomes and the [PSI⁺] prion form of Sup35p-GFP). These complexes equilibrate only several minutes after cell fusion and do so slowly. Establishment of contact between parental mitochondria is further delayed until septins relocate to the bud site, thereby causing a single mitochondrial genome to have preferential access to nearby buds. By contrast, in [psi⁻] x [PSI⁺] genetic crosses all buds receive Sup35p-GFP [PSI⁺]. Thus, septin-containing barriers restrict entities that are not limited to the cell cortex; [PSI⁺] inheritance is the norm; and biased inheritance of mitochondria is promoted by a septin-dependent checkpoint. The rate of redistribution of complexes and organelles determines whether their inheritance is symmetric.

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Spatial organization of actin and intermediate filaments on the nuclear envelope.J. Usukura¹, S. Minakata²; ¹EcoTopia Science Institute, Nagoya University, Nagoya, Japan, ²Graduate School of Engineering, Nagoya University, Nagoya, Japan

Current study aims to reveal the spatial organization of cytoskeletal actin filaments in the periphery of nuclear envelope. Cytoskeletal actin filaments in living cell have been investigated so far exclusively with fluorescent light microscopy. Therefore, observation was restricted on the stress fiber with relatively strong fluorescence and at ventral side of the cell. In order to detect real spatial structure of cytoskeleton, high voltage TEM (1000 KV), high resolution SEM and immuno-freeze etching technique were applied to unroofed whole cells.

Nuclear envelope was supplied with many cytoskeleton including intermediate filaments, actin filaments and microtubules. In particular, intermediate filaments, vimentin, extending from nuclear pore like a rosette formed complicated meshwork and covered the total surface. Many actin filaments were attached to nuclear envelope as well. Since S1 decoration showed both pointed and barbed ends clearly in the surface of nucleus, there seemed to be the terminations and origins of actin filaments on the nuclear envelope. Unfortunately, however, we were not able to trace entire actin filaments on the nuclear envelope, because all actin filaments were associated or covered with vimentin filaments on the surface of nucleus. Under careful observation of many electron micrographs, many actin filaments appeared to extend from nuclear pores. Indeed, Nesprin 1, actin binding protein, was detected in the periphery of nuclear pores. Actin filaments extending from nuclear envelope were bundled gradually to form stress fibers. These are completely new concept on nuclear related actin filaments.

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Apical targeting of the Formin Diaphanous in *Drosophila* tubular epithelia.T. Rousso¹, A. Shewan², K. Mostov², E. D. Schejter¹, B-Z. Shilo¹; ¹Molecular genetics, Weizmann Institute of Science, Rehovot, Israel, ²Department of Anatomy, University of California, San Francisco, CA

Apical secretion in epithelial tubes of the *Drosophila* embryo was shown to be mediated by the Formin family protein Diaphanous (Dia) (Massarwa et al., 2009). Apical F-actin cables that are generated by Dia serve as tracks for MyosinV-based trafficking of vesicles that will be secreted to the tube lumen. Apical localization and activity of Dia is at the core of generating apical F-actin in this system. In this work we aimed to identify and characterize the molecular machinery(s) responsible for apical localization of Dia-based actin polymerization in *Drosophila* tubular epithelia.

We show that the open conformation of Dia, which is relieved from the auto-inhibited state, facilitates efficient apical targeting, probably by exposing the N-terminal domain that is critical for localization. Moreover, binding to Rho1 mediates not only opening of Dia, but also serves as one of the physical anchors of Dia at the apical membrane.

Drosophila Dia maintained apical localization in the mammalian tubular model system of MDCK cells, and could be relocalized to the baso-lateral membranes immediately after incorporation of PI(4,5)P2 into these membranes. Consistent with this, using live imaging we show that PI(4,5)P2 as well as the PIP5 kinase, Skittles, that catalyses its production, are enriched in apical membranes of *Drosophila* tubular organs. In addition, Dia apical targeting is sensitive to PI(4,5)P2 levels in these organs. Finally, we show that the basic amino-acid composition of the Dia N-terminal domain is crucial for apical localization, suggesting that it mediates the direct binding to PI(4,5)P2.

Our results demonstrate that the tight apical localization of Dia is achieved by combining apical-biasing cues, each of which provides only very partial targeting. The ability to distinguish between apical targeting by Rho1 and PI(4,5)P2 allows to establish the hierarchy between them. While Rho1 binding alone provides partial targeting, no apical targeting by alternative cues is observed in its absence. Binding to activated Rho1 may thus facilitate the initial interaction of Dia with the apical membrane. Physical proximity to the membrane can now overcome low affinity restrictions, and allow interaction of Dia with PI(4,5)P2 that stabilizes its association with the apical membrane.

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RANKL-induced Myo1b localizes to sites of dynamic actin-membrane remodeling and is required for osteoclast formation and function.

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Osteoclasts (OCs) are large multinucleated cells whose exquisite function is the resorption of bone. To achieve this, OCs boast a specialized cytoskeleton that is unique amongst all eukaryotic cells. The necessity of a highly dynamic cytoskeleton is borne out of cyclical requirements for motility and the generation of a polarized phenotype. Despite the obvious importance of the 'membrane-cytoskeleton interface' in the establishment of OC polarization, the nature and identity of molecules that directly link the OC plasma membrane with the underlying cytoskeleton remain largely obscure. Utilizing state-of-the-art transcriptional profiling we have systematically screened for novel actin-coupling molecules that are robustly up-regulated in OCs during RANKL-driven differentiation. Among the candidates identified, we uncovered myosin 1b (Myo1b), a member of the Class-I family of small actin-based mechano-sensitive motors, as a prominent RANKL-responsive gene. Up-regulation of Myo1b expression was confirmed both at the mRNA and protein level by quantitative PCR and immunoblotting. Consistent with its involvement in supporting membrane tension and deformation, Myo1b

specifically localized to dynamic sites of actin remodelling and membrane ruffling during OC motility and bone resorption. To begin to exploit the functional contribution of Myo1b (and other class I myosins) in OC formation and function, we employed the natural compound Pentachloropseudilin (PCIP), a specific and allosteric inhibitor of myosin-I ATPase activity. We demonstrate that blockade of myosin-I motor function dose-dependently attenuates OC formation and bone resorptive capacity *in vitro*, an effect that correlates with morphological disturbances in F-actin ring organization and membrane polarization. Collectively, our findings uncover Myo1b as a novel regulator of OC formation and function, most likely serving to bridge the actin-membrane interface at dynamic sites of membrane turnover.

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Actin controls the I-BAR-membrane interaction.

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The Inverse BAR (I-BAR) domain is the N-terminal 250 amino acid of the IRSp53 protein that induce negative membrane curvature both in vitro and in cells. Generation of membrane curvature by I-BAR proteins often works together with actin dynamics. I-BAR shares its function between actin bundling and membrane binding but it is still obscured what molecular mechanisms are responsible for these functions. The aim of our project is to investigate the detailed membrane binding properties of the I-BAR of IRSp53 and its relations to the actin cytoskeleton. In vitro FRET experiments and fluorescence quenching studies were carried out between the I-BAR and liposomes made up from different lipid constructs. We have found that the I-BAR has preference to bind to the negatively charged lipids ($K_d = 1-2 \mu\text{M}$) however it can also bind to the uncharged lipids. The fluorescence quenching studies reflected that the accessibility of the I-BAR surface was higher toward the negatively charged lipids than for the uncharged ones. The I-BAR membrane interaction is controlled by the polymerization state of actin where filamentous actin stabilizes while the globular actin disrupts their interaction. The I-BAR domain influences the actin polymerization, the affect depends on the I-BAR concentration. Our results suggest that the I-BAR-actin interaction may have a crucial role in filopodia formation and regulation.

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Structure of the ZU5-ZU5-UPA-DD tandem of ankyrin-B reveals interaction surfaces necessary for ankyrin function.

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Ankyrin-R/B/G (encoded by ANK1/2/3 respectively) are a family of very large scaffold proteins capable of anchoring numerous receptors and ion-channels to specific, spectrin-containing membrane micro-domains. Hereditary mutations of ankyrins are known to be associated with diseases including spherocytosis, cardiac arrhythmia, and bipolar disorder in humans, although the underlying molecular bases are poorly understood. The middle spectrin-binding domain of ankyrins contains highly conserved ZU5-ZU5-UPA-DD domains arranged into the ZZUD tandem. Curiously, most of the disease-causing mutations in the tandem have no apparent impact on the spectrin binding of ankyrins. The high resolution structure of the ankyrin-B ZZUD tandem determined here reveals that the ZU5-ZU5-UPA domains form a tightly packed structural supramodule, whereas DD is freely accessible. Although the formation of the ZZU supramodule does not influence the spectrin binding of ankyrins, mutations altering the inter-

domain interfaces of ZZU impair the functions of ankyrin-B&G. Our structural analysis further indicates that the ZZU supramodule of ankyrins has two additional surfaces that may bind to targets other than spectrin. Finally, the structure of the ankyrin ZZUD provides mechanistic explanations to many disease-causing mutations identified in ankyrin-B&R.

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MICAL1 an F-actin-disassembly factor links membrane repair.

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Plasma membrane disruption is a common form of cell injury in mammalian tissues under physiological conditions. Cell survival depends on the initiation of a rapid (second time-scale) resealing response that is mounted only in the presence of physiological levels of extracellular Ca²⁺. Vesicle-vesicle and vesicle-plasma membrane fusion events occurring in cortical cytoplasm surrounding the defect are thought to be a crucial element of the resealing mechanism. We have previously shown that a requisite prelude to this fusion is the disassembly in local cell cortex of the physical barrier constituted by filamentous actin. However, the identity of the proteins involved in this disassembly event remained unknown. Recently, the MICAL family of enzymes have been found to associate with the cytoplasmic domain of plexins, which are large cell-surface semaphorin receptors. MICAL thereby functions as an F-actin-disassembly factor mediating actin remodeling in a variety of cell responses. We expressed MICAL1/actin -GFP in culture cells or mice muscle fibers and then subjected them to a plasma membrane disruption created by a laser. Subsequent confocal imaging revealed a striking wave and rapid (second time-scale) accumulation of MICAL1-GFP at the disruption site, followed by actin depolymerization (second time-scale). We also observed, for the first time in living cells responding to a membrane disruption, vesicle fusion events at the disruption site. We hypothesize that localized filamentous actin disassembly mediated by MICAL1 removes a cortical barrier standing in the way of the membrane-membrane contacts leading to the homotypic and exocytotic fusion events required for repair.

Cytokinesis II

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Regulation of spindle-pole-body duplication and cytokinesis by the centrin-binding protein Sfi1 in fission yeast.

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Cytokinesis partitions a mother cell into two daughter cells. The fission yeast *Schizosaccharomyces pombe* is a powerful model organism to study cytokinesis because both key regulators and the overall mechanism involving an actomyosin contractile ring are conserved. Nevertheless, many regulators of cytokinesis remain to be identified. By sequencing the genome of a mutant previously denoted as *M46*, we identified a mutation in *sfi1*, a conserved essential gene encoding a centrin-binding protein. Some *sfi1-M46* cells exhibit defects in spindle-pole-body (SPB) duplication and spindle formation, similar to those of *S.*

cerevisiae sfi1 mutants. However, the majority of fission yeast *sfi1-M46* cells can form bipolar spindles and undergo mitosis. Interestingly, cytokinesis is misregulated in *sfi1-M46*. In wild-type cells, the two daughter cells separate after constriction of the contractile ring and formation of the septum. In *sfi1-M46*, some cells assemble a second contractile ring immediately and form another septum next to the first one. Contractile-ring assembly and septum formation in fission yeast are regulated by the septation-initiation network (SIN). Because both Sfi1 and SIN components localize to the SPBs, we hypothesize that Sfi1 regulates cytokinesis via the SIN pathway. Indeed, *sfi1-M46* has synthetic lethal/sick genetic interactions with many SIN mutations. Using Cdc7-EGFP as a marker, we find that the inactivation of SIN is delayed by *sfi1-M46*, and prolonged SIN activity results in the multi-septated phenotype in *sfi1-M46*. Our results indicate that in addition to its role in SPB duplication, the centrin-binding protein Sfi1 also regulates cytokinesis. This study opens up a new direction in investigating the functions of centrin and centrin-binding proteins in yeast and higher eukaryotes.

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Polar expansion during cytokinesis.

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Vesicle trafficking and new membrane addition at the cleavage furrow have been extensively studied. Less clear, however, is the old idea that expansion at the cell poles occurs during cytokinesis. Previous studies using the adherence of kaolin or charcoal particles to the surface of a dividing a cell to track membrane dynamics showed that the poles expands during the later stages of mitosis, and this expansion was attributed to a “stretching” of the plasma membrane. We have monitored movement of lipid rafts, which migrate during cytokinesis into the furrow membrane, in dividing echinoderm eggs. While the rafts are found to be immobile by FRAP analysis throughout the cell cycle, we document bulk flow of the membrane rafts away from the poles into the furrow. We have re-examined this phenomenon and find that new membrane is added to the cell poles, likely via exocytosis, during anaphase causing the original raft rich plasma membrane to be “pushed” from the poles to the furrow coincident with the constriction of the contractile ring. This membrane addition is dependent on actin and astral microtubules and occurs significantly earlier during mitosis than membrane addition at the furrow. The membrane that is added at the polar regions is compositionally distinct from the original cell membrane in that it is devoid of GM₁, a component of lipid rafts. These results suggest that polar expansion, via new membrane addition, may provide a force for moving the plasma membrane to the furrow during cytokinesis.

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Self-organization of taxol-stabilized microtubules by spindle and midzone mechanisms.

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Adding Taxol to mitotic cytosol forces microtubule nucleation and stabilization, allowing analysis of organization mechanisms that act on stabilized microtubules. The Karsenti group used this approach to discover that dynein clusters microtubules, and aligns minus ends, to form taxol asters, and inferred similar mechanisms for spindle pole assembly (Verde et al 1991 J Cell Biol. 112: 1177-87). The Compton group used taxol asters to discover new proteins involved in spindle pole organization (Mack and Compton 2001 Proc Natl Acad Sci U S A. 98:14434-9.). We revisited this approach using mitotic cytosol from *Xenopus* eggs supplemented with glycogen to preserve physiological metabolic activity (Groen et al 2011 Mol Biol Cell. 22:3139-51). We observed self-organization of highly organized assemblies consisting of parallel bundles of microtubules arranged in circles and paired lines, that we called pineapples.

Microtubule minus ends, marked by NUMA, were always on the outside. Plus ends, marked by Aurora B, were on the inside. Both minus and plus ends were globally aligned. Based on localization of cell division proteins and response to perturbation, we believe pineapples assemble using a combination of metaphase spindle and cytokinesis midzone mechanisms. We are focusing on how plus ends cluster and align. These processes depended on the midzone kinase Aurora B, which is part of the Chromosome Passenger Complex (CPC), and the midzone kinesin Kif4, which blocks plus end polymerization in midzones, and probably other factors. We are currently trying to distinguish between three models for plus end alignment based on (i) motor-driven sliding of plus ends, (ii) maximization of bundling energy, and (iii) sideways interactions between capping complexes. We are also using pineapples to explore how the microtubule-bound proteome in mitotic cytosol responds to perturbation, and correlate this with altered morphology.

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The minus-end directed kinesin KifC1 interacts with nucleoporin Nup153 and is required for targeting the spindle assembly checkpoint proteins Mad1 and Mad2 to kinetochores.

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Cytokinesis is the final event in the cell cycle, when the segregated genome, the organelles and the plasma membrane are partitioned into two physically distinct daughter cells. It requires an intricate interplay between cytoskeletal, chromosomal, and cell cycle regulatory pathways, but also a broad range of other cellular processes, for example protein and membrane trafficking, and signalling pathways. We have previously shown that over-expression as well as knock down of the nuclear pore protein Nup153 lead to abnormal mitoses including cytokinesis failure. In search for putative Nup153 target proteins important for cytokinesis we have now identified the minus-end directed kinesin KifC1 as Nup153 binding partner. Both proteins interact directly *in vitro* and *in vivo* and co-purify in a complex isolated from human cells. The depletion of KifC1 from HeLa cells by siRNAs leads to abnormal spindles, but interestingly also to failed recruitment of the spindle checkpoint proteins Mad1 and Mad2 to kinetochores in prometaphase cells. Together our data suggest that the interplay between Nup153 and KifC1 is important for spindle organization, spindle checkpoint activity and in turn successful cytokinesis.

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Analysis of late cytokinetic defects in cells lacking an organized central spindle.

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Upon anaphase onset, cells assemble an antiparallel array of microtubules termed the central spindle that is essential for the completion of cytokinesis. Two heterodimeric complexes act as the principle organizers: Centralspindlin (comprised of MgcRACGAP and MKLP1) and PRC1/KIF4A. Disruption of either complex results in disorganization of the central spindle and cleavage failure. However, these cells are still capable of initiating and ingressing a cleavage furrow. In a better effort to understand the mechanics of cleavage failure in cells with disrupted central spindles, we examined the structure and function of cleavage furrows in cells depleted of PRC1, KIF4A or MKLP1. Measurement of furrow ingression revealed that cells depleted of PRC1 or KIF4A ingressed at the same rate as controls, whereas MKLP1-depleted cells furrowed with slightly slower velocities. Depletion of PRC1 resulted in an elongated cytoplasmic bridge that failed to form a proper Flemming body, resulting in cleavage failure within two hours. Additionally, PRC1 was required for the normal midzone localization of key cytokinetic factors,

which instead were spread throughout the intercellular bridge. MKLP1 depletion displayed similar effects, whereas midzone components were largely localized correctly in KIF4A-depleted intercellular bridges. And while these extended intercellular bridges contained microtubules, brief treatments with high concentrations of nocodazole (to reveal stable microtubules) revealed that both MKLP1 and PRC1 were required to maintain microtubule stability during late cytokinesis. Taken together, these findings suggest that PRC1 and MKLP1 play a prominent role in maintaining the native midbody architecture and normal recruitment of cytokinetic factors at the midbody.

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Interphase fission maintains genomic integrity after failure of cytokinesis in human cells.

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The century-old Boveri hypothesis predicts that failure of cytokinesis leads to aberrant cell divisions, aneuploidy, and cancer. We used modern single-cell techniques to investigate the fate of human epithelial cells after failed cytokinesis. To do this, we established a uniform population of binucleate interphase RPE1 cells that had previously segregated chromosomes (karyokinesis) but failed to divide into daughters (failed cytokinesis). After minimizing effects of DNA damage, we found a third of binucleate cells generate viable progeny. Strikingly, the majority of the progeny are euploid, with a karyotype that matches the parental line, and only a small fraction are near tetraploid. We performed confirmatory experiments that unambiguously demonstrate that euploid colonies are derived from single binucleate cells. To elucidate the mechanism of this process, we performed timelapse videomicroscopy. This revealed that binucleate cells can execute cytoplasmic fission to segregate nuclei without intervening mitosis. This fission echoes a primitive adhesion-dependent process reported in lower organisms such as *Dictyostelia*. Using fluorescent Cdt1, we demonstrate that the fission occurs during the G1 phase. The fission event does not require canonical components required for cytokinesis, demonstrating a distinct mechanism. Similar results were found in other human epithelial cell types including MCF10a breast epithelial cells and HCT116 colorectal cancer cells. Interphase fission can explain previous reports that polyploid multinucleate hepatocytes can resolve to euploid hepatocytes. We conclude that human cells have a failsafe mechanism to maintain euploidy in the face of cytokinesis failure, making subsequent aneuploidy less frequent than envisioned by Boveri.

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Proteomic Identification Reveals That FLJ25439 Overexpression-Induced Cellular Tetraploidization Is Associated with Changes in Stress-Related Protein Profiles.

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Cell cycle progression must be precisely regulated by checkpoint. It has been reported that malfunction of checkpoint mechanism would lead to aneuploidy through formation of tetraploid or polyploid cells, hallmarks of tumorigenesis. In cancer cells, the tetraploid cells may contribute to abnormal mitotic progression, which may be associated with cytokinesis failure. Tetraploidy would lead to genomic instability due to centrosome and chromosome over-replication. Until now, the mechanism of how the cells maintain the tetraploid status is unknown. Here, we identify a novel protein FLJ25439 localized to the centrosome in interphase cells and

at the midbody during cytokinesis. To investigate the function of FLJ25439, we established stable cell lines overexpressing FLJ25439. FLJ25439-overexpressers grew slower than its normal counterpart and showed dysregulated expression of p53, pRb and p21, suggesting a defect in cell cycle progression. In addition, cells with FLJ25439 overexpression displayed a tetraploid DNA content as compared to its diploid parental cells. To explore the molecular mechanism responsible for FLJ25439 over-expression, we analyzed the global protein expression profile between wild type and over-expressers using proteomics and bioinformatics approaches. Protein category profiling indicated FLJ25439 would be involved in pathways which reflect in stress, protein folding, cell cycle, and cytoskeleton regulation. Specifically, genotoxic-stress related chaperone proteins and tubulin-regulated proteins are greatly contributed to the FLJ25439 overexpression phenotypes. The results of this study pave a fundamental avenue for our further understanding of the role of this novel cytokinesis-related protein in protecting cells from environmental stress and tetraploid formation.

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Role of Mitosis-Specific Translation in *S. cerevisiae* Cytokinesis.

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It has long been known that chromosome condensation during mitosis is associated with a global repression of transcription. Indeed, by using the Pol-II mutant *rpb1-1*, we verified in *S. cerevisiae* that mRNA transcription during this phase is not required for mitosis or cytokinesis. This has left the unanswered questions of how, where, and when the proteins required during this interval of cell cycle are synthesized. It has been widely assumed that such proteins are all synthesized before the shutdown of transcription. To test this hypothesis, we arrested cells in metaphase (with nocodazole) or late anaphase (with *cdc15*) and treated them after release with the translation-elongation inhibitor cycloheximide (CHX). In the CHX-treated cells, many of known cytokinesis proteins were present at normal or near-normal levels; however, depending on when the drug was added, the cells failed to execute mitotic exit, cytokinesis, and/or abscission despite an apparently normal progression of mitosis, suggesting that synthesis of one or more proteins involved in these events is regulated by translation. Similar effects were observed with a mutation in a core subunit of eIF3 (*prt1-1*), but not with a mutation of the cap-binding protein eIF4E (*cdc33-E17*), suggesting that the translation during mitosis may be initiated by a cap-independent mechanism. Current evidence suggests that the Polo kinase Cdc5 may be a target of the putative translation regulation that is responsible for many, but not all, of the above phenotypes: in CHX-treated cells, Cdc5 was rapidly degraded despite the apparent loss of APC activation, and it disappeared from the division site; Cdc5-dependent release of Cdc14 from the nucleolus was also blocked. Interestingly, a premature release of Cdc14 induced by *pds1f ϕ* bypassed the mitotic-exit and cytokinesis defects but not the abscission defect, suggesting the presence of an additional gene(s) involved in this step. Our attempt to identify such genes by polysome profiling suggests that yeast may regulate translation of genes encoding ER and Golgi proteins in anaphase to meet the increased demand for membrane trafficking during division.

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Mitochondria in Cytokinesis.

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Cytokinesis is the final stage of cell division in which one parent cell is physically separated into two distinct daughter cells. In order to execute cytokinesis, the cell assembles a contractile ring at the equatorial cortex, which then constricts and eventually leads to division. One can imagine that such constriction and fission of the cell equator is an active event requiring energy and metabolites. Since mitochondria are organelles that function in energy production, calcium homeostasis, ROS production and lipid metabolism, we asked whether mitochondria play an active role in cytokinesis. To address this question, we performed live imaging of mammalian cell lines to observe the distribution of mitochondria during cell division. We show that mitochondria localize to the cleavage furrow after anaphase onset and remain enriched at the cell equator until the end of cytokinesis. Using small molecule inhibitors of cytoskeleton dynamics we show that the equatorial localization of mitochondria is dependent on microtubules but not actin. Furthermore, depletion of Miro1, an outer mitochondrial membrane protein that links mitochondria to microtubules, leads to mitochondrial mislocalization during division. This project will establish novel roles for mitochondria in cytokinesis and provide critical insights into the molecular basis of cytokinesis. Such work is crucial for the development of improved cancer therapies.

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Phosphorylation of MyoGEF at Thr-544 by aurora B creates a docking site for Plk1.

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We previously reported that Plk1 activates MyoGEF by phosphorylating MyoGEF at Thr-574, the phosphorylation of which promotes the localization of MyoGEF at the central spindle. It is known that binding of Plk1 to its substrates requires a docking site (S-*S-P or S-*T-P) that is phosphorylated by priming kinases, such as CDK1/cyclin B. Analysis of the MyoGEF sequence revealed that there are two potential Plk1 docking sites in MyoGEF, i.e., Thr-544 (S-*T-P) and Ser-697 (S-*S-P). Although in vitro kinase assays showed that Cdk1/cyclin B phosphorylated MyoGEF, point mutations of Thr-544 or Ser-697 to Ala did not substantially change Cdk1 phosphorylation of MyoGEF, suggesting that Cdk1 did not phosphorylate Thr-544 and Ser-697. Interestingly, in vitro kinase assays showed that aurora B could phosphorylate a MyoGEF fragment that contains Thr-544 and Ser-697. Mutation of Thr-544 (but not Ser-697) to Ala completely abrogated the phosphorylation of the MyoGEF fragment by aurora B. In addition, co-immunoprecipitation experiments showed that the T544A point mutation decreased the interaction between MyoGEF and Plk1. Our results suggest that phosphorylation of MyoGEF by aurora B can create a Plk1 docking site in MyoGEF.

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Mutations in *Cog7* affect Golgi structure, meiotic cytokinesis and sperm development during *Drosophila* spermatogenesis.

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The Conserved Oligomeric Golgi (COG) Complex plays essential roles for Golgi function, vesicle trafficking and glycosylation. Deletions of human *COG7* are associated with a rare multisystemic congenital disorder of glycosylation causing mortality within the first year of life. We have characterized the *Drosophila* ortholog of *COG7* (*Cog7*). Loss-of-function *Cog7* mutants are viable but male sterile. *Cog7* is enriched in both the Golgi stacks and the Golgi derived structures throughout spermatogenesis. Mutations in *Cog7* disrupt Golgi architecture and reduce the number of Golgi stacks in primary spermatocytes. During spermiogenesis, loss of *Cog7* impairs the assembly of the Golgi-derived acroblast in spermatids and affects axoneme architecture. Similarly to the *Cog5* homologue Four way stop (*Fws*), *Cog7* enables furrow ingression during cytokinesis. We show that the recruitment of both the small GTPase *Rab11* and the phosphatidylinositol transfer protein *Giotto* (*Gio*) to the cleavage site requires the wild type function of *Cog7*. In addition *Gio* coimmunoprecipitates with both *Cog7* and *Rab11* in testes. Together our results implicate *Cog7* as an upstream component in a *gio-Rab11* pathway controlling membrane addition during cytokinesis.

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Chemical genetics to Polo-like kinase inhibition in *Trypanosoma brucei*

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The protist parasite *Trypanosoma brucei* (*T. brucei*) is a unicellular flagellated organism that causes African trypanosomiasis in humans and livestock. *T. brucei* multiplies by binary fission and this is a highly coordinated process in time and space. Polo-like kinases (PLKs) are known to regulate different cell cycle stages in eukaryotes. We became interested in understanding how the single PLK homolog in *T. brucei* (*TbPLK*) participates in the replication of the bilobe, a cytoskeletal structure of yet unclear function, and the closely associated Flagellar Attachment Zone (FAZ), which keeps the flagellum adhered to the cell body. With the help of chemical genetics tools, we designed a mutant *TbPLK* kinase that can be selectively and quickly inhibited using a small-molecule inhibitor, whereas all other kinases are not affected. Acute *TbPLK* inhibition resulted in rapid cell growth arrest and led to replication defects in both bilobe and FAZ. During the cell cycle, *TbPLK* localizes to both structures but, upon inactivation, the kinase was not able to migrate to the FAZ anymore. We then wondered whether *TbPLK* inhibition affects FAZ assembly directly or as a consequence of bilobe replication impairment. By introducing a synchronization method based on centrifugal elutriation, we found out that *TbPLK* regulates both bilobe and FAZ replication and is necessary for FAZ length extension.

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Leukemia-associated RhoGEF (LARG) is a Novel RhoGEF in Mitosis and Required for the Proper Completion of Abscission.

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Proper completion of mitosis requires the concerted effort of multiple RhoGEFs. Here we show that leukemia-associated RhoGEF (LARG), a RhoA specific RGS-RhoGEF, is required for abscission, the final stage of cytokinesis wherein the intercellular membrane is cleaved between daughter cells. LARG co-localizes with α -tubulin at the spindle poles before localizing to the central spindle. During cytokinesis, LARG is condensed in the midbody where it colocalizes with RhoA. HeLa cells depleted of LARG display apoptosis during cytokinesis with unresolved intercellular bridges, and rescue experiments show that expression of siRNA-resistant LARG prevented this apoptosis. Moreover, live cell imaging of LARG-depleted cells revealed greatly delayed fission kinetics in abscission wherein a population of cells with persistent bridges undergo abscission-related apoptosis. The formation of a Flemming body and secondary ingression sites in cells depleted of LARG is consistent with a defect in late abscission, just prior to fission. In contrast to studies of other RhoGEFs, particularly Ect2 and GEF-H1, LARG depletion does not result in cytokinetic furrow regression nor does it affect internal mitotic timing. These results show that LARG is a novel and temporally distinct RhoGEF required for the completion of abscission.

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How do fission yeast cells sense their size?

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To maintain a certain cell size, cells often grow to a certain size before committing to cell division. A key question in this process is how do cells sense their own size? In the rod-shaped fission yeast *S. pombe*, wildtype cells grow to 14 microns long before entering mitosis. Genetic analysis has identified a pathway involved in cell size control, in which the pom1p kinase inhibits the cdr2p kinase, which inhibits wee1p. A current model for cell length sensing is that a protein gradient of pom1 emanating from cell tips regulates a "sensor" of cdr2p located in dots at the medial cortex. Here, to test this model, we pursue a quantitative analysis of pom1p and cdr2p using fluorescence intensity measurements of fluorescent protein fusions in vivo. Our data suggest that contrary to the previous model, the gradient of pom1p is not used to sense cell length. Pom1 levels do not decrease at the medial cortex as cells grow longer. Further, a pom1 mutant with an altered gradient distribution does not result in abnormal cell length. Instead, cdr2p itself may act as a sizer. Cdr2p accumulates in dots at the medial cortex overlying the nucleus; its local concentration increases with cell length, even though its total concentration does not change. The dosage of cdr2p determines the length of cells at division. Pom1p may be required primarily to restrict cdr2p to the medial cortex. Thus, we propose a new model in which the cell size is regulated by the local concentration of cdr2p near the nucleus. As cells grow to 14 microns long, a critical concentration of cdr2p at the medial cortex may contribute to triggering entry into mitosis. This system illustrates how the localization of sizer to a discrete structure (such as the medial cortex) allows the cell to sense its size.

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A novel role for Blt1p in regulation of the Septation Initiation Network.*J. W. Goss¹, T. Pollard¹; ¹MCDB, Yale University, New Haven, CT*

Cytokinesis is the final step in the cell cycle during which a dividing cell separates into individual daughter cells. While many of the proteins involved in this process have been identified, the manner in which they interact with one another and coordinate their functions is largely unknown. One such protein identified in *S. pombe* is Blt1p, which functions prior to the onset of cytokinesis to ensure recruitment of the regulatory GEF, Gef2p, to the division plane and ensure proper placement of the contractile ring. This was believed to be its only function, however, I have recently found that Blt1p also interacts with proteins involved in the septation initiation network (SIN) that are required for completion of cytokinesis and septation in fission yeast. Specifically, Blt1p directly binds to the NDR-family kinase activator Mob1 and regulates its localization to the division plane prior to the onset of septation and completion of cytokinesis. This dual role early in mitosis and late in cytokinesis suggests that Blt1p could be an important regulator throughout cell division.

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Regulation of Rho GEF Gef2 by the adaptor protein Ger1 in fission yeast cytokinesis.*Y. Zhu¹, J-Q. Wu¹; ¹Molecular Genetics, The Ohio State University, Columbus, OH*

Cytokinesis is the last step of the cell-division cycle for successful cell reproduction, which requires precise control and regulation to ensure genomic stability. Rho GTPases and Rho guanine nucleotide exchange factors (Rho GEFs) are among the key regulators of cytokinesis. In higher eukaryotes, activated Rho GEFs and GTPases facilitate division-site positioning and promote contractile-ring formation during early cytokinesis. However, such roles had not been identified in fission yeast. Our lab recently found that putative Rho GEF Gef2 helps define the division site by coordinating with Polo kinase Plo1 to recruit anillin-like protein Mid1 to the medial cortex. Here we show that a novel Gef2-related protein Ger1 shares high similarity with the COOH-terminus of Gef2. Ger1 colocalizes with Gef2 in the contractile ring and its precursor nodes at stoichiometric ratio of 1:1. Like *gef2* deletion, *ger1* deletion has genetic interactions with various cytokinesis mutants such as *plo1* and *mid1*, suggesting a role of Ger1 in early cytokinesis. However, sequence analysis finds that Ger1 has no GEF domain. Thus, Ger1 may regulate cytokinesis through Gef2 as an adaptor protein. We found that the two proteins are interdependent on their COOH-termini for the localization to cortical nodes. Furthermore, Ger1 and Gef2 interact through their COOH-termini in yeast two hybrid and Co-IP assays. We propose that the Ger1-Gef2 interaction is crucial for Gef2 localization and function during early cytokinesis in fission yeast. These and other results will highlight the existence and importance of Rho GEF adaptor proteins in regulating Rho GEF functions.

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Analysis of Cellulose Synthase Trafficking and New Cell Wall Biosynthesis During Plant Cytokinesis.*C. T. Anderson¹, C. Somerville²; ¹Biology, The Pennsylvania State University, University Park, PA, ²Energy Biosciences Institute, University of California Berkeley, Berkeley, CA*

Unlike animal cells, plant cells accomplish cytokinesis by building a double-sided wall to separate new sister cells. Plant cytokinesis involves an orchestrated series of vesicle trafficking, membrane remodeling, and wall polymer biosynthetic events, all of which can occur in less than 60 minutes in the model plant *Arabidopsis thaliana*. However, many molecular details of how

new wall synthesis is carried out and regulated remain unknown. By monitoring the subcellular localization of cellulose synthase (CESA) and microtubules in vivo using spinning disk confocal microscopy, we determined the location and trafficking patterns of CESA over time in dividing cells at high temporal resolution. We discovered that CESA is depleted from the cortical plasma membrane during mitosis and cytokinesis and becomes highly enriched at the cell plate, an intermediate structure that gives rise to the new cell wall. These results suggest that CESA secretion is largely diverted from the cortical plasma membrane to the division plane during cytokinesis. Using drug treatments to inhibit specific trafficking pathways and cell plate maturation steps during cytokinesis, we found that CESA is delivered to the cell plate well before the earliest stage at which cellulose is detectable in this compartment, suggesting that in contrast to the case at the cortical plasma membrane, CESA becomes active long after its initial delivery to the cell plate. We also determined that CESA is mainly delivered to the cell plate via a de novo secretory pathway, that normal CESA distribution in the cell plate requires an intact actin cytoskeleton and Brefeldin A-sensitive trafficking events, and that inhibitors of CESA activity do not prevent the delivery of CESA to the cell division plane. In combination with rapidly accumulating knowledge of membrane trafficking events during plant cytokinesis, these results elucidate how cellulose-producing proteins are trafficked and regulated to help build new plant cell walls, which can serve as renewable sources of food, materials, and bioenergy.

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Translocation of the Chromosomal Passenger Complex to the central spindle in anaphase requires deacetylation of INCENP.

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The Chromosomal Passenger Complex (CPC) is required for error-free chromosome segregation and for proper execution of cytokinesis. To coordinate nuclear division with cytoplasmic division, the CPC has to translocate from centromeres to the overlapping anti-parallel microtubules of the central spindle in anaphase. How this translocation is regulated is not fully understood. We found that it requires the microtubule-binding capacity of the coiled-coil (CC) domain of INCENP. An INCENP mutant lacking the coiled-coil domain localized to the cell cortex but failed to translocate to the midzone microtubules in anaphase. Cortical localization of INCENP- Δ CC promoted cleavage furrow ingression in anaphase, but gave rise to midbodies with reduced amounts of Aurora B in telophase. Importantly, central spindle localization and midbody accumulation of Aurora B was restored when the INCENP coiled-coil domain was replaced by an alternative microtubule-binding domain. Interestingly, in recent proteomics screens, INCENP was found being acetylated and the identified acetylated lysine residues appeared to reside in the coiled-coil domain of INCENP. We reasoned that the microtubule binding capacity of the INCENP coiled-coil is suppressed in (pro)metaphase by acetylation that neutralizes the positive charge of the lysine residues. Indeed, mutation of the lysine residues into glutamine to mimic constitutive acetylation, perturbed INCENP translocation to the central spindle. We therefore propose that translocation of the CPC to the central spindle in anaphase not only requires dephosphorylation, as shown before, but also deacetylation of INCENP.

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Microvilli are disassembled to provide membrane for cleavage furrow ingression.

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Cell shape change often requires cell surface growth, but the source of new membrane fueling this growth is in many cases unknown. A decades-old hypothesis proposes that unfolding

microvilli can provide new membrane for cell surface growth during cytokinesis, phagocytosis, wound healing, and cell spreading, although this has never been definitively demonstrated. Here we show that microvilli serve as a membrane source for cleavage furrow growth during cellularization, the first complete cytokinetic event in the *Drosophila* embryo. We have characterized the density and morphology of microvilli that coat the embryo surface at the start of cellularization by scanning electron microscopy (SEM), and we calculate that microvilli contain enough membrane to support all furrow growth. Accordingly, we see many microvilli before cellularization, but almost none after. Using 3D imaging of live cells over time, we find that microvillar membrane is depleted slowly at the start of cellularization and is depleted rapidly later in the process. Remarkably, the rate of microvillar membrane depletion follows the biphasic kinetics of furrow ingression. Finally, quantitative analysis correlating our SEM and live-cell imaging data shows that microvillar membrane depletion and microvilli density decrease linearly as furrow length increases throughout cellularization. To then directly show that microvillar membrane contributes to furrow growth, we labeled and tracked microvillar membrane as it moved along the cell surface and into ingressing furrows. This work shows that microvilli are disassembled to provide membrane for furrow ingression and suggests that the rate of microvillar disassembly may govern furrow ingression kinetics.

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Global lipid profiling of dividing cells reveals the significance of sphingolipids during cytokinesis and at the midbody.

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Although cells undergo dramatic shape changes during cytokinesis, the role of the plasma membrane and lipids is poorly understood. It is known that a few lipids localize to the cleavage furrow, and that the specific distribution of these lipids is necessary for cleavage. However, the full spectrum of lipids that may be involved in cytokinesis, and their functions are still unclear. Therefore, to identify further lipids that are involved in cytokinesis, we employed LC-MS based global lipid profiling. Specifically, we synchronized HeLa cells at cytokinesis and S-phase. We compared the lipid profiles of cells at S-phase and cytokinesis and identified lipids that change during cytokinesis. To gain insights into the role of lipids during late stages of cytokinesis, we also analyzed the lipid content at the midbody region, which is where cleavage occurs. We isolated midbodies and compared the lipid content of this region to that of cell lysate and cells at cytokinesis. This analysis revealed primarily accumulation of different dihydroceramides, and ceramides during cytokinesis as well as in the midbody region. In a parallel study to address the functional roles of lipids in cytokinesis, we assembled a small library of small molecules that inhibit different steps of lipid biosynthesis and evaluated their effects on cytokinesis. We found that inactivation of glucosyl ceramide synthase (GCS), either with the small molecule PPMP or by RNAi, causes failure of cleavage furrow ingression. We identified individual lipids that are enhanced or depleted due to GCS inhibition. We found that PPMP treatment primarily causes accumulation of C16- and C22-ceramide whereas siGCS treatment causes depletion of glucosyl ceramide species. We show that GCS inhibition results in the mislocalization of actin and the ERM proteins, key cytoskeletal proteins that connect the plasma membrane to the actin cortex. We propose that an imbalance between ceramides and glucosylated ceramides can trigger cortical changes, possibly mediated by interactions between ceramides and ERM proteins, which can in turn cause changes in the actin cytoskeleton. Our data provide evidence linking ceramides and other ceramide-related lipids to cytokinesis, and add a new class of lipids to the growing list of membrane components that play a role in cell division.

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G-protein-coupled receptors participate in cytokinesis.

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Cytokinesis, the last step during cell division, is a highly coordinated process that involves the relay of signals from both the outside and inside of the cell. We have a basic understanding of how cells regulate internal events, but how cells respond to extracellular cues is less explored. In a systematic RNAi screen of G-protein-coupled receptors (GPCRs) and their effectors, we found that some GPCRs are involved in cytokinesis. RNAi knockdown of these GPCRs caused increased binucleated cell formation, and live cell imaging showed that most formed midbodies but failed at the abscission stage. OR2A4 localized to cytokinetic structures in cells and its knockdown caused cytokinesis failure at an earlier stage, likely due to effects on the actin cytoskeleton. Identifying the downstream components that transmit GPCR signals during cytokinesis will be the next step and we show that GIPC1, an adaptor protein for GPCRs, may play a part. RNAi knockdown of GIPC1 significantly increased binucleated cell formation. We also found that RNAi of Dopamine receptor D3 (DRD3) inhibits endocytic sorting as well as cytokinesis. The antihypertensive drug Prazosin stabilizes a normally transient interaction between DRD3 and the coatome COPI, a complex involved in membrane transport, and shifts endosomal morphology entirely to tubules, disrupting cargo sorting. Understanding the molecular details of GPCRs and their interaction proteins in cytokinesis regulation will give us important clues about GPCRs signaling as well as how cells communicate with their environment during division.

Mitosis II

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The epigenetic basis of centromere identity and maintenance.

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The basic element for chromosome inheritance, the centromere, is specified in mammals by an undefined epigenetic mark rather than by DNA sequence. Using targeted replacement in human cells and fission yeast, centromere position is shown to be maintained indefinitely by chromatin assembled with CENP-A, the histone variant that replaces histone H3 at functional centromeres. Chromatin containing the CENP-A or histone H3 carrying the CENP-A targeting domain (CATD) is shown to template its cell cycle-dependent replication through action of the chaperone/chromatin loader HJURP/Scm3. Nucleation of kinetochore assembly onto CENP-A chromatin is shown to require either direct recruitment of CENP-C by CENP-A's short carboxy-terminal tail or stabilizing binding other components, including CENP-B, by CENP-A's amino-terminal-most tail. Thus, a conserved, two-step mechanism for is identified for both human and fission yeast in which CENP-A-containing chromatin is the epigenetic mark of centromere identity by templating its own replication and nucleating subsequent kinetochore assembly.

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Analysis of the function of the chromosomal passenger complex in regulating spindle and central spindle formation in *Drosophila*.

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The Chromosomal Passenger Complex (CPC), consisting of the Aurora B kinase and three regulatory subunits Borealin, Survivin/Deterin and INCENP, is a highly conserved complex which is required for regulating many distinct aspects of cell division, such as mitotic chromosome structure, spindle assembly and disassembly, kinetochore-microtubule attachment, central spindle formation and cytokinesis. We are investigating the functions of the CPC during cell division in *Drosophila*, primarily its role in central spindle formation and cytokinesis.

Quantitative analyses of the spatio-temporal properties of GFP-fusions to each of the four *Drosophila* CPC homologues confirms their similar cell cycle regulated distribution in embryonic mitoses, highlighting the presence of a population of the CPC on the mitotic spindle between prometaphase and metaphase, and the dramatic redistribution of the CPC from centromeres to interpolar microtubules at the onset of anaphase.

In order to dissect the specific role of the CPC on the spindle, we have utilised a microinjection approach. Previous work in humans has identified a region of INCENP required for targeting the CPC to the spindle. Injection of a polypeptide corresponding to this region of DmINCENP into syncytial embryos expressing AuroraB-GFP confirms a delay in the accumulation of the CPC on the spindle around the site of injection and a gradation of effects further from the injected area. Injection of the polypeptide into embryos expressing α -Tubulin-GFP line also demonstrates a reduction in the amount of microtubule nucleation within the spindle. This is consistent with the known role of the CPC in promoting spindle formation and demonstrates that injection of this domain provides a tool for further investigating how the CPC contributes to this process.

We are also using the GFP-CPC embryos to understand the molecular details by which the CPC redistributes to interpolar MTs at anaphase onset. We have developed a method, based on incubation of GFP-CPC embryos with inhibitors of cell cycle progression, to isolate embryos in a metaphase- or anaphase-like state, subjecting these extracts to co-immunoprecipitation and mass spectrometry. Through this, we have identified putative novel interactors of the CPC. We are currently analysing the function of these proteins and their localisation during mitosis.

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Functional states of bivalent kinetochores during prometaphase congression.

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To achieve metaphase alignment, bivalent chromosomes in meiotic crane-fly spermatocytes typically move slowly at maximal velocities of $\sim 0.5\mu\text{m}/\text{min}$ from off-equator positions to the spindle equator during the course of prometaphase. In such congression, the leading kinetochores of one homologue move toward (P) a distant pole, and the trailing kinetochores of the other homologue move away (AP) from a near pole. Crane-fly bivalents do not exhibit directional instability (oscillating back and forth between P and AP movement), characteristic of chromosomes during congression in vertebrate mitosis. Hence, our objective was to determine

the applicability of the mechanism of congression in well-studied vertebrate mitosis to non-oscillating bivalents. Specifically, we wanted to ascertain whether leading kinetochores engage in P motility and trailing AP partners are in a neutral state, as observed in vertebrate mitosis by Khodjakov and Rieder (1996. *J Cell Biol.* 135:315-327). We used a laser microbeam to cut one of the three prometaphase bivalents of spermatocytes into two “univalents”, each having the two sister kinetochores of their homologue plus attached chromatin that varied in size, depending on the location within the bivalent where the cut was made. Live cell imaging following the cut and frame-by-frame analysis of digital movies revealed new findings about released kinetochores. Both sets of bivalent kinetochores (one pair of sisters to one pole, and the other pair to the opposite pole) exhibit rapid P movement immediately after the operation. There was no detectable difference in the motile capabilities of leading kinetochores and trailing kinetochore subsequent to the operation. Moreover, rapid-than-normal post-op P velocities are suggestive that univalent kinetochores had converted from a functional state in which microtubule polymerization takes place at kinetochore microtubule plus ends to a depolymerization state consistent with pac-man motility. Maximal P movement of normally congressing bivalent kinetochores is slower [$\sim 0.5\mu\text{m}/\text{min}$ to the pole] than the average flux rate [$\sim 0.8\mu\text{m}/\text{min}$], whereas P movement of released univalent kinetochores is faster [$\sim 2.0\mu\text{m}/\text{min}$] than the flux rate. Thus, in crane-fly spermatocytes, where chromosome movement is dominated by traction fiber mechanics, the release of bi-oriented kinetochores from their normal bipolar tension during prometaphase caused conversion to a pac-man state. For related work, see LaFountain, et al, 2012. *MBoC* 23(16):3133-3142.

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A quest for proteins that bind the flared ends of microtubules.

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Electron tomography suggests that fibrils form direct connections between centromeric chromatin and the flaring plus ends of kinetochore-associated MTs. We have therefore sought proteins that bind better to curled oligomers of tubulin than to normal MTs. Using Vinblastine to polymerize tubulin into “curls” and Taxol to stabilize normal MTs, we have examined the proteins that can be pulled down from lysates of human cell with these two forms of assembled tubulin. Electrophoresis shows that both sets of proteins are complex, but liquid chromatography and mass spectrometry have allowed the identification and approximate quantification of large numbers of proteins from each kind of pull-down. Extracts of either U2OS or RPE1 cells were prepared from both log-phase cultures and from populations enriched for mitotic cells by Nocodazole arrest and mitotic shake-off. Extracts, proteins pulled down by tubulin polymers, and proteins released from the pull-downs by high salt were subjected to extensive tryptic digestion, chromatographic separation on an Acquity UPLC system, and then analysis with an Orbitrap mass spectrometer. MS/MS data were searched against a human protein database using MASCOTT, and the abundance of each protein identified was estimated by spectral counting. Among the thousands of proteins pulled down, we have focused on components of the cytoskeleton. Several actin-binding proteins clearly prefer tubulin curls to MTs, as do a few kinesins and MT-associated proteins. Centromere Binding Protein F (CENPF) is prominent in the latter group, and given its known properties as an important kinetochore protein that contains long stretches of alpha-helical coiled coil, we have focused our principal attention here. c-DNAs that encode parts of the CENPF molecule have been expressed in bacteria or SF9 cells. Polypeptides from the N-terminal region of the protein bind better to curls than to MTs, while the C-terminal region of the protein prefers MTs. We are working to characterize these

binding reactions and to learn the impact of protein fragment expression on the growth of human cells in culture.

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Formin mDia3 regulates kinetochore movements in mitosis.

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The mammalian diaphanous-related (mDia) formin proteins are well known for their actin-nucleation and filament-elongation activities in mediating actin dynamics. They also directly bind to microtubules and regulate microtubule stabilization at the leading edge of migrating cells. By using mammalian cultured cells, we have profiled spatiotemporal dynamics of the formin mDia3 in mitosis and have found that mDia3's kinetochore association is important for metaphase chromosome alignment, a process in which kinetochores form stable attachments with growing and shrinking microtubules. In addition, with high resolution live imaging and quantitative analysis of kinetochore movements, we have observed apparently altered kinetochore oscillations in cells expressing a phosphomimetic mDia3 mutant that has reduced ability to bind to microtubules, suggesting that the formin mDia3 could contribute to the regulation of kinetochore-bound microtubule dynamics, via its own microtubule-binding activity, and/or in coordination with its interaction partners at microtubule plus ends.

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Dissecting the kinetochore throughout *Toxoplasma gondii* mitosis and cell cycle.

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The obligate intracellular parasite *Toxoplasma gondii* exhibits closed mitosis, as chromosome segregation occurs within an intact nuclear envelope. In addition, the nucleolus is maintained and condensation of chromosomes is restricted, as such distinct structural changes are absent during mitosis. Moreover, the kinetochores are clustered and the chromosomes remain anchored to the spindle poles through their centromeres throughout the cell cycle. This persistent clustering and anchoring is likely functioning in maintaining a full set of chromosomes throughout division. This phenomenon appears to be critical for bona fide chromosome segregation, yet the mechanism by which this is organized remains unknown. Thus, in an effort to define kinetochore-mediated clustering and anchoring of chromosomes, we are focusing on two microtubule-binding proteins, TgNuf2 and its binding partner TgNdc80, subunits of the Ndc80 complex. A conserved kinetochore component from yeast to humans, the Ndc80 complex is an essential component of the kinetochore, required for the establishment of stable microtubule attachments, chromosome segregation, and kinetochore assembly. We are currently investigating the role of these proteins during *T. gondii* cell division, specifically focusing on chromosome anchoring and segregation. The generation of a conditional TgNuf2 knockout revealed this kinetochore component is essential for proper chromosome segregation, as demonstrated by the loss of the nucleus at a high incidence. Moreover, the conditional knockout revealed the spindle pole remains intact. Loss of TgNuf2 revealed microtubules are required for the anchoring of chromosomes during mitosis, but are dispensable for kinetochore clustering. Additionally, we are in the process of mapping the *T. gondii* kinetochore proteome through affinity purification, using strains expressing TgNdc80 and TgNuf2 fusions, to fully characterize the composition of the kinetochore. TgNuf2 and TgNdc80 were isolated in reciprocal immunoprecipitations, demonstrating their interaction, while we are validating

potential additional kinetochore proteins. These findings will contribute to a greater understanding of chromosome anchoring and segregation.

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A Nuclear-Derived Proteinaceous Matrix Embeds the Microtubule Spindle Apparatus during Mitosis.

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The concept of a spindle matrix has long been proposed; however, whether such a structure exists and its molecular and structural composition has remained controversial. In *Drosophila* we have recently identified two nuclear proteins, Chromator and Megator, from two different nuclear compartments that interact with each other and that redistribute during prophase to form a molecular complex that persists in the absence of polymerized tubulin. Chromator is localized to polytene chromosome interbands during interphase whereas Megator occupies the nuclear rim and the intranuclear space surrounding the chromosomes. Chromator has no known orthologs in other species; however, Megator is the homolog of mammalian Tpr. The Megator/Tpr family of proteins is highly conserved through evolution and structural homologs are present from yeast to humans. Moreover, Megator and human Tpr have been demonstrated to have a shared function as spatial regulators of spindle assembly checkpoint proteins during metaphase. In this study using a live imaging approach in *Drosophila* syncytial embryos we demonstrate that these nuclear proteins reorganize during mitosis to form a highly dynamic, viscous spindle matrix that embeds the microtubule spindle apparatus, stretching from pole-to-pole. We show that this "internal" matrix is a distinct structure from the microtubule spindle and from a Lamin B containing spindle envelope, and by injection of 2000 kDa dextrans, that the disassembling nuclear envelope does not present a diffusion barrier. Furthermore, when microtubules are depolymerized with colchicine just prior to metaphase the spindle matrix contracts and coalesces around the chromosomes suggesting that microtubules act as "struts" stretching the spindle matrix. Additionally, we demonstrate that the spindle matrix protein Megator requires its coiled-coil amino-terminal domain for spindle matrix localization suggesting that specific interactions between spindle matrix molecules are necessary for them to form a complex confined to the spindle region. The demonstration of an embedding spindle matrix lays the groundwork for a more complete understanding of microtubule dynamics and of the visco-elastic properties of the spindle during cell division. Supported by NSF grant MCB0817107.

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Regulation of a microtubule depolymerizing kinesin by importin α contributes to spindle size scaling during *Xenopus* development.

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Early development of many animals is characterized by rapid cell divisions in the absence of growth, generating progressively smaller cells during embryogenesis. The mitotic spindle must adapt to the changing cell dimensions in order to properly segregate chromosomes to daughter cells, but how spindle size and architecture are modified is not understood. To identify physiological mechanisms that operate during development of the frog *Xenopus laevis*, we prepared cytoplasmic extracts from embryos at different developmental stages that are capable of reconstituting mitotic spindle assembly in vitro. Large spindles ~40 μm in length formed in extract derived from stage 3 (4 cell) embryos, while stage 8 (~4000 cell) extract spindles were ~20 μm , recapitulating the scaling of spindle size observed in vivo. Mixing of the two extracts

generated spindles of intermediate size, further indicating the existence of a biochemical scaling mechanism that is not the direct result of physically confining the spindle. We found that microtubules in stage 8 extract undergo catastrophe more frequently than those in stage 3 extract, and stage 8 spindles specifically recruit the microtubule depolymerizing kinesin-13 kif2a, suggesting that a decrease in microtubule stability contributes to spindle scaling during development. Interestingly, kif2a contains a nuclear localization sequence and interacts with the transport receptor importin α , which inhibits its microtubule depolymerizing activity in vitro. In vivo, postranslational modifications lead to a redistribution of importin α to a membrane fraction of stage 8 embryos. This suggests that the increasing ratio of plasma membrane to cytoplasm during development may act as a sink to sequester importin α and concomitantly increase kif2a activity, thereby reducing spindle size. In support of this model, manipulating kif2a or importin α levels in developing embryos could decrease spindle size in stage 3 blastomeres or increase spindle size in stage 8 blastomeres. Small spindles became randomly oriented in large cells, highlighting the importance of spindle scaling for polarized cell divisions during development.

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Spindle assembly requires suppression of microtubule plus-end dynamics by the unprocessive kinesin-8 Kif18B.

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During mitosis, a bipolar spindle is assembled from dynamic microtubules to segregate a replicated set of chromosomes among two daughter cells. Spindle bipolarity is established by motor proteins that act on both interpolar and astral microtubules to push and pull spindle poles apart, respectively. Whether compressive forces generated by the interaction of astral microtubules with the cell cortex inhibit spindle formation is largely unexplored. Here, we show that the kinesin-8 Kif18B is required for efficient bipolar spindle assembly, characterize its motile properties and ability to regulate microtubule plus-end dynamics. Kif18B is a human kinesin-8 that concentrates at astral microtubule plus-ends by hitchhiking with the +TIP EB1. Using a single molecule TIRF assay, we find that Kif18B is unlike canonical kinesin-8s in that it is highly unprocessive, shows periods of fast diffusive movement, and interacts only weakly with the microtubule lattice. Despite these properties, Kif18B shows striking enrichment at the extreme plus-end of some astral microtubules where the motor locates in front of EB1 comets. By analyzing the effect of Kif18B on the behavior of dynamic microtubules, we show that Kif18B, like the related motor Kif18A, suppresses microtubule plus-end dynamics in a manner potentiated by EB1. In cells, Kif18B depletion causes abnormal elongation of astral microtubules and an increase in the number of cells with monopolar spindles. This phenotype is exacerbated by pharmacological inhibition of kinesin-5/Eg5, a motor that pushes spindle poles apart. Collectively, these data suggest that Kif18B facilitates spindle formation by suppressing the growth of astral microtubules which in turn allows for efficient spindle pole separation by plus-end-directed motors that slide interpolar microtubules apart.

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Temporally distinct roles for TACC3/ch-TOG/clathrin microtubule crosslinkers during mitosis.*L. Cheeseman¹, I. Prior¹, S. Royle¹; ¹Cellular and Molecular Physiology, University of Liverpool, Liverpool, United Kingdom*

Equal segregation of chromosomes between daughter cells during mitosis is essential to avoid aneuploidy, a hallmark of cancer. A TACC3/ch-TOG/clathrin complex was recently found to crosslink and stabilize kinetochore fiber (K-fiber) microtubules (MTs), thereby contributing to efficient mitosis. TACC3 and ch-TOG were described previously to promote MT nucleation and polymerization in early mitosis, but it is unclear whether this function is clathrin-independent.

Here, we distinguish between roles of the complex at early versus late mitosis by using 'knock-sideways' (KS), a rapamycin-based removal technique to rapidly reroute specific proteins to the mitochondria.

KS of TACC3 resulted in complete removal of the protein from the metaphase spindle within 10 minutes of rapamycin application. Its mitotic partners ch-TOG, clathrin and GTSE1 were also removed and localized to the mitochondria, whereas the unrelated spindle proteins NuMA, HURP and Eg5 were unaltered. Similarly, rerouting of clathrin light chain fully removed TACC3 and associated proteins, indicating that there is no clathrin-independent TACC3 on the metaphase spindle.

KS of TACC3 at early mitosis caused severe delays in chromosome alignment, with most cells failing to achieve metaphase within 3 hours of rapamycin application. Spindle regrowth following nocodazole treatment indicated that this delay was likely due to reduced MT polymerization.

Intriguingly, KS of TACC3 at metaphase (after a normal prometaphase) delayed anaphase, indicating that the TACC3/ch-TOG/clathrin complex has an ongoing function in K-fibers after fiber maturation and chromosome alignment. In these cells, we found that the spindle checkpoint was reactivated. Furthermore, inter-kinetochore distance was reduced by ~40%, suggesting a partial loss of K-fiber tension. Correlative light-electron microscopy of metaphase K-fibers 10 and 30 minutes post-rapamycin application revealed a loss of MT crosslinkers. Interestingly, K-fiber MT numbers and spacing remained unaltered. These results suggest that tension may contribute to silencing the spindle checkpoint independently of kinetochore attachment.

Our results show that the TACC3/ch-TOG/clathrin complex contributes to spindle function in several ways: firstly, by promoting MT nucleation and polymerization at early mitosis to mature K-fibers, secondly by maintaining K-fiber tension, thereby silencing the spindle checkpoint. We also demonstrate the advantages of rapid methods for removal of spindle proteins over slower methods, such as RNAi.

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Modeling the dynamics and structure of the mitotic spindle.*J. Bruges¹, D. Needleman¹; ¹Harvard University, Cambridge, MA*

The mitotic spindle is an array of microtubules that segregate chromosomes during cell division. It has been difficult to validate models of spindle assembly due to a lack of quantitative measurements in these structures. To understand the spindle architecture and dynamics, we experimentally test different models for spindle assembly, by quantitatively comparing measured and predicted spatio-temporal correlation functions of microtubule density, orientations, and internal stresses. These measurements are precise enough to formulate a complete theory for the dynamics and structure of the spindle. Our theory quantitatively predicts the overall shape of the spindle, the orientation and polarity of microtubules. Our results show that the spatial

regulated microtubule nucleation and a local interaction of microtubules are sufficient to account for spindle morphogenesis.

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Evolution and Genetic Architecture of the First Mitotic Spindle in *C. elegans*.

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The architecture and dynamics of sub-cellular structures show remarkable variations between species, but little is known about the evolutionary or mechanistic basis of this diversity. Examining intraspecies variation can provide valuable evolutionary insights because differences between species arise from differences between individuals within species, but the extent of intraspecific variation of sub-cellular traits is unknown. Mutations are the ultimate source of variation between individuals. Understanding how spontaneous mutations effect sub-cellular processes shows what phenotypes are evolutionary accessible and provides a “baseline” of how these traits would change in the absence of selection.

We are using the first embryonic division in *C. elegans* to study the evolution of the mitotic spindle. We developed a high-throughput microscopy platform and automated image analysis software that allows us to obtain quantitative information on the structure and dynamics of the spindle from thousands of embryos in hundreds of lines. We have found extensive standing genetic variation among natural isolates of *C. elegans* for all traits we have measured including: cell size; the size and motion of pronuclei; the length, motion, and speed of elongation of the spindle; the size of centrosomes; the positioning of the cleavage plane. We are studying the genetic architecture of these traits by performing a genome wide association analysis (aided by Erik Anderson, Princeton), by characterizing a panel of recombinant inbred advanced intercross lines (aided by Matt Rockman, New York University), and by investigating these same traits in a previously performed genome wide RNAi screen (Sonnichsen, et al, Nature 2005).

We are also studying how spontaneous mutations modify the spindle by characterizing cell division in a panel of mutation accumulation (MA) lines - created by propagating an initially genetically identical set of lines at low effective population size for ~250 generations (aided by Charlie Baer, University of Florida). Comparing the spectrum of variations in MA lines to the spectrum of variations we observe among natural isolates allows us to draw inferences about how selection and population dynamics combine with raw mutational inputs to shape the spindle in *C. elegans*.

Our preliminary results are very promising and suggest that combining cell biology, biophysics, and quantitative genetics will produce novel evolutionary and mechanistic insights.

1107

Towards a MAP interactome: Using a predictive logistic regression model to identify novel *Drosophila* mitotic MAPs.

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The nucleation, length and dynamics of microtubules (MTs) that constitute the mitotic spindle are determined by MT associated proteins (MAPs). Our lab recently undertook a multi-disciplinary study, combining proteomics, functional screening and bioinformatics, to find proteins capable of binding to and altering the properties of MTs during mitosis in the early *Drosophila* embryo. However, although this work identified 270 MAPs, 2D gel analysis showed the presence of at least 600 MAPs in this tissue suggesting our list of MAPs is far from complete. In addition, in order to progress from a list of MAPs to an understanding of the way in

which they determine spindle self-organisation, the way in which these MAPs interact with one another in the cell needs to be determined.

To move towards this goal, we have used reciprocal BLAST to identify *Drosophila* homologues of MAPs originally found through biochemical techniques in other model species (humans, mouse, rat and *A. thaliana*), interrogating the BIOGRID protein-protein interaction (PPI) database with this list to produce a single PPI network containing 1134 putative *Drosophila* MAPs. In order to predict the likelihood of each of these proteins binding MTs and having a function during mitosis we have developed a logistic regression model that uses multiple features such as protein interactions, gene expression data, domain composition, along with data from a compilation of genome-wide mitotic RNAi screens, training the model against both positive and negative datasets.

The final output of this model is a ranked list of predicted MAPs with uncharacterised roles in mitosis. Strikingly, thirteen of the top fifty putative MAPs interact directly or indirectly with one another, and sub-cluster with protein kinases that have known roles in cell cycle regulation, and with other putative MAPs in our list. A functional analysis of this cluster of proteins using RNAi in *Drosophila* S2 cells demonstrates roles for at least ten of the high-ranking putative MAPs in the regulation of spindle formation, centrosome number and chromosome segregation, strongly suggesting that we have identified previously unrecognised mitotic MAPs with related function.

1108

A novel and essential asymmetric centrosome protein in *Drosophila*.

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Asymmetric cell division is critical during development. It is the mechanism by which stem cells renew themselves while at the same time produce a population of differentiated cells with the potential to make multiple cell types. Asymmetric cell division involves complex coordination between the cellular cortex, centrosomes and the mitotic spindle. Centrosomes in mitotic cells are inherently asymmetric because one cell will inherit the older (mother) centrosome while the other cell will inherit the newer (daughter) centrosome. During stem cell divisions the asymmetry of the centrosomes is coordinated directly with the asymmetry of cell fate. Neural stem cells (neuroblasts, NB) inherit the daughter centrosome at each division. Conversely, male germline stem cells (GSC) inherit the mother at each asymmetric division. We have identified a novel *Drosophila* centrosomal protein that associates specifically with daughter centrosomes in asymmetrically dividing NBs and GSCs. In NBs this protein localizes to the centriole. In contrast, distribution of this protein is not asymmetric in symmetrically dividing cells. In symmetrically-dividing ganglion mother cells (GMCs) and cells of the embryo, it localizes to the periphery of the pericentriolar matrix (PCM) as defined by Cnn staining. We screened for EMS-induced mutations, and identified a nonsense deletion in the middle of the ORF. This mutation appears to be a null allele. Mutation of the gene and RNAi knockdown cause mislocalization of asymmetric determinants in larval NBs and lethality at early pupal stages. Together, these results show that this novel asymmetric centrosome protein is essential for asymmetric division and animal development.

1109

Examining the Spatial Organization of the Centromere Throughout the Cell Cycle.

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The kinetochore is the primary microtubule-binding site on the chromosome and governs accurate chromosome segregation during cell division. Kinetochores form during mitosis on a specialized chromatin domain called the centromere. In higher eukaryotes, the location of the

centromere is thought to be epigenetically specified; the proteins that comprise the centromere determine its identity independent of the DNA sequence upon which it is built. The centromere is a large multiprotein complex. Understanding how these proteins act in concert to maintain the identity of the centromere through rounds of cell division and how the cell reads out the location of the centromere remain open questions. Understanding centromere and kinetochore function requires knowing how individual protein subunits of the centromere and kinetochore are spatially arranged and how this organization changes over the course of the cell cycle.

We present a technique called Colocalization and In-situ Correction of Aberration for Distance Analysis (CICADA) and use it to examine the spatial organization of the centromere and how it changes through the cell cycle at 10 nm length scales using standard widefield fluorescence microscopy. In particular, we examine centromeres before DNA replication, just after DNA replication and in metaphase of mitosis in order to understand how centromere organization might influence its major functions of self-propagation and providing a platform for mitotic kinetochore assembly.

1110

ILLUMINATING AURORA A'S *in vivo* BIOCHEMISTRY.

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Aurora kinase A (AurA) is a member of the Aurora family of mitotic serine/threonine kinases first discovered in a *Drosophila* mutagenesis screen for activities regulating the centrosome cycle. As a major player in the control of mitosis, AurA has many roles including regulating asymmetric cell division, mitotic entry, centrosome maturation, centrosome dependent and independent microtubule nucleation and organisation and anaphase spindle dynamics. Although AurA's activation and role in bipolar spindle assembly are known to be dependent on its interactions with TPX2 and CEP192 in mammalian systems, generally, however, the *in vivo* biochemistry underpinning many of AurA's cellular functions has yet to be elucidated. Here we have utilised a co-immunoprecipitation and mass spectrometry approach to identify potential cell cycle phase specific regulators of AurA activity, localisation and degradation in a comparative study between synchronised populations of human cells and *Drosophila* embryos and cell culture. Over 600 novel AurA-specific interactors have been identified, many of which in the human screen are specific to a single cell cycle phase, allowing us to infer how AurA functions change as the cell cycle progresses. In addition, the *Drosophila* screen identified a potentially significant interphase AurA function in regulating muscle and neuron development. A selection of interactors from both screens are now being validated and studied in detail in order to investigate the physiological role of their interaction with AurA.

1111

FUNCTIONAL SIGNIFICANCE OF POLO-LIKE KINASE 1 PHOSPHORYLATIONS REVEALED BY CHEMICAL GENETICS.

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Mitosis is coordinated by carefully regulated phosphorylation and protease activity. Polo-like kinase 1 (Plk1) is a core regulator of mitosis. We previously developed a chemical genetic system to allow specific interrogation of Plk1 function in human cells. Here, we extend this system using genetic complementation to evaluate the functional significance of previously identified Plk1 phosphorylation sites *in vivo*. With this method, we are able to specifically target one of the Plk1 alleles using chemical inhibition. The other allele is resistant to treatment and

can be mutated to test the functionality of this mutated version of Plk1. In our experiments, we mutated Plk1 at known phosphorylation sites to examine their ability to rescue known roles of Plk1. As a control, we showed that wildtype Plk1 is able to rescue normal functions of Plk1. Surprisingly, Plk1 mutations at most phosphorylation sites were also able to rescue including: T6V, S103A, S330A, S375A, S450A, S461A, T464V, and T498V. Interestingly, cells containing the Plk1- S450A, S461A, T464V, and T498V mutants preferentially expressed this mutated Plk1 over other alleles, suggesting that the inability to phosphorylate these residues increased protein stability.

Plk1-T214V, however, was unable to rescue multiple Plk1 functions: cells grow and proliferate more slowly, are unable to progress normally through mitosis, have defective spindle maturation, and have a reduced ability to enter mitosis following DNA damage. We next sought to identify the kinase responsible for this posttranslational modification. T214 is immediately followed by a proline, making Cdk1 an attractive upstream candidate kinase. However, two assays have indicated that Cdk1 is not responsible. First, *in vitro*, Cdk1 did not significantly phosphorylate purified Plk1. Also, when the proline is mutated to alanine, normal mitotic functions of Plk1 are not affected, indicating that the upstream kinase is not proline-directed as suspected. We next will determine when this site is phosphorylated in cells.

Previously, T210 was considered to be the essential phosphorylation site for Plk1 activation; however, our results demonstrate that multiple phosphorylation events are essential for regulation of this kinase. A threonine at corresponding residues to T214 is widely conserved in kinase T-loops. Identification of additional regulators of Plk1 will have implications in our understanding of how kinases and other proteins are regulated in the cell cycle.

1112

Novel mitotic signalling crosstalk between PI3K-Akt pathway and Plk1.

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Polo-like kinase 1 (Plk1) controls multiple aspects of mitosis, whereas a kinase cascade from phosphatidylinositol 3-kinase (PI3K) to Akt regulates cell growth, proliferation, survival and motility. Here, we report an unexpected role of the PI3K-Akt signalling pathway in regulating mitotic Plk1 function. We demonstrated that Plk1 is phosphorylated at Ser99 in a manner that requires the activities of PI3K and Akt. This phosphorylation occurs specifically in mitosis but independently of Plk1-Thr210 phosphorylation, a process essential for Plk1 activation. Plk1-Ser99 phosphorylation creates a docking site for 14-3-3 γ and binding of 14-3-3 γ stimulates the catalytic activity of Plk1. 14-3-3 γ silencing or the replacement of wild-type Plk1 by a Ser99-phospho-blocking mutant leads to a prometaphase/metaphase-like arrest due to the activation of the spindle assembly checkpoint (SAC). These results suggested that PI3K-Akt pathway facilitates Plk1-Ser99 phosphorylation, which stimulates Plk1 catalytic activity through the formation of a Plk1-14-3-3 γ complex. This activation pathway is required for proper progression from metaphase to anaphase.

1113

A transition between two types of oscillators during the *Xenopus laevis* early embryonic cell cycle.

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During *Xenopus laevis* early embryonic development, the first cell cycle is long and the subsequent 11 cycles are short and highly accurate, raising the question of how an oscillator can be initially tunable yet precise afterwards. By reconstructing the oscillatory dynamics from single embryos, we found that the positive feedback is much stronger in the first cycle, but becomes minimal in the subsequent cycles, supporting a transition between a positive-plus-negative feedback oscillator to a negative-feedback-only oscillator. The negative feedback is highly ultrasensitive and could contribute to the robustness of the oscillator when the positive feedback is weakened. It is critical for the first cycle to be sufficiently long, as the embryos with shorter first cycle period upon drug treatment have dramatically reduced viability. This reduction in viability can be rescued by co-treatment with cycloheximide to extend the first cycle period back to its normal length. Using a mathematical model that fits our experimental data, we compared the systems level properties of the two oscillator design. The presence of positive feedback in the first cycle allows the oscillatory period to be tunable without changing the amplitude of Cdk1 activity, and down-regulating the positive feedback in the subsequent cycles helps to increase the precision of the oscillatory period. Thus, embryos may be altering the underlying architecture of cell cycle feedback regulation to meet two different developmental objectives during early development.

1114

Resolution of the yeast septin ring reorganization by single molecule microscopy.

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The septins are a family of cytoskeletal GTPases with conserved essential functions in cell division and polarization. Heteromeric nonpolar octamers of yeast septin proteins localize to a ring-like structure at the mother-bud neck in dividing yeast cells. During cell division, this ring-structure separates several cell compartments of the mother cell from the daughter cell and ensures asymmetric cell division.

Emerging first as a ring at the future bud site, the structure assumes an hourglass-like arrangement during bud growth. At the onset of cytokinesis the hourglass-like structure splits into two rings that are distributed to the daughter and mother cell.

The dynamics and organization of this process are regulated by cell signaling and the respective terminal protein in the individual complexes. Fluorescence polarization measurements suggest that individual complexes within the structure undergo a 90-degree shift during reorganization. Electron tomography data on the other hand point towards a coexistence of two populations of differently organized filaments. How this essential structure that is paradigmatic for septin assembly in all cells is assembled and reorganized from individual complexes remains unclear.

Here we imaged both ends of the 35 nm long individual septin complexes in the septin structure at the yeast mother-bud neck by two- and three-dimensional single molecule super-resolution

microscopy. We could gather thousands of individual localizations at the septin ring and found dramatic changes in septin organization at different points during the cell cycle.

We find that the initial septin ring has an opening of around 382 nm (+/- 70 nm), which then widens to around 659 nm (+/- 50nm) before splitting into two rings. Our results reveal previously inaccessible insight into how this gigantic multiprotein complex is assembled from individual, 35 nm long septin octamers and changes during cell cycle progression.

1116

An essential mitotic role for the DNA replication protein, Cdt1.

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The cell division cycle requires complete and precise duplication of the entire genome in S phase followed by accurate chromosome segregation in mitosis. The formation and stability of kinetochore-microtubule (kMT) attachments during mitosis depends on the Ndc80 complex, Ndc80 (hsHec1), Nuf2, Spc24 and Spc25. In the approximate middle of the central coiled-coil rod domain of the complex is a "hinge" site produced by a ~40 amino acid loop in Hec1. We have identified the Cdt1 DNA replication licensing protein as an essential partner for Ndc80 during prometaphase through interaction with the Hec1 loop domain.

Cdt1, a protein first identified for its critical role in DNA replication origin licensing during G1 phase, is normally degraded during S phase but re-accumulates in G2. We now demonstrate that human Cdt1 has a distinct and essential function in prometaphase. We employed multiple strategies to temporally separate Cdt1's replication function from this new mitotic function to ensure that the mitotic arrest in Cdt1-depleted cells cannot be attributed to incomplete DNA replication. G2-specific depletion of Cdt1 arrests cells in late prometaphase due to abnormally unstable kinetochore-microtubule attachments and Mad1-dependent spindle assembly checkpoint activity. Cdt1 localizes to kinetochores during mitosis through interaction with Hec1. Cdt1 binds the unique loop extending from the rod domain of Hec1, and this loop is also required for kinetochore-microtubule attachment. Mutation of the loop prevents Cdt1 kinetochore localization and arrests cells in prometaphase. High resolution fluorescence microscopy indicates that Cdt1 binding to the Hec1 loop domain is required for a large microtubule-dependent conformational change in the Ndc80 complex in vivo. Thus Cdt1 binding to Hec1 represents a novel form of Ndc80 regulation in vertebrates essential for kinetochore-microtubule attachment. The Hec1 binding domain in Cdt1 overlaps with regions involved in binding some of Cdt1's replication partners such as geminin and Cdc6. These findings suggest that Cdt1 participates in multiple independent interactions that are separated both temporally and spatially. We suggest that the acquisition of multiple essential cell cycle functions by Cdt1 facilitates coordinated cell cycle progression to preserve genome stability.

1117

Cortical nodularity controls signaling pathways to link cell growth and division.L. Deng¹, J. B. Moseley¹; ¹Department of Biochemistry, Dartmouth Medical School, Hanover, NH

Cell cycle progression is coupled to cell growth, but the molecular connections between these processes are largely unknown. In fission yeast, the kinases Cdr1 and Cdr2 promote mitotic entry and receive upstream cues related to polarized cell growth. To define the molecular connections between Cdr1/2 and cell polarity, we performed a comprehensive pair-wise yeast two-hybrid screen. From the resulting interaction network, we found that the protein Skb1 interacted with both Cdr1 and the Cdr1 inhibitory target Wee1. Consistent with these physical interactions, Skb1 inhibited mitotic entry through negative regulation of Cdr1 in genetic epistasis experiments. Skb1 localized to the cytoplasm and to stable nodes at the cell cortex, similar to Cdr1 and Cdr2. Surprisingly, Skb1 nodes were distinct structures from Cdr1/2 nodes, and artificial targeting of Skb1 to Cdr1/2 nodes delayed entry into mitosis. Thus, distinct cortical nodes prevent the inhibitory interaction of Skb1 and Cdr1 to ensure proper cell cycle progression. Moreover, we identified a novel protein Slf1 that co-localized and physically interacted with Skb1. We found that the number of cortical Skb1 nodes scaled with cell size and Slf1 promoted both Skb1 node formation and mitotic entry in a dosage-dependent manner. This suggests that regulation of Slf1 during cell growth may promote node formation and mitotic entry by sequestering Skb1 from its inhibitory target Cdr1. From these results, we propose that the formation of stable node structures in the cell cortex controls signaling pathways to link cell growth and division.

1118

Mitofusin 1 is degraded at G2/M phase through ubiquitylation by MARCH5.Y-Y. Park¹, H. Cho¹; ¹Department of Biochemistry, Ajou university, Suwon, Korea

Background: Mitochondria exhibit a dynamic morphology in cells and their biogenesis and function are integrated with the nuclear cell cycle. In mitotic cells, the filamentous network structure of mitochondria takes on a fragmented form. To date, however, whether mitochondrial fusion activity is regulated in mitosis has yet to be elucidated.

Findings: Here, we report that mitochondria were found to be fragmented in G2 phase prior to mitotic entry. Mitofusin 1 (Mfn1), a mitochondrial fusion protein, interacted with cyclin B1, and their interactions became stronger in G2/M phase. In addition, MARCH5, a mitochondrial E3 ubiquitin ligase, reduced Mfn1 levels and the MARCH5-mediated Mfn1 ubiquitylation were enhanced in G2/M phase.

Conclusions: Mfn1 is degraded through the MARCH5-mediated ubiquitylation in G2/M phase and the cell cycle-dependent degradation of Mfn1 could be facilitated by interaction with cyclin B1/Cdk1 complexes.

1119

***Cryptococcus neoformans* can undergo cell enlargement and reversible ploidy change during infection.**M-S. Fu¹, K. Nielsen¹; ¹Department of Microbiology, University of Minnesota, Minneapolis, MN

Cryptococcus neoformans is a major life-threatening human fungal pathogen that grows as a budding yeast. Recently, we showed that *C. neoformans* can enlarge its total cell size 5-10 fold during infection. These enlarged cells are defined as "titan" cells. Normal-size cryptococcal cells are haploid, but titan cells have an increased ploidy (tetraploid or octoploid) with single nucleus.

We hypothesize that titan cell production involves a process similar to endoreduplication or endomitosis, performed by plants and animals, in which mitosis is precluded or cytokinesis is not completed and the cells undergo extra rounds of DNA replication resulting in polyploidy and cell enlargement. Normally, endoreduplicated plant and animal cells rarely return to mitotic division. However, titan cells are able to undergo cell division and generate small daughter cells with cell size similar to normal-size cryptococcal cells. Interestingly, flow cytometric analysis showed that the majority of the progeny cells contained 1N DNA content (haploid) when titan cells isolated from mice were grown in standard laboratory conditions. These results showed that polyploid titan cells were able to give rise to haploid daughter cells. A similar phenomenon of endoreduplication and depolyploidization has also recently been observed in mammalian tumor cells upon exposure to chemotherapy. In the studies presented here, the nuclear division of normal-size and titan cells was visualized by fluorescent-labeling with mCherry or GFP fusions to the nucleolar protein (Nop1), alpha tubulin (Tub1), and DNA using histone H4. In normal-size cryptococcal cells, a single nucleolus was co-localized with DNA during interphase. However, the Nop1 signal was diffuse and did not localize with DNA during metaphase, suggesting an open or semi-open mitosis similar to mammals and in contrast to the model yeast *Saccharomyces cerevisiae*. Interestingly, titan cells were classified into two groups based on their nucleolar localization. The majority of titan cells contained a single nucleolus, but unlike normal-size cells, the Nop1 signal did not become diffuse and was co-localized with DNA in daughter cells during mitosis. A small fraction of titan cells contained two distinct nucleoli within long tube-shaped DAPI signal not associated with budding. These data suggest that titan cells have an unusual nucleus and may not follow the typical nuclear division seen in normal-size cryptococcal cells. This unusual nucleus in *C. neoformans* titan cells may allow an atypical division that produces haploid daughter cells from polyploid mother cells.

1120

Constitutively Active-RSK2 induces chromosomal instability through diverse mechanisms.

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The 90KDa ribosomal S6 Kinases (RSKs) are family of Ser/Thr kinases that lie downstream of the Ras-MAPK cascade and they are found to play an important role in cancer progression. In relation to cancer, RSK2 expression was shown to be elevated in breast and prostate tumor samples. To investigate the involvement of RSK2 in tumorigenesis and tumor development, we took advantage of two different constitutively active RSK2 (CA-RSK2) constructs. We hypothesized that increased activity of RSK2 might induce chromosomal instability (CIN) which further contributed to tumorigenesis and tumor progression. We first analyzed the activities of two CA-RSK2 forms. In spite of inhibition of MEK by treatment with U0126, we observed an increase in the level of highly phosphorylated RSK2 at Ser380 in both CA-RSK2 construct transfected cells. As expected, we observed that cells expressing CA-RSK2 showed increase in number of chromosome bridge and lagging chromosome during anaphase. In addition, we observed that expression of CA-RSK2 increased micronuclei after mitosis. These results indicate that CA-RSK2 induced mis-segregation of chromosomes resulting in increase of CIN. Consistently, we observed that CA-RSK2 expression enhanced abnormal chromosome number in HeLa cells. Regarding underlying mechanism of CIN, we first analyzed the Spindle Assembly Checkpoint (SAC) protein activity by treating the cells with Nocodazole, an inducer of microtubule depolymerization, and found out that CA-RSK2 decreased mitotic cells as compared with that of control, suggesting partial impairment of SAC activity. In addition, when MCAK and CA-RSK2 were co-transfected, we observed that MCAK decreased CA-RSK2-

induced lagging chromosome and chromosome bridge of anaphase, suggesting the involvement of kinetochore microtubule here. Further investigating on the mechanisms underlying the chromosomal instability caused by CA-RSK2 is undertaken.

1121

The Histone-fold CENP-T-W-S-X complex induces positive supercoils into DNA.

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The centromere is the genomic locus where the kinetochore is assembled to facilitate faithful chromosome segregation during mitosis through formation of a dynamic interface with microtubules from the mitotic spindle. Centromeres are specified by sequence-independent epigenetic mechanisms in most eukaryotes and centromere-specific histone H3 variant CENP-A serves an important epigenetic marker for the centromere specification. However, centromeric chromatin is much complicated and there should be additional features, which are involved in the centromere specification and kinetochore assembly. We recently identified the CENP-T-W-S-X complex, which forms a unique nucleosome-like structure with the histone-fold. Whereas we demonstrated that tetramer formation of CENP-T-W-S-X is essential for kinetochore assembly in both in vivo and in vitro, it is still unclear how the CENP-T-W-S-X tetramer contacts to DNA and the nucleosome-like structure is established.

Here, we examined how the CENP-T-W-S-X complex contacts DNA. The CENP-T-W-S-X complex efficiently binds to ~100 bp DNA. Remarkably, we found that the CENP-T-W-S-X complex introduces positive supercoils into plasmid DNA, whereas the canonical histones-containing regular nucleosome induces negative DNA supercoils. We like to discuss about biological significance of this unique structure.

1122

Epigenetic Regulation of Rsf-1, a Subunit of Remodeling and Spacing Factor Complex in Mitosis.

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The RSF complex has been identified as an ATP-dependent nucleosome remodeling and spacing factor that facilitates the transcription on the DNA template packaged into chromatin. The RSF complex is composed of two subunits, SNF2h ATPase and Rsf-1. Notably, Rsf-1 is found to be overexpressed in a variety of cancers, in which high Rsf-1 level is often correlated with aggressiveness of cancers and poor prognosis. However, how the chromatin remodeling activity is related to cancer development remains largely unknown. Here, we showed that in early mitosis Rsf-1 localizes throughout the mitotic chromosomes but changes its localization through progression into late mitosis. Depletion of Rsf-1 by siRNA induces a severe defect in chromosomal congression as well as in the timing of cohesion resolution. We found that knockdown of Rsf-1 causes a severe imbalance in histone modifications in mitosis. An extensive immunofluorescence study also indicates that Rsf-1 is required for proper localization and movement of histone deacetylases and HAT during mitotic progression. Together, we suggest Rsf-1 as a novel epigenetic regulator in mitosis.

1123

Segregation of extra-chromosomal DNA in fission yeast.H. Yu¹, Y. Barral¹; ¹Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

Extrachromosomal DNA (ecDNA) is prevalent in various eukaryotic organisms. It has been shown that the asymmetric retention of ecDNA is a crucial mechanism of cellular rejuvenation during asymmetric mitosis of budding yeast *Saccharomyces cerevisiae*. Nothing is known about the fate of ecDNA in symmetric dividing cells. Here we establish the fission yeast *Schizosaccharomyces pombe* as a model system for studying ecDNA behaviour in symmetric mitosis. We show that in *S. pombe*, ecDNA particles are localized predominantly at nuclear periphery, probably through the LEM domain proteins Lem2 and Man1. During mitosis, ecDNA particles exhibit condensin-dependent dynamic clustering, and are segregated asymmetrically in the two apparently equivalent daughter cells. Non-random asymmetry is abolished in mutants of Lem2, Man1, condensin as well as genes involved in transcription and silencing. Our data suggest that *S. pombe* regulate ecDNA segregation and dynamics through a combination of mechanisms that involve nuclear structure and dynamics, chromatin condensation, transcription and heterochromatin assembly.

1124

Dissecting the role of non-coding RNAs in pericentric heterochromatin formation in human cells.W. L. Johnson¹, W. T. Yewdell¹, A. F. Straight¹; ¹Biochemistry, Stanford University, Stanford, CA

During mitosis, pairs of replicated chromosomes must remain linked until anaphase to ensure proper attachment to the mitotic spindle and equal segregation of the genome. The primary sites for cohesion between sister chromatids are the heterochromatic outer regions of the centromere, called pericentric heterochromatin. Disruption of pericentric heterochromatin leads to defects in cohesion, chromosome segregation errors and aneuploidy. In fission yeast and plants, the assembly of pericentric heterochromatin requires transcription of repetitive sequences flanking centromeres. Recent evidence suggests that pericentric transcription also contributes to centromere function in vertebrates, but how pericentric RNAs interact with the proteins that initiate and maintain heterochromatin and how these interactions contribute to cohesion and chromosome segregation is poorly understood. Using *in vivo* RNA labeling techniques, we have found that RNA is bound to the pericentric regions of human mitotic chromosomes. This RNA is encoded by pericentric DNA repeat sequences, is single-stranded and colocalizes with heterochromatin protein 1 alpha (HP1 α). We show that HP1 α binds to pericentric transcripts *in vivo*, and that HP1 α and the histone 3 lysine 9 methyltransferases SUV39H1, SUV39H2 and SETDB1 directly bind RNA *in vitro*. We propose a model in which pericentric RNA transcripts directly recruit factors that initiate and maintain heterochromatin at centromeres.

1125

An Alternative Mechanism of Action for Paclitaxel in Breast Cancer.L. M. Zasadil¹, B. A. Weaver¹; ¹Cell and Regenerative Biology, University of Wisconsin-Madison, Madison, WI

Paclitaxel is a microtubule-stabilizing drug that binds to the β -tubulin subunit and accumulates within cells. As a chemotherapeutic agent, paclitaxel is used for the treatment of a variety of cancers including those of the breast. Currently, no biomarker is available to predict which

patients will benefit from paclitaxel treatment. Paclitaxel studies in cells have focused primarily on high concentrations of the drug that significantly inhibit microtubule dynamics, resulting in long-term mitotic arrest. Our preliminary measurements of paclitaxel concentrations in breast tumors indicate that the clinically relevant concentrations of paclitaxel to study in cell culture are in the low nM range, and cause more subtle effects on microtubule dynamics. Cells exposed to low concentrations of paclitaxel complete mitosis with minimal delay, but frequently contain multipolar spindles. In both chromosomally stable breast cancer cells and those that exhibit chromosomal instability (CIN), low concentrations of paclitaxel are sufficient to increase the incidence of chromosome missegregation. These data suggest that the mechanism of cell death due to low dose paclitaxel is likely to be due to abnormal mitoses that result in uneven division of genetic material, as opposed to mitotic arrest caused by high dose paclitaxel. They also predict that CIN cells may exhibit differential sensitivity than chromosomally stable cells to clinically relevant concentrations of paclitaxel. Recent studies have found that chromosomally stable cells must acquire tolerance for a nondiploid genome in addition to CIN to become viable aneuploid cells. This suggests that CIN cells are better adapted to proliferate despite chromosome missegregation, giving them increased resistance to death following paclitaxel treatment when compared to chromosomally stable cells. However, it has also been shown that low rates of chromosome missegregation are well tolerated, at least in some cell types, but high rates of missegregation lead to rapid cell death. Since CIN cells have an existing level of chromosomal missegregation, which is exacerbated by treatment with low doses of paclitaxel, these cells may be more likely than chromosomally stable cells to die after paclitaxel treatment as a result of losing both copies of a vital chromosome. We are currently testing the differences in response to low dose paclitaxel between chromosomally stable and CIN cells to determine whether chromosomal stability status can be used as a biomarker to predict which tumors are most likely to be sensitive to treatment with paclitaxel.

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A single-molecule assay of ubiquitination/deubiquitination in cell extracts.*Y. Lu¹, M. Kirschner¹; ¹Harvard Medical School, Boston, MA*

Ubiquitination is the major mechanism for regulated protein degradation, and is closely associated with various diseases such as cancer and neural degenerative diseases. Kinetics of the reaction has been exploited by the cell to regulate or time physiological events such as the cell cycle where a series of substrates of the APC (a major E3 ligase) undergo sequential ubiquitination and proteasome degradation. It is unclear which mechanism sets up the reaction order, and how the order is affected by modifications to the APC. In order to study the regulatory mechanism, we designed a single molecule assay to capture transient intermediates and to measure the reaction constants in both purified reaction systems and in cell extracts. In this assay, ubiquitin, E3 ligase (APC) and substrates are fluorescently labeled respectively. The polyubiquitination and deubiquitination kinetics on substrate molecules was measured and associated with the binding of E3 ligases. Several technical improvements were made to ensure successful implementation of this assay in the presence of μM concentration of fluorescent-ubiquitin. Due to rapid deubiquitination and degradation, no kinetic measurements could be made before in cell extracts using conventional methods. Our single molecule assay allows the examination of the reactions mechanism and kinetics in a real cytosolic environment of cell extracts. Using this assay, we found that initial ubiquitination of substrates greatly enhances its binding with E3, thus allows further ubiquitination and degradation through a positive feedback mechanism. This mechanism allows the E3 to efficiently locate substrate molecules in crowded cellular environment, and is important for setting up the correct order of degradation. Using a modified method, we also studied how the proteasome recognizes and degrades ubiquitinated substrates in a reconstituted ubiquitin-proteasome system.

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Single-cell analysis of the mitotic size control system in fission yeast.

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Cell cycle progression is coupled to cell growth through a series of checkpoints that monitor size, time, and/or environmental cues. Rod-shaped fission yeast cells enter into mitosis upon reaching a critical cell size, similar to size-dependent checkpoints in other cell types. Size measurement depends on the mitotic inhibitor Pom1, which forms a gradient enriched at cell poles. Pom1 targets the mitotic inducer Cdr2, which localizes to the cell middle. Pom1 levels in the cell middle decrease as cells grow, allowing Cdr2-dependent entry into mitosis upon reaching a critical size. Here, we used single-cell analysis to resolve the role of Pom1 in cell size homeostasis. The mitotic size control system generates a reproducible cell size at division that is largely independent of cell size at birth, meaning that wild-type fission yeast cells born smaller or larger than normal will still divide at the typical size of 14 μm . In contrast, cell size at division in *pom1* mutants was dependent on cell size at birth, indicating that Pom1 is required for dynamic cell size control. Further, our data suggest that a backup 'timer' emerges to control cell cycle progression in the absence of the Pom1 'sizer.' This Pom1-Cdr2 sizer has been linked to cell geometry due to its cellular distribution. To test this connection, we developed a fluorescent single-cell reporter that measures cell size independently of cell shape. Cell sizes at birth and division were not affected by changes in cell geometry, but rather plasticity in the Pom1-Cdr2 system maintained geometry-independent cell size control. To test the limits of this system, we engineered a strain that reversibly becomes multinucleated through repeated mitoses without intervening cytokinesis. In these cells, Pom1 and Cdr2 displayed an unexpected compartmentalization at the cell cortex, and this distribution drove synchronized mitoses in a single cell with altered size control dynamics. Our combined data identify a central role for the Pom1-Cdr2 system in the dynamic control of cell size homeostasis. This system coordinates cell cycle progression with changes in cell size and shape that occur during growth.

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The Hippo Pathway targets Rae1 (an inhibitor of Cdh1-APCC) to regulate mitosis and establish organ size.

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The Hippo Tumor Suppressor Pathway acts as a master regulatory pathway to coordinate proliferation, growth, and apoptosis in *Drosophila* and vertebrates. Loss of core pathway components (Serine-Threonine kinases Hippo and Warts, and also Salvador and Mats) and over-activation of the crucial pathway target Yorkie (a transcriptional regulator) promote cell division, cell growth, and cell death resistance, resulting in increased organ size. However, organ homeostasis typically engages mechanisms to ensure that variations in proliferation do not alter organ size. How the pathway integrates restricting proliferation within an "organ size checkpoint" remains a major unanswered question. Using *Drosophila* *In vitro* Expression Cloning (DIVEC), we performed a genome-wide kinase screen to identify Hippo and Warts substrates using a gel-shift assay. We identified Rae1, a reported inhibitor of the Cdh1-activated form of the Anaphase Promoting Complex/Cyclosome (APCC). We show that Rae1 acts in the Hippo Pathway to regulate both proliferation and organ size in *Drosophila*. Tissue culture studies show that Hippo signaling promotes phosphorylation and degradation of Rae1

downstream of Warts. *In vivo Drosophila* genetic interactions confirm that this negative regulation of Rae1 is important in Hippo-mediated restriction of organ size. Loss of Rae1 reduces organ size, decreases cyclin A and cyclin B levels, and impairs S-phase entry and causes mitotic arrest of those cells that do enter the cell cycle. These data suggest a role for Rae1 in both S-phase entry and mitotic progression. Exogenous Rae1 increases cell proliferation, cyclin A and cyclin B levels, and organ size. Importantly, reducing Cdh1 gene dosage suppresses the reduced organ size upon Rae1 loss or Hippo over-expression, implying that Cdh1 is important to these organ size phenotypes. We propose a model that Hippo signaling promotes Cdh1-APCC activity to restrict proliferation and organ size by relieving its Rae1-mediated inhibition. Work by others established that reducing the gene dosage of Yorkie suppresses organ overgrowth upon loss of Hippo signaling. Reducing Rae1 gene dosage also dramatically suppresses organ overgrowth. Surprisingly, we also see catastrophic tissue lethality arguing that tissue overgrowing from loss of Hippo requires a minimum threshold of Rae1 to survive. Tissue lethality upon reducing Rae1 occurs in the context of loss of Merlin, Expanded, Hippo, or Warts but not over-expressing Yorkie, highlighting a fundamental difference between loss of core components of Hippo signaling and Yorkie activation. This “synthetic lethality” is reminiscent of oncogene/non-oncogene “addiction” models and implicates Rae1 as a therapeutic target for cancers in which Hippo signaling is dysregulated.

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Dissecting the cell size monitoring system in fission yeast by quantitative analysis of the Pom1 concentration gradient.

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In the rod shaped fission yeast, like in many other organisms, cell division is tightly coordinated with cell growth and allows cell division to occur at a constant cell length. This control relies in part on a geometric cell size monitoring system based on cortical concentration gradients of the DYRK kinase Pom1 that emanates from the cell tips. Pom1 modulates CDK1 (Cdc2) activity by negatively regulating the Cdr2 kinase localized at the cell middle; this inhibition is thought to be alleviated by cell elongation. However the mechanistic nature of the Pom1 gradient readout responsible for mitotic entry remains to be described.

We have shown that the establishment and maintenance of Pom1 gradients involves a cycle of dephosphorylation and autophosphorylation: Pom1 is locally dephosphorylated at cells tips by the Tea4-Dis2 (type 1 phosphatase) complex, revealing a lipid-binding domain that mediates Pom1 binding to the plasma membrane. As Pom1 diffuses along the membrane, Pom1 autophosphorylates at multiple sites to promote its release into the cytoplasm.

We have developed an Image J plugin for semi-automatic quantitative analysis of the Pom1 gradients. Using this tool our analyses revealed a correlation between cell length and the size of the central domain with low Pom1 signal and high Cdr2 signal, suggesting the size control of this medial cell zone is critical for regulating mitotic entry. We use two experimental approaches to manipulate this medial cell zone: first we make use of a chemical genetic approach based on an analogue sensitive allele of Pom1 that can be specifically inhibited. Using suboptimal doses of the inhibitor, we can manipulate the shape of the gradient and study its impact on mitotic commitment and Cdr2 localization. Second, by generating Pom1 alleles with increasing numbers of auto-phosphorylation sites mutated, we show a progressive spreading of the Pom1 gradient and a progressive increase in cell size at division according to the number of mutated sites. This indicates that the multiple autophosphorylation sites impact on the gradient shape. Together, these approaches combined with quantitative analysis provide new tools to dissect the Pom1 gradient readout responsible for mitotic entry.

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Bod1 is a novel inhibitor of Protein Phosphatase 2A-B56 during mitosis.

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Bod1 is a small, 22 kDa, protein that is required for resolution of syntelic attachments, proper chromosome alignment and proper phosphorylation of MCAK by Aurora B in mitosis. Here we demonstrate that Bod1 is a novel inhibitor of PP2A-B56 during mitosis. Bod1's sequence is similar to Ensa and Arpp-19, two proteins recently identified as inhibitors of PP2A-B55, which are required for proper entry into mitosis. Bod1-GFP immunoprecipitates possess phosphatase activity that is inhibited by 2 nM okadaic acid. This activity is markedly increased if the Bod1/PP2A complex is dissociated in the reaction by addition of salt and detergent. Bod1 binds specifically to the PP2A-B56 holoenzyme and not PP2A-B55. Binding of Ensa and Arpp-19 to PP2A-B55 requires a conserved Asp and phosphorylation by Gwl kinase at a neighboring Ser. Similar residues, T95 and D98, are critical for Bod1 function; expression of Bod1 T95A or D98A mutants fail to rescue Bod1 siRNA depleted cells whereas expression of a T95E mutation rescues as well as wt Bod1. Cdk1 phosphorylates Bod1 at T95 in vitro and the T95E mutant immunoprecipitates PP2A-B56 more strongly than either wt or T95A Bod1. Taken together, these data indicate that phosphorylation of Bod1 at T95 induces specific binding and inhibition of PP2A-B56. These data suggest that loss of Bod1 might therefore cause hyperactive PP2A-B56 and defects in mitotic progression. siRNA depletion of Bod1 from HeLa cells results in increased PP2A-B56 localisation to centromeres, causes loss of phosphorylation of MCAK and PBIP, delocalization of Plk1 and Sgo1, and defects in chromosome biorientation and cohesion. The Bod1 phenotype can, in part, be rescued by co-depletion of B56, consistent with the idea that Bod1 inhibits PP2A-B56 activity. Our results demonstrate that Bod1 is a critical modulator of PP2A-B56 activity and that together Bod1, Ensa and Arpp-19 form a new class of mitotic PP2A inhibitors.

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The Inhibitory Role of Aurora A in Mitotic Entry in the Early Embryonic Cell Cycle.

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Cyclin-dependent kinase 1 (CDK1) is the master kinase that directs a wide array of mitotic events, ranging from nuclear envelope breakdown to chromosome separation. Other kinases—Aurora A and Polo-like kinase 1 (Plk1), for example—serve additional roles at mitosis. Research in the past decade has suggested that positive feedback loops underlie the activation of CDK1, Aurora A and Plk1. My current studies using *Xenopus* embryonic extracts, however, has revealed a more complicated network exists among these kinases. Through some initial data, I found that Aurora A may not only act as an activator, but also as an inhibitor to fine tune the activation of CDK1. By removing, inhibiting or overexpressing endogenous proteins, “single-cell” approaches using *Xenopus* extracts enable us to test the behavior of modified cell cycle control systems. We discovered that adding purified Aurora A protein into cycling extract caused mitosis delay in a dose-dependent manner. Newly synthesized Aurora A protein produced after the addition of *in vitro*-transcribed mRNA also delayed mitosis, excluding any potential artifacts caused by protein contaminants. Neither depleting nor inhibiting Aurora A severely affected proper mitotic timing in the first cycle, but accelerated the second and the third cycles by 10-20 minutes, suggesting that losing Aurora A activity has a cumulative effect on cell cycle progression. To narrow down through which leg(s) Aurora A exerts its inhibitory effect on CDK1 activation, Wee1 kinase was depleted to weaken its double-negative feedback loop with CDK1.

Mitosis in this extract was delayed, but to a lesser extent compared to overexpressing Aurora A without depleting Wee1. This result suggests that Aurora A may not function through activating Wee1 to inhibit CDK1, but through inhibiting the Cdc25 phosphatase pathway to prevent the timely activation of CDK1. In summary, we have identified a novel inhibitory effect of Aurora A on the mitotic onset in early embryonic extract. This work has significant potential to reveal how the connection of the M-phase-control system that ensures mitosis is regulated properly and with tremendous precision. Since all M-phase kinases are commonly overexpressed in tumors and are implicated in carcinogenesis, gaining an understanding of the systems-level rules of these signaling cascades will provide new and critical insights into how proliferative diseases arise. It may also enable the identification of proteins that could be the most efficacious targets in clinical therapies for cancer and other diseases.

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A universal correlation between protrusion direction and division axis of cells in 3D matrices.

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The regulation of cell mitosis is critical for normal development of tissues and organs. Uncontrolled mitosis has been implicated in natural genetic variations, progression of cancer and human aging. Previous studies investigating the control of the cell division orientation were performed with cells cultured on 2D substrates. However, many types of cells divide in 3D matrices, in particular fibroblast and fibrosarcoma cells which are located in collagen I-rich connective tissues. Adding a third dimension to the culture by employing a three-dimensional (3D) matrix could better recapitulate the microstructure, mechanical properties and biochemical signals of the 3D extracellular matrix (ECM) for both normal and pathologic tissues. Here, we employed single live-cell imaging assay, shRNA technique and quantitative image analysis to probe the regulation of cell division in 3D collagen matrices. Our results showed that cells exhibit different modes of cell mitosis in 3D matrix compared with their counterparts on 2D substrates. Results also revealed a strong correlation between the direction of the last protrusion before cell mitosis and the orientation of cell division. Such correlation held for vastly different types of cells, including fibrosarcoma, breast cancer and human embryonic kidney cell lines. This correlation did not change as the density of collagen matrix varied. Moreover, depleting the expression of integrin receptors, inhibiting the activities of Src kinase or matrix metalloproteinase did not abolish the correlation between the directions of the last protrusion and cell division. These results suggested that there is a universal correlation between the cell protrusion direction and the division orientation of cells in 3D collagen matrices. This study provides novel insights into the mechanisms governing the control of cell mitosis in a 3D cultural environment.

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Pds5 is required for sister chromatid resolution at mitosis.

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The correct segregation of chromosomes at mitosis is vital for maintaining genome integrity in eukaryotic cells. Errors in this process can result in aneuploidy and ultimately lead to the development of cancer or birth defects. The core components of the cohesin complex include Smc1 and Smc3 (members of the structural maintenance of chromosomes family of ATPases),

scc1 (a member of the kleisin family of proteins) and scc3 (SA1 and SA2 in vertebrates). However, additional proteins such as Pds5A and Pds5B are also known to regulate sister chromatid cohesion although their role remains unclear. Using an RNAi approach, our results show that Pds5 proteins are required for the removal of cohesin both from the arms of the sister chromatids and from the centromeric region. Our data suggest that Pds5 may be important for the recruitment of proteins that trigger the removal of cohesin from the chromosomes and initiate sister chromatid disjunction.

1134

Spatial organization of the Ran pathway in mitotic human cells.

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During spindle assembly, microtubules are enriched around chromatin by a process which, in many systems, is driven by the GTPase Ran. The Ran pathway is thought to establish a reaction-diffusion network that generates spatial gradients in the behaviors of soluble proteins. However, the validity of this model of Ran activity is still unclear because the spatial distributions of the activities of all the relevant components in the Ran pathway have not been measured. We developed a novel form of fluorescence fluctuation spectroscopy capable of measuring the concentration, diffusion, and interactions of fluorescently labeled proteins simultaneously at hundreds of locations throughout cells. We are using this technique to study the behaviors of Ran, Importin α , Importin β , RanGAP1, and cargo proteins by expressing them tagged with GFP in human mitotic cells. Our preliminary results show spatial gradients in protein behaviors throughout mitotic cells, and produce surprising new insights about the principles underlying the spatial organization of the Ran pathway.

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Ki-67, a PP1 interacting protein and chromosome periphery organizer.

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Ki-67 is a proliferation antigen that is expressed during all cell cycle phases except the G0 phase. Staining for Ki-67 has been used very widely to identify cells that maintain the ability to proliferate amongst populations of differentiated cells. Although Ki-67 is one of the most popular proliferation markers and widely used in cancer diagnosis and prognoses, its biological function is still unknown. We have identified Ki-67 as the evolutionary ancestor of Repo-Man (a PP1 targeting subunit and substrate specifier) and we have shown that Ki-67 itself is a PP1 interacting protein.

Ki67 is a nuclear protein in interphase and re-localizes around the periphery of the mitotic chromosomes from prometaphase until telophase. It thus belongs to the so-called perichromosomal layer compartment.

Lack of Ki-67 was previously reported to prevent the localization of hKLP2 to the mitotic chromosomes. Here we have identified Ki-67 as an important factor in nucleolus disassembly during mitosis and as a key organizer of the chromosome periphery.

G1, G1-S, and S Phase Regulation

1136

Positional Memory of Quiescent Cells.

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Improper entry into or exit from quiescence can lead to a variety of human diseases, including cancer. A long-standing debate has raged about whether quiescent cells occupy a distinct G0 phase outside of the proliferative cell cycle, or whether they are simply deposited along a continuous G1 phase at the point where they last encountered growth signals. Mechanistically, our previous work demonstrated that the mammalian transition from quiescence to active proliferation is determined at the restriction point (R-point) by an Rb-E2F bistable switch. Stochastic activation of this switch can account for the heterogeneity in cell cycle entry observed among a population of isogenic cells. Here, using single cell experiments, we demonstrate that quiescent embryonic fibroblasts are distributed at varying distances from the R-point, and that after this positional distribution is altered by growth stimulation, these cells can memorize their new positions for several days. In other words, our work supports a model in which quiescent (G0) cells display G1-like positional heterogeneity, and can memorize these “quiescent positions” in the absence of growth signals. This cellular memory of quiescent positions could be critical for the self-renewal of stem and progenitor cells and the development of cancers arising from cancer stem cells.

1137

In Vivo Detection of Cell Cycle Kinetics in Mice.

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BrdU (Bromodeoxyuridine) can be used to study cell cycling in living cells both in vitro and in vivo. BrdU is a thymidine analogue which replaces thymidine during DNA replication (S phase of the cell cycle) and can be detected by specific monoclonal antibodies. Combining BrdU, proliferation dyes (VPD450) and cell surface markers allows for the detection of cell cycle within specific cell populations in vivo and in vitro. We aimed to analyze the cell cycle characteristics of distinct cell populations in bone marrow, spleen and thymus. BALB/c and C57Bl/6 mice were injected with 1 mg of BrdU (IP) for 24-96 hrs. Cell preparations from each of these organs were stained with antibodies to identify T, B, and macrophage cell types. Thymus and bone marrow displayed the highest levels of BrdU incorporation, while the spleen showed the lowest. Spleen incorporated BrdU at approximately 10%, in which the main population of cycling cells were either B220+ cells or Mac-1+ macrophages. In the thymus, the CD4/CD8 double positive and double negative immature T cells showed the highest percentage of BrdU positive cells (incorporating approximately 40%). In contrast, single positive CD4, CD8, and CD3 positive mature T cells incorporated the least as would be expected. The bone marrow compartment incorporated the most BrdU (53%). This compartment is made up of B cells, macrophages, T cells and hematopoietic progenitors. The B cells macrophage and HSC progenitors accounted for the majority of the BrdU+ cells. Further dissection of the bone marrow compartment for the HSC progenitor KLS (Ckit+/Sca-1+/Lineage-) showed that this population incorporated BrdU at 35-50%. These cells were loaded with VPD450 then sorted for KLS and plated on a feeder layer for 18-30 hours to determine cell proliferation trends post sort. The cells were harvested and assayed for the loss of BrdU and VPD450 peak formation. The progenitors lost 15% of their BrdU in the first 30 hrs proving the cells were actively cycling in culture. All together, these data demonstrate that combining analysis of BrdU incorporation in conjunction with cell surface markers and cell proliferation dyes enables the dissection of cell cycle status of distinct

hematopoietic cell types in vivo and in vitro. These techniques could be adapted to other organs or cell systems comprised of heterogeneous cell types.

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Kinetics of early growth response proteins in human periodontal fibroblast wound healing model.

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Early growth response (EGR) is a nuclear transcription factor implicated in the regulation of cell proliferation. We previously suggested that high molecular weight EGR-1 was not present in the nuclear fraction and the expression of native EGR-1 showed a peak at 60 min with both of FGF-1 and -2 on Western blotting analyses. Wound healing in the connective tissue is a highly orchestrated process that involves inflammation, cell proliferation, reorganization of the extracellular matrix and tissue remodeling. However, there is little information on the EGR expression in human periodontal fibroblasts during wound healing. In this study, we investigated the kinetics of EGR-1 and EGR-2 in a human periodontal fibroblast wound healing model. Three periodontal culture fibroblasts derived from three different teeth were first pre-incubated for two days in serum-free Dulbecco's modified Eagle medium (DME). After scraping with a P-200 pipette chip, periodontal culture fibroblasts were incubated for one, two or three days in DME supplemented with 10% FCS. EGR-1 and EGR-2 were expressed in the cell layer near the wound, and expression was downregulated upon wounding. Strong immunoreactions for EGR-2 were observed throughout the cell layer after one day. After two days, EGR-1 was expressed around the edge of the wound, and expression of EGR-2 decreased gradually. Although EGRs in periodontal fibroblasts are generally glycosylated, this expression is not correlated with scraping treatment. These results demonstrate the inducible nature of EGR proteins in damaged human periodontal tissues, and suggest a potential important role for EGR-1 in fibroblast growth and mobility during wound healing or tissue regeneration in vivo.

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Variability of cell cycle duration is not random in MDCK cells.

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MDCK cells, canine kidney epithelial cells, can differ phenotypically, yet are related by divisions. We analysed cell cycle duration of individual MDCK cells and their progeny to decipher if cellular differences arise. Single cells were either seeded in elastic matrix or on rigid 2-dimensional substrate, where they were transfected with either non-targeting or ninein targeting siRNAs oligos. The individual nuclei and their progeny were tracked, and lineage trees were generated.

We find that in both culture environments the cell cycle duration is highly variable within a single cellular lineage. This effect was strongest for cells grown within the elastic matrix. But, down-regulation of the mother centriolar and centriole-microtubule anchoring factor ninein strongly decreased this variance. These results suggest that the variability of cell cycle duration is not random but may depend on both external and internal cues. They might also reflect the existence of pathways dedicated to the generation of cell diversity.

Analysis of the lineages reveals the presence of a recurring pattern, whereby three out of the four progenies of a grandmother cell behave similarly, while the fourth one shows more variable cell cycle duration. However, subtrees without the pattern also exist and the daughters of such a grandmother cell can generate subtrees, where this pattern emerges de novo. Further, the capacity to generate this pattern segregates unequally between daughter cells. The frequency and magnitude of this pattern are reduced upon ninein down-regulation. Thus, our results suggest that cells influence the generation of irregularities in cell cycle duration.

Together, these results indicate that MDCK cells can divide asymmetrically segregating non-randomly some determinants of interphase duration. Furthermore, the impact of these determinants on proliferation is modulated by the nature of the environment.

1140

How Does the Cell Cycle Respond to Environmental Change?

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S. cerevisiae cells rely on environmental nutrients and internal stores for the raw materials they need to grow and build daughter cells. Because it takes longer for the cell to build a daughter than to duplicate its genome, the cell cycle must lengthen to accommodate reductions in growth rate. However, it is unclear whether specific programs exist to accomplish this in a regulated way. We hypothesized that Start, the cell cycle program that restricts S-phase entry to cells of a threshold size, is also used to lengthen the cell cycle when environmental nutrients become scarce. To test this hypothesis, we observed the growth of *S. cerevisiae* cultures by microscopy after we deprived them of essential nutrients instantaneously, noting each cell's position relative to Start. Here, we report three observations made using this approach.

1. Each *S. cerevisiae* cell contains sufficient internal reserves to grow, pass Start, and produce a viable daughter when deprived many single nutrients (e.g. $(\text{NH}_4)_2\text{SO}_4$, glycerol, metals, etc.). Many cells complete more than one cell cycle after withdrawal of these nutrients, then they arrest in G1, before Start.
2. Glucose is an exception to this rule: its withdrawal induces immediate growth arrest, irrespective of cell cycle position. In the arrested state, cell size remains constant for hours and cells neither bud nor divide. If glucose is re-supplied, arrested cells resume growth and progress through the cell cycle without delay or loss of viability. These results suggest while Start can accumulate cells in G1 when environmental conditions worsen generically, glucose-grown cells have an additional, dominant "emergency brake" that can halt the cell cycle at any position when glucose is withdrawn.
3. Consistent with this interpretation, we have identified mutations that remove the glucose-responsive "emergency brake." These mutants react to glucose withdrawal differently if they are in different phases of the cell cycle: newborn cells fail to pass Start; cells that have passed Start continue to grow, divide, and arrest in the next G1. Current work aims to identify molecular components and regulators of the "emergency brake" and understand the fitness costs associated with not engaging it properly during environmental transitions.

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Cdk1 inhibitors rapidly diverge their phosphorylation sites but maintain phosphodegron function.

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The general architecture of the cell cycle is conserved but the molecular details underpinning this network are highly divergent. For example, all cell cycles described so far involve a mutual antagonism between the Cyclin Dependent Kinase (Cdk1) and a stoichiometric inhibitor of Cdk1 (Cki), but the Cki molecule cannot be recognized across lineages by sequence homology. Upon reaching a certain threshold of cyclin accumulation, Cdk1 phosphorylates the Cki to create a phosphodegron thus creating a double negative feedback loop. In *S. cerevisiae*, the Cki phosphodegron is generated by a complex processive phosphorylation cascade. This cascade has been proposed to set the thresholds for the G1-S transition and mitotic-exit; generate switch-like behavior for Cki destruction; and filter noise from other kinase signalling pathways. However, the arrangement of Cdk1 consensus sites in the degron of the Cki has diverged extensively. We have characterized divergent degrons *in vitro* and *in vivo*. Even the most divergent degrons retain function when imported into *cerevisiae* and support viability. The kinetics of destruction of divergent degrons is strikingly similar *in vivo* despite huge differences in phosphorylation efficiency and processivity *in vitro*, indicating that this system has substantial robustness to change. However, the signal processing fidelity of divergent degrons seems to have degraded in terms of discrimination between Cdk1 input and spurious inputs from mating pathway kinases.

1142

Prion-like behavior regulates cyclin transcript localization and cell-cycle control.

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Mechanisms for positioning mRNAs in the cytoplasm is well established in early development but are not well understood in cycling cells. We show here that a specific G1 cyclin transcript is clustered and not uniformly distributed in the cytoplasm of large multinucleate cells. This anomalous cyclin transcript localization results from prion-like behavior of an RNA-binding protein, and deletion of a poly-glutamine stretch in this protein results in random transcript localization. These multinucleate cells, like many tumor cells, are remarkable in that nuclei cycle asynchronously despite sharing a common cytoplasm. Notably randomization of cyclin transcript significantly diminishes nucleus-to-nucleus differences in the local levels of mRNAs and synchronizes cell-cycle timing. Thus, autonomous and variable nuclear cycling in a common cytoplasm arises due to prion-like behavior of an RNA-binding protein. Many RNA-binding proteins from yeast to humans exhibit polyQ expansions and areas of low structural complexity, suggesting that polyQs may be a physiological mechanism for non-random positioning of transcripts and generating variability in cell cycle timing.

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 γ -tubulin has a significant role in inactivating APC/C^{Cdh1} at the G₁/S boundary.

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Our lab has found that γ -tubulin has a role in regulating the anaphase promoting complex/cyclosome (APC/C) in interphase. In *Aspergillus nidulans*, a cold-sensitive mutant

allele of γ -tubulin, *mipAD159*, causes a nuclear-autonomous failure of inactivation of the APC/C at some point between late mitosis and S phase when grown at a restrictive temperature, resulting in constitutive destruction of cyclin B and removal of nuclei from the cell cycle (Nayak *et al.*, 2010, *J. Cell Biol.*, 190, 317-330). In many organisms Cdh1 activates the APC/C in G₁, targeting S-phase cyclins for destruction. This prevents the onset of S phase and APC/C^{Cdh1} must, thus, be inactivated for S phase to be initiated. In *A. nidulans*, cyclin B is an S-phase cyclin and this raised the possibility that *mipAD159* causes a failure of inactivation of APC/C^{Cdh1} at the G₁/S transition. We identified, GFP-tagged, and deleted the *A. nidulans* homolog of *cdh1*, and we designate the gene *cdhA* and its product CdhA. CdhA-GFP localizes to the nucleoplasm and spindle pole body (SPB) in late G₂, disappears shortly before mitosis, returns to both locations at the beginning of G₁, and disappears from the SPB at G₁/S. We found that *cdhA* is not essential, but CdhA targets cyclin B for destruction in G₁ and is required to prevent cyclin B from accumulating in subapical cells. CdhA is not, however, required for the localization of cyclin B to the SPB. To determine if *mipAD159* caused failure of inactivation of APC/C^{CdhA}, we created a strain carrying *mipAD159* and the *cdhA* deletion. We found that cyclin B accumulated in all nuclei. These data reveal that γ -tubulin plays an important role in inactivation of APC/C^{CdhA} at the G₁/S boundary. Accumulating data suggest that localization of mitotic and cell cycle regulatory proteins to polar microtubule organizing centers such as centrosomes and SPBs is important for their activity and, consequently, their roles in mitosis and cell cycle progression. We, therefore, examined the localization of CdhA in strains carrying *mipAD159*. We found that CdhA failed to dissociate from the SPB in nuclei that failed to accumulate cyclin B and cyclin dependent kinase 1. These data suggest that *mipAD159* causes failure of dissociation of CdhA from the SPB resulting in continuous APC/C^{CdhA} activity and failure to enter S phase. Since *mipAD159* is a recessive allele (Jung *et al.*, 2001, *Mol. Biol. Cell*, 12, 2119-2136), we hypothesize that γ -tubulin interacts with a protein or protein complex that is required for CdhA destruction or dislocation from the SPB. Supported by NIH grant GM031837.

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Cyclin-specific docking motifs promote phosphorylation of yeast signaling proteins by G1/S Cdk complexes.

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Cyclin-dependent kinases (Cdks) drive the eukaryotic cell cycle. In *S. cerevisiae*, a single Cdk (Cdc28) associates with nine different cyclins, which drive specific events at distinct cell cycle stages. Although Cdk activity is generally highest at later stages (S and M), some Cdk substrates are phosphorylated predominantly at the G₁/S transition. These early Cdk substrates include two signaling proteins in the pheromone response pathway, Ste5 and Ste20, whose phosphorylation is driven preferentially by G₁/S cyclins (Cln1 and Cln2) and not by G₁, S, or M cyclins. The basis of this cyclin specificity was previously unknown.

We recently found that both Ste5 and Ste20 have recognition sequences, or “docking” sites, for the G₁/S cyclins. These docking sites bind specifically to Cln1 and Cln2, but not to other cyclins (Cln3, Clb5, Clb2). They strongly promote Cln1/2-driven phosphorylation of each substrate in vivo, and function largely independent of position and distance to the Cdk sites. They are also functionally interchangeable, as the Ste20 docking site can be used to drive phosphorylation of Cdk sites in Ste5. We exploited this functional modularity to re-wire the Cdk regulatory control of pheromone signaling: i.e., by providing a docking site plus Cdk phosphorylation sites that flank a critical functional domain, we could transfer Cdk regulation from Ste5 onto Ste20. We then used this re-engineered circuit as a tool to identify functional Cln1/2 docking sites in several other Cdk substrates. The number of sites discovered suggests that docking likely facilitates substrate

recognition in many G1/S Cdk targets. Finally, we found that these docking sites enhance substrate phosphorylation in a cyclin-specific manner, and that the cyclin preference can be switched by using a different type of docking site (i.e., an "RxL" motif that binds B-type cyclins). Moreover, we find that an artificial docking interaction can allow any cyclin (i.e., G1, S, M) to drive in vivo phosphorylation of a substrate that is normally G1/S-specific.

Collectively, our results show that some Cdk substrates are intrinsically capable of being phosphorylated by several different cyclin-Cdk forms, but they are poor substrates in vivo without a cyclin-specific docking motif. These findings provide the first examples of docking motifs specific to G1/S cyclins, and they help explain how some Cdk phosphorylation events can be timed to occur early in the cell cycle rather than later.

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Mechanical cell cycle checkpoints mediate control of cell proliferation in epithelial tissues.

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The control of cell cycle progression in single eukaryotic cells has been well studied from yeast to humans. However, little is known about how proliferation is regulated in the context of epithelial tissues, where cells are mechanically coupled with each other and are constrained by their neighbors, e.g. during development and wound healing.

Here we study the cell cycle progression of Madin-Darby canine kidney (MDCK) cells in response to mechanical manipulations and pharmacological inhibition of the MEK-ERK signaling pathway. Using live cell imaging and advanced image analysis, we find that cell cycle duration correlates with cell size and that there is a characteristic cell size required for G1/S-transition. Moreover, the proliferation rate within a tissue is increased or reduced in a predictable manner by stretching or compressing the tissue, respectively, thereby accelerating or delaying cell cycle progression. We conclude that mechanical checkpoints during cell cycle progression are employed to control proliferation in tissues and maintain tissue integrity.

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Inhibition of TGF- β signaling promotes proliferative activity of neonatal cardiomyocytes.

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Mammalian cardiomyocytes irreversibly withdraw from the cell cycle and become terminally differentiated shortly after birth. The mechanism underlying this event remains largely unknown. It is already known that the TGF- β family is a major cytokine in regulating cell growth, differentiation, adhesion and apoptosis in many cell types. In this study, we examined whether and how TGF- β signaling regulates the proliferative activity of neonatal cardiomyocytes. We investigated the effects of TGF- β 1 or SB-431542, a potent T β RI/ALK5 inhibitor, on the proliferative activity of neonatal rat cardiomyocytes. We found that TGF- β 1 stimulation inhibits cardiomyocyte proliferation significantly, while SB-431542 treatment promotes this activity of cardiomyocytes remarkably. To clarify the mechanisms by which TGF- β signaling regulates cell cycle progression, we examined whether the expression or subcellular localization of cell cycle regulators, which control the G1 progression and G1/S transition, are affected on TGF- β signaling alteration. Exposure to TGF- β 1 in cultured cardiomyocytes induced binucleated cardiomyocytes and accumulation of p27 in nuclei. Treatment of cultured cardiomyocytes with

SB-431542 resulted in a significant decrease in nuclear localization of p27. Taken together, our findings suggest that TGF- β signaling regulates the proliferative activity of neonatal cardiomyocytes via the cell cycle regulator p27.

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The role of Origin Recognition Complex in chromatin organization.

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The Origin Recognition Complex (ORC) is a hetero-hexameric complex required for the initiation of DNA replication in eukaryotes. Apart from its role in replication initiation, ORC plays important roles in several other cellular processes including centrosome duplication, sister chromatid cohesion, chromosome segregation, cytokinesis and heterochromatin silencing. While a lot of progress has been made in elucidating the function of ORC in DNA replication, its role in chromatin organization remains to be clearly elucidated. Our lab has identified a novel protein, ORC-Associated (ORCA/LRWD1), that is a critical component of the pre-replicative complex. ORCA associates with different ORC subunits and with ORC2 directly. The association with ORC2 is required for the stability of ORCA. Furthermore single molecule analysis indicates that one molecule of ORCA associates with one molecule of ORC, one molecule of Cdt1 and two molecules of geminin. Association of ORCA with Cdt1 is during mitosis and G1 while the association with geminin is in post-G1 cells. Knockdown of ORCA leads to defects in ORC loading onto chromatin indicating that ORCA stabilizes ORC on chromatin. We demonstrate that ORCA is required for progression of cells from G1 to S phase of cell cycle. Our data collectively point towards a role of ORCA in regulating cell cycle possibly by modulating the association of ORC with chromatin. The role of ORCA and the different ORC subunits in chromatin organization will be discussed.

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Yeast S-phase B-type cyclin Clb6 is a substrate in late G1 for the cyclin-dependent kinases Cdc28 and Pho85.

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In budding yeast *Saccharomyces cerevisiae*, the S-phase B-type cyclins Clb5 and Clb6 are transcribed in the G1-phase of the cell cycle, where they bind to the cyclin-dependent kinase (CDK) Cdc28, but the CDK activity is kept off by Sic1, a CDK inhibitor (CKI). Although Clb5 and Clb6 are homologous, the N-termini of the proteins are divergent. The N-terminus of Clb6 contains a D-box motif (R-x-x-L-x-x-x-N) and a KEN box motif, both of which may serve as signals for regulated proteolysis by the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase. The N-terminus of Clb6 also contains three potential SP/TP phosphorylation sites that are thought to contribute to the regulation of Clb6 protein stability regulated by the SCF^{Cdc4} ubiquitin ligase later in the cell cycle in S-phase. We have set out to study the regulation of Clb6 protein stability in late G1 by initially investigating the phosphorylation of Clb6. Wild-type Clb6 and a mutant Clb6, with the three potential N-terminal phosphorylation SP/TP sites mutated to alanine, were prepared by *in vitro* transcription/translation and labeled with ³⁵S-Methionine. Active Cdc28 and Pho85 kinases were isolated out of yeast extracts made from *cdc4-1* temperature-sensitive mutant yeast cells arrested in late G1 at 37°C. In kinase assays,

we have observed that wild-type Clb6 was a substrate for both Cdc28 and Pho85 kinases, whereas the phosphorylation site mutant Clb6 was not. The observed phosphorylation was eliminated by the addition of calf intestinal phosphatase. Initial results have revealed that the cyclin partners of Cdc28 responsible for the phosphorylation of Clb6 are both the G1-phase cyclins Cln1 and Cln2, where Cln2-Cdc28 may be the primary source of kinase activity. Clb6 phosphorylation was found not to be dependent upon S-phase cyclin-CDK activity, such as Clb5-Cdc28 or Clb6-Cdc28 complexes isolated from *cdc4-1* late G1 arrested cells. We are currently investigating which cyclin partner(s) of Pho85 can phosphorylate Clb6 in late G1. Finally, using the phosphorylated form of wild-type Clb6 protein as the target substrate, we plan to perform *in vitro* APC/C^{Cdh1} assays to explore whether phosphorylated Clb6 is targeted by regulated proteolysis in late G1.

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Reconstitution of DNA replication licensing on the chromatin fiber.

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Eukaryotic DNA replication initiates at multiple regions of genomic DNA, which is called replication origin. The proper determination of the sites of replication origins is important for stable maintenance of genomic information. Onto replication origins, origin recognition complex (ORC) located throughout the cell cycle in budding yeast, and MCM complex is loaded during G1 phase with the help of Cdc6 and Cdt1. The complex consisting of ORC, MCM, Cdc6, and Cdt1 is called pre-replicative complex (pre-RC), and the formation of pre-RC licenses the replication origin to initiate DNA replication during the next S phase.

Although the assembly process of these proteins on origin has been studied by genetic and biochemical researches, the architecture of ORC or pre-RC on origin has not been understood well. In addition, eukaryotic genome DNA forms chromatin fibers with histones, so that, for understanding the molecular mechanism of the licensing of DNA replication *in vivo*, it is essential to analyze ORC and subsequent pre-RC formation on chromatin fibers.

Here, we reconstituted chromatin fibers from yeast core histones and DNA fragments containing yeast origin sequence and analyzed the interaction between ORC and the chromatin fibers. We found that purified ORC binds to the origin-containing chromatin more stably than to naked DNA and forms a nucleosome-free region at origins by interacting with histones. Molecular imaging using atomic force microscopy (AFM) revealed that ORC associates with adjacent nucleosomes. Moreover, the stable chromatin binding of ORC required linker DNA. Thus, ORC establishes origin-specific interactions by binding both to nucleosome-free-origin DNA and to neighboring nucleosomes. We are also trying to form pre-RC on the reconstituted chromatin, and will discuss how the formation of chromatin fibers on origin DNA affects pre-RC formation.

Mitotic Spindle-Structure/Organization

1150

How Cell Size Regulates Spindle Size.

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A mitotic spindle can assemble and function to segregate chromosomes in cells of widely varying sizes, but the extent to which spindle size is regulated directly by cell size is unknown. During *Xenopus laevis* embryogenesis, the 1.2 millimeter diameter egg cleaves rapidly to form

smaller blastomeres, and after an initial plateau phase, spindle size changes linearly with cell size. Major challenges to uncovering physical mechanisms of spindle scaling include recapitulating the broad range of cell sizes found in the embryo, and decoupling cell size and developmental stage. To overcome these issues we developed a system for making synthetic cell-like compartments of defined sizes in vitro. By encapsulating *Xenopus* egg extracts we can create artificial cells of varying size that support spindle assembly. We find that the steady-state length and area of *X. laevis* extract spindles is dependent on the size of the compartment they are assembled in. Specifically, encapsulated spindles possess both an upper and lower size boundary and a linear scaling range. Interestingly, although *X. laevis* and *X. tropicalis* spindles have different steady-state sizes in bulk extract and large compartments, both spindle types converge on the same lower size limit in the smallest compartments. Multiple features of *Xenopus* spindle length regulation under confinement closely match embryo data demonstrating at least in part that spindle scaling can occur independent of developmental regulation. Intriguingly, spindle size tracks more closely with cell volume than with cell geometry, suggesting that spindles respond to the amount of material available in the cell. We propose a limiting substrate model to explain the linear response of spindle size to cell size during frog embryogenesis.

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Fission yeast Psr2 helps establish initial spindle bipolarity and subsequent proper chromosome segregation.

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Proper chromosome segregation during mitosis is of paramount importance for proper genetic inheritance to daughter cells from mother cells. Fission yeast model organism has proven to be a powerful tool in which to study the mechanisms that control spindle bipolar structure and chromosome segregation. Mis-segregation of chromosomes can lead to aneuploidy and chromosome instability, which are hallmarks of cancer cells and other human diseases. Chromosome segregation is accomplished by the mitotic spindle, composed of microtubules, microtubule-associated proteins (MAPs), motors and other regulatory proteins. The spindle is a bipolar structure, with microtubules emanating from opposite poles crosslinking and overlapping at the spindle midzone in an antiparallel manner. Spindle bipolarity establishment is generally thought to be accomplished by the motor kinesin-5. However, kinesin-5 can equally generate parallel or antiparallel microtubules. What then organizes antiparallel microtubules required for spindle bipolarity? In a screen for fission yeast Spindle Assembly Defective mutants, we discovered Psr2 (Pole Separation Regulator 2). Psr2-deletion leads to transient monopolar spindle, longer spindle at metaphase-anaphase transition, and subsequent significant chromosome segregation defects.

Psr2p localizes to the spindle pole body (SPB), and its localization is dependent on the conserved Sad1p/SUN protein and Psr1p. Psr1-deletion has been shown in our lab to have transient monopolar spindle, prolonged prophase-metaphase, and chromosome segregation defects, similar to Psr2-deletion. In addition, Psr1p binds the conserved Mal3p/EB1, and presumably microtubule plus ends emanating from the opposite poles. This leads to antiparallel microtubules to initial spindle bipolarity. Psr2 and Psr1 may interact in the same pathway. Interestingly, Psr1p also requires Psr2p to localize to the SPB, but only specifically at the start of mitosis through early anaphase. Our findings give better understanding of the mechanism for prophase bipolar spindle establishment, and its importance for subsequent faithful chromosome segregation.

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Furry is a positive regulator of microtubule acetylation in mitotic spindle.

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During mitosis, microtubules form the bipolar spindle that separates chromosomes into two daughter cells. Tubulin subunits of mitotic spindle are subject to several post-translational modifications, including acetylation of α -tubulin at Lys-40. Tubulin acetylation is a "stable marker" of microtubule lattice and affects the function of microtubule-binding kinesin motors. Whereas several regulators of tubulin acetylation have been identified, functional significance and regulatory mechanisms of tubulin acetylation in mitotic spindle are still largely unknown. Furry (Fry) is an evolutionarily conserved protein from yeast to human. Previous studies showed that Fry is involved in polarized cell growth, morphogenesis, and cell cycle progression in yeast and invertebrates. We previously showed that mammalian Fry is required for proper formation of spindle microtubules and precise alignment of mitotic chromosomes and that Fry has the potential to directly bind to microtubules and localizes on the mitotic spindle. However, it is unclear how Fry affects the microtubule function during mitosis. In this study, we show that Fry is a positive regulator of microtubule acetylation. Knockdown of Fry reduced the level of spindle microtubule acetylation in HeLa cells. Transient expression of the N-terminal fragment of Fry induced microtubule hyperacetylation in interphase cells. These results indicate that Fry has the potential to up-regulate the level of microtubule acetylation via its N-terminal region. Moreover, we identified SIRT2 tubulin deacetylase as a novel Fry-binding protein. Fry bound to SIRT2 via its N-terminal region and this interaction was increased in mitotic cells. Fry did not bind to a catalytically inactive mutant of SIRT2, which suggests that Fry inhibits SIRT2 activity by binding to the catalytic domain of SIRT2. *In vitro* microtubule deacetylation assays revealed that Fry inhibits SIRT2 activity via its N-terminal region. Together these findings suggest that Fry is involved in the positive regulation of spindle microtubule acetylation through localization on the spindle microtubules and inhibition of the tubulin deacetylase activity of SIRT2.

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Mars and Mei-38: The roles of two spindle assembly factors in *Drosophila* syncytial embryos.

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The microtubules (MTs) that comprise the mitotic spindle are generated from multiple sites from within the cell. Condensed mitotic chromosomes themselves have long been proposed as a source of MTs, including those emanating directly from kinetochores. TPX2 and HURP are two key proteins implicated in the formation and maintenance of kinetochore MTs in vertebrates. Both have been implicated in promoting MT polymerisation and stabilisation and their presence in complex together is suggestive of co-operative function. However, their exact mechanistic roles in chromatin mediated MT formation have yet to be determined.

Drosophila melanogaster provides an excellent biological system for the study of mitotic spindle formation, in which biochemical and proteomic analyses can be complemented with genetic and cell biological techniques. Mars has been proposed as the *Drosophila* homologue for HURP, while our research group, and others, have proposed Mei-38 as a potential TPX2 homologue. We are investigating the comparative roles of Mei-38 and Mars with the aim of understanding their contributions to chromatin mediated MT formation.

During interphase of embryos expressing GFP-fusions, both Mei-38 and Mars are present in the nucleoplasm, like TPX2 but unlike the cytoplasmically localised HURP. Both proteins show similar localisation patterns to spindle MTs throughout mitosis, with Mei-38 additionally present at centrosomes. We show that Mei-38 is able to promote MT polymerisation and bind to and bundle MTs in vitro. Equivalent experiments with recombinant Mars demonstrate MT binding properties, but no evidence of MT polymerisation or bundling. Surprisingly, the absence of Mei-38 does not have a huge significance on spindle formation or embryonic development. Flies are viable and fertile, though spindles exhibit a delay in metaphase-anaphase progression and a decrease in spindle length. In comparison, Mars null flies are female sterile, with embryos failing at the first mitotic cycle. A hypomorphic Mars mutant reveals multiple spindle failures including frequent centrosome detachment, monopolarity and short spindles.

By employing the use of GFP to immunoprecipitate interacting MAPs, double mutants and interfering antibody injections, we aim to establish the role each protein plays in building kinetochore microtubules.

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The *Arabidopsis* augmin complex contains two plant specific subunits and plays a critical role in microtubule organization during cell division.

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Microtubules are organized into the bipolar spindle array to ensure accurate segregation of chromosomes during cell division. It has been realized that in animal cells centrosome-independent and microtubule-dependent microtubule nucleation directly contributes to spindle assembly, in addition to those initiated from the centrosomes and chromosomes. It has been shown that the γ -tubulin complex is targeted to spindle microtubules via the augmin complex in order to initiate microtubule nucleation and consequently to amplify microtubules in animal spindles. In higher plant cells that lack the centrosome, we tested whether an augmin complex functioned in spindle microtubule nucleation and organization. Using AUG3 which has limited homology with the human augmin subunit HAUS3 as the bait, we successfully purified the augmin complex from the seedling of *Arabidopsis* and identified eight subunits (AUG1 to AUG8) based on mass spectrometry analysis. Among them, AUG7 and AUG8 are plant specific proteins whose homologs can only be found in the plant kingdom, while the other six subunits (AUG1 to AUG6) show weak similarity to their animal counterparts. They exhibit a localization pattern similar to that of γ -tubulin during mitosis. Insertional mutations of *aug1*, *aug2*, *aug3*, *aug4* and *aug5* at the corresponding loci were only inherited by heterozygous plants, suggesting these AUG subunits to be essential. We isolated a sterile *aug7* homozygous mutant in which *AUG7* was expressed at a considerably lower level than the wild-type control. This mutant exhibited phenotypes of severe growth retardation. γ -Tubulin was barely detected in spindles and phragmoplasts of the *aug7* cells, and concomitantly these microtubular arrays were disorganized. We conclude that plant cells deploy an evolutionarily conserved augmin-dependent mechanism to organize microtubules during the mitosis and cytokinesis.

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The Three-dimensional Organization of the *Xenopus laevis* Meiotic Spindle Resolved Using Electron Tomography.

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Understanding the packing density, length, spacing, and polarity of the microtubules (MTs) in the vertebrate spindle is important for extending our understanding of the spindle functionality. Light microscopy studies in *Xenopus laevis* egg extract propose a dynamic tiled array of MTs that are cross-linked and focused during bipolar spindle assembly. However, the three-dimensional (3D) organization of the vertebrate spindle at the level of individual MTs is still unresolved. Using electron tomography it is now possible to image large areas and we aim to image half of the large *Xenopus laevis* meiotic spindle, as assembled in the well established cell-free egg extract, and to resolve its 3D organization. The quality and location of the spindles in a sample are assessed using light microscopy, and correlative light and electron microscopy is used to retrieve spindles of interest after sample processing. Well-formed, isolated spindles are high-pressure frozen, freeze substituted and processed into plastic for electron tomography. Spindles are serial sectioned parallel to their long axis and the sections are screened on a transmission electron microscope to verify MT orientation and chromosome integrity. For samples that fulfill these criteria, high magnification supermontage tilt series are acquired on a FEI Tecnai F30 transmission electron microscope, and reconstructed into large tomograms. Tomograms from serial sections are stacked together into a large 3D volume. MT and chromosome masses are modeled using the Filament Editor tool in Amira. To date, a partial volume of the spindle extending from pole to pole to midzone has been acquired, and reconstructed. Modeling of the data is currently underway. We will present our current model of the spindle from which we hope to gain insight into the detailed spatial organization of microtubules in the vertebrate spindle.

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The inter-relationship between spindle assembly pathways in *Drosophila* syncytial embryos.

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Mitotic spindle formation is an example of an inherently flexible and robust biological system, where at least three well-described molecular mechanisms of microtubule (MT) generation act co-operatively to ensure faithful chromosome segregation. The predominant source of MTs for the spindle are the centrosomes, electron dense organelles that recruit the MT nucleator, gamma-tubulin. Despite their predominance, it has been shown that centrosomal MTs are dispensable for spindle formation in some experimental systems. Generation of MTs also occurs in the vicinity of mitotic chromatin, both through stabilisation of MTs by the activation of spindle assembly factors (SAFs) and through nucleation from kinetochores. MTs contributing to the mitotic spindle are also generated from pre-existing spindle MTs through the action of the Augmin protein complex.

While we know much about the molecular details of these three pathways, little is known about how they interact, and the mechanisms by which they can compensate for the loss of each other. Simultaneous perturbation of multiple pathways can be used to advance our understanding of these phenomena, however not without complications; a purely genetic approach often results in lethality, making it impossible to analyse spindle formation itself, while RNAi gradually reduces protein function over a number of days, leading to pleiotropic effects that are difficult to analyse. Instead, we have employed a combined approach of analysing MT

organisation during spindle formation in embryos carrying mutations in genes required for specific MT generation pathways, or in embryos injected with interfering antibodies against these proteins. Loss of centrosomal MTs, through inactivation of the core centriolar protein Spd2, results in the formation of multiple MT nucleating sites, whose MTs nonetheless coalesce to form a functional bipolar spindle. In contrast, removal of Augmin results in a significant delay in MT generation at the onset of mitosis, with astral MTs eventually connecting to kinetochores to form a bipolar spindle.

We will further discuss our results, and their implications for the formation of centrosomal, chromatin mediated and Augmin generated MTs.

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A novel role for the IFT B complex in mitotic spindle assembly.

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Intraflagellar transport (IFT) is a bidirectional transport system required for ciliogenesis. IFT proteins form multimeric complexes, A and B, which transport pre-assembled ciliary components along the axoneme while riding microtubule motors. Cilia disruption causes ciliopathies, characterized by cystogenesis and misoriented cell division. Interestingly, several IFT proteins localize to mitotic spindle poles, suggesting a role for these proteins in mitosis. In fact, we previously showed that IFT B complex member, IFT88, is part of a dynein-1 driven mitotic transport complex required for spindle orientation. (Delaval, Bright et al, NCB, 2011). However, nothing is known about the remaining ciliary IFT B complex members' mitotic function. We addressed this by comparing IFT B complex size during mitosis and interphase, and found that the complex is maintained in mitosis even in the absence of cilia. Furthermore we identified mitotic interactions between several IFT B complex members and dynein-1, confirming the existence of a mitotic IFT B/dynein-1 complex. These results provided an argument for characterizing mitotic functions for additional IFT B complex proteins (eg. IFT57, IFT52, and IFT20). Like IFT88, IFT57 and IFT20 depletions caused several mitotic defects including disrupted spindle poles, non-congressed chromosomes, increased mitotic index, etc. Surprisingly, IFT52 displayed none of these phenotypes. Because these mitotic disruption phenotypes resemble dynein-1 depletion we tested whether IFT depletion affects dynein-1 function. Surprisingly, dynein-1 levels are increased specifically during mitosis when IFT88 or IFT57 is depleted, while IFT52 depletion decreases dynein-1. With this striking result we conclude that IFT proteins are not passive dynein-1 passengers, but are in fact actively regulating dynein-1 during mitosis. This work raises the important possibility that mitotic, as well as cilia defects could contribute to ciliopathies.

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Spindle Length Scales with XMAP215-dependent Microtubule Growth Rates in Agreement with a Mass-Balance Model.

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Although we have extensive knowledge of individual proteins that modulate microtubule dynamics, we still lack an understanding of the mechanisms by which their collective properties give rise to a spindle with a defined size and shape. Here, we describe spindle size using a mass balance model, which implies that rates of gain and loss of microtubule polymer are equal for a spindle of constant size. Microtubule mass balance depends on microtubule nucleation,

growth and lifetime. To test this model, we modulate microtubule growth independently of other kinetic parameters by using mutants of the XMAP215 microtubule polymerase in *Xenopus* spindles. We show that spindle length increases linearly with microtubule growth velocities. Taken together, the combined theory and experiment allow us to relate the molecular activity of the polymerase directly to spindle length. We conclude that spindle length is determined by microtubule mass balance in a liquid crystal drop of constant microtubule density.

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Tension-dependent dynamic microtubule model for metaphase and anaphase phenomena.

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We present a simple theoretical model that captures key phenomena of eukaryotic chromosome segregation such as metaphase chromosome oscillations, microtubule (MT) attachment error correction, and anaphase chromosome separation. During metaphase, chromosome pairs align near the center of a bipolar MT spindle and oscillate as the MTs attaching them to the cell poles polymerize and depolymerize. Simultaneously, the cell fixes misaligned chromosome pairs that are attached to only one cell pole by some tension-dependent mechanism. Finally, in anaphase, chromosome pairs separate as each chromosome in the pair is pulled by depolymerizing MTs toward its respective cell pole. Despite the ubiquity of these phenomena, their details vary from system to system. Instead of including all known components to develop a comprehensive, species-specific description, we introduce a minimal model based on fundamental properties of MT kinetics. We use the tension-dependence of the rates of single MT polymerization/depolymerization kinetics measured by Akiyoshi et al. [1] and assume that the same functional dependence holds for the compression dependence. We apply these kinetics to many MTs connected to kinetochores to form a spindle, and solve this stochastic model numerically and by a master equation approach. We find that tension dependence of the rate constants enhances the speed at which a single chromosome is pulled by MTs during anaphase- or error-correction-like behavior. Furthermore, we show that the force-velocity curve for the single chromosome attached to dynamic MTs exhibits behavior reminiscent of a phase transition: at high loads, large tension fluctuations induce the MTs to spontaneously switch from a depolymerizing state into a polymerizing state. The system is hysteretic; upon decreasing the load on a chromosome being pushed by growing MTs, the system remains in the polymerizing state for loads far smaller than those required to initially induce polymerization. This behavior leads to the chromosome oscillations we observe in the two-chromosome system. Interestingly, in the two-chromosome system, we observe breathing oscillations, which are not captured by any other existing model of chromosome oscillation. Altogether, our model reflects generic features of three phenomena in eukaryotic mitosis and leads to an understanding of their underlying mechanisms. The minimal model can also be used to understand how different components affect these phenomena via the rate constants.

[1] Akiyoshi et al. (2010) *Nature* 468, 576-579.

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Force-balance model of suppression of multipolar division in cancer cells with extra centrosomes.

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Cancer cells often possess extra centrosomes which have the potential to cause cell death due to catastrophic multipolar division. Many cancer cells, however, are able to escape multipolar mitosis by clustering the extra centrosomes to form bipolar spindles. The mechanism of centrosome clustering is therefore of great interest to the development of anti-cancer drugs because the de-clustering of extra centrosomes provides an appealing way to eliminate cancer cells while keeping healthy cells intact. We present a physical model assuming 1) dynamic centrosomal microtubules interact with chromosomes by both pushing on chromosome arms and pulling along kinetochores; 2) these microtubules interact with force generators associated with actin/adhesion structures at the cell boundary; and 3) motors act on anti-parallel microtubules from different centrosomes. We find via computer simulations that chromosomes tend to aggregate near the cell center while centrosomes can be either clustered to form bipolar spindles or scattered to form multipolar spindles, depending on the strengths of relative forces, cell shape and adhesion geometry. The model predictions agree with data from cells plated on adhesive micropatterns and from biochemically or genetically perturbed cells. Furthermore, our model is able to explain various microtubule distributions in interphase cells on patterned substrates.

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Establishing the copy number and centromeric architecture of the centromere-specific histone Cse4p.

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Considerable debate exists regarding the copy number of the centromere-specific histone H3 variant Cse4p and the physical conformation of the centromeric locus in budding yeast. Estimates of Cse4p copy number per kinetochore, mainly obtained using fluorescence microscopy in cells expressing Cse4-GFP fusion protein, range from exactly one Cse4p molecule per kinetochore to 6-8 molecules. Although biochemical studies point to Cse4p incorporation exclusively at the CEN locus, whether the structure containing Cse4p molecule/s is a hemisome, two hemisomes, a canonical nucleosome, or a heterotypic nucleosome remains unknown. To obtain Cse4p count in live cells, we conducted stepwise photobleaching measurements of single budding yeast kinetochores carrying Ndc80-GFP(S65T) in live cells. Objective statistical analysis of photobleaching time series allowed us to determine the molecular brightness for GFP within the yeast nucleus in mid-anaphase arrested cells. This molecular brightness is consistent with 6-7 Ndc80 molecules per kinetochore in anaphase. Comparison of Ndc80-GFP signal in metaphase and anaphase also revealed that there is little change in the molecular count for this complex over these phases of the cell cycle, as reported before. As reported before, the metaphase and anaphase ratio of Ndc80-GFP and Cse4-GFP is 3.5 and 4 respectively. Therefore, we conclude that there are 2 copies of Cse4p per yeast kinetochore in both metaphase and anaphase. We are developing related microscopy techniques including FRET to fully establish the physical conformation of the Cse4p molecules at the centromeric locus. Our goal is to establish the physical architecture of the foundation of the kinetochore-microtubule attachment.

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Kif18A and Kif4A suppress the growth of different subsets of spindle microtubules to antagonistically control chromosome alignment.

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The alignment of chromosomes at the equator of the microtubule-based mitotic spindle is one of the most conspicuous steps of the cell division process, however, the mechanisms that control alignment are not understood. Mitotic chromosome movements are intimately coupled to the growth and shortening of bundles of microtubules, called K-fibers, which attach chromosomes to the spindle via a specialized protein structure called the kinetochore. K-fiber dynamics must be spatially controlled to align and maintain chromosomes at the spindle equator. Two kinesin-like motor proteins, Kif18A (kinesin-8) and Kif4A (kinesin-4), play critical roles in providing this control. Interestingly, these two kinesins are both plus-end directed motors that suppress microtubule growth *in vitro*, yet oppositely affect chromosome alignment. We demonstrate that Kif18A and Kif4A suppress different subsets of microtubules within the spindle and that targeting the Kif4A motor to the plus-ends of K-fibers can functionally compensate for the loss of Kif18A activity. Collectively, these data suggest that similar modifications of microtubule dynamics can have profoundly different effects when applied to different classes of spindle microtubules.

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Elevated polar ejection forces stabilize kinetochore-microtubule attachments.

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Chromosome biorientation promotes congression and generates elevated tension that stabilizes kinetochore-microtubule (kt-MT) interactions. Molecular motors also contribute to chromosome alignment, but it is unclear how motor forces impact kt-MT attachment stability. A critical force that acts on mitotic chromosomes is the kinesin-10-dependent polar ejection force (PEF), which has been proposed to facilitate congression by pushing chromosome arms away from spindle poles. Here we describe a live-cell PEF assay in which expression levels of the chromokinesin NOD (*D. melanogaster* kinesin-10) are experimentally controlled. Application of the assay revealed that PEFs hyper-stabilize syntelic kt-MT attachments in a dose- and motor-dependent manner. Aurora B kinase activity is unaffected by NOD overexpression indicating that elevated PEFs overwhelm Aurora B-mediated correction of syntelic attachments. We expect the PEF assay to be a valuable tool for defining how forces regulate kt-MT interactions and error correction in living cells.

Cilia and Flagella II

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Screening a Million Mutations to Identify Novel Ciliary proteins.

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Cilia are primordial microtubule-based organelles found on most vertebrate cell types. Motile cilia perform well-understood roles, such as enabling sperm locomotion, and clearing debris from respiratory airways. How non-motile (primary) cilia enable sensory physiology (vision, smell, hearing) and modulate various signaling pathways (Wnt, Hedgehog, PDGF) important for development, are, in comparison, less well understood. Primary cilia are implicated in many human disorders including polycystic kidney disease and Bardet-Biedl syndrome, and disrupting their functions collectively results in retinal degeneration, kidney cyst formation, obesity, brain malformations, and various other ailments. To uncover novel proteins required for the formation and function of primary cilia, we have undertaken an innovative, high-throughput screen for cilia structure and function using a library of 2,000 mutagenized *C. elegans* strains, which altogether carry about 1 million mutations. As the genomes of these mutant strains are sequenced and annotated, we are able to rapidly identify the mutation responsible for the observed ciliary phenotype, eliminating the rate-limiting cloning step required in genetic screens. Using this approach, we have identified *C. elegans* strains with novel mutations that result in gross defects in cilia structure, which we identify with a simple, commonly used dye-filling (dyf) assay, in which worms are incubated in a lipophilic dye (e.g. Dil) that fills ciliated sensory neurons through ciliary membranes that are (normally) exposed to the environment. We are also identifying mutants with abrogated ciliary functions, identified by assaying cilia-dependent behaviors, including starvation pattern, dauer formation and CO₂ avoidance. Detailed molecular and cellular analysis of the most interesting candidates (genes that are novel and possess a human orthologue) from this screen in both *C. elegans* and mammalian tissue culture will provide new insights into the factors required for cilium biogenesis, structure and function, a fundamental cellular process that also has important biomedical implications.

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Characterization of BBC73, a *Tetrahymena thermophila* ortholog of the human EFHC1.

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The basal body is a microtubule-based cellular structure found widely in eukaryotes. At its core the basal body contains a radial array of nine triplet microtubules. Basal bodies reside at the cell surface where they act to form motile cilia or the immotile, sensory primary cilia. Dysfunctional basal body or ciliary components have been implicated in a number of human diseases known as ciliopathies. We identified Bbc73 in a *Tetrahymena* basal body proteome (Kilburn, Pearson *et al.* 2007. *J. Cell Biol.* **178**:905 – 912). Bbc73 is highly conserved from ciliates to humans. Mutations in the human ortholog EFHC1 was been found to cause juvenile myoclonic epilepsy (Suzuki *et al.* 2004. *Nature Genetics.* **36**: 842 - 849). We have localized Bbc73 to both the basal bodies and the cilia at endogenous levels in *Tetrahymena*. Immunoelectron microscopy further revealed that Bbc73 mainly localizes to the microtubule scaffold of the basal bodies. We also demonstrated that the N terminal domain of Bbc73 is responsible for its localization by deletion mutant analysis. We have also identified a paralog of Bbc73 in *Tetrahymena* genome. This protein (Bbc60) exhibits a localization pattern similar to Bbc73. A complete genomic knockout

analysis of *BBC73* is being constructed with the expectation that the phenotypes of the null strain will contribute to understanding the function of this protein.

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Investigation of a novel cilia-related gene K04F10.2/KIAA0556.

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Multiple proteomics and genomics approaches have been used to identify the molecular parts list of cilia and flagella. However, the specific cilia-related functions of many of these components remain unknown. Previously, K04F10.2 was identified as a candidate cilia-related gene in *C. elegans*, exhibiting specific expression in ciliated sensory neurons. Evolutionarily conserved, K04F10.2 encodes a protein with motifs homologous to galactose binding domains. GFP-tagged K04F10.2 is enriched at the ciliary transition zone (TZ) and at the more proximal basal body region. Although K04F10.2 null mutants possess grossly normal cilium structure and ultrastructure, overexpression of K04F10.2 results in a dye-filling abnormality (Dyf), short cilia, cilia that are misaligned, and abnormally short dendrites. Genetic analyses using double mutants revealed a synthetic interaction (SynDyf) between K04F10.2 and Joubert syndrome *Arl13b/arl-13*, but not with TZ-associated *nphp-4* and *mks-5* alleles. Consistent with the nematode data, KIAA0556/K04F10.2 knockout mice possess grossly normal cilium structure. However, these mice present with hydrocephalus, indicating possible defects in motile cilia. To further understand the molecular basis of KIAA0556/K04F10.2 function, efforts are currently underway using affinity proteomics to identify the constituents of KIAA0556-containing complexes. Together, these data implicate K04F10.2 as a novel TZ-enriched protein with functions distinct from canonical MKS and NPHP modules.

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Identifying causative mutations in *Chlamydomonas* flagellar mutants using whole genome sequencing.

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Whole-genome sequencing (WGS) provides a platform for the identification of mutations that produce a mutant phenotype (Dutcher *et al.*, 2012). Given the growing number of ciliopathies in human patients, identification of mutants in a genetically tractable organism produces validation and an avenue to studying the mechanistic role of proteins. We use Illumina sequencing to identify the mutational profiles of three *Chlamydomonas* mutant strains: *fla9*, *pf7*, and *imp3*. Each lane of sequencing produces about 300M reads and about 92% of the JGI version 5.3 genome shows >25-fold coverage. Multiple mutants are loaded into a lane. On average, each mutant has 35,000 to 50,000 changes relative to the reference genome. Using a library of ~250,000 polymorphisms assembled from WGS of ten mutant and wild-type strains, we identify approximately 5,000 strain-specific changes. We focus on exonic and splice site mutations as potential causative mutations. The *fla9* temperature-sensitive flagellar assembly strain maps to 450 kb on chromosome 17. This region has three mutations with coverage ranging from 60-122 fold. We show by the isolation of an intragenic revertant and transgenic rescue that a mutation in *IFT81*, which encodes a Complex B intraflagellar transport protein, is responsible for the phenotype. The mutation causes the loss of an exon that has no effect on IFT train frequency or velocity at 21°C, but fails to assemble flagella at 33°C (Iomini *et al.*, 2001). The short flagella

mutant strain, *pf7*, maps to 400 Mb on chromosome 17. This region has five mutations with coverage ranging from 13-85 fold. We show by the isolation of intragenic revertants that a mutation in *CCDC40*, which encodes a coiled-coil domain protein, is responsible for the phenotype. The Ccdc40 protein has been implicated in the assembly of the nexin link and primary ciliary dyskinesia in humans, dogs, and zebrafish. The *imp3* mutant strain, which was identified via a role in mating (Goodenough *et al.*, 1976), shows a severe flagellar regeneration phenotype. It was mapped to chromosome 2, which contains a single change. We show by rescue that a nine base pair deletion, which encodes the terminal conserved amino acids YFL in the catalytic subunit of a protein phosphatase 2A (PP2A), is responsible for both phenotypes. WGS is a quick and inexpensive means to identify the genes responsible in the large collection of flagellar mutants in *Chlamydomonas*. This approach will facilitate the understanding of flagellar assembly and signaling.

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Post-Transcriptional Mechanisms for Cytoskeletal and Ciliary Formation.

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The desiccated microspore of *Marsilea* (fern) is a meiotic product that undergoes rapid development upon hydration to produce 32 spermatozoids. The single cell in the spore undergoes 9 successive divisions in 4.5 h to produce 39 cells, 32 of which are spermatids. Each spermatid differentiates to become a coiled gamete with an elongated nucleus, an elaborate cytoskeleton and ~140 cilia. The gametes are released 11 h after the spore is placed in water. We are interested in how these cells undergo profound morphological change. Our earlier work shows that development is controlled post-transcriptionally. The gametophyte processes stored pre-mRNA by splicing retained introns and polyadenylating the transcripts, and then translates these mRNAs at precise times during development. We used RNAseq to elucidate transcriptional mechanisms controlling development. PolyA-RNA was isolated from 'early' (1-2h), 'middle' (3-5h), and 'late' (6-8h) intervals and sequenced using Illumina technology. *De novo* transcriptome assemblies were generated for each sample using Trinity software. We obtained >234,000 transcripts from all samples. To construct unigenes and reduce potential artifacts, the longest transcript from each isoform cluster was selected. They were grouped into contigs (unigenes) for use in reference transcriptomes in downstream analysis. BLAST and Gene Ontology (GO) comparisons with known genomic databases provided similarity and homology identities with contigs in our datasets. The Tuxedo suite was used to calculate FPKM values for relative abundance. GO enrichments show an increase in the numbers of rare transcripts as development proceeds. The early transcriptome featured components participating in DNA replication, RNA processing, mitosis, and cell cycle regulation. GO enrichment analysis of the late transcriptome reveals an over-abundance of centrosomal, ciliary and microtubule components, RNA splicing, RNA localization, and mRNA transport. GO enrichment shows the middle transcripts as a rich mix of early and late transcriptomes, with fewer than expected contigs being unique to this transitional period. RNAseq combined with RNAi silencing assays reveal that the gametophyte has two pools of stored pre-mRNAs and employs a bi-phasic strategy in controlling translation. The first pool of pre-mRNAs is processed and translated rapidly for the cell division phase of development. The second pool is then processed and translated for the *de novo* formation of basal bodies, cytoskeletal formation, nuclear remodeling, cell elongation and ciliogenesis in the forming spermatozoids. Supported by NSF grant 0842525 to SMW.

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Ciliogenesis depends on the splicing of retained introns in the male gametophyte of *M. vestita*.T. C. Boothby¹, R. S. Zipper¹, S. M. Wolniak¹; ¹University of Maryland, College Park, MD

Post-transcriptional regulation of RNA is a hallmark of many rapidly developing systems. We are interested in mechanisms that mediate the storage and temporal utilization of RNA during rapid development. To address these questions, we use the transcriptionally-silent male gametophyte of *Marsilea vestita*, which starts as a single cell and produces 7 sterile cells and 32 spermatozooids in 11 h. Each spermatid undergoes *de novo* basal body assembly and produces ~140 cilia affixed to a complex cytoskeleton. We showed previously that several splicing factors are regulated in a cell type specific manner, leading us to speculate that splicing plays a regulatory role in the translation of stored RNA. We performed RNAseq and assembled transcriptomes *de novo* from early, middle, and late developmental stages of spermatogenesis. We developed a Visual Basic for Applications program that identified a subset of intron retaining transcripts (IRTs) and their fully spliced isoforms, a large portion of which are involved in spermatid specific events such as basal body formation and ciliogenesis. Analyses of potential IRTs revealed that a large proportion of 5' and 3' splice signals correspond to evolutionarily conserved U2 consensus sequences. Inhibition of splicing with Spliceostatin A (SSA) perturbed development, resulting in failed spermatid differentiation, and the absence of basal body formation, cytoskeletal organization, and ciliogenesis. SSA and α -amanitin treatments confirm that the appearance of fully spliced isoforms is a spliceosome-dependent, but transcriptionally-independent event. RNAseq and isoform specific RT-PCR reveal that splicing of different IRTs occurs at different times during development. Phenocopies resulting from RNAi knockdowns of specific IRTs were seen only after splicing would normally occur. Thus, IRTs stored in the gametophyte are not translatable, but they become translatable following removal of their retained introns. The abundance of RNA encoding splicing homologs present in the precatalytic or catalytic spliceosome was examined. Surprisingly, RNA encoding splicing factors present in the precatalytic spliceosome was predominantly abundant late in development, whereas RNA encoding factors present in the catalytic spliceosome was abundant early in development. RNAi knockdowns of these splicing factors confirmed that RNA encoding components of the catalytic spliceosome are required earlier in development than RNA encoding precatalytic components. These results suggest that precatalytic splicing factors are present as stored proteins at the onset of development, and could be associated with stored RNA. The association of IRTs with spliceosomes at various stages of maturity, coupled with the translation of catalytic splicing factors prior to precatalytic splicing factors provide an elegant means by which to regulate differential timing of intron removal necessary for controlling the translation of stored RNA essential for spermatid differentiation. Supported by NSF grant 0842525 to S.M.W.

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Proteomic analysis of flagellar transition zones.D. R. Diener¹, G. B. Witman², J. L. Rosenbaum¹; ¹Molec. Cell. Dev. Biol., Yale University, New Haven, CT, ²Cell and Dev. Biology, UMass Medical School, Worcester, MA

The transition zone (TZ) of cilia and flagella is a specialized region of the organelle that forms the boundary between the basal body and the axoneme and is part of the barrier separating the cell body and flagellar compartments. An expanding list of proteins involved in ciliopathies have been mapped to the TZ through a variety of genetic, immunofluorescence, and protein interaction techniques. In a global approach to identifying components of the TZ, we have isolated TZs from the biflagellate alga *Chlamydomonas*. Prior to mitosis, these cells resorb their

flagella, leaving behind a small vesicle of TZ membrane containing the central hub characteristic of the *Chlamydomonas* TZ. Although the flagellar microtubules depolymerize during flagellar resorption, nine projections emanate from the hub and in many cases retain attachments to the membrane, suggesting that a TZ scaffold *sans* microtubules but including membrane and membrane connectors persists. We have purified these TZs by gradient centrifugation. Electrophoretic analysis of the isolated TZs shows they are enriched in centrin, a known component of the TZ. The initial proteomic analysis of this preparation identified 115 proteins, approximately 50 of which are also in the *Chlamydomonas* flagellar proteome; however, axonemal proteins, such as dyneins and radial spokes, were not present. Nine known ciliopathy proteins were identified, including CEP290/NPHP6, NPHP1, MKS1 and a tectonic homologue. Validation of putative TZ proteins that have human orthologues is underway. Supported by NIH Grant #GM014642 to JR and a gift from the Grousbeck Family Foundation to JR and GW.

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Meckelin functions in basal body orientation and cortical unit organization in *Paramecium tetraurelia*.

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Meckelin (MKS3), a conserved transmembrane protein found at the base of the cilium, functions in ciliogenesis, centriole positioning and ciliary gating. Mutations in *MKS3* result in the ciliopathy Meckel Syndrome (MKS). Previously our lab depleted *MKS3* mRNA levels in the ciliate *Paramecium tetraurelia* using RNAi. Depletion resulted in global defects such as short and missing cilia, disruptions to the ciliary membrane and localized defects in basal body organization on the dorsal surface of the cell.

The surface of *Paramecium* consists of highly organized cortical units arranged in longitudinal rows called kineties. Each cortical unit contains one or two basal bodies, from which cilia arise, and a set of microtubule rootlets that emanate from the basal body in a fixed orientation. The post-ciliary microtubule rootlet arises from the right side of the basal body and projects towards the posterior of the cell while the transverse microtubule rootlet arises from the left side of the basal body and projects laterally toward the adjacent kinety. The cortical units are separated from each other by sets of ridges.

Our current work suggests that depletion of *MKS3* mRNA also results in disruptions of the cortical unit organization, the submembranous cytoskeleton and the basal body associated microtubules. The most prominent type of disruption is the insertion of a single unit between two cortical rows creating a cluster of cortical units. A second type of cortical cytoskeletal disruption is the insertion of a partial row of cortical units resulting in the formation of an abbreviated kinety. Disruptions of this type vary from several cortical units in length to disruptions extending for the majority of the cell's dorsal surface.

Furthermore, the orientation of the post-ciliary rootlet and transverse microtubule are changed with *MKS3* RNAi. The microtubule rootlets no longer project in their predicted orientations. The mis-oriented rootlets are seen where there are disruptions in basal body organization and the orientation of the rootlets varies between and within observed areas of disruption.

In addition to its established role in ciliogenesis and basal body positioning, our work supports the idea that Meckelin functions to establish the cortical units and to provide polarity to the basal bodies during cell division. The disruptions suggest that the link between Meckelin and the cytoskeleton serves to orient the basal bodies within the individual cortical units and in doing so, provides the proper environment for ciliogenesis.

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A novel function of the the centriolar satellite protein SSX2IP/hMsd1 in targeting BBS4 to the ciliary compartment.

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Primary, non-motile cilia fulfil essential functions in transducing mechanical or chemical stimuli into intracellular signals in the majority of differentiated human cells. Defects in cilia assembly (ciliogenesis) lead to a wide range of human diseases, commonly termed ciliopathies, such as the Bardet-Biedl syndrome or polycystic kidney diseases. The primary cilium is organized by a microtubule based core structure, the axoneme. This unique microtubule array consists of nine parallel microtubule doublets originating from the basal body. Basal bodies derive from centrioles that migrate to the cell cortex in cells exiting the cell cycle, e.g. during differentiation. Here, we characterise the function of a previously unknown centriolar satellite protein, SSX2IP/hMsd1, in the assembly of primary cilia. Using differential proteomics in maturing *Xenopus* oocytes, we recently identified SSX2IP/hMsd1 as a maturation factor for mitotic centrosomes in cycling cells (Baerenz et al., submitted). We now show that during late phases of the cell cycle, SSX2IP/hMsd1 as well as the centriolar satellite marker PCM-1 preferentially localize to mother centrioles. This observation suggested a role of SSX2IP in basal body assembly, which are known to arise from the mother centriole that is targeted to the plasma membrane. Consistent with that, we show that SSX2IP localises to the basal body of primary cilia in human RPE-1, murine NiH3T3 cells and medaka embryos. Importantly, knockdown of SSX2IP in RPE-1 cells leads to defects in ciliogenesis resulting in cilia with significantly reduced axoneme length. We provide evidence that SSX2IP promotes efficient recruitment of the ciliopathy-associated protein Cep290, to both centrosomal satellites and the basal body. Moreover, loss of SSX2IP impairs the localization of the BBSome component BBS4. BBS4 relocalizes during ciliogenesis from centriolar satellites to the ciliary compartment. We show that loss of SSX2IP interferes with BBS4 primary cilia relocalization and limits its ciliary entry. Our experiments establish SSX2IP as a new regulator of primary cilia formation in human cells and reveal a new link between centriolar satellites and cilia assembly.

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A microRNA control of primary cilia formation.

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Primary cilia are antenna-like organelles projecting from cell membrane. They are critical for several signaling pathways and related to many genetic diseases. MicroRNAs are reported to play important roles in multiple biological and pathological processes, such as embryo development and tumorigenesis. CP110, a protein that caps the distal side of the centrioles, is inhibitory to ciliogenesis, whereas actin dynamics has recently been shown to affect cilia formation and growth by inducing the centrosomal accumulation of ciliogenic vesicles. Whether microRNAs could contribute to primary cilia formation, however, is not known. Here we found that overexpression of a microRNA (miR-129-3p) induced robust ciliogenesis in the presence of serum and increased cilia length in the absence of serum, whereas blocking miR-129-3p with sponge inhibited the serum starvation-induced cilia formation. CP110 was predicted to have two potential target sites of miR-129-3p in its 3' UTR by TargetScan software. Repression of CP110 by miR-129-3p was confirmed by luciferase assay and Western blotting. As CP110 RNAi was much less efficient than the overexpression of miR-129-3p in both the cilia induction and

elongation, we investigated additional targets of miR-129-3p. We found that actin cytoskeleton was largely disorganized upon the overexpression of miR-129-3p. Wound healing assay indicated a repression of the branched F-actin formation. Because a microRNA can destabilize its target mRNA, we used gene chip assay and TargetScan software analyses to screen for miR-129-3p targets in actin dynamics-related cilia formation. Four actin-related genes, ARP2, TOCA1, ABLIM1 and ABLIM3 were identified. RNAi of these genes phenocopied the effects of miR-129-3p overexpression. miR-129-3p was highly expressed in mouse tissues abundant in primary cilia, including renal tubules, retina and brain. In order to explore the in vivo functions of miR-129-3p, morpholino oligonucleotides against miR-129-3p was microinjected into zebrafish embryos. Developmental abnormalities including curved body, pericardial oedema and defective left-right asymmetry were observed. Both the cilia number and length were reduced as well in the Kupffer's vesicle and the pronephros. Taken together, our results suggest that miR-129-3p controls ciliation by downregulating both CP110 and positive regulators of branched F-actin.

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CEP162 is the axoneme-recognition protein promoting ciliary transition zone assembly.
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Assembling the transition zone (TZ) around nascent ciliary microtubules nucleated from centrioles is essential for ciliogenesis, but how microtubules are recognized to promote TZ assembly at the base of a growing cilium is unclear. By quantitatively dissecting centrosome proteomes into domain-specific subcategories, we identified a centriole-bound, microtubule-associated protein, CEP162, as the TZ-specification factor. CEP162 is associated with centriole distal ends, where it recognizes local ciliary microtubules, and interacts with the core TZ component CEP290. Loss of CEP162 disrupts TZ formation and ciliogenesis. However, abolishing centriolar tethering allows CEP162 to remain associated with axoneme tips throughout ciliogenesis, and ectopically recruit TZ components, generating extra-long cilia with exceedingly swollen tips that burst and release ciliary contents. CEP162 is thus an axoneme-recognition protein tethered at centriole distal ends to promote and restrict TZ formation specifically at the cilia base.

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Mouse Kif7, a ciliopathy protein and regulator of Sonic Hedgehog signaling, has microtubule depolymerization activity.

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In vertebrates, primary cilia are required for the Sonic Hedgehog (Shh) signaling, which plays crucial roles in embryonic development and tissue homeostasis. The Kinesin-4 family protein Kif7 acts in the center of the Shh pathway, between the Smo membrane protein and the Gli transcription factors. Here we show that Kif7 moves to the tip of primary cilia upon pathway activation, paralleling the movement of Gli and Sufu. In the absence of Kif7, Gli2 and Sufu are mislocalized within cilia. Our data suggest a Shh signaling complex, which includes Kif7-Gli-Sufu, acts at the tip of primary cilia. In addition to these roles in the Shh pathway, we find that *Kif7* mutant cilia are significantly longer than that of the wild type. The mutant cilia have microtubule structural defects and tubulin acetylation and glutamylation are decreased in the

distal part of the mutant axoneme. Coupled with the absence of these post-translational modifications, *Kif7* mutant cilia are more labile in response to cilia retraction stimuli. Analysis of genetic interactions between *Kif7* and *IFT* genes confirms the importance of Kif7 in maintaining cilia structure. We show that, like conventional kinesins, Kif7 can form homodimers and bind to microtubules. Consistent with the localization of endogenous Kif7 to the tips of primary cilia, ectopically expressed Kif7 associates with the plus-end of microtubules. Interestingly, unlike other Kinesin-4 family proteins, purified Kif7 protein promotes microtubule disassembly *in vitro*. Thus we have identified Kif7 as a microtubule depolymerizing kinesin that acts in the primary cilia to regulate the length of axonemal microtubules and couples cilia growth to tubulin modification. We propose that dual functions of Kif7 as a core component of the Shh pathway and a ciliary motor may explain the connection between mammalian Shh pathway and the primary cilia.

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PF19 encodes the catalytic subunit of katanin, p60, and is required for assembly of the flagellar central apparatus in *Chlamydomonas*.

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For motile eukaryotic cilia and flagella the central apparatus plays a crucial role in generating wild-type waveforms and beat frequency. While important and significant advances have recently been made in the field of ciliogenesis, we still do not know how the central pair of microtubules and associated projections are assembled in the axoneme. The basal bodies provide a template for the assembly of the doublet microtubules, and intraflagellar transport (IFT) provides a mechanism for transport of axonemal components into the growing cilium. What is not known is how the central pair of microtubules is nucleated or how their associated polypeptides are assembled. We previously demonstrated that *Chlamydomonas* mutants with defects in the p80 subunit of katanin (*pf15*) lack the central pair of microtubules. Here we report that the *Chlamydomonas pf19* mutation results in a single amino acid change within the p60 catalytic subunit of katanin (L277S), and that this mutation ablates microtubule severing activity. L277 is highly conserved in p60 from *Chlamydomonas* to mammals and is within the AAA domain, only 28 amino acids upstream from the Walker A nucleotide binding motif. The *pf19* mutant has paralyzed flagella that lack the central apparatus. Using a combination of mutant analysis, RNAi-mediated reduction of protein expression, and *in vitro* assays, we demonstrate that the p60 catalytic subunit of the microtubule severing protein katanin is required for central apparatus assembly in *Chlamydomonas*. In addition, we show that in *Chlamydomonas* the microtubule severing activity of p60 katanin is not required for stress-induced deflagellation or cell cycle progression. The *pf15*, *pf19*, and *pf15pf19* double mutants are all viable and show no sign of cell cycle defects. In addition, all three mutant strains have no defects in flagellar autotomy. Therefore, katanin does not appear to be required for deflagellation in *Chlamydomonas*. As a first step towards defining a mechanism for katanin's role in central pair assembly, we are conducting a structural analysis of central pair assembly in wild-type cells, and localizing the p80 and p60 katanin subunits during assembly. This work was supported by NIH GM66919 to E.F.S.

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C. elegans ciliary architecture depends on molecules that regulate tubulin polyglutamylation levels.

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Cilia are sensory organelles found on most cell types in the human body. In humans, ciliary defects lead to pleiotropic diseases called ciliopathies. Although all cilia are built by microtubule (MT) based intraflagellar transport (IFT) and have common features, cilia also display specializations in form and function. The molecular mechanisms that modulate IFT to generate such specializations in ciliary architecture are unknown.

To study how cilia diversity is generated, we use the nematode *Caenorhabditis elegans*. The hermaphrodite possesses 60 ciliated sensory neurons (CSNs), and the male has an additional 52 sex-specific CSNs. We compared two types of morphologically and functionally distinct CSNs: amphid channel neurons and cephalic male (CEM) neurons. Using high-pressure freeze fixation, transmission electron microscopy and electron tomography, we found significant differences in MT architecture between amphid and CEM cilia. In amphid channel cilia, the middle segment contains MT doublets. However, because the B-tubules of each doublet terminate abruptly at the end of the middle segment, the distal segment of amphid channel cilia contain only A-tubule singlets. By contrast, B-tubules in CEM cilia middle segments split off from doublets such that both A- and B-tubules continue as distinct MT singlets in the distal segment. It is unknown how this difference in architecture contributes to specialized sensory functions in these distinct neuron types.

To understand how this ultrastructural difference is generated, we examined the role of tubulin posttranslational modifications (PTMs) on ciliary MT architecture and IFT motors. We used genetic analysis, immunocytochemistry, and in vivo imaging of IFT motors to characterize the *ttl* family of glutamylases and the *ccpp-1* deglutamylase. We found that loss of these molecules, which regulate MT polyglutamylation levels, causes defects in CEM MT singlet numbers, MT doublet stability, and the motility of specific ciliary kinesins.

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A novel *Chlamydomonas* mutant, *tpg2*, reveals a conserved 177-kDa protein crucial for the localization of TTLL9, an enzyme that catalyzes tubulin polyglutamylation in the axoneme.

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Tubulin undergoes polyglutamylation on the surface of microtubules in various cellular elements such as axons, axonemes, centrioles and mitotic spindles. This modification is catalyzed by proteins belonging to the tubulin tyrosine ligase-like protein family (TTLL). We have recently found that the *Chlamydomonas* mutant *tpg1* lacks TTLL9, has a greatly reduced amount of polyglutamylated alpha tubulin in the axoneme, and is deficient in the functioning of a specific inner-arm dynein (Kubo et al. 2010, Curr. Biol. 20, 441-445, Kubo et al. 2012, Cytoskeleton, submitted). In this study, we analyzed *tpg2*, a novel mutant phenotypically similar to *tpg1*, and found that it has a mutation in a gene encoding a 177-kDa flagellar protein (p177), which is

widely conserved but of unknown function. The flagella of *tpg2* lack both p177 and TTLL9. Immunoprecipitation assays revealed that p177 is complexed with TTLL9 in both the cytoplasm and the flagella. Furthermore, experiments using *fla10*, a temperature-sensitive mutant deficient in kinesin-II, suggested that the TTLL9-p177 complex is transported in the axoneme by intraflagellar transport. These and other results indicate that p177 is essential for the localization and function of TTLL9 in the axoneme.

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A Role for Gas8 in Motile Cilia and Left-Right Asymmetry.

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Cilia are microtubule based organelles that project from the surface of a cell. Cilia dysfunction in humans can lead to a wide variety of diseases that are collectively referred to as "ciliopathies." While our understanding of ciliopathies has progressed over the past decade, it remains far from complete, with previously uncharacterized mammalian genes constantly being shown to have ciliary roles. For example, work on the *Chlamydomonas reinhardtii* gene, PF2, revealed defects in the Nexin-Dynein Regulatory Complex (NDRC) and cilia motility. The Growth Arrest Specific 8 (*Gas8*) gene product is the mammalian homologue of PF2 and is associated with the NDRC where it is thought to play a role in cilia and flagella motility. To date, no *in vivo* mammalian studies on *Gas8* have been reported, thus its significance in mammalian systems and disease remains undetermined. To this end, we have generated a *Gas8* mutant mouse model. Analysis of cilia on ependymal cells of the lateral ventricles of the brain in *Gas8* mutants indicates a severe defect in cilia motility and hydrocephalus. Also interesting is the fact that a number of *Gas8* mutants present with *situs inversus*, implicating *Gas8* in left-right body axis patterning and nodal cilia motility or signaling. Previous *in vitro* studies indicate that *Gas8* localizes to the axoneme of the motile cilia and base of primary cilia and is implicated in sonic hedgehog signaling where it is proposed to be a positive regulator of the pathway activator smoothed. Based on our preliminary data, we hypothesize that *Gas8* is required for proper function of the NDRC and cilia motility; furthermore we hypothesize that *Gas8* is required for nodal cilia motility and/or signaling at the embryonic node.

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Structural and biochemical properties of the Outer-Dynein-Arm Docking Complex (ODA-DC) in *Chlamydomonas* flagella.

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Outer dynein arms in cilia and flagella are bound to specific loci on outer-doublet microtubules with a regular spacing of 24 nm. Their binding to the doublet microtubules is mediated by the outer dynein arm docking complex (ODA-DC) present at the base of the outer arm. In *Chlamydomonas*, the ODA-DC is composed of three subunits, DC1 (ODA3), DC2 (ODA1) and DC3 (ODA14). ODA-DC binds to specific loci on the outer-doublets of mutant axonemes that lack outer arm dynein, suggesting that it is responsible for the binding of outer arm dyneins to specific sites. To understand the basis for this crucial function of the ODA-DC, we examined the structural and biochemical properties of ODA-DC using both recombinant and native proteins. Low-angle rotary shadowing indicated that the recombinant ODA-DC has an oval shape, ~25

nm in length. Chemical crosslinking of axonemes from a mutant lacking outer arms revealed that the native ODA-DCs associate with each other on the doublet microtubules. A Hill plot for the binding of recombinant ODA-DC to *oda1* axonemes (lacking the ODA-DC) indicates that the binding is cooperative. These data suggest that the periodic and cooperative binding of ODA-DC molecules on the doublet microtubules is based on an end-to-end association of the ODA-DCs. Furthermore, thin-section EM analysis of *oda1* axonemes mixed with excess amounts of recombinant ODA-DC suggests that the recombinant ODA-DC molecules were preferentially bound to the correct sites on the doublet. Thus, there may be additional factor(s) that specify the ODA-DC binding sites on the doublet microtubules.

Microtubule Dynamics and Its Regulation II

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DCX recognizes longitudinal curvature, not the nucleotide state of the lattice, during microtubule end-tracking.

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Doublecortin (DCX) is a protein that stabilizes microtubules (MTs) in developing neurons and nucleates 13-pf MTs in vitro. Using fluorescence microscopy assays, we previously showed that the binding of DCX to MTs is optimized for the lateral curvature of the 13-pf lattice. Using assays with dynamic MTs, we discovered that DCX binds to polymerization intermediates at growing MT ends. DCX shares its microtubule binding site with EB1, the canonical MT end-tracking protein, and we wondered if DCX tracks MT ends by the same mechanism as EB1, namely by recognition of the GTP state of MT ends. Unlike EB1, DCX does not bind preferentially to microtubules assembled with GTP analogs. Rather, we have found that DCX shows high affinity for regions of longitudinal curvature on MTs, while EB1 did not. This behavior could explain the high affinity of DCX for nucleation intermediates and MT tips, which have been shown to flare outward. These findings indicate the existence of two distinct features of microtubule ends, nucleotide state and longitudinal curvature, which can be sensed independently by different proteins. Our results demonstrate that DCX templates new 13-pf MTs through preferential associations with the sides of nucleation intermediates of the MT lattice.

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Acetylation of EB1 regulates chromosome dynamics in mitosis.

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The faithful chromosome segregation during mitosis is orchestrated by dynamic interaction between spindle microtubule plus-ends and the kinetochore. Here we show that PCAF, a kinetochore-associated acetyltransferase, acts as a negative modulator of microtubule stability through acetylation of EB1, a protein that controls the plus ends of microtubules. PCAF acetylates EB1 at its C-terminal tail and disrupts the stability of a hydrophobic cavity on the dimerized EB1 C-terminal, which was previously reported to interact with plus-end tracking proteins containing the SxIP motif. As determined with an acetylation site-specific antibody, EB1 acetylation is dramatically increased in mitosis and localized to the spindle microtubule plus-ends. Surprisingly, persistent acetylation of EB1 delays metaphase alignment, resulting in impaired checkpoint silencing. Consequently, suppression of Mad2 overrides mitotic arrest induced by persistent EB1 acetylation. Thus, our findings identify dynamic acetylation of EB1 as

a molecular mechanism to orchestrate accurate kinetochore-microtubule interactions in mitosis. These results establish a previously uncharacterized regulatory mechanism governing localization of microtubule plus-end tracking proteins and thereby the chromosome dynamics during cell division. Currently, we are delineating how EB1 acetylation regulates the temporal dynamics of specific EB1-TIP interactions at the kinetochore during chromosome movements.

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VE-cadherin Signaling Induces EB3 Phosphorylation to Suppress Microtubule Growth and Stabilize Adherens Junctions.

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The endothelium, lining the vessel wall forms a semi-permeable restrictive barrier that regulates tissue fluid homeostasis and transmigration of blood cells. Vascular Endothelial (VE)-cadherin homotypic adhesion controls endothelial barrier permeability through assembly of adherens junctions (AJs). The functions of actin and microtubule (MT) cytoskeleton are critical for the integrity of AJs; however, how VE-cadherin homotypic adhesion induces the cytoskeletal changes remains unknown. Here we addressed the question whether VE-cadherin adhesion influences MT dynamics through modulation of function of End Binding (EB) protein, the most conserved and ubiquitous family of +TIPs, and whether these changes in MT dynamics are themselves critical for AJ integrity. By using time-lapse confocal imaging and PlusTipTracker software, we showed that VE-cadherin mediated adhesion modulates steady-state MT dynamics by suppressing MT growth. Destabilization of VE-cadherin adhesion by extracellular Ca^{2+} depletion or addition of the small inhibitory peptide (RVDAE), which binds to VE-cadherin extracellular 1 module and disrupts VE-cadherin trans-interaction was accompanied by activation of Src-phospholipase C (PLC)_v 2 signaling and concomitant synthesis of Ins-1,4,5-trisphosphate (IP₃). In addition, destabilization of VE-cadherin adhesion induced reversible phosphorylation of serine 162 in EB3. Inhibition of both Src and PLC as well as depletion of PLC_v2 inhibited EB3 dephosphorylation following loss of VE-cadherin adhesion. Consistent with increased cytosolic $[Ca^{2+}]_i$ in endothelial cells subjected to extracellular Ca^{2+} depletion protocol, downregulation of calcineurin (CaN), the Ca^{2+} -dependent phosphatase, or pre-treatment of cells with the CaN inhibitors also prevented dephosphorylation of EB3. This S162 phospho-switch was required to destabilize the EB3 dimer and suppress MT growth. The phospho-defective S162A EB3 mutant in contrast induced MT growth in confluent endothelial monolayers and disassembled AJs. Thus, VE-cadherin outside-in signaling regulates cytosolic Ca^{2+} homeostasis and EB3 phosphorylation, which induces assembly of AJs. These results identify a novel function of VE-cadherin homotypic interaction in modulating endothelial barrier through the tuning of MT dynamics.

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Control of chromosome stability by PCAF-EB1-TIP150 axis.

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Microtubule is a key cytoskeleton network essential for cellular plasticity and dynamics in all eukaryotes. Recent study suggests that mutation of BRCA2 can lead to chromosome instability by perturbation of PCAF activity at the centromere. Although microtubule acetylation is essential for cellular plasticity, it has remained elusive how kinetochore microtubule plus-end dynamics are regulated by PCAF acetylation. Here we demonstrate that TIP150 regulates

kinetochore-microtubule attachments by promoting spindle microtubule plus-end stability. Suppression of TIP150 by siRNA results in mitotic delay with misaligned chromosomes and perturbation in chromosome biorientation. TIP150 is a tetramer that binds an EB1 dimer through the C-terminal domains and overexpression of the C-terminal TIP150 or disruption of TIP150-EB1 interface by a membrane-permeable peptide perturbs accurate chromosome segregation. Remarkably, the EB1-TIP150 interaction is regulated by PCAF acetylation, and persistent acetylation perturbs accurate metaphase alignment and spindle checkpoint satisfaction. Consequently, suppression of BubR1 overrides mitotic arrest induced by persistent EB1 acetylation but exhibits aneuploidy. Thus, our findings identify a mechanism in which dynamic TIP150-EB1 hexamer governs kinetochore microtubule plus-end plasticity and the temporal control of TIP150-EB1 interaction by PCAF acetylation ensures chromosome stability in mitosis.

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TBCB and EB1 crosstalk in microtubule dynamics.

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Tubulin folding cofactors (TBCs) are a set of different proteins discovered as key elements in the so-called "postchaperonin" tubulin folding pathway. TBCs are responsible for the achievement of the quaternary conformation of the $\alpha\beta$ -heterodimer after tubulin monomers have reached their tertiary structure. In vivo, these proteins are implicated in microtubule dynamics through their ability to dissociate the tubulin heterodimer. TBCB and TBCE are two well-conserved α -tubulin interacting proteins playing important roles in vivo as revealed by the plethora of human disorders in which they are implicated. TBCB shares with TBCE two similar domains, a CAP-Gly, and a UBL domain, but the cytoskeleton-associated protein glycine-rich (CAP-Gly) domains are localized at the C-terminal position in TBCB and at the N-terminal position in TBCE. In this work, we have used a multidisciplinary approach to study the molecular mechanism of TBCB's regulation of microtubule dynamics. We have established that the last three amino-acid residues of TBCB protein are crucial for TBCE interaction and an efficient tubulin dimer dissociation. The overexpression of the mutated form of TBCB lacking the DEI/M-COO⁻ motif, similar to the EEY/F-COO⁻ element in EB1 and related proteins, produces a massive microtubule destruction in vivo suggesting its role in autoinhibition. Using extensive biophysical and biochemical approaches, we unmasked the molecular mechanism by which TBCB controls microtubule depolymerization by means of EB1. In addition, we show for the first time that TBCB interacts directly with cytosolic chaperonin containing TCP-1 (CCT) during the folding process of α -tubulin. All the results obtained from this work led us to propose three different models to explain the mechanism for which the C-terminal tail of TBCB protects microtubule from depolymerization, the role of TBCB in tubulin folding as a CCT cofactor, and the mechanism by which the deregulation of TBCB activity induces the microtubule catastrophe in living cells.

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Evolving tip structures can explain age-dependent microtubule catastrophe.

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Microtubules are key structural and transport elements in cells. The dynamics at microtubule ends are characterized by periods of slow growth, followed by stochastic switching events termed “catastrophes”, in which microtubules suddenly undergo rapid shortening. The mechanistic basis of catastrophe is not known. To investigate microtubule catastrophe events, we performed 3D mechanochemical simulations that account for interactions between neighboring protofilaments. We found that there are two separate factors which contribute to catastrophe events in the 3D simulation: the GTP-Tubulin cap size, which settles in to a steady-state value that depends on the free tubulin concentration during microtubule growth, and the structure of the microtubule tip. Importantly, 3D simulations predict, and *in vitro* microscopy experiments confirm, that microtubule tip structures evolve during microtubule growth. Specifically, we found that the lengths of the individual protofilaments within a microtubule become more tapered over time as the microtubule grows. Along with the evolving microtubule tip structures, catastrophe events increase in frequency as a function of microtubule age, both experimentally and in simulation. Thus, the likelihood of a simulated catastrophe event is intimately linked to the changing physical structure of the growing microtubule tip. The link between tip structure and catastrophe is supported by experimental and simulation results which demonstrate that disruption of the naturally evolving microtubule tip structure by the depolymerizing kinesin MCAK also disrupts the age-dependent catastrophe process. These results have important consequences for catastrophe regulation in cells, as microtubule-associated proteins could promote catastrophe events in part by modifying microtubule tip structures.

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XMAP215 and EB1 Act in Synergy to Promote Microtubule Growth.

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In cells, a complex network of microtubule-associated-proteins regulates the dynamic growth and shrinkage of microtubules that is essential for division and migration. *In vitro* approaches with purified components have helped to elucidate the mechanisms and the effects of individual microtubule plus-end-localizing proteins (+TIPs) on microtubule dynamics. Because microtubule dynamics observed *in vitro* with individual +TIPs does not account for the dynamics observed *in vivo*, it is important to study the combined effects of +TIPs. Here we show that two well-studied +TIPs — microtubule plus-end-tracking protein EB1, and the microtubule polymerase XMAP215 — act together to strongly promote microtubule growth to rates never before observed with purified proteins. Unexpectedly, we find that the combined effects of XMAP215 and EB1 are highly synergistic, with acceleration of growth well beyond the product of the individual effects of either protein. The synergy remains after EB1’s C-terminal 20 amino acids have been removed, showing that it does not rely on any of the canonical EB1 interactions. The increase in growth rates is accompanied by a strong enhancement of microtubule catastrophe, thereby rendering the fast and dynamic microtubule behavior typically observed in cells.

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EB proteins sensitize microtubules to drugs by increasing catastrophes.

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End Binding (EB) proteins, master regulators of dynamics at the plus ends of microtubules, can modulate the interaction of various microtubule targeted agents (MTAs) with microtubules. In this study, we investigated the effects of stabilizing and destabilizing MTAs on the dynamics of EB-associated microtubules. We found that low nanomolar concentrations of destabilizing MTAs, both in cells and in vitro, can induce a seemingly non-dynamic state of microtubules, which we termed the “balancing point”. Microtubule-destabilizing MTAs induce the “balancing point” by promoting very short phases of microtubule growth and shortening, without changing the EB binding and turnover at the microtubule ends. In the absence of EB proteins, MTAs failed to induce the “balancing point” state, suggesting that EB proteins sensitize microtubule ends to MTAs. In cells, MTAs can induce “balancing point” even when the function of microtubule rescue factors such as CLIP-170 is inhibited. Laser photoablation experiments showed that microtubule-destabilizing MTAs affected not only microtubule growth but also depolymerization: a severed microtubule plus end was stable or showed only very limited shortening, suggesting that the microtubule structure might be altered. A microtubule stabilizer, Paclitaxel, on the other hand, did not induce “the balancing point” condition either in cells or in vitro, although it did increase the frequency of catastrophes. This was due to the fact that paclitaxel also increased the frequency of rescues. In vitro studies showed that paclitaxel introduced stabilized lattice regions that could serve as points of repeated microtubule rescue. A common mechanism for the action of MTAs on the dynamics of EB-associated microtubules is an increase in the catastrophe frequency.

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A +TIPs census in mammalian genome uncovers numerous crosslinkers between microtubules and various cellular structures.

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Microtubule plus end tracking protein (+TIPs) are structurally and functionally diverse factors that accumulate at the growing microtubule ends, regulate their dynamics and serve as linkers between microtubules and various cellular structures. Among a few +TIPs that can recognize growing microtubule ends autonomously, the End Binding (EB) family plays a dominant role at the microtubule plus ends by recruiting numerous other +TIPs. However, our current knowledge about the function of +TIPs is limited because we lack a complete picture of the composition of the ‘+TIPtome’. Here, we established a procedure to systematically search for EB partners by combining proteomics and bioinformatics methods. We have obtained ranked lists of potential candidates, experimentally tested a subset of these proteins for EB binding and plus end tracking and significantly extended the list of EB partners that have the capacity to accumulate at the growing microtubule ends, including membrane-, centriole- and actin-associated proteins as well as kinases and a small GTPase. These proteins should be taken into account in future when considering molecular mechanisms that govern the interplay between microtubules and other cellular structures. We found that one of the newly identified

+TIPs, a centriolar protein CEP104, interacts with the centriolar capping complex CEP97/CP110 and regulates ciliogenesis. Another newly identified +TIP, AMER2, can serve as a reporter of microtubule-plasma membrane interactions. By using this reporter, we are able to directly confirm the long standing idea that cortical actin inhibits the interaction between microtubules and the plasma membrane. In a more general context, a combination of proteomics and motif ranking approaches can be applied to other types of protein binding motifs to uncover their proteome-wide occurrence and their contribution to the global protein interaction networks.

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Assembly of a millimeter-sized microtubule aster in a model cytoplasm.

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The cytoplasm of large egg and embryonic cells are organized primarily by radial arrays of microtubules called asters. Prior to cell division, asters expand to cover the entire millimeter-sized cytoplasm in 20 minutes. Such rapid exploration of cell shape and size is thought to be crucial for positioning of centrosomes and the cleavage plane (Wuhr et al., 2010, *Curr Biol* 20, 2040-45). It is unknown how microtubules (MTs) assemble at such a large distance from their presumable nucleation centers or centrosomes. We hypothesize three distinct processes that contribute to addition of MT at the aster periphery: (1) MT polymerization at plus-ends, (2) MT transport and (3) MT-stimulated MT nucleation. We used interphase *Xenopus* egg extract with either centrosomes or beads coated with Aurora A antibody (Tsai & Zheng, 2005, *Curr Biol* 15, 2156-63) to recapitulate aster growth. Through time lapse microscopy, we quantified the distribution of growing plus ends of MTs and found it to be uniform within the expanding aster. MT transport, observed by fluorescent speckle microscopy, was minimal. We explored the consequences of noncentrosomal MT nucleation on aster growth by constructing a biophysical model of plus-end distribution. Our model suggests that MT-stimulated MT nucleation, when coupled with plus-end dynamic instability, increase the rate of aster expansion. In the depleting limits of a hypothetical MT nucleator, the model offers an explanation to how MT density is maintained at the aster periphery. Our work begins to explore the biophysical mechanisms underlying the assembly of a millimeter-sized cytoskeletal network.

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Golgi outpost mediated microtubule nucleation is necessary for outgrowth and stability of *Drosophila* dendritic arborization neurons.

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Microtubule nucleation is essential for proper establishment and maintenance of axons and dendrites. The centrosome is thought to be the primary site of nucleation in most cells; however, *Drosophila* dendritic arborization (da) neurons do not contain centrosomes, and therefore provide a model system to study acentrosomal microtubule nucleation. Here we investigate the origin of microtubules within the terminal branches of Class IV da neurons, which produce an elaborate dendritic arbor. Using EB1-GFP to track the growing plus end of the microtubule in vivo, we find that microtubule organization differs dramatically in the terminal branches compared with the primary branches. EB1-GFP comets grow towards the distal tip of the terminal branches and originate from specific sites within the arbor. Intriguingly, we observe that EB1 comets often emanate from stationary Golgi outposts positioned throughout the arbor, but especially at branch points. In vitro assays also reveal that partially purified Golgi outpost vesicles can directly nucleate microtubules. We find that gamma-tubulin along with CP309, the *Drosophila* homologue of AKAP450, are necessary for this process. Together these results

provide the first evidence that individual Golgi outposts can act as acentrosomal microtubule nucleation centers throughout the dendritic arbor. Golgi outpost mediated nucleation contributes not only to the complex organization of the microtubule cytoskeleton, but also to the growth and maintenance of the entire dendritic arbor.

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An inherited beta-tubulin mutation disrupts microtubule dynamics, kinesin-microtubule binding interface and causes polymicrogyria, CFEOM, and axon dysinnervation.

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Microtubules are cytoskeletal polymers that perform a multitude of functions during development of the central nervous system. They assemble from $\alpha\beta$ -tubulin heterodimers in a polarized fashion and form dynamic networks inside the cell. The vast majority of higher eukaryotes utilize multiple α - and β -tubulin isoforms. However, the role of specific tubulin genes during neuronal development remains elusive. Missense mutations in *TUBB2B*, encoding β -tubulin isoform 2B segregate with a range of neurological phenotypes associated with impaired neuronal migration, suggesting a key role for *TUBB2B* in this process. We identified a novel inherited heterozygous mutation in *TUBB2B* that results in congenital fibrosis of the extraocular muscles (CFEOM), previously linked to mutations in *TUBB3*, and polymicrogyria. This mutation causes an E421K amino acid substitution, decreased production of TUBB2B-E421K polypeptide, and impaired folding of tubulin heterodimers *in vitro*. Using *in vitro* biochemical assays we showed that the successfully folded mutant $\alpha\beta$ -tubulin heterodimers are incorporated into microtubule polymers. We modeled the E421K mutation in budding yeast *S. cerevisiae*, a powerful tool for studying the effects of specific tubulin mutations on microtubule function. While the tubulin heterodimers bearing the E421K substitution co-assembled into microtubules, they altered their dynamics leading to increased stability of microtubule polymers, and decreased kinesin localization due to disruption of the kinesin-tubulin binding interface. E421K- β -tubulin incorporates into microtubules and alters their biochemical properties, thus it has unique characteristics not seen for previously identified *TUBB2B* mutations. Furthermore, the exogenous expression of Tubb2b-E421K in developing colossal projection neurons affected homotopic connectivity without perturbing neuronal production and migration. These observations reveal that the E421K substitution results in axon dysinnervation as a primary phenotype as observed in affected individuals. Our results provide mechanistic insights into why E421K and not other *TUBB2B* mutations results in axon dysinnervation, highlighting the overlapping and divergent functions of β -tubulin isoforms during development of the central nervous system in humans.

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Microtubules Are Involved in Pneumolysin-Induced Pulmonary Endothelial Hyperpermeability.

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The cytoskeleton plays a crucial role in maintaining endothelial barrier function. Indeed, cytoskeletal reorganization changes the cell shape and provides the structural basis for either an enhancement or a loss of endothelial barrier function. Our previous data have demonstrated

a critical involvement of microtubule (MT) disassembly in thrombin- and nocodazole-induced EC barrier dysfunction and have indicated that MT dynamics is an early event in the circuit of the reactions leading to changes in pulmonary EC barrier permeability. Antibiotics-induced release of the pore-forming virulence factor pneumolysin (PLY) in patients with pneumococcal pneumonia results in its presence days after lungs are sterile and is a major factor responsible for the induction of permeability edema. Here we investigated the involvement of MTs into PLY-induced endothelial dysfunction and we examined whether the TNF-derived TIP peptide, which was previously shown to protect from PLY-induced hyperpermeability, could prevent PLY-induced changes in cytoskeletal organization. The endothelial MT network is heterogeneous, which is consistent with the dynamic characteristics of its constituent MT. The MT population can be divided into two subpopulations: dynamic MTs and acetylated MTs, the latter of which are more stable and more resistant to the effects of external factors than the former ones. The density of the MT network is the highest in the internal cytoplasm, but diminishes gradually in the direction of the cell margin. PLY induced a partial MT depolymerization of the dynamic and stable MT network at the cell periphery. Treatment with the TNF-derived TIP peptide increased the dynamic MT density near the cell edge, but did not influence the density of stable acetylated MTs. Quantitative analysis of MT density in the 5- μ m zone from the cell margin revealed that dynamic MTs decreased more than twice upon PLY action, but increased over basal levels upon TIP peptide pre-treatment. The TIP peptide completely rescued dynamic MTs near the cell edge and restored the density of stable acetylated MTs upon PLY treatment. As such, PLY caused de-polymerization of both dynamic and stable MTs. The TNF-derived TIP peptide can effectively rescue both dynamic and stable MTs from the pore-forming toxin's action and should therefore be further evaluated as a potential therapeutic agent for treating pulmonary permeability edema associated with severe pneumonia. Supported by NIH grants R01HL094609 (R.L.), RO1HL067307 (A.D.V.), GHSU CVDI and AHA 11SDG7670035 grants (E.A.Z.), RFBR grants 09-04-00363 and 12-04-00488 (I.B.A.).

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Microtubule (MT)-dependent regulation of muscle length.

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Many muscle diseases are characterized by smaller, weaker myofibers, highlighting the fact that muscle size is critical for muscle function. To study the regulation of muscle size, we used the model organism, *Drosophila melanogaster*, a system that permits *in vivo* cell biological studies with an additional means to assess muscle function at later stages of development. Many aspects of morphogenesis determine overall muscle size, including nuclear number, cell volume, and myofiber length. We have focused on how muscle length is regulated because we have shown that larvae with shorter muscles exhibit significantly impaired muscle function. Through a combination of mutational analysis and RNAi-based screens, we have identified a number of factors that affect muscle length. Mutations in, or depletion of, Lis1, NudC, Rapsynoid (Raps/Pins), the minus-end directed MT motor Dynein Heavy chain (Dhc), and its regulatory light chain (Dlc), all result in muscles that are shorter than controls. Conversely, mutations in, or depletion of, the plus-end directed motor Kinesin heavy chain (Khc), its regulatory light chain (Klc), and Ensconsin (Ens), exhibit significantly longer muscles compared to controls. Finally, we have identified Sunday Driver (Syd) as a factor that coordinates Kinesin and Dynein activities as they pertain to muscle length determination. Although homozygous *syd* mutants produce muscles of the proper length, double heterozygotes of *syd* and *khc* have longer muscles than controls, and double heterozygotes of *syd* and *dhc* have shorter muscles than controls. This suggests that the adapter protein, Syd, is simultaneously regulating Dhc and Khc to influence muscle length. Collectively, these data suggest that muscle length is regulated by a

MT-dependent process mediated by motor protein complexes that are coordinated by Syd to facilitate proper extension of the muscle pole.

1195

Understanding the three-dimensional organization of microtubule coils in human platelets.

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Blood platelets circulate through blood vessels. They have as primary function to survey the integrity of the vascular system and are activated in response to blood vessel injuries. Microtubule coils are the major structural elements responsible for platelet characteristic disc shape and the only example of a circumferential marginal band existing in mature human cells. This tight microtubule assembly is found along the long axis of discoid platelets, beneath the plasma membrane. Upon activation, platelets lose their lentiform shape and within a few seconds they become more rounded and start to project pseudopodia. This activation is crucial for their spreading, aggregation and the formation of platelet plugs. The associated shape change is correlated with the reorganization of the internal cytoskeleton including the constriction of microtubule coils into smaller rings around centrally concentrated organelles. For a long time it has been thought that the marginal band consists of a single microtubule polymer that is wound in 8 to 12 coils, the so-called single microtubule model (reviewed in Hartwig and Italiano, 2003). Visualization of microtubule growth in living platelets recently revealed that the marginal band is a dynamic structure composed of multiple microtubules assembled in a bidirectional pattern favoring a multiple microtubule model (Patel-Hett *et al.*, 2008).

To decisively understand the three dimensional organization of the platelet marginal band at the single-microtubule resolution, we use electron tomography on plastic embedded sections from cells cryofixed by high pressure freezing. We reconstruct the complete microtubule cytoskeleton in human platelets at the resting and different activation states. Using data extracted from electron tomograms about the number, the length distribution, the polarity and the three dimensional organization of microtubules and other parameters collected from the literature, we are able to perform first principle computer simulations using Cytosim, a program developed for stochastic simulations of cytoskeletal fibers, diffusible activities and flexible objects in a confined environment. There, our goal is to understand the dynamic maintenance of the marginal band at resting state and its reorganization upon platelet activation.

1196

Exploring microtubule dynamics in vivo and its role in tissue biogenesis.

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Cytoskeletal dynamics control vast numbers of cellular processes including cell polarity and cell division both of which are critical for tissue biogenesis. A complex developmental process, tissue biogenesis requires that the constituent cells undergo division, differentiation, movement, and reorganization of adhesions. Despite playing a central role, the dynamic properties of microtubules (MTs) in tissue biogenesis have not been studied.

We hypothesized that MT dynamics are differentially regulated throughout tissue biogenesis, and that this regulation is important for the differentiation and function of specific tissues. To test this idea, we generated a *C. elegans* strain in which GFP-tagged tubulin is expressed

specifically in cell lineages giving rise to the egg-laying apparatus (vulva), from larval stages to adult. This system allows us to measure and compare MT dynamics in cells of the same lineage at different developmental stages within the same organism. Intact living worms are immobilized and mounted for time-lapse real-time confocal imaging of MT dynamics in 3 or 4 dimensions with high spatial and temporal (1 sec) resolution. Interestingly, MTs from differentiated cells have a higher growth rate than those of precursor cells (0.1534 +/- 0.0028 vs. 0.1199 +/- 0.0033 $\mu\text{m}/\text{sec}$), and display long biphasic growth excursions, slowing as they near the cell periphery. Precursor cells' MTs have shorter growing phases and higher catastrophe and rescue frequencies.

To define the molecular mechanism of differentiation state-specific variations in MT dynamics, we depleted MT- and tubulin-binding proteins by RNAi and assessed vulva formation and function. By varying the timing of RNA introduction, we distinguished whether proteins are essential for tissue biogenesis (i.e. cell division, migration, etc.), the function of the fully developed tissue, or both. Our targeted RNAi screen identified two proteins with inverse phenotypes: CLS-1^{CLASP2} is specifically required for tissue biogenesis and KLP-7^{MCAK} is required for tissue function. Furthermore, depletion of a given protein at different developmental stages resulted in differential effects on MT dynamics and therefore terminal phenotypes.

Thus, we have developed a powerful model system to investigate the role of MT dynamics in tissue biogenesis using intact animals. We further show that MT dynamics are differentially and selectively regulated during cell differentiation and that this regulation is fundamental to development of multi-cellular organisms.

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Different casein kinase isoforms as important regulating factors of plant microtubular functioning.

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It was demonstrated that different members of the casein kinase (CK) I family play a basic role in regulation of plant microtubule (MT) organization and dynamics. Bioinformatic analysis revealed the presence of 18 CK1-like kinases in *Arabidopsis thaliana*. The spatial structures of catalytic domains of CK1s from *Rattus norvegicus* (α , β , γ 1-3, δ , and ϵ) and 18 homologues from *Arabidopsis* were reconstructed. Superimposition of catalytic domains of CK1 (α , β , γ 1-3, δ , and ϵ) from *R. norvegicus* and *A. thaliana* homologues confirms high structural similarity for 13 plant CK1-like kinases: KC1D, CKI1, CKL2, CKL3, CKL4, CKL5, CKL6, CKL7, CKL8, CKL9b, CKL10, CKL12 and CKL13. It was found that CK1-specific inhibitor D4476 interacts with rat CK1 δ and all 13 plant homologues in similar ATP-competitive manner. Ligand docking, molecular dynamics and chemogenomic analysis of D4476 complexes with rat CK1 δ and 13 CK plant isoforms revealed similar molecular mechanisms of ligand binding. Respectively, it was shown that regulation of plant MT dynamics by CK1 depends on its ability to bind tubulin or MT-associated proteins. D4476 had a strong effects on growth and morphology of *A. thaliana* primary roots. It was found that root growth and development was sensitive to all tested (0.1-50 μM) concentrations of D4476. The effects of this inhibitor on MT organization (spatial reorientation of cortical MTs) were proved with confocal laser scanning microscopy on GFP-MBD (GFP-Map4) expressing *Arabidopsis* root cells. Thus, obtained results demonstrate the plant CK1 members can regulate MT behavior through direct tubulin (isoform CKL6) and EB1 (isoforms CKL2 and CK1D) phosphorylation. We identified motifs presumably involved in MT-

binding of plant CK1D and CKL2 with EB1 and CKL6 with tubulin. These results were also confirmed by GeneVestigator data on CKL6, CKL2 and CK1D expression in *A. thaliana* roots.

1198

Nitration of plant α -tubulin tyrosines in nitric oxide cell signaling.

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Nitric oxide (NO) regulates numerous cellular processes in plants; however, the exact mechanisms of NO signalling are still poorly studied. Protein post-translational modifications realize direct NO action, affecting proteins' structure, function and/or protein–protein interactions. Among the candidates for NO-downstream effectors are cytoskeletal proteins because of their involvement in many processes regulated by NO. We demonstrated for the first time the occurrence of α -tubulin tyrosine nitration in *Nicotiana tabacum* suspension culture BY-2 cells under physiological conditions using tubulin immunoprecipitation and Western-blot analysis (Yemets et al., 2011, Plant Sci, 181(5):545–554). The colocalization of α -tubulin and 3-nitrotyrosine on preprophase bands, mitotic spindles and fragmoplasts in untreated BY-2 cells was revealed by immunofluorescent microscopy with antibodies against α -tubulin and 3-nitrotyrosine. It was shown, that treatment with NO donor sodium nitroprusside decreased number of mitotic cells. Hence, α -tubulin tyrosine nitration can be considered as important mechanism for the regulation of microtubular dynamics.

1199

Plant acentriolar cortical microtubule array reorganization uncovers cellular processes governing array regulation.

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The acentriolar microtubule arrays in *Arabidopsis* hypocotyl cells organize into functional patterns that are critical for cellular morphogenesis. To elucidate the mechanisms required for array patterning, we synchronously induced the formation of transverse co-aligned microtubule arrays in epidermal cells using plant hormones. We defined four general classes of cortical microtubule array pattern in the box-shaped epidermal cells prior to hormone exposure. Quantification of plus-end polymerization trajectories revealed a striking 3:1 asymmetry in the number of microtubules polymerizing out of the outward-facing (periclinal) array versus those moving into the array from the lateral (anticlinal) side faces of the cell. After induction, we identified a specific spatial and temporal sequence of microtubule array changes that lead to transverse co-alignment from all four initial array patterns. Cells appear to shift their arrays by rapidly correcting the orientation asymmetry and by introducing transverse microtubules from the lateral side faces of the cell. Transverse patterning initiated around the cell's midzone and progressed to the apical and basal ends. The longitudinally oriented polymers at the cell's apical and basal ends were the last elements of microtubule pattern to change. We conclude that transverse array organization is driven primarily by the introduction of new transverse microtubules from the lateral side faces of the cell. Application of the hormone induction assay to *Arabidopsis* CLASP and Katanin mutants indicates opposing roles in the mechanism of transverse microtubule array patterning.

1200

The response of the microtubule cytoskeleton to extreme deformations of the cell*E. Buchman¹, O. Haddad¹, D. Fudge¹; ¹Integrative Biology, University of Guelph, Guelph, ON, Canada*

This study sought to investigate the response of the microtubule cytoskeleton to extreme deformations of the cell, on the order of 50% cell strain. Using a custom built live-cell imaging and stretching chamber, we employed epi-fluorescence microscopy and GFP conjugated proteins to illuminate either full microtubules (GFP-tubulin) or growing microtubule plus ends (GFP-EB1) in MDCK cells cultured on silicone strips. We found that the microtubule cytoskeleton is remarkably resilient to strains up to 50%, undergoing only minor changes in network organization (ie. alignment of filaments with the perturbation axis), while maintaining their characteristic dynamic instability. Following stretch, microtubule bending fluctuations were found to decrease in amplitude, though they showed a tendency to recover towards prestretch values in only fifteen minutes. Our analyses of microtubule bending fluctuations suggest that similar analyses may serve as a proxy for investigating the distribution and magnitude of forces in the cytosol by way of measuring nonequilibrium shape changes in cytoskeletal filaments. Furthermore, this study has implications for the use of cells and microtubule networks in 'smart-materials' and bioengineering applications involving high strain.

1201

Conformational Changes in Tubulin in GMPCPP and GDP-taxol Microtubules Observed by Cryo Electron Microscopy.*H. Yajima¹, T. Ogura², R. Nitta¹, Y. Okada¹, C. Sato², N. Hirokawa¹; ¹Department of Cell Biology and Anatomy, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, ²Biomedical Research Institute and Biological Information Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan*

Microtubules are dynamic polymers that stochastically switch between growing and shrinking phases. Microtubule dynamics are regulated by GTP hydrolysis by β -tubulin, but the mechanism of this regulation remains elusive because high-resolution microtubule structures have only been revealed for the GDP state. Here we solved the cryo-EM structure of microtubule stabilized with a GTP analogue guanylyl 5'- α,β -methylenediphosphonate (GMPCPP) at 8.8 Å resolution by developing a novel cryo-EM image reconstruction algorithm. In contrast to the crystal structures of GTP-bound tubulin relatives such as γ -tubulin and bacterial tubulins, significant changes were detected between GMPCPP and GDP-taxol microtubules at the contacts between tubulins both along the protofilament and between neighboring protofilaments, contributing to the stability of the microtubule. These findings are consistent with the structural plasticity or lattice model, and suggest the structural basis not only for the regulatory mechanism of microtubule dynamics, but also for the recognition of the nucleotide state of the microtubule by several microtubule-binding proteins, such as EB1 or kinesin.

1202

Luminal localization of α -tubulin K40 acetylation by cryo-EM analysis of Fab-labeled microtubules.

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The $\alpha\beta$ -tubulin subunits of microtubules can undergo a variety of evolutionarily-conserved post-translational modifications (PTMs) that provide functional specialization to subsets of cellular microtubules. Acetylation of α -tubulin residue Lysine 40 (K40) has been correlated with increased microtubule stability, intracellular transport, and ciliary assembly, yet a mechanistic understanding of how acetylation influences these events is lacking. Using the anti-acetylated tubulin antibody 6-11B-1 and electron cryo-microscopy, we demonstrate that the K40 acetylation site is located inside the microtubule lumen and thus cannot directly influence events on the microtubule surface, including kinesin-1 binding. Surprisingly, the monoclonal 6-11B-1 antibody recognizes both acetylated and deacetylated microtubules. These results suggest that acetylation induces structural changes in the K40-containing loop that could have important functional consequences on microtubule stability, bending, and subunit interactions. This work has important implications for acetylation and deacetylation reaction mechanisms as well as for interpreting experiments based on 6-11B-1 labeling.

1203

A New Motion-Detection Method Reveals Key Regulatory Steps of Microtubule Dynamics in Growth Cone Motility from Only a Pair of Images.

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Microtubule dynamics is critical to many morphogenetic processes, including growth cone guidance and wound healing. However, our understanding of its regulation, especially the step of the assembly cycle that might be targeted during cell shape remodeling, is limited. Here we present a new method to identify these steps and analyze their spatial and temporal regulation in live cells. Using a GFP-tagged plus-end-tracking protein EB3 as a probe, this method analyzes motion and appearance/disappearance of labeled microtubule ends at any given time, and deduces the corresponding step of microtubule assembly at each end. Specifically, plus-end labels from two sequential images are first pseudocolored with separate colors and merged to generate a microtubule assembly map. Color-coded ends in the map are then identified and segregated based on object recognition algorithms into four dynamic steps: growth, rescue, catastrophe, and pause. Furthermore, morphological analysis provides quantitative data on microtubule assembly such as growth rate and direction at growing microtubule ends. To validate the method, we have confirmed the representation of these dynamic steps by the analysis of history and fate of identified microtubule ends. In addition, we have shown that our results are comparable to the ground truth data derived from manual analysis as well as to the quantitative data obtained by the commonly used tracking approach. Thus, this new method can be used in many cellular processes involving rapid shape remodeling when traditional tracking is not feasible. To illustrate this application, we have investigated microtubule assembly in growth cones during turning and branching. From the assembly map generated at each time point, we have found that microtubule growth direction in the central domain correlates well with future growth cone orientation. Moreover, we have found that redistribution of rescue and catastrophe is associated with changes in microtubule growth direction prior to morphological polarization, suggesting key regulation sites in directed growth cone motility.

Thus, this computer vision method offers a unique and quantitative approach to visualize and analyze microtubule dynamics and can be extended to studying spatial regulation of microtubule assembly in responses to cell signaling as well as in other complex morphogenetic processes.

1204

The structural basis of microtubule dynamics.

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Microtubules are one of the principle classes of cytoskeletal filaments and are critical for a diverse array of cellular processes, including intracellular trafficking, the generation and maintenance of cell morphology, and cell division. Microtubules exhibit the property of dynamic instability: rather than existing as static assemblies, the filaments stochastically switch between phases of growth and shrinkage through the addition and dissociation of tubulin subunits. These dynamics are capable of performing work and are critical for microtubule function. Cell division in particular is powered by the polymerization and depolymerization of microtubules, processes which are specifically targeted and disrupted by several successful anti-proliferative therapeutics. It has been known for decades that tubulin's binding and hydrolysis of GTP underlies dynamic instability, yet a molecular mechanistic explanation remains elusive, largely because the structural details of tubulin's conformation and interaction with nucleotide in the context of the microtubule lattice are unknown. Through technological improvements in cryo-electron microscopy sample preparation, instrumentation, and image processing, we have obtained reconstructions of GMPCPP (a non-hydrolyzable GTP analog) and GDP microtubules with better than 5 Å resolution in the absence of drugs and other stabilizing factors, sufficient to resolve all elements of tubulin involved in and affected by lattice contacts and to directly visualize the positions and orientations of bound nucleotides. We integrate these results into a physical model for the interrelationship between nucleotide state, tubulin lattice contacts, and microtubule stability.

Neuronal Cytoskeleton II

1205

Centrosome amplification causes microcephaly.

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The presence of more than two centrosomes in a mitotic cell- commonly referred to as centrosome amplification- has been proposed to impact on the homeostasis of tissues in the context of tumour formation by triggering chromosome instability. One of the first evidence of centrosome amplification being readily tumourigenic came from an investigation at the scale of a fly organism. In the fly model, defective orientation of the division axis driven by extra centrosomes was sufficient to confer to neural stem cells, NSCs, a tumourigenic potential. To explore this possibility in a vertebrate brain tissue, we have now generated the first mouse model where embryonic NSCs carry extra centrosomes by manipulating the machinery of centrosome duplication. We show that centrosome amplification is at the origin of a microcephaly that can be explained by defects in the progression of NSCs through mitosis. We have rapidly excluded the possibility that microcephaly relies on impaired positioning of the mitotic spindle like in drosophila, or on defects in primary ciliogenesis. Instead, we provide compelling evidences that the presence of extra centrosomes at the onset of mitosis results in

cell cycle lengthening through mitotic delay, as well as in the transmission of an abnormal number of chromosomes to the progeny that is poorly tolerated as evidenced by a massive cell death. By computational modeling, we provide further evidences of the likelihood of this scenario to explain the extent of the observed microcephaly. Surprisingly, however, such defects in the NSC population are not sufficient to prevent the production of all the populations of neurons in good shape and order, but only affect the number of neurons produced. Altogether, our results support the concept that extra centrosomes can be at the origin of microcephaly by conferring growth disadvantages and survival defects to the NSC population. This scenario would have to be considered while elucidating the mechanisms responsible for brain size reduction, especially in the context of neuro-developmental disorders caused by mitotic spindle pole abnormalities.

1206

T-cells Mediate Substantia Nigra Neurodegeneration in Parkinson's Disease.

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Parkinson's disease (PD) patients exhibit marked inflammation in the substantia nigra (SN) with extensive and selective death of their dopaminergic (DA) neurons. We find that in primary murine neuronal cultures, SN and locus coeruleus (LC) neurons can be induced to express cell surface MHC-I by the proinflammatory cytokine gamma-interferon, L-DOPA, or conditioned medium obtained from murine microglia exposed to alpha-synuclein or neuromelanin (NM). In addition, SN DA murine neurons can process the foreign protein ovalbumin to a smaller antigenic peptide that is presented by their MHC-I and trigger their own destruction by CD8+ killer T-cells that recognize the presented antigen. Studies using human pathological samples show that DA SN and norepinephrinergic locus coeruleus (LC) neurons from adult human PD patients and age-matched controls express MHC-I as detected by immunostaining using specific antibodies directed against HLA-1 and microglobulin β 2. Moreover, we detect the presence of CD8+ T cells in association with NM containing DA neurons in patients with PD. Based on these results, we propose three requirements for neurodegeneration in PD; 1) MHC-I presentation, 2) antigen display, and 3) the presence of an appropriate antigen-specific cytolytic T-cell population- processes that may occur most commonly in older individuals in whom the appropriate T-cells have proliferated and for whom the blood brain barrier can be compromised. The immunologically based mechanism proposed for neurodegeneration in PD is consistent with slow disease progression, the presence of inflammatory cytokines, and the association with aging observed in PD.

1207

Regeneration of *Drosophila* sensory neuron axons and dendrites in the PNS and CNS.

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Both cell-intrinsic and extrinsic pathways govern axon regeneration, but only a limited number of factors have been identified. Moreover, it is not clear whether dendrites regenerate nor to what extent axon regeneration is evolutionarily conserved. We found, like the axons of mammalian sensory neurons, the axons of certain *Drosophila* dendritic arborization (da) neurons are capable of substantial regeneration in the periphery but not in the central nervous system (CNS), and activating the Akt pathway enhanced axon regeneration in the CNS. Moreover,

those da neurons capable of axon regeneration also displayed dendrite regeneration, which is cell type specific, developmentally regulated and associated with microtubule polarity reversal. Dendrite regeneration is restrained via inhibition of the Akt pathway in da neurons by the epithelial cell derived microRNA bantam, but facilitated by cell autonomous activation of the Akt pathway. Our study begins to reveal mechanisms for dendrite regeneration, which depends on both extrinsic and intrinsic factors including the Pten-Akt pathway that is also important for axon regeneration. We have thus established an important new model system, the fly da neuron regeneration model that resembles the mammalian injury model, with which to study and gain novel insights into the regeneration machinery.

1208

Mechanoprotection in neurites involves a pre-stressed spectrin cytoskeleton.

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Many somatosensory neurons have evolved specialized molecular sensors that convert mechanical stress into behavioral responses. The genetics, development and physiology of the touch receptor neurons (TRNs) in *Caenorhabditis elegans* nematodes are especially well characterized and this animal has the particular advantage that the TRNs can be studied both in living animals and dissociated in culture. Like other somatosensory neurons, the TRNs use ion channels to convert mechanical stress into electrical signals and ultimately appropriate behaviors. Whereas the protein partners that form these mechanosensitive channels have been known for some time, the mechanism by which mechanical force is propagated from the skin to the TRN and to the channel is essentially unknown.

To investigate the transmission of forces in *C. elegans* TRN neurites, we focused on the structural protein β -spectrin. Spectrin forms tetramers and is a major cytoskeletal component of eukaryotic membranes that integrates the lipid bilayer to the actin cytoskeleton. In red blood cells, spectrin has been shown to unfold during shear stress (Krieger, PNAS, 2011) while tetramerization is critical for cell shape elasticity (Sleep, BiophysJ, 1999). Our results show that mutations in the tetramerization domain of *C. elegans* β -spectrin leads to defective neuron morphology in moving worms and therefore we hypothesized that spectrin might play an important role in bearing and transmitting mechanical load within TRNs. We combined single cell force spectroscopy experiments on isolated neurons, optomechanical manipulation of TRNs, and FRET-based strain measurements (Grashoff, Nature, 2010) in living animals to characterize the role of spectrin in maintaining neurite mechanical integrity. Our data indicate that resting TRNs are understress and that spectrin is held under constitutive tension in living animals. Based on these results and behavioral data on β -spectrin mutants, we speculate that β -spectrin-dependent pre-tension is required for efficient response to external mechanical stimuli.

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G β -Microtubule Interaction is Critical for Neuronal Differentiation and Neurodegeneration.

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Assembly and disassembly of microtubules (MTs) is critical for axon and dendrites formation, and for neurite outgrowth. During neuronal degeneration, the architecture of MTs and proteins

associated with them are severely altered. We have shown earlier that the $\beta\gamma$ (beta-gamma) subunit of G proteins binds to tubulin stimulating MT assembly. More recently, we found that nerve growth factor (NGF)-induced neuronal differentiation of PC12 cells promotes the interactions of $G\beta\gamma$ with microtubules. Overexpression of $G\beta\gamma$ in PC12 cells induced neurite formation in the absence of NGF. The goal of the present study is to understand whether interference with the $G\beta\gamma$ /MT-mediated pathway inhibits neuritogenesis and causes neurodegeneration. Since polyisoprenylation and further carboxy-terminal processing (methylation) of γ subunits are important for the interaction of $G\beta\gamma$ with MTs, we used L-23 and L-28, which are both inhibitors of polyisoprenylated methylated protein methyl esterase (PMPMEase), an esterase enzyme of the polyisoprenylation pathway, to conduct the study. In addition, GRK-ct peptide (the carboxy terminus of G protein-coupled-receptor kinase) which is known to inhibit $G\beta\gamma$ -dependent signaling by binding to and sequestering $G\beta\gamma$ was also used to determine whether $G\beta\gamma$ is necessary for MT organization and neurite outgrowth. PC12 cells were treated with NGF over the course of three days, followed by overnight treatment with L-28, L-23 or the prototypical molecule, PMSF. Subcellular fractionation, co-immuno-precipitation, and confocal-scanning microscopy were used to analyze the samples. We found that neurite formation was not affected significantly in the presence of PMSF or L-23 (10 μ M). On the other hand, L-28 (10 μ M) significantly reduced neurite outgrowth, MT formation, and $G\beta\gamma$ labeling. We found that GRK-ct peptide (10 μ M, 1h), had a very dramatic effect on morphology and survival of NGF-differentiated PC12 cells, causing neurite and MT disruption, and severe degeneration. Our results show that the $G\beta\gamma$ -MT interacting pathway is critical for neuritogenesis, and that both inhibitors of PMPMEase, and GRK-ct peptide could be used as valuable tools to study the mechanism of neurite outgrowth and neurodegeneration and for designing strategies to develop effective drugs against neurodegenerative disorders.

1210

Unraveling the role of neuron sub-cellular mechanical properties in Traumatic Brain Injury.

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The cerebral cortex is estimated to contain 15-33 billion neurons, each connected by synapses to several thousand other neurons. Neurons interact with the brain extracellular matrix (ECM) and communicate with one another by means of long protoplasmic fibers called axons, which carry trains of signal pulses called action potentials. During head injuries or violent accelerations, neurons can be severely damaged and diffuse axonal injury (DAI) is one of the most common and important pathologic features of traumatic brain injury (TBI) [1]. The susceptibility of axons to mechanical injury appears to be due to their viscoelastic properties and their high organization in white matter tracts. Recently, integrin-mediated activation of Rho has been suggested as a key player of diffuse axonal injury reported in mild TBI [2]. However, due to the natural complexity of the neuronal network, cellular and molecular mechanisms of TBI as well as the propagation of forces is still not well understood. Based on the assumption that cell deformation is the initiating event of TBI [3], we investigate the role of the mechanical properties of sub-cellular components of neuronal cells.

First, we have developed a new hydrogel to control and tune independently ECM stiffness, neuron morphology, protein type and density, which are major environment factors in cellular mechanotransduction [4]. Then, we have developed a magnetic tweezers set-up to probe the mechanical behaviour of the cell body and the neurite part.

Cryogenic primary neuron cells were grown on laminin-coated lines deposited on hydrogels to obtain a robust bipolar morphology. We have characterized the viscoelastic properties of sub-cellular components of neuronal cells with creeping experiments and our results show distinct

rheological behaviours that may explain the mechanical vulnerability of axons observed in DAI. By using immunostaining experiments and specific pharmacological agents, we have determined the role of each cytoskeletal components in the mechanical behaviour of both sub-cellular parts.

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Explant Model For The Study of Neuronal Injury Processes.

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Unlike the neurons of the peripheral nervous system (PNS), neurons within the central nervous system (CNS) do not regenerate spontaneously. Inhibitory cues present in the environment and intrinsic growth ability of the injured neurons determine the extent of axonal regeneration. Mechanisms involved in this process include retrograde transport of injury signals, local translation of necessary proteins, and anterograde transport of molecules. Studies of these mechanisms will be beneficial in enhancing our understanding of neuronal regeneration, and guiding regeneration in the CNS. We have developed a simple mouse neonate hippocampal explant model to study these neuronal response to injury. As for dissociated cultures, this model enables the control the cellular environment free from systemic effects. However, the preexisting in vivo interconnectivity within the hippocampus is preserved. This allows a more accurate interpretation of results from studies on neuronal injury regeneration. For explantation, the hippocampus of one day old mouse was dissected and cultured on a Matrigel coated glass surface. Tissue was nourished by neurobasal medium with B27 serum. To assess axonal regeneration, the CA1 region of the tissue was injured, and compared to the tissues without any damage. In addition, dynamics of the growth of control and injured axons were quantified for both the injured and control explants. Results suggest that pre-conditioning or injuring the explants enhances the outgrowth of axons after injury. Current studies are investigating mechanisms by which the retrograde transport of injury signals is regulated. This work has important implications for the regulation of neuronal plasticity during neuronal development and in response to neuronal injury.

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Mutations in p53 and NTF4 Genes are not associated with Retinal Ganglion Cell (RGC) Death in Glaucomatous Beagles.

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Glaucoma is a second leading cause of irreversible vision loss in the world and can affect all age groups as well as all populations. It is characterized by the elevated intraocular pressure (IOP), optic disc changes and retinal ganglion cell (RGC) death. The precise mechanism of RGC death and progressive degeneration of optic nerve in glaucoma is not understood. Although the genetic basis of the majority of cases of glaucoma in human was not known, it has been suggested that apoptosis is the common pathway that leads to the death of RGCs in glaucoma and that neurotrophin 4 (NTF4) protein plays a role in the protection of RGCs by activating tyrosine kinase receptors. Additionally, one previous study suggested that p53 codon

72 polymorphism (R72P) might have a greater susceptibility to apoptosis in some ethnic population. Glaucoma also occurs in dogs and the primary glaucoma in beagles is inherited as an autosomal recessive trait. Although recently a candidate gene has been isolated, the mechanism underlying RGC death is not understood. In order to understand whether the same p53 and NTF4 pathway mechanism is involved in an animal model of glaucoma, we have isolated NTF4 gene from dog and analyzed both p53 and NTF4 genes for mutations in glaucomatous animals. Our analyses failed to identify any disease causing mutations in both genes with the exception of two heterozygous polymorphisms in NTF4 gene (CGA to GGA in the third codon and GCG to GCA in 49th codon). One of the polymorphisms changed the third codon (exon 1) from arginine to glycine whereas the second one (exon 2) is a silent change (alanine to alanine). However, these are not pathogenic changes because they are also present in normal animals and are not segregated with the disease. These results suggest that impaired neurotrophin signaling or compromised trophic support to the retina and p53 mediated apoptosis may not be the underlying mechanism of RGCs death in a canine model of glaucoma. Our study underscores the importance of further investigations for more comprehensive understanding of the specific form of RGC death in glaucoma.

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SIL1, a causative gene of Marinesco-Sjogren syndrome, plays an essential role in establishing the architecture of the developing cerebral cortex.

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Marinesco-Sjogren syndrome (MSS) is a rare, autosomal-recessive neurodegenerative disorder that is characterized by mental retardation, cerebellar ataxia, cataracts and progressive myopathy. The SIL1 gene encodes an endoplasmic reticulum resident co-chaperone that regulates the function of the heat-shock protein family chaperone HSPA5. Recently, mutations in SIL1 were identified as a major cause of MSS. Here, we report a novel in-frame 15 bp-deletion in the SIL1 coding region that results in a 5 -amino acid deletion in the encoded protein. To understand the role that SIL1 plays in the pathophysiology of MSS, we examined the effect of altering SIL1 expression and the effect of expressing three different MSS-linked SIL1 mutants, including the newly identified deletion mutation, during brain development. Knockdown experiments using the in utero electroporation method revealed that reducing SIL1 expression leads to an inhibition of neuron migration during corticogenesis. Expressing RNAi-resistant SIL1 reversed the migration defect; however, the MSS-linked SIL1 mutants that were tested did not rescue migration. Moreover, we found that HSPA5 also plays a role in neuron migration during corticogenesis. Biochemical analyses revealed that SIL1 proteins bearing an MSS-linked mutation exhibited weaker binding affinity to HSPA5, and perturbing the protein-protein interaction between SIL1 and HSPA5 led to impaired in vivo neuron migration. Additional analyses revealed that the defective neuron migration induced by MSS-linked SIL1 mutations is due at least in part to abnormal neuron morphology. These data suggest that the MSS-linked mutations abrogate the ability of SIL1 to interact with (and therefore regulate) HSPA5 and that impaired HSPA5 function may lead to abnormal neuron migration, which may subsequently contribute to the mental retardation that is observed in MSS patients.

1214

Centrosomal role of spastin-NA14 interaction in the pathogenesis of hereditary spastic paraplegia.

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Hereditary spastic paraplegias (HSPs) are a group of inherited neurological disorders characterized by the cardinal feature of lower-extremity weakness and spasticity due to a length-dependent axonopathy of corticospinal upper motor neurons. The most frequent form of HSP results from mutations of the SPG4 gene product, spastin. This protein is a ubiquitous member of the AAA (ATPase associated with diverse cellular activities) family that binds to and severs microtubules. While spastin participates in crucial cellular processes, its mechanistic role in HSPs remains unclear. Spastin interacts with NA14, a major specific target for auto-antibodies in Sjögren's syndrome nuclear autoantigen 1 (SSNA1). Our analysis of endogenous spastin and NA14 proteins in HeLa cells and primary rat cortical neurons show a clear localization to centrosomes, with NA14 localizing specifically to centrioles. NA14 knock-down in stable cell lines dramatically affects cell division, in particular cytokinesis. Furthermore, overexpression of NA14 and spastin in neurons significantly increases axon outgrowth and branching, and enhances neuronal differentiation. However, the number of centrosomes is not modified. Taken together, our data suggest that NA14 acts as a small adaptor protein regulating spastin localization to centrosomes, likely leading to temporal regulation of microtubule-severing activity. Future analyses of the spastin-NA14 interaction will be crucial to understand the functional role of spastin and how its dysfunction may underlie SPG4.

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The cellular prion protein mediates amyloid- β and TNF α -induced cofilin-actin rod formation through NADPH oxidase.

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In neurites of neurons undergoing hypoxia or treated with excitotoxic glutamate, soluble forms of β -amyloid peptide (A β) or the proinflammatory cytokine TNF α , each inducing oxidative stress, actin undergoes dynamic remodeling into rod-shaped cofilin-saturated actin filament bundles (rods) that might explain the common pathologies of familial and sporadic Alzheimer disease (AD). Rods can occlude neurites, blocking transport and disrupting synaptic function. Rods from stressed neurons contain dithiothreitol-sensitive cofilin dimers and oxidation of cysteines at positions 39 and 147 is required to form rods in vivo. Here we show that SDS-stable A β dimer/trimer (250 pM A β d/t) or TNF α (2.9 nM) induces rods in the same population (20%) of hippocampal neurons, but not in neurons lacking the cellular prion protein (PrP^c). A higher percentage of PrP^c-null neurons form rods after treatment with glutamate or mitochondrial inhibitors (e.g. antimycin A), suggesting these inducers generate reactive oxygen species (ROS) by a different mechanism from that of A β d/t or TNF α . This finding was confirmed with MitoQ, a mitochondrial ROS scavenger, which inhibited rod formation by 50% in response to glutamate or antimycin A, but did not inhibit rods induced by A β d/t or TNF α . Furthermore, TG6-44, an NADPH oxidase (NOX)-2/4 inhibitor, completely blocked neuronal rod formation in response to A β d/t but did not block the rod response to antimycin A. PrP^c is a component of detergent

resistant membrane rafts; about 50% of rods induced by A β d/t or TNF α associate with enlarged (coalesced) raft domains that are visualized with a G_M1-ganglioside label. We conclude that A β d/t and TNF α induce rod formation via a PrP^c-dependent signaling pathway involving production of ROS by NOX2/4. Because A β -induced cognitive deficits in rodent models of AD require the presence of PrP^c and brain injections of A β d/t at the maximal rod-inducing concentration induce cognitive deficits, rods may mediate cognitive impairment. Cofilin-actin rods bridge the amyloid and cytokine hypotheses for AD.

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Expression of alternative first exon isoforms of ankyrin-G in brain regions associated with bipolar disorder.

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Bipolar disorder (BD) is a mood disorder characterized by cyclic episodes of mania and depression. BD is common and can be severely disabling. Multiple independent genome-wide association studies have implicated the ANK3 gene in BD. ANK3 encodes the ankyrin-G (AnkG) protein, which mediates the attachment of integral membrane proteins to the spectrin-actin cortical cytoskeleton. AnkG has three alternative first exons (called here, nt1-3), encoding conserved N-termini for families of isoforms generated by additional variable splicing. Nt-2 and nt-3-containing AnkG isoforms are concentrated at axon initial segments (AIS) and nodes of Ranvier (NOR). Previous studies have suggested that the nt2 was forebrain specific and nt3 was cerebellum specific. Although the molecular mechanism through which variation in AnkG confers susceptibility to BD is unknown, the SNPs most highly associated with BD risk are closest to these neuronal alternative first exons. It was surprising that risk of BD might result from altered function in the cerebellum. We have therefore begun to reexamine AnkG diversity and expression patterns using new antibodies specific for the alternative first exons and immunostaining of rodent brain sections. We found evidence for widespread expression of both AnkG-nt2 and nt3 throughout the forebrain, including in regions associated with BD such as the hippocampus, amygdala, striatum, anterior cingulate cortex, thalamus and prefrontal cortex. In the hippocampus, where principal cells and interneurons are highly segregated, AnkG-nt3 isoforms appears to be absent from pyramidal cells, but highly expressed in a subpopulation of interneurons and in dentate granule cells. Ongoing experiments are aimed at identifying additional forebrain cells types in these regions of interest that express nt3 containing AnkG isoforms. Clarifying the cellular and subcellular localization of these variants may give insight into how changes ANK3 could confer susceptibility to BD.

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Effect of short vs. long term stress on cytoskeletal plasticity.

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Stress, which is defined as a real or interpreted threat to the physiological or psychological integrity of an individual, can result in psychological, behavioral or even physical changes. On a cellular level, stress affects neurogenesis and can cause atrophic retraction of the neuronal dendritic arbor in regions within the brain such as the medial prefrontal cortex (mPFC) and hippocampus which are responsible for executive function, learning and memory. It has been proposed that stress induced neural remodeling may contribute to psychiatric illnesses including depression, anxiety and post-traumatic stress disorder (PTSD). Previous long term stress studies have shown that cytoskeletal dendritic retraction occurs in the CA3 region of the hippocampus but in mice there are no studies that have focused on changes in neural plasticity

within the hippocampus as a result of short term stress. This study examines the effects that variable time restraint stress has on cytoskeletal plasticity in pyramidal neurons. Wild type C57BL/6 mice were placed in Plexiglas restraints for either a 30 minute, 1 or 5 hour time duration for a time span of 2, 5 or 21 days. Changes in neural plasticity were measured in the hippocampal CA3 region and mPFC (pre-limbic and cingulate) at level II-III. Verification of a stress response was evaluated using immuno-fluorescence by measuring the level of c-Fos, an intermediate early gene which is up-regulated in response to stress induced stimuli in the CNS, and BrdU to measure neurogenesis. Changes in cytoskeletal rearrangement of the pyramidal apical dendrites were measured and evaluated using Golgi stained sections. Our results indicate that changes in the cytoskeletal morphology within the pyramidal neurons begin to occur at shorter time points than have been previously reported.

1218

Using *Xenopus laevis* to study regulatory elements of the medium neurofilament gene *in vivo*.

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Building an axon requires highly regulated changes in the expression of key structural genes. For example, the developmental onset of the expression of the medium neurofilament (NF-M) subunit is tightly coordinated with neuronal differentiation. This tight regulation, operating at both the transcriptional and post-transcriptional levels, is mediated through the binding of specific proteins to elements within the gene and its transcribed mRNA. The identification of these elements and the proteins that bind them requires an *in vivo* model system in which the activity of genes bearing mutated prospective control regions can be rapidly screened within the context of the developing nervous system. We modified the ϕ -C31 integrase-mediated *Xenopus* transgenesis protocol to generate transgenic *Xenopus laevis* using direct plasmid injection into 2-cell stage embryos. This plasmid contained an *attB* site to facilitate its integration into the *Xenopus* genome and a *DsRed2* or *EGFP* reporter gene driven by the *NF-M* promoter. To reduce position effects, the transgene was flanked by two tandem repeats of chicken *HS4* insulator sequences. Injection of 100 pg of this plasmid into embryos yielded strong reporter protein expression in differentiated neurons at the onset of endogenous NF-M expression. Replacing the *NF-M* promoter with a neuronal β -tubulin (*N β T*) one confirmed strong reporter protein expression driven by specific promoters. Using this method, we identified domains within the 3'-untranslated region of the *NF-M* gene that were required for protein expression *in vivo*. These domains have been previously shown *in vitro* to differentially bind hnRNP K, an RNA binding protein essential for both NF-M protein expression and axon outgrowth. Thus, this method provides a useful tool for the study of regulatory elements of the *NF-M* gene in the developing vertebrate nervous system. *Funded by NSF IOS 951043.*

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Optimizing neuron adhesion and growth by choosing the right chamber slide surface.

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Isolated neurons from normal and diseased tissues of the mammalian Central Nervous System (CNS) are routinely cultured *in vitro* to study neurogenesis and to identify factors for drug screening that promote or inhibit neurogenesis. Culturing primary neurons *in vitro* is particularly challenging because they do not continue to proliferate after dissociation and survival of primary neurons is dependent on cell adhesion and differentiation. While many cells prefer culture surfaces with high surface energies (i.e. hydrophilic surfaces), neuronal cells require the additional presence of functional binding groups on the growth surface. Consequently, glass

and plastic surfaces must be adequately modified with specific surface chemistries. In this study, we examined the correlation between cell adhesion of PC-12 and primary neuron cells and multiple chamber slide surface modifications. The chamber slide (Thermo Scientific) surfaces, available in conventional glass, a proprietary chemically modified glass (CC²), and a cell culture treated plastic surface (Permanox), were evaluated with and without a fresh polylysine coating. We were able to demonstrate that polylysine coating on glass and plastic surfaces were sufficient in supporting the attachment and growth of the PC-12 cells and primary neurons. Interestingly, the chamber slide with CC² chemical modification functionally mimics the effect of polylysine coating without the use of the actual chemical. In contrast, glass and cell culture treated plastic surfaces alone were unable to support neuronal growth. Furthermore, the quantification of primary amines on all surfaces indicated that the CC² surface yielded sufficient amine surface density to facilitate neuronal cell adhesion and growth. In conclusion, the amount of primary amines on culture surfaces directly correlates with the attachment and survival of neuronal cells. The CC² chamber slide surface provides an optimal tool for imaging study of neuronal cell culture.

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MST3 kinase regulates neuronal cytoskeleton during development.

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Dendrite arborization and synapse formation are essential for establishing the neural circuitry for proper brain functioning. Defects in a growing number of genes are associated with neurodevelopmental disorders such as autism. Kinases play important roles in regulating the molecular components during synapse assembly and maintenance. We use chemical genetic methods to determine kinase signaling pathways essential for brain development.

MST3 kinase is a member of the Ste20-like (Sterile 20- like) kinases in mammals. MST3 is present in the dendrites and axons of the developing rat hippocampal neurons. A splice variant of MST3, MST3b, enhances axonal growth as well as regeneration. We asked if MST3 functions in dendritic development in hippocampal neurons. Using siRNA to knock-down MST3 as well as dominant-negative (kinase-dead) MST3 expression, we found that MST3 is essential for dendrite and dendritic spine development. Loss of MST3 in the first postnatal week causes a drastic increase in dendritic filopodia number and length and impairs dendrite arborization, whereas loss of MST3 in the third postnatal week leads to loss of dendritic spines and an increase in dendritic filopodia. MST3 siRNA expression in vivo by in utero electroporation of plasmids also resulted in loss of dendritic spine and increased filopodia, implicating that it plays essential functions in the formation and maintenance of dendritic architecture in vivo as well as in cultures. Downstream effectors of MST3 are not well-known. Using chemical genetic kinase substrate identification method we identified 15 potential substrates for MST3 and their phosphorylation sites. Several of these novel substrates are known regulators of cytoskeleton regulation. Most of the phosphorylation sites we identified are highly conserved among other species, implying important conserved molecular functions. By expressing phospho-mutant and phospho-mimetic forms of a subset of the novel substrates, we tested which one of these may be downstream of MST3 in dendrite morphogenesis. We found that phospho-mutant TAO kinase TAO1 or TAO2 expression or shRNA knockdown of TAO2 alone is sufficient to cause increased dendritic filopodia, reminiscent of MST3 loss of function. TAO2 kinase is a known regulator of microtubule and actin cytoskeleton and is implicated in autism. Our data implicates MST3 in regulating neuronal development by altering neuronal cytoskeleton and limiting dendritic filopodia via phosphorylating TAO2.

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Cofilin in Long Term Depression and Long Term Potentiation: a Reassessment.*B. Calabrese¹, J-M. Saffin¹, S. Halpain¹; ¹Division of Biological Sciences, UCSD, La Jolla, CA*

Previous studies suggested that during LTD actin filament severing by cofilin induces spine shrinkage, and during LTP inhibition of cofilin-mediated actin severing permits spine enlargement. However, we suggest that both types of structural plasticity are associated with decreased cofilin activity, but that the means for achieving this inhibition significantly affects the outcome for spine morphology. Results presented here support a model in which constitutive cofilin-mediated actin severing is critical for spine maintenance, and a reduction -- not an increase -- in endogenous spine cofilin concentration during LTD leads to spine shrinkage. Conversely, during LTP increased LIM kinase activity increases cofilin Ser-3 phosphorylation, which in turn activates phospholipase D to stimulate actin polymerization and spine enlargement. Overall, these results implicate novel mechanisms for the role of cofilin in both growth and shrinkage of synapses during circuit plasticity.

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Down regulation of PAI-1 by valproic acids modulates astrocyte-dependent regulation of neurite outgrowth from cultured rat primary cortical neuron.*K. Cho¹, H. Ko¹, K. Kwon¹, S. Lee¹, J. Park¹, S. Jeon¹, K. Kim¹, J. Cho², S. Han¹, C. Y. Shin¹; ¹Konkuk University, Seoul, Korea, ²Gachon University, Incheon, Korea*

Valproic acid (VPA), a histone deacetylase inhibitor that is used for the treatment of epilepsy and bipolar disorders, promotes neurite extension, neuronal growth and has neuroprotective effect in neurodegenerative diseases. One of the essential regulators of neurite extension in CNS is tissue plasminogen activator (tPA). Being a serine protease, tPA plays regulatory roles such as neurite outgrowth, synaptic plasticity and long term potentiation in CNS. Most of the cells in CNS express tPA and the activity of tPA is regulated by an endogenous inhibitor plasminogen activator inhibitor-1 (PAI-1), which is expressed mainly in astrocytes. In this study, we investigated the effects of VPA on the activity of tPA/PAI-1 system in cultured rat primary neuron and astrocytes. VPA concentration-dependently increased neurite outgrowth in co-culture of astrocyte and neuron but not in pure neuron. In pure cultured neuron VPA did not affect tPA activity, however, VPA decreased PAI-1 activity in rat primary astrocytes. The decrease in PAI-1 activity in cytosol as well as extracellular secretion of rat primary astrocytes induced up-regulation of tPA activity. An inhibitor of tPA activity, tPA STOP as well as exogenous addition of PAI-1 inhibited VPA-induced neurite outgrowth. Although astrocyte culture supernatants obtained from wild type mice induced neurite outgrowth, culture supernatants obtained from tPA knock-out mice did not induce neurite outgrowth. Alternatively, culture supernatants obtained from PAI-1 knockout mice increased neurite extension more than wild type culture supernatants. The decrease in PAI-1 activity and increased neurite extension was regulated via JNK mediated post-transcriptional pathway. Regulation of tPA/PAI-1 activity in astrocyte by VPA may affect both physiological and pathological processes governed by this neuromodulator system.

Voltage-gated Channels and Neurotransmission

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Modulation of rat Eag1 potassium channels by 14-3-3 θ .

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To further understand the nature of 14-3-3 regulation of ion channels, we employed the yeast two-hybrid screening of a rat brain cDNA library and identified 14-3-3 θ as a candidate interacting protein of the brain-specific rat ether-a-go-go (rEag1) K⁺ channel. Data from in vitro yeast two-hybrid and GST pull-down assays suggested that the interaction with 14-3-3 θ was mediated by both the N- and the C-termini of rEag1. GST pull-down assay also showed that among the six brain-expressing 14-3-3 isoforms, 14-3-3 θ was the preferred binding partner of rEag1. Co-precipitation and co-localization of the two proteins were demonstrated both in heterologous HEK293T cells and in native hippocampal neurons. Furthermore, the 14-3-3 antagonist Difopein effectively disrupted the interaction of these two proteins. Truncation analyses of rEag1 revealed that the N- and the C-terminal 14-3-3 θ interaction sites were mainly located at the PAS domain and the CNBHD region, respectively. Parallel truncation analyses of 14-3-3 θ further indicated that the C-terminal helices α F, α G, and α H were essential in mediating the association with rEag1. Electrophysiological studies showed that co-expression with 14-3-3 θ led to a suppression of rEag1 K⁺ currents. However, neither the total protein level nor the surface expression of rEag1 proteins was significantly altered by the over-expression of 14-3-3 θ . These data imply that 14-3-3 θ binding may render a fraction of the channel locked in a non-conducting state.

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Imaging voltage with microbial rhodopsins.

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In the wild, microbial rhodopsin proteins convert sunlight into a transmembrane voltage, which provides energy for their host. We engineered microbial rhodopsins to run in reverse: to convert membrane potential into a readily detectable optical signal. When expressed in a neuron or a cardiac myocyte, these voltage-indicating proteins convert electrical action potentials into visible flashes of fluorescence, allowing us to make movies of electrical activity in cells, tissues, and live animals. We developed techniques for simultaneous optical stimulation and recording of electrical activity in cells, as well as computational techniques for observing electrical propagation with microsecond time resolution. Upon expression of the voltage indicator in *E. coli*, we discovered that bacteria generate electrical spikes too. We find that electrical dynamics in cells are far more widespread than was previously thought.

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Development of optogenetic methods to study neuronal connectivity.

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We are interested in quantifying the effects of test compounds on neuronal transmission and the development of neuronal cell circuits utilizing optogenetic methods. This will be an important in vitro tool to screen for environmental pollutants, and for drug discovery efforts to combat “chemo-brain” condition, which is a loss of cognitive ability developing in cancer patients following chemotherapy, and other forms of memory loss and dementia, including Alzheimer’s and aging.

We successfully cultured primary, hESC and iPSC-derived neurons in a high throughput format (96 well), transduced them with lentiviruses encoding light-activated channel rhodopsin (ChR2) fused with yellow or mCherry fluorescent proteins, assayed their depolarization (activation) with fluorescent calcium indicators and analyzed the neuronal activity via automated microscopy and image analysis techniques. We showed that ChR2-expressing neurons fire in response to blue light, and that the activity of the neurons in our system can be modulated (inhibited or activated) by long or short term treatments with pharmacological drugs. We have also correlated the calcium based activity assessment with expression and colocalization of pre- and postsynaptic markers using immunolabeling.

Our preliminary results demonstrate that the optogenetic approach allows for synchronized neuronal activation in cultures grown in multi-well plates, and may facilitate characterization of neuronal connectivity relevant to “chemo-brain”, memory-loss, and dementia- related dysfunction.

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R7BP modulates GIRK channel activity by allosterically regulating RGS proteins in Hippocampal Neuron.

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G protein-activated inward rectifying K⁺ (GIRK) channels hyperpolarize neurons to inhibit synaptic transmission throughout the nervous system. By accelerating G protein deactivation kinetics, the regulators of G-protein signaling (RGS) protein family modulate the timing and magnitude of GIRK activity and function. Despite many investigations, whether RGS proteins modulate GIRK activity in neurons by mechanisms involving kinetic coupling, collision coupling, or macromolecular complex formation have remained unknown. Here we show that GIRK modulation occurs by channel assembly with R7-RGS/Gβ5 complexes under allosteric control of R7 RGS-binding protein (R7BP). R7BP is a novel palmitoylated SNARE-like protein that binds heterodimers containing any member of the R7 subfamily of RGS and the Gβ5 protein, which accelerate deactivation of Gi/o-class G proteins in vitro. However, the consequences and neuronal functions of R7BP forming complex with R7RGS/ Gβ5 in neuron were still unclear. We found that elimination of R7BP occludes the Gβ5 subunit that interacts with GIRK channels. R7BP bound R7-RGS/Gβ5 complexes and Gβγ dimers interact noncompetitively with the intracellular domain of GIRK channels to facilitate rapid activation and deactivation of GIRK currents. By disrupting this allosterically-regulated assembly mechanism, R7BP ablation

augments GIRK activity. This enhanced GIRK activity increases the drug effects of agonists acting at G protein-coupled receptors that signal via GIRK channels, as indicated by greater antinociceptive effects of GABA(B) or mu-opioid receptor agonists. These findings show that GIRK current modulation in vivo requires channel assembly with allosterically regulated RGS protein complexes, which provide a novel target for modulating GIRK activity in neurological disorders in which these channels have crucial roles, including pain, epilepsy, Parkinson's disease and Down's syndrome.

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Role of voltage-gated sodium channels in axon initial segment maintenance.

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In central neurons, the axon initial segment (AIS) is enriched with multiple types of ion channels, including Na⁺, K⁺ and Ca²⁺ channels in association with scaffolding protein and cytoskeletal components. In particular, the high density of voltage-gated sodium channels (Nav) drives the initiation of action potentials at the AIS. The AIS also forms a boundary between the axonal and somatodendritic compartments that has an essential role in maintaining neuronal polarity. Many studies have established the crucial role of the scaffolding protein ankyrin G (ankG) in ion channel clustering and polarity maintenance. Using cultured rat hippocampal neurons in culture and cortical organotypic slices, we observed that the knockdown of Nav by shRNA in mature neurons led to a decrease in ankG concentration in the AIS. As expected, the segregation of other ankG-interacting proteins (such as Neurofascin-186) was also disrupted in the AIS. These results suggest that, in addition to well-known role in the generation of action potentials, Nav channels may act in the maintenance of AIS structural integrity.

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KCNQ2 targeting to the axon initial segment is disrupted in a transgenic mouse model of severe neonatal-onset epileptic encephalopathy.

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KCNQ2 and KCNQ3 are homologous voltage-gated potassium channel subunits that form neuronal "M-channels". M-channels activate slowly in the voltage range between rest and action potential threshold, thereby integrating synaptic inputs and modulating neuronal excitability. Certain KCNQ2 and KCNQ3 mutations cause dominantly inherited forms of epilepsy and myokymia; by contrast, other, de novo, KCNQ2 mutations have been found in a severe syndrome of epileptic encephalopathy. KCNQ2 and KCNQ3 are conspicuously enriched at the axonal initial segment (AIS) of many central neurons, but the time course of their arrival at the AIS has been little studied. We have performed immunofluorescence labeling and immunoblotting for these subunits in tissue samples and hippocampal neuronal cultures during development. Labeling of mouse hippocampal sections shows that in pyramidal cells, KCNQ2 is detectable at P4 and is highly concentrated within the distal portion of AISs by P15. KCNQ3 arrival at the AIS appears slightly delayed, suggesting the existence of two distinct populations of channels rather than only KCNQ2/3 heteromers. Within neocortical pyramidal neurons, KCNQ2 and KCNQ3 are expressed in the distal two-thirds of the AIS, and most concentrated at the distal tip, the location at which action potentials are initiated. Additionally, in KCNQ2 mutant mice which are a model of KCNQ2 encephalopathy (Peters et al., Nat Neurosci. 2005; Millichap and Cooper, Epil. Curr., 2012) KCNQ2 was completely undetectable at the AIS, and was found instead in perinuclear and dendritic aggregates suggestive of ER retention. KCNQ3 was redistributed to the KCNQ2-labelled aggregates, but was detected at low levels within the AIS.

These results indicate that certain KCNQ2 mutations may act as dominant-negative trafficking traps, preventing functional surface expression of subunits co-expressed from wild-type alleles and thereby leading to severe, encephalopathic phenotypes.

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The Anaphase Promoting Complex Regulates GABAergic Synaptic Transmission at the *C. elegans* Neuromuscular Junction.

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Neuronal communication is critical for information processing, storage, and transmission within the nervous system. This communication, which occurs at specialized cellular junctions called synapses, is tightly regulated, and misregulation of this synaptic transmission is linked to neurological and neurodegenerative diseases. The ubiquitin enzyme system is a vital regulator of synaptic transmission. It controls the abundance of many synaptic proteins, and its dysfunction is observed in many neurological disorders. Previously, the Anaphase Promoting Complex (APC), a well characterized ubiquitin ligase and cell cycle regulator, was identified as a modulator of synapse development and synaptic transmission at glutamatergic synapses through its ability to control synaptic protein abundance. However, the mechanisms of APC action and its potential for impacting signaling at other synapse types are unknown. Here, we show that the APC regulates GABA transmission at the *C. elegans* neuromuscular junction (NMJ), a specialized synapse where a balance of excitatory cholinergic and inhibitory GABAergic signaling from presynaptic motor neurons controls postsynaptic muscle activity. In behavioral assays assessing NMJ signaling, loss-of-function mutants in several APC subunits exhibit increased muscle contraction, as indicated by hypersensitivity to the acetylcholinesterase inhibitor aldicarb. This defect can be rescued by expressing a functional copy of the defective APC gene in GABA neurons using a cell type-specific promoter. Increased muscle excitation also occurs following GABA neuron-specific APC inhibition by ectopic expression of the APC inhibitor hEmi1. In addition, APC mutants exhibit convulsions in the presence of the GABA antagonist, pentylentetrazole, which induces this behavior only in GABA signaling defective worms. Quantitative fluorescence imaging further revealed an increase in synaptobrevin-GFP at presynaptic sites in GABAergic but not cholinergic neurons of APC mutants, as well as an increase in the postsynaptic GABA_A receptor UNC-49. Preliminary electrophysiological analyses of endogenous NMJ currents indicate that APC mutants exhibit normal EPSC but decreased IPSC frequencies, supporting a presynaptic role for the APC in regulating GABA signaling. Finally, fluorescence colocalization studies indicate that these effects may be due to APC activity at presynaptic sites, as we observed strong colocalization between a GFP-tagged APC subunit and synaptobrevin-RFP in GABAergic motor neurons. Taken together, these data suggest a model in which the APC acts at GABAergic motor neuron presynapses to promote GABA release. Current studies are aimed at identifying relevant substrates of the APC that mediate its effects at the NMJ.

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Investigation of the relationship between FSHR-1 and the Anaphase Promoting Complex in regulating synaptic transmission at the *C. elegans* neuromuscular junction.

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Neuronal communication occurs at cellular junctions called synapses. The signaling at each synapse is tightly regulated, and misregulation of this synaptic transmission occurs in neurological diseases. The ubiquitin enzyme system controls synaptic transmission by regulating synaptic protein activity and abundance. We previously identified the Anaphase Promoting Complex (APC), an ubiquitin ligase, as a critical regulator of synaptic transmission at the neuromuscular junction (NMJ) in *C. elegans*. At the *C. elegans* NMJ, a tight balance of excitatory (cholinergic) and inhibitory (GABAergic) signaling controls muscle contraction; however, the mechanisms by which the APC regulates this transmission are unknown. Here, we identified FSHR-1 as a potential APC substrate at the NMJ. FSHR-1 is a G protein-coupled receptor homologous to the follicle stimulating hormone, luteinizing hormone, and thyroid stimulating hormone receptors in mammals. In *C. elegans*, FSHR-1 is highly expressed in neurons and the intestine and, along with roles in germline differentiation and pathogen susceptibility, is required for synaptic transmission at the NMJ. However, the specific functions and regulators of FSHR-1 in neurons are unknown. Previous research has shown *fshr-1* loss of function mutants have decreased muscle contraction, as indicated by their resistance to paralysis induced by the acetylcholinesterase inhibitor aldicarb, suggesting that FSHR-1 promotes signaling for muscle contraction. Given that this *fshr-1* mutant phenotype is opposite to that seen in animals lacking APC function, and since the FSHR-1 protein contains an APC recognition motif, we tested whether FSHR-1 and the APC function in the same genetic pathway at the NMJ. We found that double mutants lacking both the APC and *fshr-1* exhibit aldicarb resistance, similar to *fshr-1* single mutants. Thus, FSHR-1 may function downstream of the APC in regulating muscle contraction. Neuronal expression of FSHR-1 in *fshr-1* mutants restores aldicarb sensitivity to at least wild type levels, and neuronal over-expression of FSHR-1 in wild type animals induces aldicarb hypersensitivity. These results suggest that FSHR-1 function in neurons is sufficient to promote signaling for muscle excitation. Current co-localization experiments using transcriptional fusions are aimed at determining if FSHR-1 is expressed in the same NMJ cell type where the APC functions. Preparation for biochemical analyses is also underway to test if FSHR-1 is a direct APC substrate at the NMJ. Given the high conservation of neuronal protein structure and function between worms and vertebrates, by better understanding the proteins that control *C. elegans* neuronal communication, we can begin to address human neurological diseases.

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Synaptotagmin 12 is necessary for homeostatic synaptic plasticity at the *Drosophila* neuromuscular junction.

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At the neuromuscular junction (NMJ), decreased postsynaptic neurotransmitter receptor sensitivity initiates a homeostatic increase in neurotransmitter release from presynaptic motoneurons. This increase in presynaptic release precisely offsets the defect in neurotransmitter receptor sensitivity and restores normal postsynaptic excitation in the continued presence of the perturbation. This form of homeostatic plasticity is evolutionarily conserved and has been observed in organisms ranging from *Drosophila* to mouse and human.

Currently, little is known about the underlying molecular mechanisms that achieve this robust and accurate form of homeostatic plasticity. We are taking advantage of the forward genetic tools in *Drosophila* to identify genes that, when mutated, block the induction or expression of homeostatic plasticity. As part of an ongoing screen, we have discovered that mutations in the *Synaptotagmin 12* (*Syt12*) gene disrupt synaptic homeostasis at the *Drosophila* NMJ. *Syt12* is an evolutionarily conserved member of the synaptotagmin family. While Synaptotagmin 1 functions as a calcium sensor for neurotransmitter release, there is evidence indicating that *Syt12* does not bind calcium ions. Currently, the functions of *Syt12* and other calcium-insensitive synaptotagmins remain largely unknown. We will present a genetic analysis of *Drosophila Syt12*, providing evidence that *Syt12* is necessary for normal basal synaptic transmission and homeostatic synaptic plasticity. We demonstrate that loss of *Syt12* causes a minor defect in presynaptic neurotransmitter release and completely blocks the rapid induction and sustained expression of synaptic homeostasis. Use of cell type-specific RNAi and genetic rescue demonstrates that *Syt12* function is necessary in motoneurons for normal basal synaptic transmission and synaptic homeostasis. An analysis of synapse morphology demonstrates that synaptic growth, assayed by the number of synaptic boutons at the NMJ, is normal in *Syt12* mutants. Thus, we have identified a novel function for a calcium-insensitive member of the synaptotagmin family and extended our understanding of how homeostatic synaptic plasticity is executed at a model glutamatergic synapse.

Signaling Networks Governing Cell Migration II

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Digitalis-derived compound exhibits anti-proliferative activity on hepatocarcinoma cells by down-regulating the expression of farnesoid x receptor.

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Farnesoid X receptor (FXR), bile acid-activated nuclear receptor, is a pivotal factor maintaining bile acid homeostasis. We have previously shown that FXR plays a crucial role in the proliferation of human hepatocellular carcinoma cell line, HepG2, Huh7, HLE: FXR stimulates cell proliferation by suppressing the p16/INK4a expression whereas downregulation of the FXR stimulates the p16/INK4a expression, resulting in the suppressed cell proliferation. Moreover, in contrast to hepatocellular carcinoma cells, downregulation of the FXR in primary hepatocytes causes decrease of p16/INK4a expression, resulting in the stimulated cell proliferation. Thus, it is expected that we can establish the cancer therapy by suppressing the FXR expression in liver. Glucodigifucoside is a compound extracted from digitalis. We have recently shown that the compound suppresses cell proliferation of human leukemia cell line HL-60. In the present study, we investigate whether or not the compound affects cell proliferation of human hepatocellular carcinoma cell line, HepG2.

1.0x10⁵ cells of HepG2 seeded on 60-mm dish were treated with 0.0079- 0.79 uM glucodigifucoside for 72 h, and cell number was counted. Indeed, cell proliferation was dramatically inhibited by glucodigifucoside (IC₅₀=0.59uM). Moreover, FXR level of glucodigifucoside- treated cells was significantly lower than that of control cells, while p16/INK4a level was increased by glucodigifucoside treatment.

In conclusion, digitalis-derived compound glucodigifucoside exhibits anti-proliferative activity on hepatocarcinoma cells in FXR and p16/INK4a-dependent manner.

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Role of Rho GTPases in Regulation of Cell Migration and Polarization in Human Corneal Epithelial Cells.

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Objective. Rho GTPases are conserved proteins in eukaryotic known to mediate the signaling from cell surface adhesion complexes to molecular motors and cytoskeletal structures in the cells, and hence determine cell motility. Epithelial cell migration is defective in many ocular surface diseases. Even though Rho GTPases have been linked to cell migration, their roles in the ocular surface remain unknown. This study aimed to determine the role of various Rho GTPases in human corneal epithelial directional cell migration and polarization.

Methods. Dominant-negative Rho GTPase or siRNA targeting Rho GTPase were transfected into cultured human corneal epithelial large T antigen (HCET) cells, and wound closure rate were evaluated by scratch wounding assay and non-traumatic cell migration assay. Cell polarization was observed by immunofluorescence staining for Golgi body.

Results. Out of the Rho GTPases (RhoD, Rac1, Cdc42, RhoJ, TC10 and Chp) tested, Cdc42 and RhoJ had distinctive effects on cell migration. Cells transfected with dominant-negative plasmids or siRNAs targeting Cdc42 or RhoJ have slower migration rates (in each assay) compared to control cells. In confluent HCET which commenced migration for 1 hour, the ratio of polarized cells to total cells was significantly reduced in cells with siRNA targeting Cdc42 and to a lesser extent for RhoJ, compared to scrambled siRNA, despite similar amounts of silencing on western blots.

Conclusion. Cdc42 and RhoJ have a facilitative role in 2-D cell migration in-vitro in HCET cells. Cdc42 and RhoJ are important for cell polarization in the early phase of in-vitro cell migration, but Cdc42 probably plays a relatively more important role. There may be distinctive downstream targets that specifically link these two Rho GTPases to cellular machinery for motility. These findings may have application in therapy of ocular surface disease with aberrant or deficient cell migration.

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A novel PDGF-mediated RhoG pathway in vascular smooth muscle cells.

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Remodeling of the vascular smooth muscle cytoskeleton is essential for cell motility involved in the development of diseases such as atherosclerosis and restenosis. It is well known that platelet-derived growth factor (PDGF) is a major stimulus for the abnormal migration and proliferation of vascular smooth muscle cells (VSMCs) and contributes critically to vascular disease. Our results show that PDGF promotes the rapid activation of RhoG in rat vascular smooth muscle cells (A7r5). RhoG is one of the small GTPases of the Rho family and its function has been correlated previously with many of the cellular processes regulated by PDGF, including proliferation, survival and migration and Rac1 activation. Our hypothesis is that PDGF-induced changes in the morphology and behavior of VSMCs are mediated by RhoG. Here we

describe the initial characterization of a novel signaling pathway that activates RhoG downstream of PDGF. This PDGF-mediated RhoG activation is dependent of Src and PI3K activity. Both RhoG and Rac1 are activated by PDGF independently. Our results also show that one of the early phenotypic responses to PDGF, the formation of actin-rich dorsal ruffles, is modulated by RhoG activity. In addition, RhoG activation downstream of PDGF modulates cell migration in a process that involves the guanine-nucleotide exchange exchange factor (GEF) Trio.

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Characterization of PLD2 GEF activity.

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We have demonstrated phospholipase D2 (PLD2) is a guanine nucleotide exchange factor (GEF) for Rac2 and determined the PLD2 domains and amino acid site(s) responsible for its GEF activity. Experiments using GST fusion proteins or GST-free counterparts, purified proteins, revealed that the PX domain is sufficient to exert GEF activity similar to full length PLD2. The PLD2-GEF catalytic site is comprised of hydrophobic pocket of residues namely F107, F129, L166, and L173, all of which are in the PX domain. A nearby R172 is also important in the overall activity. PX mutants comprising any of those 5 amino acids fail to have GEF activity, but still bind to Rac2 and their lipase activity was mostly unaffected. In addition to the PX domain, a region in the PH domain (I306-A310) aids in the PX-mediated GEF activity by providing a docking site to hold Rac2 in place during catalysis. The physiological relevance of this novel GEF in cell biology is demonstrated here in chemotaxis and phagocytosis of leukocytes, as the specific PX and PH mutants abolished cell function. Hence PLD2 is a unique GEF with the PX being the major catalytic domain for its GEF activity, whereas the PH domain assists in the PX-mediated activity. Not only is PLD2 unique in terms of activity but also in terms of regulation. PLD2 is an enzyme that bears two activities: a Guanine-nucleotide Exchange Factor (GEF) and a lipase that reside in the N-terminus (PX/PH domains) and the C-terminus (HKD domains), respectively. PLD2's GEF activity yields Rac-GTP (from Rac2-GDP) and the lipase activity yields phosphatidic acid (PA) (from phosphatidylcholine). PA at low concentrations and early times (3-5 min) promotes GTP binding to Rac2, while Rac-GTP upregulates PLD2's lipase activity. This positive feedback by the two reaction products on their alternate activities serves to mount a rapid and early cellular response. However, as PA accumulates at later times (>30 min), this scenario does change. At high concentrations, PA binds to PLD2 at an allosteric site, specifically the PX/PH tandem, which is required for GEF activity and impedes efficient binding and interaction with its substrate, Rac2-GDP. This leads to a termination of PLD2's GEF activity. In addition to this inter-regulation, tyrosine kinase Janus Kinase 3 (JAK3) plays a key role in switching PLD2 from a GEF to a lipase.

Thus, this study reveals for the first time the catalytic site that forms the basis for the mechanism behind the GEF activity of PLD2. Also, this is the first report of a temporal inter-regulation of a dual enzymatic activity that resides in the same molecule with profound biological consequences in cell growth and cell migration.

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Neuronal Thy-1 induces astrocyte migration via a signaling pathway that includes FAK, PI3K, Tiam-1 and Rac1.M. Kong¹, A. Alvarez¹, A. Cárdenas¹, N. Muñoz¹, A. Valdivia², A. F. Quest¹, L. Leyton¹;¹Molecular and Cellular Biology, Facultad de Medicina-Universidad de Chile, Santiago, Chile,²Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Astrocytes in the normal brain are immobile quiescent cells. However, upon brain injury, they become reactive, retract their processes, polarize and acquire front-to-rear asymmetry typical of migratory cells. Effects of the extracellular matrix (ECM) on migration have been well characterized in vitro, but how neurons modulate the ability of reactive astrocytes to migrate remains unknown. Thy-1 is an abundant neuronal glycoprotein that interacts with $\alpha\beta 3$ integrin and syndecan-4 in astrocytes to promote strong astrocyte adhesion to the ECM through signaling events that involve FAK, PKC α and RhoA activation. Cell migration is a highly regulated process that requires adhesion and is controlled by RhoA, Cdc42 and Rac1 GTPases, which in turn are regulated by guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP. Thus, the aim of our study was to determine whether prolonged astrocyte stimulation with Thy-1 augmented migration after initially promoting adhesion and to shed light on the molecular mechanisms involved. Immunofluorescence analysis was used to evaluate cell polarization, whereas migration was tested in wound-healing assays. Here, we demonstrate that prolonged, rather than short-term Thy-1 exposure induces astrocyte polarization and migration. Then, to identify the signaling pathways involved in Thy-1-induced migration, pharmacological and genetic inhibitors were employed. PI3K activation downstream of FAK was evaluated by Western blotting. Tiam1 and Rac1 involvement were assessed by affinity-precipitation assays. Phosphorylation of Akt, a downstream effector of PI3K, increased significantly after incubation with Thy-1. This effect was not observed in the presence of a FAK inhibitor indicating that FAK activation is upstream of PI3K. In addition Tiam1 was activated and Rac1 activity increased after 30 min. Moreover, pre-incubation with PI3K or Rac1 inhibitors, as well as overexpression of a dominant negative form of Rac1, decreased directional cell migration to basal levels. In summary, our data indicate that Thy-1 induces astrocyte polarization and migration by activating signaling pathways that involve FAK, PI3K, Tiam1 and Rac1. These observations are likely to be relevant to wound healing and regeneration in the central nervous system.

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Rap1 localization is dynamically controlled during immune cell transmigration in *Drosophila*.D. Siekhaus^{1,2}, O. Moffitt², M. Haesemeyer², R. Lehmann²; ¹IST Austria, Klosterneuburg, Austria, ²NYU School of Medicine, New York, NY

Both metastatic cancer cells and immune cells pursuing infections must penetrate the endothelial barrier presented by the blood vessel wall to reach tissues. This transmigration requires the Rap1 GTPase to modulate Integrin affinity. We have defined a new model system in *Drosophila* to dissect the genetic pathways that control this process. Using live imaging and genetics, we have discovered that *Drosophila melanogaster* immune cells penetrate an epithelial, DE-Cadherin-based tissue barrier during their embryonic developmental movements into the tail. A mutant in RhoL, a GTPase homolog that is only expressed in *Drosophila* macrophages at this stage, specifically blocks this invasive step but not other aspects of guided

migration. The invasion defect in rhoL mutants can be rescued by lowering embryonic DE-Cadherin levels, arguing that RhoL is required to allow hemocytes to penetrate adherens junctions. We also show that RhoL is localized to the cell surface and mediates Integrin adhesion in hemocytes. We observe that RhoL mutants fail to transport the Integrin activator Rap1 from a cytoplasmic membrane containing concentration to the leading edge during invasion. We have identified 4 mutants in secretory pathway components with defects in this invasive migration, one of which has been linked to metastasis in mice. These genes are expressed specifically during the invasive phase and turn off thereafter. Rap1 localization is found to be dynamically cycling to the cell surface at this time. However after this, Rap1 is found mostly in an intracellular concentration even in wild type. We have thus identified RhoL as a new regulator of invasion, adhesion and Rap1 localization. We propose that RhoL is part of a regulated intracellular trafficking pathway required to shuttle the Rap1 Integrin activator to and from the cytoplasmic edge, permitting the dynamic shifts in adhesion required for hemocytes to invade between adherens junctions. This capacity for invasion and Rap1 relocalization appears to be developmentally regulated. Intriguingly, vertebrate Rap1 also relocalizes to the cell surface to increase integrin affinity. Our work establishes the utility of *Drosophila* for identifying novel components of transmigration and for understanding the *in vivo* interplay of cells with the barriers they penetrate.

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Cadherin 6 is necessary for Rho dependent apical detachment during neural crest cell epithelial to mesenchymal transition.

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Epithelial to mesenchymal transitions (EMTs) are critical events during embryonic development, but also drive several pathologies, most notably carcinoma metastasis. Neural crest cells (NCCs) undergo EMT to become migratory as a part of their normal development and zebrafish NCCs are an ideal model to study EMT *in vivo*. We defined a series of cell behaviors that drive motility during zebrafish NCC EMT, and are analyzing how specific molecules control these behaviors in the intact embryo. Cadherin 6B (Cad6B) is important for chick NCC EMT, however conflicting reports have come from studies of its function, leaving its precise roles in EMT unclear. We investigated the function of Cad6 (the zebrafish ortholog of Cad6B) during NCC EMT *in vivo*. We found *cad6* mRNA is expressed in NCCs and in the neuroepithelium during EMT. Morpholino knockdown of Cad6 resulted in accumulation of cranial NCCs in the neuroepithelium as well as disruption of craniofacial structures, suggesting Cad6 is required for EMT. Live imaging after Cad6 knockdown showed that NCCs fail to detach from the apical midline, a critical early event in EMT. Detachment is normally preceded by the accumulation of F-actin, suggesting that actomyosin may be involved. To investigate this hypothesis, we examined the function of Rho GTPase, a regulator of actomyosin whose role in EMT has also been controversial. We used live biosensor imaging of active Rho *in vivo*, and manipulation of Rho signaling to show that apical detachment is driven by Rho mediated actomyosin contraction. To test whether Cad6 affects Rho activation during detachment we imaged active Rho following Cad6 knockdown. While the level of Rho activation in the NCC apical tail is unchanged following Cad6 knockdown, Rho appears to be activated in a broader area and apical detachment does not occur. This suggests that Rho signaling must be tightly spatiotemporally controlled for proper NCC EMT and suggests that Cad6 functions to direct focal activation of Rho and actomyosin contractility in NCC EMT. This pathway may function in parallel to a recently described role for Cad6B in which it combines with non-canonical BMP signaling to activate LIMK to promote EMT.

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A Novel Arf6—ERK Pathway is Required for Migration of Metastatic Breast Cancer Cells.*J. Freed^{*1}, C. V. Shaffer^{*1}, C. C. Moore¹; ¹Department of Pharmacology, University of the Sciences, Philadelphia, PA*

* = co-first authors. The research presented here supports a model whereby Arf6 is critical to the mechanism of CXCR4 dysregulation in metastatic breast cancer cells. CXCR4 is a chemokine receptor essential for select neuronal, cardiovascular, and hematopoietic cell migration towards SDF (CXCL12), and is now recognized to promote cancer metastasis. Dysregulation of the SDF-CXCR4 axis in nonmotile primary tumor cells confers an aberrant migratory capacity and promotes metastatic homing of tumor cells to distal SDF-expressing organs. Metastasis is the major cause of mortality in cancer patients, therefore these findings have led to vigorous attempts to identify molecular factors that contribute to CXCR4 dysregulation in cancer. Previously we identified Arf6 as a novel regulator of the SDF-CXCR4 axis, whereby it enhances both CXCR4 cell surface levels and CXCR4 signaling to membrane-delineated ERK. Here we identified a novel Arf6—ERK pathway required for migration of metastatic breast cancer cells. Specifically, we determined the steepness and duration of SDF gradient that is associated with robust CXCR4 signaling to cortactin, an actin-binding protein with known involvement in cancer cell migration and invasion. Utilizing this defined gradient, we assessed the effects of mutational or GEF-mediated Arf6 activation, siRNA-mediated Arf6 knockdown, and MEK inhibition on CXCR4-mediated migration in response to co-stimulation with SDF and collagen, as measured by transwell cell motility assays. Our results demonstrate that in noninvasive MDA-MB-361 and MDA-MB-468 cells, Arf6 activation unmask a migratory phenotype which is blocked by MEK inhibition with PD98059 and U0126. Additionally, in highly invasive MDA-MB-231 and BT-549 cells, siRNA-mediated knockdown of endogenous Arf6 and MEK inhibition with PD98059 and U0126 significantly reduce the migratory phenotype. These responses are specific to CXCR4-mediated migration as suggested by blockade with a CXCR4 antagonist or neutralizing antibody, AMD3100 and 12G5 respectively, and no change of cell adhesion or FBS-mediated migration. These results provide insight into the role of a novel Arf6—ERK pathway in regulating CXCR4-mediated migration, and support the model that Arf6 is critical to the mechanism of CXCR4 dysregulation in metastatic cancer cells. These studies were supported by NIH grant GM-097718, AFPE sponsored AACP New Investigator grant, and start-up funds from USP.

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ARF6 and Shp2 phosphatase regulate CXCR4 phosphorylation and signaling to ERK.*A. Jancina¹, C. C. Moore¹; ¹Department of Pharmacology, University of the Sciences, Philadelphia, PA*

CXCR4, a chemokine G protein-coupled receptor, is essential for select neuronal, cardiovascular, and hematopoietic cell migration towards its ligand SDF. It has also been determined to be a major co-receptor for HIV-1 entry, and CXCR4 gene mutations lead to WHIM syndrome, an immunological disease. More recently, CXCR4 has been implicated in cancer and is now recognized to play a critical role during metastasis. Primary non-motile tumor cells with dysregulated SDF-CXCR4 axis adopt migratory phenotype and metastasize to distal SDF-expressing organs. Metastasis is the major cause of mortality in cancer patients, therefore it is important to identify the machinery that regulates CXCR4 in tumor cells. Normally, following SDF stimulation, CXCR4 is rapidly phosphorylated by G protein-coupled receptor kinases (GRKs) or protein kinase C (PKC). CXCR4 phosphorylation initiates adaptor recruitment, promoting receptor uncoupling from G proteins leading to receptor desensitization, and promoting receptor trafficking to sites of endocytosis leading to receptor downregulation.

Previously we identified ARF6 as a novel regulator of SDF-CXCR4 axis, whereby it inhibits removal of CXCR4 from cell surface following agonist stimulation, and potentiates CXCR4 signaling to membrane-delineated ERK. Here we addressed mechanism by which ARF6 regulates CXCR4 trafficking. Specifically, we assessed effects of time, SDF dose, deglycosylation, serine/threonine/tyrosine phosphatase inhibitors on CXCR4 phosphorylation, and the ability of ARF6 to modulate CXCR4 phosphorylation. Here, we demonstrated that ARF6 inhibits CXCR4 phosphorylation in a dose- and time-dependent manner, and upon CXCR4 deglycosylation. ARF6 inhibition of CXCR4 phosphorylation is unaltered in presence of phosphatase inhibitors OA and PHS1. We also assessed the effects of PHS1, and siRNA-mediated ARF6 and Shp2 knockdown on ERK signaling, upon gradient SDF sensing. We identified a novel gradient SDF→CXCR4→Shp2→sustained ERK pathway, and demonstrated that Shp2 is differentially regulated upon SDF gradient sensing. These data provide further insight into the mechanism by which CXCR4 is regulated, and support a working model that ARF6 and Shp2 modulate CXCR4 phosphorylation and signaling in metastatic cancer cells, leading to dysregulated termination of CXCR4 signaling and trafficking of CXCR4 to endocytic sites.

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Coupling of excitable signaling and cytoskeletal systems mediates cell migration.

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Many cells migrate by stochastically extending protrusions such as pseudopodia or lamellipodia. These protrusions are associated with activations of signaling responses as well as the actin based cytoskeleton. It is not clear what triggers the signaling and cytoskeletal activities in the absence of stimuli or how each contributes to the cellular projections. Here we show that the signaling and cytoskeletal events are in slow and fast excitable systems, respectively, which can act independently. During migration the active phase of signaling coincides with the formation of large projections, but whose leading edges expand in a series of steps corresponding to short bursts of cytoskeletal activity. Blocking the signaling system inhibits large extensions and impairs migration although the cytoskeleton system continues to oscillate rapidly and the cell periphery undulates. These observations suggest that engagement of the "idling" motor force of the cytoskeleton by the intermittent, more prolonged activity of the signaling system acts as the pacemaker for cell migration.

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EGFR and Integrin $\alpha 2\beta 1$ Dependent Invasiveness of Lung Adenocarcinoma Cells that Survived 10 Gy Ionizing Radiation.

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Ionizing radiation (IR) enhanced tumor invasiveness is emerging as one mechanism responsible for the limited benefit of radiotherapy. However, the mechanism is unclear yet. In our previous study, we showed that subcloned lung adenocarcinoma cells (A549-P cells; abbreviated as P cells) that survived 10 Gy IR (A549-IR cells; abbreviated as IR cells) acquired high invasiveness *in vitro*. Here, we tried to identify the mechanism by which IR cells gain the increased invasiveness by examining altered gene expression and signaling pathway in IR cells compared

to P cells. To simulate the microenvironment *in vivo*, cells for analysis were embedded in 3-dimensional (3D) collagen type I matrices. Given that integrins play crucial roles for tumor cells by directly binding components of extracellular matrix and providing the traction necessary for cell motility and invasion, several integrins expression patterns in IR cells were compared with those in P cells. RT-PCR and immunoblotting results demonstrated that integrin $\alpha 2$ and $\beta 1$ expressions were elevated in IR cells. Knock-downs of their expressions by siRNA separately, or function blocking of integrin $\alpha 2\beta 1$ resulted in round morphology and abrogation of IR cells invasion. Additionally, immunofluorescence staining by antibody that specific to activated integrin $\alpha 2\beta 1$ indicated its activation only in collagen matrices, but not on glass substrate. Therefore, integrin $\alpha 2\beta 1$ was supposed to be an essential receptor that mediated cell spreading and invasion in 3D collagen. The epidermal growth factor receptor (EGFR) represents the main target for non-small cell lung cancer (NSCLC) therapy, as its overexpression or constitutive activation contributes to malignancy and correlates with poor prognosis. Indeed, in IR cells, activity of EGFR was significantly enhanced. Treatment of IR cells with selective EGFR tyrosine kinase inhibitor PD168393 showed that cell invasion, EGFR activation, extracellular signal-regulated kinase (ERK1/2) activation and integrin $\beta 1$ expression were inhibited in a dose-dependent manner. Taken together, our results suggest that overexpression of integrin $\alpha 2\beta 1$ and activation of EGFR cooperatively promoted higher invasiveness of IR resistant lung cancer cells, which could be the alternative targets to combine with radiotherapy.

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Examining the Role of mTOR in T Cell Proliferation and Migration Under Immunosuppression by Rapamycin.

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mTOR (mammalian target of rapamycin) is a serine/threonine protein kinase that has a role in cell growth and metabolism. mTOR is also the core of two functionally distinct complexes, mTORC1 and mTORC2. mTORC1 has a prominent role in cell growth and proliferation while mTORC2 has been shown to affect cell proliferation and cytoskeletal organization. However, the role of mTOR signaling in T cell migration is not fully understood. Studies were conducted to determine the effects of rapamycin, an immunosuppressant that targets mTOR, on Jurkat T cell proliferation and migration. Rapamycin treatment of Jurkat cells was expected to inhibit cell proliferation and cell migration due to its inhibition of normal mTOR signaling. Jurkat T cells were stimulated with PMA (50 ng/ml) and PHA (10ug/ml) in the presence or absence of rapamycin (5, 10, 20, 50 nM; 12, 24, and 48 hours). Cell counts at 12, 24, and 48 hours showed a concentration-dependent inhibition of cell proliferation by rapamycin; no significant effects were seen beyond 20 nM of rapamycin. Phosphorylated ERK expression was decreased in activated T cells treated with rapamycin as determined by Western blot analysis. Rapamycin also inhibited Jurkat T cell migration toward CXCL12. Results show that even in combination with activating agents, rapamycin inhibits T cell proliferation and migration, suggesting that mTOR signaling may also have an effect on ERK signaling and T cell chemotaxis.

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FXR regulates the cell proliferation of human kidney-derived cell line HK-2.

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Farnesoid X receptor (FXR) is a pivotal factor maintaining bile acid homeostasis and highly expressed in the liver, kidney, intestine, and adrenal gland. We have previously revealed that FXR controls the proliferation of hepatocellular carcinoma cells: FXR stimulates cell proliferation by suppressing the p16/INK4a expression whereas downregulation of the FXR stimulates the p16/INK4a expression, resulting in the suppressed cell proliferation. In the present study, we investigate whether or not FXR also plays a crucial role in the proliferation of human kidney-derived cell line, HK-2.

The treatment of HK-2 with FXR siRNA reduces the level of p16/INK4a expression resulting in the stimulation of cell proliferation. HGF treatment down-regulates the FXR expression resulting in the stimulation of cell proliferation, however, p16/INK4a expression level is elevated by HGF. In conclusion, FXR negatively regulates cell proliferation of kidney cells. HGF positively regulates kidney cell proliferation by downregulating the FXR expression in p16/INK4a-independent manner.

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Role of Inositol 1,4,5-triphosphate Receptors in Dendritic Cell migration and Antigen Presentation.

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Immature dendritic cells (DCs) reside in peripheral tissues where they sample their environment for the presence of antigens (Ags) by coupling locomotion to endocytosis. Upon encounter with infectious agents, they switch to a mature state and initiate the adaptive immune response by activating T cells in lymph nodes. Whereas mature dendritic cell migration to draining lymph nodes is driven by various chemokine receptors and adhesion molecules, the factors that drive the migration of immature DCs in peripheral tissues remains to be understood.

In migrating cells, calcium has a multifunctional role in directional sensing, polarization, cytoskeleton reorganization, traction force generation and relocation of focal adhesions. The main organelle that controls calcium-related cell functions is the endoplasmic reticulum (ER), which is a major calcium store. ER calcium is released upon binding of the second messenger, Inositol 1,4,5-triphosphate (IP3) to its receptors IPR1,2,3, which correspond to calcium channels located at the ER membrane. In this study, we address the role of the ER and IP3R in cell migration and antigen presentation by DCs.

We have found that Xestospongine C, an IP3R inhibitor, impairs the migration of immature DCs suggesting that ER calcium stores play a role in the sampling function of DCs. Using lentivirus-encoded shRNA, we silenced each type of IP3R (IP3R type 1,2 and 3) and analyzed their role in DC migration and polarization, cytoskeleton redistribution (actin, myosin IIA) and in antigen presentation. Interestingly, our results suggest that IP3R3 plays an important role in the function of immature DCs.

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Cellular pathways involved in epithelial-to-mesenchymal transitions in neural crest cells.

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Neural crest cells are a population of multi-potent progenitor cells in the developing vertebrate embryo that undergo an epithelial-to-mesenchymal transition (EMT) from the dorsal neural tube and migrate extensively to generate diverse derivatives. Abnormal development of neural crest cells (NCC) can lead to neurocristopathies or even cancers. Both internal biochemical signals and external mechanical factors play essential roles in facilitating neural crest cell EMT. How cells modulate their adhesion machinery and dynamically reorganize their actin cytoskeleton to respond to the mechanical features of their external environment during EMT is not well understood. To evaluate the role of the actomyosin cytoskeleton during neural crest cell EMT and migration, midbrain neural folds that contain premigratory neural crest cells were dissected out from chick embryos, explanted into chamber slides, and incubated to allow for the formation of migratory neural crest cells. Time-lapse imaging revealed that midbrain neural crest cells spread out from the center of these explants with a velocity around 0.4 $\mu\text{m}/\text{min}$ and form a layer of cells with lamellipodial structures. To elucidate cellular pathways controlling EMT and migration, chemical inhibitors (blebbistatin, Y-27632, latrunculin, nocodazole) that perturb molecular cascades regulating cellular structures were employed. Effects of these perturbations on neural crest cell EMT and migration were quantified in terms of the spreading rate of the explants, velocity distributions of individual cells and vorticity of collectively moving cell groups. We observed that blebbistatin treatment reduces the overall velocity of moving neural crest cells to negligible levels. Moreover, migratory neural cells developed rounder cell bodies, and lamellipodia were transformed into filopodia at the periphery of the extract. These results potentially implicate a role for non-muscle myosin II, the target of blebbistatin, in chick midbrain neural crest cell EMT and migration. Experiments on non-muscle myosin II loss-of-function will further validate this finding. Actin crosslinkers such as α -actinin also participate in pathways affected by these cytoskeletal inhibitors through their regulation of focal adhesion formation and cytoskeletal organization, thereby modulating cell stiffness and migration. We have documented the changes in the distribution of the actin-crosslinking protein α -actinin in migratory neural crest cells after the treatment of explants with these inhibitors. Collectively, our studies give insight into specific cellular pathways regulating neural crest cell EMT and migration and the impact on various biophysical parameters upon perturbation of these pathways.

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Reversible host cell transformation by *Theileria annulata* uncovers crosstalk between inflammatory cytokine signalling and actin cytoskeleton dynamics.

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Oncogenic transformation of mammalian cells is mostly irreversible and leads to aberrant cell regulation including enhanced cytoskeleton dynamics, cell motility and invasiveness. In contrast, oncogenic host cell transformation by the intracellular protozoan parasite *Theileria annulata* and the acquisition of metastatic properties by the host cell can be attenuated by drug-mediated elimination of the parasite. We are using this reversible model of cancer to study transformation-dependent actin cytoskeleton dynamics and cell motility.

Mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4), alternatively named HGK or NIK, is a Ste20 family germinal centre serine/threonine kinase. MAP4K4 regulates cytoskeleton

dynamics and cell motility on one hand, and TNF α -regulated glucose metabolism on the other hand. Cells transformed by *Theileria* produce a cocktail of cytokines including TNF α and display morphological alterations and aberrant motility *in vitro* and actively disseminate *in vivo*. Here we show that host MAP4K4 is recruited to the parasite surface and accumulates in actin-rich host cell lamellipodia in a parasite-dependent manner. Elimination of the parasite leads to decreased MAP4K4 protein expression and kinase activity, whereas silencing of MAP4K4 in infected cells by siRNA impairs lamellipodia formation and cell motility. MAP4K4 expression in infected cells increases in the presence of conditioned medium or when non-conditioned medium is substituted with recombinant TNF α . TNF α -induced MAP4K4 expression is mediated through JNK, while silencing MAP4K4 can block JNK activation. Thus, parasite-induced TNF α secretion stimulates MAP4K4 expression and JNK pathway activation through a positive feedback. Interestingly, unlike MAP4K4 protein, mRNA expression levels of MAP4K4 increased after parasite elimination. We explain this conundrum by two mechanisms: First, intracellular *T. annulata* sequestered host cell p53, which after parasite elimination is released to the nucleus to induce MAP4K4 expression. Second, elimination of parasite enhances miRNA targeting of MAP4K4-3'UTR sequence, suggesting parasite regulation MAP4K4 through transcriptional and post-transcriptional mechanisms.

Thus, using a reversible cancer model system we revealed a novel, tightly controlled crosstalk between inflammatory cytokine signalling and actin cytoskeleton dynamics, which is likely involved in human inflammatory and motility disorders.

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Electrospun Poly-L-Lactic acid substrate alters migration and signaling pathway regulation in astrocytes.

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The body cannot naturally recover completely from traumatic spinal cord injury due to the rapid formation and prolonged presence of the glial scar resulting in patient paralysis. Although the glial scar supports the damaged barrier between cerebrospinal fluid and circulatory blood it also creates a barrier to neuronal repair through reactive astrogliosis and astrocyte expression of chemorepellants such as neurocan. Prior work indicates that astrocytes grown on electrospun Poly-L-Lactic acid (PLLA) fibers produce less neurocan and thus PLLA fiber substrates create a chemically permissive environment for neuronal repair and formation of functional connections. Here we used a novel cloning cylinder based assay to measure migration on both random and aligned spun PLLA fibers. Changes in migratory behavior are determined through measurement of the samples feret's diameter (major and minor axis) as well as total sample entropy. To simulate migratory stimulation and inhibition, non-hydrolyzable GTP analogues and phosphatidyl-inositol-3-kinase inhibitors are utilized as respective on and off switches for migration. Migratory signaling pathways differ on random and aligned fibers through application of stimulatory and inhibitory treatments as evidenced by altered migratory parameters. Differences in cellular response on random and aligned fibers indicate that the PLLA substrate type is the determining factor in regulation of migratory behavior as well as alteration of signaling pathways.

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RACK1 and vimentin complex to regulate FAK during endothelial invasion.

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Angiogenesis is defined as formation of new blood vessels from preexisting ones and is critical for many physiological and pathological processes including, but not limited to, wound healing and tumor growth. To identify molecules relevant to angiogenesis, we performed a proteomic screen comparing invading versus non-invading endothelial cells (ECs) using angiogenic growth factors (GFs) and sphingosine 1-phosphate (S1P) in three-dimensional collagen matrices. We found up-regulated levels of Receptor for Activated C Kinase 1 (RACK1) and the major intermediate filament protein, vimentin that correlated with increased EC invasion. Because both RACK1 and vimentin have been linked to focal adhesion kinase (FAK), we investigated whether this pathway played a role in EC invasion. RACK1 depletion reduced invasion responses in ECs, which was associated with attenuated activation of FAK. Knockdown of vimentin blocked invasion responses and significantly decreased levels of phosphorylated and total FAK. Treatment of ECs with pharmacological inhibitor of FAK dose-dependently reduced invasion indicating a crucial role for FAK activity during EC invasion. Because RACK1 and vimentin were both up-regulated with S1P treatment, required for EC invasion, and regulated FAK, we tested whether they complexed together in ECs. RACK1 complexed with vimentin, and GFs enhanced this interaction. To further understand the interplay between RACK1, vimentin, and FAK, we tested whether RACK1 and vimentin associated with FAK during three-dimensional endothelial invasion. We observed that RACK1, vimentin and FAK complexed together in invading endothelial cultures. Moreover, depletion of RACK1 decreased association between vimentin and FAK, suggesting that RACK1 was required for stabilizing vimentin-FAK interaction during EC sprouting. Taken together, these results demonstrate that pro-angiogenic signals (S1P and GFs) converge to enhance expression and association of vimentin and RACK1 which act upstream to regulate FAK, resulting in successful endothelial sprout formation in three-dimensional collagen matrices.

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Investigating the role of methylation in neural crest migration.

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Neural crest cells are multipotent cells that migrate extensively to give rise to diverse structures throughout vertebrate embryos. Research has shown that a network of transcription factors specifies neural crest precursors at the borders of the neural plate, and that histone methylation regulates expression of these transcription factors. Our objective was to determine whether protein methylation also regulates neural crest migration. Indeed, knock down of an essential methylation cycle enzyme, S-adenosyl homocysteine hydrolase (SAHH), by morpholino electroporation into chick embryos impedes neural crest migration. Moreover, pharmacological inhibition of SAHH in chick neural tube cultures causes a vast reduction in the number and polarity of migratory neural crest cells, suggesting that methylation is essential for neural crest migration. In embryos, newly emerging and actively migrating cranial neural crest cells exhibit elevated levels of lysine-methylated proteins by immunofluorescence. Further analysis of individual migratory neural crest cells shows methyl-lysine immunoreactivity in the cytoplasm, suggesting a role for non-histone protein methylation in migratory neural crest cells. Proteomic profiling of cytoplasmic proteins with mono- and di-methylated lysines in premigratory and migratory neural crest cells has identified 188 putatively methylated proteins. A large subgroup of these proteins regulate the cytoskeleton, and post-translational methylation of these proteins

may be involved in regulating their function to control neural crest cell motility. Several of the proteins identified were found only in the migratory population, raising the possibility that changes in methylation status may be able to dynamically regulate neural crest migration. We have characterized the localization of three of these proteins, actin depolymerizing factor, myosin 9 and tropomyosin-1-alpha, in neural crest cells and are working to identify the methylated residue within each of these proteins. Functional analysis of these proteins is also underway to determine the importance of their methylation for neural crest migration.

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Deletion of LRP5 prevents intraretinal angiogenesis caused by VLDLR mutations.

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This study aims to understand the underlying mechanisms for the opposite retinal vasculature phenotypes in the loss-of-function mutant mice of low-density lipoprotein receptor-related protein 5 (LRP5) and very low-density lipoprotein receptor (VLDLR), and to characterize the retinal vasculature defect in mice lacking both LRP5 and VLDLR. Fundus examination, fluorescein angiography, immunostaining, and 3-dimensional retinal vasculature reconstruction are performed to evaluate the molecular and cellular alterations of retinal vasculature in mutant animals. Our recent studies indicate that VLDLR and LRP5, members of the LDL receptor family, play opposite roles during intraretinal angiogenesis. A loss of LRP5 in mice leads to an undergrowth of the intraretinal vasculature, associated with the formation of clusters of endothelial cells (ECs) in the nerve fiber layer, and a failure of ECs to migrate into deeper retinal layers. In contrast, VLDLR knockout mice show retinal ECs penetrate through the photoreceptor cell layer to cause an overgrowth of the intraretinal vasculature and subretinal neovascularization. However, the genetic hierarchy of pro-angiogenic signals mediated by LRP5 versus anti-angiogenic signals mediated by VLDLR during retinal angiogenesis remains unclear. We have tested whether LRP5 is crucial or dispensable for the neovascularization caused by VLDLR gene mutations by generating and characterizing the LRP5/VLDLR double knockout (DKO) mice. Our results reveal that retinal vessels fail to penetrate into the photoreceptor layer in DKO mice, and an enormous number of ECs clusters appear in the nerve fiber cell layer. Thus, the presence of LRP5 is a prerequisite for the overgrowth of retinal vasculature in VLDLR knockout mice. More importantly, a functional loss of LRP5 is sufficient to prevent neovascularization caused by the VLDLR knockout mutation. LRP5 may be an effective target for inhibiting intraretinal neovascularization.

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Analysis of signaling pathways mediating in vitro reendothelialization.

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The molecular mechanisms mediating the process of large vessel reendothelialization have clinical relevance to recovery from balloon denudation and stent implantation. However, the details of the pathways underlying reendothelialization are incompletely understood, in part because of the lack of an *in vitro* model of reendothelialization that permits biochemical analysis. To fulfill the need for such a model of reendothelialization and other wound healing processes, we developed a simple method to apply a reproducible high density of linear scratch wounds to cell monolayers. This method leaves a high proportion of cells at wound edges to initiate migration and thus permits sensitive detection of biochemical changes selectively occurring in this migrating cell population. The key component of the method is a comb-like

device that, when applied to a cell monolayer in a rectangular tissue culture plate, leaves 1.5 mm wide strips of cells separated by 1 mm gaps, with minimal abrasion of the underlying culture surface. We employed this method to perform detailed biochemical characterization of the response of human umbilical vein endothelial cells (HUVECs) to wounding. Intracellular signaling pathways were analyzed using multiplexed bead-based immunoassays and Western blotting at several time points following scratch wounding. Among the MAPK signaling pathways, selective engagement of the Erk1/2 pathways was observed, with elevated pErk1/2 and pMEK1 within 30 min of wounding, followed by a gradual decline in signal. Increased phosphorylation was also observed for components of the Akt signaling pathway, including Akt, p70S6K, and GSK3 α/β , with similar kinetics as for pErk1/2. Secretion of cytokines by wounded or intact HUVEC monolayers was also analyzed using multiplexed, bead-based immunoassays. Increased secretion of the pro-inflammatory cytokines GRO α , IL-8 and MCP-1 was detected at 30 and 90 min after wounding, but levels of those cytokines normalized after 4 h. In contrast, secretion of angiopoietin-2 and endothelin-1 was decreased at 30 and 90 min after wounding. The results demonstrate that upon wounding, endothelial monolayers respond with a burst of signals that favor growth and migration, promote immune cell recruitment, and reduce vasoconstriction. Thus, this high density cell scratch method provides an *in vitro* model of reendothelialization that enables biochemical characterization of the key signaling nodes determining a pathological or favorable wound healing response.

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Bicarbonate-mediated nematode sperm maturation involves soluble adenylyl cyclase (sAC) activity.

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The sperm must undergo a maturation process called sperm capacitation in mammals and sperm activation in nematodes, which consists of morphological, biochemical and physiological changes in sperm necessary to acquire fertilizing potential. It has demonstrated that bicarbonate is essential for mammalian sperm capacitation. Different from flagellated sperm, nematode sperm are crawling cells and their motility is driven by the cytoskeleton composed of major sperm protein (MSP). Bicarbonate is required for *Ascaris* sperm activation; however, its role remains unknown. Our studies showed that sperm in bicarbonate-free buffer could not extend the normal lamellipodium. In addition, the increase of the bicarbonate concentration could promote the MSP cytoskeleton assembly in the absence of sperm activator, These data suggest the bicarbonate is required for the MSP cytoskeleton assembly. Subsequently, we found the Anion Exchanger (AE) and CFTR were involved in nematode sperm activation because the inhibitors of AE and CFTR blocked the sperm activation, indicating that bicarbonate entry into sperm cells is mediated by AE and CFTR channels. Furthermore, the cAMP level increase was observed during sperm activation and the increase of bicarbonate concentration significantly raised the intracellular cAMP level, indicating that bicarbonate might directly stimulate soluble adenylyl cyclase (sAC) activity and generate the cAMP, as observed during flagellated sperm capacitation. While pre-treatment of sperm with inhibitor of phosphodiesterase, which degrades cAMP into 5'-AMP, tremendously increased the intracellular cAMP level and in turn inhibited the sperm activation. These data suggested the microdomain organization of cAMP signaling is important for nematode sperm activation. Taken together, our data demonstrated that bicarbonate might function in nematode sperm to stimulate an evolutionarily conserved soluble adenylyl cyclase generating cAMP as second messenger for signal transduction during sperm activation, though the morphology of nematode sperm and their molecular machinery for motility are unlike that in flagellated sperm.

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Repression and Habituation In Diatom Light-stimulated Motility Responses.S. A. Cohn¹, T. S. Kordes¹, A. L. Wolske¹; ¹Biological Sciences, DePaul University, Chicago, IL

Most pennate diatoms move using a form of gliding motility with the ability to change their direction of movement based on the local light irradiance detected at the tips of the cells (Diat. Res. 14:193; 19:167). Light exposure at the ends of cells can cause cells to either reverse direction and move out of the light, or move into the light, depending on the wavelength and intensity of the light exposure. Our work has confirmed that this light-stimulated effect is characteristic for each species with regards to both wavelength and irradiance sensitivity. In order to test for lingering effects of the light exposures (i.e. if initial exposures enhance or repress the responses of subsequent exposures) we measured the direction change response times for leading-end irradiations after the cells were previously irradiated at the leading or trailing ends. We found that high-energy irradiation at the trailing tip of *Craticula cuspidata* cells causes a rapid but quickly diminishing repression of direction change. For example, compared to a control leading-end response time of 14 ± 2 sec for blue light (145nm), an exposure to the trailing end 5 sec prior to the leading end exposure increases the response time to 147 ± 57 sec. Exposure with red light (165nm) 5 sec prior to the leading end exposure also generates a repression (97 ± 36 sec, compared to 25 ± 5 sec for control cells). This repression by prior trailing end exposures is almost completely gone within 30 sec. A prior exposure of *Craticula* cells on the leading end causes about a 1.5-fold repression of a second leading end exposure that peaks 20-60 sec after exposure and lasts for about 75 sec. *Pinnularia viridis* cells show similar types of responses, with blue trailing end exposures increasing subsequent response times to 187 ± 30 sec, compared to control response times of 14 ± 2 sec; the repression by prior front end exposures are 2-2.5 fold and peak about 15 sec after exposure. *Stauroneis phoenicenteron* cells also show rear-end repression, increasing response times from 41 ± 6 sec to 166 ± 40 sec. The peak of the trailing end repression varies between the three species we tested, with the repression in *Craticula* cells peaking at 5 sec after light exposure, compared with 10-15sec for *Pinnularia* cells, and 20 sec for *Stauroneis* cells. The repression effect generated by front end exposure can also be habituated by multiple exposures. For, example, initial blue front end exposures in *Craticula* repress response time from 18 ± 1 sec to 31 ± 4 sec, but exposing cells to sequential irradiations reduces subsequent response times back to control levels (20 ± 1 sec) after 10-12 consecutive exposures. This work was supported by the DePaul College of Science and Health, and equipment purchased through NSF Grant IBN-9982897.

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Inversin is required for fibroblast polarity and directional cell migration.

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Inversin is an enigmatic protein with various interacting partners and functions, some of which are associated with the primary cilium, a microtubule-based, sensory organelle controlling a number of signaling pathways during development and in tissue homeostasis. Inversin regulates Wnt signaling by targeting Dishevelled (Dvl) for degradation and critically regulates developmental processes in vertebrates (Lienkamp et al., 2012). However, little is known on the

roles of Inversin in cytoskeletal reorganization and regulation of key proteins in cell polarity processes during directional cell migration.

Here we investigated the role of Inversin in these processes using cultures of mouse embryonic fibroblasts (MEFs) derived from *inv*^{-/-} and *inv*^{+/+} animals. Initially, we confirmed that both *inv*^{-/-} and *inv*^{+/+} MEFs form primary cilia, and that Inversin localizes to the primary cilium in *inv*^{+/+} MEFs as evidenced by IFM analysis with anti-Inversin antibodies and by transfection with GFP-Inversin. Further, *inv*^{-/-} MEFs displayed severely compromised migratory abilities and cytoskeletal rearrangements in wound healing assays, including distorted lamellipodia formation and cilia orientation. Transcriptomic analysis on differentially expressed genes in co-regulated gene networks revealed that several pathways are affected in *inv*^{-/-} MEFs, including Wnt signaling and regulating actin organization and focal adhesions, as compared to *inv*^{+/+} MEFs. Further, Dvl-1 and Dvl-3 localized to MEF primary cilia, and β -catenin/Wnt signaling was elevated in *inv*^{-/-} MEFs, which also showed reduced ciliary localization of Dvl-3. Furthermore, *inv*^{-/-} MEFs display dramatically altered activity and/or localization of RhoA, Rac1, and Cdc42 GTPases in addition to aberrant expression and targeting of the Na⁺/H⁺ exchanger NHE1 and of ERM proteins to the edge of cells facing the wound. Many of these defects were rescued by re-expression of GFP-Inversin in *inv*^{-/-} MEFs, supporting the conclusion that Inversin plays a critical role in controlling the migratory behavior of fibroblasts, at least in part through transcriptional regulation of genes involved in Wnt/PCP signaling and pathways controlling cytoskeletal organization.

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Two Hox Genes Coordinate the Expression of a Transmembrane Protein MIG-13 that Specifies Anteroposterior Polarity in Cell Migration.

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Directed cell migration is fundamental for metazoan development. In *C. elegans*, two homobox genes, *lin-39* and *mab-5*, respectively control anterior migration of Q neuroblast on the right side and posterior migration of Q neuroblast on the left side of the animal. However, their targets and underlying cellular mechanism remain elusive. Here, we found that MIG-13, an evolutionarily conserved transmembrane protein, functions cell-autonomously in specifying anteroposterior cell polarity, and MIG-13 is a novel target of LIN-39 and MAB-5. In QR descendants, anterior cell polarity is reduced in *mig-13* or *lin-39* mutant; LIN-39 binds to 5' UTR of *mig-13* and the binding is essential for *mig-13* expression and anterior migration. In QL lineage, MAB-5 inhibits *mig-13* expression, and ectopic expression of *mig-13* disrupts posterior cell polarity and reduces posterior migration. Our data thus revealed that LIN-39 and MAB-5 coordinate the expression of MIG-13 in migrating cells to specify anteroposterior polarity.

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Functional role of let-7 microRNA family during alcoholic liver injury.

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Background: microRNAs are endogenous molecules which constitute a new class of negative regulators of gene expression. Ethanol is an etiologic agent in hepatic disease and new

evidence implicated that ethanol-sensitive microRNAs (miRNAs) are indeed regulatory masterswitches during liver injuries. The current study was aimed to characterize the functional role of specific miRNAs during hepatic injury in intragastric overfeeding mice *in vivo* and LPS treated human hepatic stellate cells (HSCs) and normal hepatocytes (N-Heps) *in vitro*. Methods: miRNA expression in 4 weeks chronic alcohol feeding mouse liver specimen and control liver tissue, as well as in LPS treated hepatic HSCs and N-Heps was assessed using a hybridization based microarray. Expression of selected mature miRNAs was evaluated by real-time PCR. The effect of selected miRNA on cell migration was assessed in hepatic stellate cells using miRNA precursor to increase cellular expression. Tissue remodeling potentials were further measured by matrix metalloproteinase activity using real-time PCR and zymogen gel assay. Results: Along with the enhanced hepatocellular apoptosis after 4 weeks ethanol feeding, we identified significantly up-regulated 0.8% and down-regulated 0.9% of known miRNA compared to controls. The down-regulated miRNAs, including let-7 family members, were further verified by Taqman real-time PCR assay. Treatment of hepatic stellate cells and N-Heps with LPS (20 ug/ml) for 48 hours induced a significant decrease of let-7b, as well as a moderate down-regulation of let-7a. Over-expression of let-7b and let-7a blocked LPS-induced cell migration in both HSCs and N-Heps tested. Bioinformatics and dual-luciferase reporter assays identified toll-like receptor 4 (TLR4) as a direct target of let-7 miRNAs in both HSCs and N-Hep cells. While TLR4 expression in HSCs and N-Hep cells was significantly increased after LPS treatment, inhibition of let-7b and let-7a also resulted in an increase of TLR4 level, and enhanced HSC and N-Hep cell motility. Additionally, modulation of let-7b and let-7a also altered expression of matrix metalloproteinases 2 and 9, mediators involved in tissue remodeling during alcoholic liver injury. Furthermore, the expressions of TLR4, MMP-2 and MMP-9 were significantly altered in chronic ethanol feeding mouse liver specimens compared to controls. Conclusion: Our results show that miRNAs are critical regulators of hepatic cell migration and remodeling during alcoholic liver injury. The findings provide new insight into the function of specific miRNAs and the mechanisms of alcohol induced liver injury and tissue repair.

Cytoskeletal Membrane Interactions

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Molecular basis for CARMIL function in lamellipodia.

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Coordinated intracellular rearrangement of the actin cytoskeleton in response to extracellular cues is an important theme in cell motility. CARMIL is emerging as a crucial regulator of lamellipodia-based motility, linking GTPase signaling to actin filament dynamics at the leading edge. CARMIL's role in regulating lamellipodium formation and maintenance makes it an important player in tumor metastasis, neuronal development, and cell motility. However, the molecular basis for CARMIL's role in these cellular processes is unclear. The objective of this study is to determine the molecular mechanism by which CARMIL affects lamellipodia formation. We primarily used the methods of x-ray crystallography and small-angle x-ray scattering (SAXS), as well as biochemical and biophysical approaches for this objective. The main results of this study are: (1) we demonstrate for the first time that CARMIL can bind specific types of lipids *in vitro*. (2) Using site-directed mutagenesis, we identify several residues involved in membrane interaction. (3) Maximum lipid-binding activity requires a dimerization domain, which does not directly contribute to membrane binding. (4) X-ray crystallographic studies of CARMIL address the structural basis for membrane binding by CARMIL. Our main conclusions are that CARMIL is capable of directly binding the plasma membrane, leading to

co-localization at the lamellipodium leading edge with its known binding partners Capping Protein (CP) and Myosin I to regulate actin dynamics. CARMIL's lipid-binding specificity provides a possible mechanism for further spatiotemporal regulation of lamellipodia. CARMIL dimerization may play a role in signal transduction by CARMIL from the membrane to the actin cytoskeleton, in addition to enhancing membrane interaction. Our results addressing CARMIL's mechanism of action in lamellipodial dynamics will likely also be relevant to its role in neuronal development and tumor metastasis.

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Structural and functional bases for auto-inhibition of IRSp53 and activation by Cdc42.

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Cell migration is a key step in tumor invasion and metastasis, involving dynamic reorganization of the actin cytoskeleton and membranes. BAR domain proteins play important roles in such processes by linking membrane and actin cytoskeleton dynamics to signaling pathways controlling actin cytoskeleton reorganization. IRSp53 is a BAR domain protein that has received significant attention because of its critical involvement in lamellipodia- and filopodia-based cell motility under the control of Rho-family GTPases, making it a key player in tumor metastasis. However, little is known about the mechanisms of regulation of BAR domain-containing proteins in general and IRSp53 in particular. IRSp53 is a multi-domain protein, containing an N-terminal inverted BAR (I-BAR) domain that senses and/or induces negative membrane curvature and a C-terminal SH3 domain that binds proline-rich regions in a wide range of actin cytoskeleton proteins. The flexible linker joining the I-BAR and the SH3 domains is rich in proline residues and contains a semi-CRIB motif that has been implicated in Rho-family GTPase binding. Here, we reveal the structural and functional bases for IRSp53 regulation, including autoinhibitory interactions and activation by Cdc42. Using a combination of structural and biochemical methods, we show that the CRIB motif of IRSp53 is unique in that it provides a binding platform for both the SH3 domain of IRSp53 (autoinhibition) and GTP-Cdc42 (activation). As a consequence, we now refer to this motif as the "CRIB-Pro motif". Using a FRET assay, we further show that full-length IRSp53 undergoes a large conformational change upon binding of GTP-Cdc42, consistent with the release of autoinhibitory interactions. A crystal structure of a complex between GTP-Cdc42 and the CRIB-Pro motif shows that the proline-rich region of the motif interacts with Cdc42 through a surface different from that of canonical CRIB domains. Mutagenesis studies in vitro and in vivo corroborate the importance of these interactions, indicating that the CRIB-Pro motif is uniquely adapted to serve as Cdc42-dependent switch to relieve SH3-mediated autoinhibitory interactions in IRSp53.

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Actin turnover in lamellipodial fragments.

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Actin treadmilling is the central mechanism driving cell motility. While its biochemical components are largely known and their properties studied extensively in vitro, quantitative details of actin dynamics in vivo are not completely understood. The actin treadmill in its purest form exists in lamellipodia, and its biophysics is well studied in fish epithelial keratocytes. We focus on lamellipodial fragments from keratocytes, which lack a cell body but retain the ability to crawl with the speed and persistence of whole cells. We measured spatial distributions of F-

actin, G-actin and uncapped barbed ends of actin filaments and found that the F-actin density decreases away from the leading edge, G-actin density increases away from the leading edge, and that the uncapped barbed ends, though most dense at the leading edge, are distributed across the whole lamellipodium. We compared these data with a simple mathematical model describing F-actin rearward flow, G-actin diffusion and distributed actin assembly and disassembly. Model predictions quantitatively agree with data and, interestingly, predict that most actin monomers at the leading edge must be unavailable for polymerization in order to account for the observed motility speed. A more elaborate model incorporating (i) sequestration of monomers by β -thymosin, (ii) ADP-ATP exchange and (iii) oligomers, which diffuse and disassemble into monomers, can quantitatively match observed distributions and speeds. Our results provide a quantitative understanding of lamellipodial actin turnover and make predictions for pathways of actin disassembly and concentrations of actin accessory proteins. Most importantly, the model predicts that actin turnover in the lamellipodium is local, in contrast to the widely-assumed global transport of G-actin across the whole cell.

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Amoeboid motility is dependent on the extent of cell confinement and regulated by intracellular pressure.

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Mesenchymal cell motility, driven by actin polymerization and focal adhesion assembly, had been widely implicated in biological cell migration. However, some cells such as leukocytes and tumor cells were found to migrate with bleb-like protrusions initially devoid of F-actin, and independent of cell-substrate adhesion. The mechanism underlying this amoeboid-like motility remains vague.

In this study, neutrophil-like differentiated HL60 cells were confined between two polyacrylamide substrates embedded with fluorescent beads. Cells were found to exhibit two distinct modes of motility depending on the availability of cell-substrate adhesions: lamellipodia-driven (mesenchymal) motility in the presence of fibronectin-coated substrates; and bleb-driven (amoeboid) motility in the absence of fibronectin-coated substrates. Three-dimensional forces involved during cell migration were also calculated from bead displacements.

We also found that mesenchymal migration speed is dependent on substrate rigidity, with largest cell speed at intermediate substrate rigidity. Conversely, amoeboid migration speed is dependent on the gap size between substrates, with maximal cell speed occurring at intermediate gap size. Amoeboid cell migration was modelled computationally and results suggest amoeboid motility is regulated by intracellular pressure and the strength of cell-membrane to actin-cortex adhesion. Collectively, our results provide insights into mechanistic differences in mesenchymal and amoeboid motility which could potentially aid in developing therapies to prevent cancer metastasis.

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The balance between cytoskeletal dynamics and membrane tension determine the number of leading edges in keratocytes.

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Rapidly motile cells such as neutrophils and keratocytes polarize to generate a single front and rear in order to sustain productive forward movement. However, other less persistent cell types, such as fibroblasts and many types of epithelial cells, frequently have multiple, apparently independent, protrusive fronts. The mechanisms that limit polarization to a single front and rear for rapidly motile cells are largely unknown. Here we use embryonic zebrafish keratocytes to investigate how a purely lamellipodial cytoskeleton self-organizes to regulate protrusion number and overall cell polarity. We have observed that keratocytes derived from 4 days post fertilization (dpf) embryos often form multiple protrusions, suggesting that the motility machinery in these cells polarizes differently from “single front” keratocytes such as those found at 2dpf. Preliminary experiments with the “multiple front” 4dpf keratocytes indicate that they can be induced to form a single leading edge upon increasing membrane tension with hypoosmotic shock. Furthermore, F-actin density is decreased in 4dpf cells as compared with 2dpf keratocytes. These results suggest that the dynamics of the actin cytoskeleton can exist in at least two different regimes, depending on whether membrane tension or the intrinsic turnover of the actin cytoskeleton limits polymerization. Using RNA sequencing, we have measured gene expression levels in 2dpf and 4dpf keratocytes and found several cytoskeleton-related genes that are differentially expressed. Future studies will attempt to identify the molecular basis for how keratocytes regulate their polarization.

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Local shape control by myosin II guides endothelial cell morphology and migration in 3D environments.

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Cell shape is fundamental to myriad cellular functions, from neuronal connectivity to erythrocyte gas exchange. In angiogenesis, vascular endothelial tip cells adopt a highly extended ‘arboreal’ morphology while migrating through three dimensional extracellular matrices, displaying numerous dynamic pseudopodial branches that explore the environment and guide their movement. To gain insight into the function and regulation of endothelial cell morphology during migration, we developed a novel image analysis method for quantifying cell shape and subcellular protein localization over a range of length and time scales. At the cell scale this revealed a cyclical relationship between cell branching and cell migration efficiency, whereby phases of exploratory branch extension and cell body translocation are temporally separated. Perturbations of myosin II function or regulation break this cycle, altering branch number, directionality and structure, and hindering migration by preventing the transition between the exploratory and migratory phases. Quantitative analysis of the cell morphologies displayed during migration, and in the presence of myosin II perturbations, implied a local mechanism of cell shape regulation by actomyosin contractility. We investigated this mechanism at the sub-cellular scale by quantifying both subcellular fluorescence localization and local membrane geometry. This revealed a relationship between myosin II accumulation and membrane curvature, further supporting a local mechanism of cell shape control. Our analysis thus shows that regulation of actomyosin contractility contributes to complex cell morphologies both locally

and globally, helping to guide endothelial cell pathfinding during angiogenesis. This provides a link between nanometer-scale cytoskeletal organization and cell-scale geometry, helping to bridge the length-scale gap between small spatiotemporal length scale molecular mechanisms and the final, larger length-scale output of cytoskeletal function, namely cell morphology and migration.

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Interplay between cytoskeletal forces, membrane tension, hydrostatic pressure, and shape in rapidly migrating cells.

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In motile cells, membrane tension and shape are believed to emerge from the interplay of cytoskeletal forces and the hydrostatic pressure difference across the membrane. In particular, it is believed that actin polymerization pressure drives the formation of thin cell protrusions such as the lamellipodia and filopodia, while the hydrostatic pressure difference promotes the emergence of rounded blebs, but does not contribute to lamellipodia. To get insight into the balance of forces at the membrane interface, we measured membrane tension (with tether assay), protrusion velocity, and the 3D shape (by fluorescence displacement method) of migrating fish epidermal keratocytes subjected to volume perturbations and cytoskeletal drugs. Hypoosmotic treatment (medium dilution) induced cell swelling, slight increase in membrane tension and significant increase in protrusion velocity, while hyperosmotic treatment (addition of sucrose to the culture medium) induced cell shrinkage, transient decrease of membrane tension, and sustained decrease in protrusion velocity. The increase of protrusion velocity upon hypoosmotic treatment was not due to regulation of protrusive machinery by ion concentration, because treatment with the diluted medium supplemented with sucrose (to compensate for osmotic pressure difference) had no such effect. Inhibiting myosin-dependent contractility with blebbistatin induced cell flattening and fragmentation, reduced protrusion velocity and resulted in the appearance of cell populations with membrane tension both higher and lower than the control values. Remarkably, hypoosmotic treatment normalized blebbistatin-treated cells by restoring their tension, protrusion velocity, and shape. Across these differently treated groups of cells, as well as within each group, no clear positive or negative correlation between protrusion velocity and membrane tension was observed, but instead high protrusion velocity seemed to coincide with increased cell volume and/or elevated vertical profile. We propose that hydrostatic pressure together with myosin-dependent contractility maintain elevated cell profile, thus facilitating diffusion of actin subunits and increasing the lamellipodia protrusion velocity. Supported by Swiss National Science Foundation and Swiss SystemsX.

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Revisiting lamella hypothesis and myosin II-induced actin disassembly using a high-resolution single-molecule speckle microscopy with a new actin probe polymerizable to formin-assembled actin filaments.

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Single-molecule speckle (SiMS) microscopy has been a powerful method to analyze actin dynamics *in vivo* by tracking single-molecules (speckles) of EGFP-actin. However, there have been warnings that EGFP-actin might influence actin dynamics and interfere with processive actin polymerization by formins. To overcome the problems, we developed improved methods of

SiMS microscopy using an actin probe labeled with a fluorescent DyLight dye on lysine side chains. By using an *in vitro* microscopic assay, we verified that DyLight (DL)-actin retains the ability to collaborate with formin and profilin. To deliver DL-actin into cells, we developed a method that enables incorporation of DL-actin in the cytoplasm at a very low density with a high success rate. In cells, DL-actin labeled cellular actin networks with improved photostability and brightness. These favorable properties of DL-actin in combination with the Speckle TrackerJ software extends the application of speckle analysis to *in vivo* nanometer-scale displacement analysis. We reevaluated whether two F-actin populations with distinct retrograde flow velocities coexist in lamellipodia, as postulated in the lamella hypothesis (Ponti et al, *Science*, 2004), including short-lived speckles (lifetime <10 sec) that moved within a few hundred nanometers. Our data show that F-actin undergoes uniform retrograde movement with a monotonous flow speed regardless of their lifetimes. We also addressed the role of actin-myosin contraction on actin turnover in lamellipodia and lamella with a myosin II ATPase inhibitor blebbistatin. Our new method extends SiMS analysis to monitoring actin dynamics with nanometer-scale and order-of-a-second resolution, which is useful for reevaluating remaining issues in previous studies.

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Arp2/3 Inhibition induces amoeboid-like protrusions in MCF10A epithelial cells by reduced cytoskeletal membrane coupling and focal adhesion assembly.

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Here we demonstrate that Arp2/3 regulates a transition between mesenchymal and amoeboid protrusions in MCF10A epithelial cells. Using genetic and pharmacological means, we first show Arp2/3 inhibition impairs directed cell migration. Arp2/3 inhibition results in a dramatically impaired cell adhesion, causing deficient cell attachment and spreading to ECM as well as an 8-fold decrease in nascent adhesion assembly at the leading edge. While Arp2/3 does not play a significant role in myosin-dependent adhesion elongation, mature focal adhesions undergo large scale movements against the ECM suggesting a reduced ECM coupling. Cell edge protrusions occur at similar rates when Arp2/3 is inhibited but their morphology is dramatically altered. Persistent lamellipodia are abrogated and we observe a markedly increased incidence of blebbing and unstable pseudopods. Micropipette-aspiration assays indicate that Arp2/3-inhibited cells have a weak coupling between the cell cortex and the plasma membrane, and suggest a potential mechanism for increased pseudopod and bleb formation. Pseudopods are not sensitive to inhibition of formin or myosin II activity. Collectively, these results indicate that Arp2/3 is not necessary for rapid protrusion of the cell edge but plays a crucial role in assembling focal adhesions required for its stabilization.

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Examining the Roles of Myosin II and Dynein Motors in Nucleokinesis of Migrating Neurons Using Small Molecule Inhibitors.

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Migration of neurons from their germinal zone to final location is an essential process in the developing brain. Following polarization of a developing neuron, nucleokinesis occurs in a two-stroke process. Nucleokinesis can be temporally defined by centrosome movement into the dilation of a leading neurite followed by nuclear translocation. The roles the actin and

microtubule network play in this process, and which generates force on the cell, are currently debated. We are examining the cytoskeleton elements and motors involved in neuronal migration through *ex vivo* time-lapse imaging using migrating cerebellar granule neurons as a model, conditionally controlling motors using small molecule inhibitors. Previous work from our lab shows pharmacological inhibition of the myosin II motor using blebbistatin severely retards movement of the centrosome. Using HPI-4 (hedgehog pathway inhibitor 4, Ciliobrevin A) to inhibit dynein, dynamics of the centrosome, neurite dilation, and nuclear movement were compared to inhibition of myosin II. In addition to stopping centrosome movement, blebbistatin treatment stops movement of the neurite dilation. Inhibition of both myosin II and dynein show different effects on stopping movement of the mother versus daughter centriole.

In addition to tracking centrosome and nuclear movement, we are examining potential modes of crosstalk between the actin and microtubule cytoskeleton and associated motors in nucleokinesis. We have imaged venus-dynein and myosin II-venus dynamics under conditional drug inhibition of the motor protein of the opposite cytoskeleton network, and under cytoskeletal perturbation using jasplankinolide, taxol, and nocodazole. Live imaging shows venus-dynein flows towards the dilation in the leading process of migrating neurons, and accumulates behind the nucleus after somal translocation. This shows a contrast to myosin, which is shown to stay enriched in the leading process of neurons during nuclear migration.

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Dynamics of cancer-cell filopodia on substrates of different stiffness.

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Cell motility is highly modulated by substrate stiffness. While filopodia plays a crucial role in cell migration, the effects of environmental stiffness on filopodial dynamics remain unclear. In this work, we present the filopodial dynamics on substrates of different stiffness using a novel platform. The platform consists of an optical system called structured illumination nano-profilometry, which has a lateral resolution of 140 nm and allows filopodia observation in living cells without labeling. Human lung adenocarcinoma cells CL1-5 were cultured on substrates primarily composed of polyvinyl chloride. The substrate stiffness was tuned by addition of a plastic softener to yield Young's modulus ranging from 20 to 60 kPa. MTT assay shows that the viability of cells cultured on our substrates was similar to those cultured on commonly used elastomers such as polydimethylsiloxane. Time lapsed live cell images were acquired and for each cell the filopodial density, defined as the total number of filopodia divided by the perimeter of the cell, and the length of individual filopodia were calculated. We found that after cultured on softer substrates for 24 hours, cancer cells exhibited a significantly larger density and longer length of filopodia compared with those cultured on stiffer surface. Analyzing the temporal change of individual filopodial length reveals that a softer surface promotes cancer cells to consistently protrude filopodia. The effects of stiffer substrates on the filopodial dynamics were inhibited by treating the cells with blebbistatin. These results suggest that filopodial dynamics are closely modulated by the adhesion strength of cells. Our data quantitatively relate filopodial dynamics with environmental stiffness and should shed light in the understanding and treatment of cancer metastasis.

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Primary Zebrafish Keratocyte Explant Cultures: A Model for EMT and Epithelial Wound Healing.

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The control of collective cell migration of keratocyte sheets in explant culture is of interest in epithelial wound healing and is dependent on the gene expression profile of keratocyte explant cultures over time. In a zebrafish genome array, ~17.5% of the probe sets were differentially expressed greater than 2-fold (at significant levels) between 1 and 7 days in explant culture. Among the differentially expressed genes were many of the biomarkers for epithelial-mesenchymal transition (EMT), including keratin to vimentin, E-cadherin to N-cadherin, and loss of junctional protein expression. Supporting evidence for EMT is seen in the morphological change and rearrangement of the actin cytoskeleton during explant culture with a visible disassembly of the cell sheet. Additionally, immunofluorescent assays reveal that E-cadherin function and expression appears to decrease over time with a corresponding, although weaker, increase in N-cadherin expression by 7 days. Consistent with these morphological changes, a variety of wound healing-related genes and actin regulatory proteins were also found to be differentially expressed. To validate the microarray data, quantitative PCR (qPCR) on several genes from the array was performed. In addition, qPCR analysis of differential mRNA expression of TGF β and TNF α at various time points between 0 and 7 days of explant culture indicates that the peak of differential expression of both cytokines occurred at 3 days and, therefore, was not detectable in the microarray data. Collectively, our data establish that an EMT process is occurring during zebrafish keratocyte explant culture and support the use of this system as a wound healing model.

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Orientation of actin filaments and microtubules in teleost retinal pigment epithelial cells, and effect of the lectin, concanavalin A, on melanosome motility.

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Retinal pigment epithelial cells of teleosts contain numerous melanosomes (pigment granules) that exhibit light-dependent motility. In light, melanosomes disperse out of the RPE cell body into long apical projections that interdigitate with rod photoreceptors, thus shielding the photoreceptors from bleaching. In darkness, melanosomes aggregate through the apical projections back into the cell body. Previous research has demonstrated that melanosome motility in the RPE cell body requires microtubules, but in the RPE apical projections, actin filaments are necessary and sufficient for motility. Here we characterize the distribution of actin filaments and microtubules in isolated fish RPE cells. Both cytoskeletal polymers are found in the cell body and throughout the length of apical projections. Myosin S1 labeling and platinum replica shadowing of dissociated RPE cells revealed that actin filaments present in bundles within RPE apical projections are uniformly oriented with barbed ends towards the distal tips. Treatment with the tetravalent lectin, concanavalin A, which has been shown to suppress cortical actin flow by crosslinking cell-surface proteins, inhibited melanosome aggregation and stimulated ectopic actin polymerization, but did not block melanosome dispersion. These results suggest that dispersion of melanosomes from the cell body into apical projections could be accomplished by a barbed-end directed myosin motor. Inhibition of aggregation but not

dispersion by ConA crosslinking is consistent with the idea that melanosome aggregation could occur via passive attachment of melanosomes to actin filaments undergoing retrograde flow.

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Thrombomodulin is an ezrin-interacting protein that controls epithelial morphology and promotes collective cell migration.

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Objective: Thrombomodulin (TM) is an integral membrane protein involved in cell-cell adhesion through its extracellular lectin-like domain; here, we investigate how TM is connected to the cytoskeleton and its biological functions in epithelial cells.

Methods: A magnetic bead-assisted protein isolation kit was used to identify the interacting proteins of the TM cytoplasmic domain. The cytosolic proteins that bound to green fluorescent protein (GFP)-tagged TM were isolated and analyzed by mass spectrometry. This preliminary experiment suggested that ezrin might associate with TM cytoplasmic domain. By using in vitro binding assays, we analyzed the interaction of the TM cytoplasmic domain with ezrin and mapped the ezrin-binding site on the TM cytoplasmic domain. We examined the association of TM with ezrin and actin filaments in epithelial cells and further analyzed the effects of TM knockdown on cell morphology and migration. In addition, we explored the potential role of TM-ezrin interaction in collective cell migration during wound healing.

Results: The in vitro binding assays showed that the TM cytoplasmic domain bound directly to the N-terminal domain of ezrin. Mutational analysis of the TM cytoplasmic domain identified 522RKK524 as important ezrin-binding residues. Immunoprecipitation experiments showed that TM interacted with ezrin in HaCaT and A431 epithelial cells. Confocal microscopy analysis of A431 cells revealed that TM colocalized with ezrin at cell-cell adhesion sites, where actin filaments densely associated with the plasma membrane. Knockdown of endogenous TM expression by RNA interference induced morphological changes and accelerated cell migration in A431 cells. Moreover, epidermal growth factor, upstream of ezrin activation, stimulated the interaction between ezrin and TM. In mouse skin wound repair, TM and ezrin were highly expressed in neo-epidermis, implying that both proteins are key molecules in re-epithelialization that requires collective cell migration of epithelial cells. Finally, exogenous expression of TM in TM-deficient A2058 melanoma cells promoted collective cell migration, but ezrin knockdown abrogated this phenotype.

Conclusion: TM, which associates with ezrin and the actin cytoskeleton, maintains epithelial morphology and promotes collective cell migration.

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Profilin-1 downregulation promotes mammary tumor growth and dissemination but not metastatic colonization.

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Profilin1 (Pfn1), a ubiquitously expressed actin-binding protein, plays indispensable role in migration and proliferation of normal cells. Seemingly contrary to these essential cellular functions of Pfn1, its expression is downregulated in breast cancer, the significance of which is unclear. This study for the first time examines the functional consequences of Pfn1

downregulation in breast cancer cells in vivo. Expression profiling of Pfn1 in human breast cancer specimens of various histopathological stages correlate lower Pfn1 expression levels with propensity to metastasize. In xenograft models of human breast cancer cells, we find that both mammary tumor growth and metastasis are enhanced upon loss of Pfn1 expression. Our data suggest that Pfn1 depletion promotes breast cancer metastasis mainly through facilitating emigration of tumor cells from the primary tumor site. Consistent with this implication, we further show that upon Pfn1 depletion, breast cancer cells acquire a hyper-invasive signature (marked by MMP9 upregulation, faster invasion through collagen matrix). In Pfn1-deficient cells, hyperinvasiveness involves a PI3K-PI(3,4)P2 signaling axis. Finally, in striking contrast to its action of promoting primary tumor growth, loss of Pfn1 strongly inhibits metastatic outgrowth of disseminated cells. These data not only demonstrate that Pfn1 plays an essential role in metastatic colonization process, but may also suggest that tumor microenvironment influences how loss of Pfn1 dictates cellular phenotype.

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Coupling cell function with cell migration: the example of dendritic cells.

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Dendritic Cells (DCs) patrol peripheral tissues in search for pathogenic antigens (Ag) to capture, by integrating macropinocytosis and cell motility. We used micro-fabricated channels to unravel the mechanisms that allow the spatiotemporal regulation of these two biological processes. We found that DCs display a discontinuous migration mode that includes high motility phases, during which macropinosomes form at the cell front, and low motility phases during which they are resorbed and their content transported to endolysosomes. We further show that low motility phases result from the association of the actin-based motor protein Myosin II with the MHCII-associated Invariant Chain (Ii), a key regulator of Ag processing and presentation. Ii transiently diverts Myosin II from the cell rear to the cell front, resulting in decreased motility but enhanced macropinocytosis and Ag transport to endolysosomes. Accordingly, while Ii-deficient DCs migrated faster but displayed decreased ability to capture external Ag, DCs over-expressing Ii exhibited the opposite phenotype. Remarkably, artificial decrease of DC motility strongly enhanced their Ag capture capacity, whereas macropinocytosis inhibition resulted in increased DC velocity, indicating that these two processes are tightly coupled. Thus, the displacement of Myosin II from migration to endocytosis enables immature DCs to coordinate these two functions in time and space, most likely optimizing their ability to scan peripheral tissues. Because Myosin II controls the motility of many cell types, we foresee that diversion of this motor protein by cell-specific components might be an evolutionarily conserved mechanism that enables cells to couple their function to their migratory capacity.

Intermediate Filaments

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The structure of vimentin linker 1-2 as determined by SDSL-EPR and identification of the vimentin central rod domain elementary structure.

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Intermediate filaments (IFs) are a diverse group of cytoskeletal proteins located in both the nucleus and cytoplasm. The dominant structural feature of all IFs is the alpha helical central rod

domain. In this report, we present spectroscopic data to show that linker 1-2 of vimentin, a type III IF, adopts an alpha helical parallel helices structure and thus links and continues the structures identified for the end of rod 1B and former rod 2A. Using both room temperature and low temperature methods we have identified coiled-coil structure within rod 1B and the shift from coiled-coil to parallel helices at the end of rod 1B. We have similarly characterized the parallel helices structure of rod 2A. With these data in hand, we find that the structure of linker 1-2 is consistent with a parallel helices structure. Additional spectroscopic techniques to measure the distance between spin labels placed at each end of linker 1-2 support an extended not globular structure, consistent with parallel helices structure. The identification of linker 1-2 as parallel helices completes the molecular characterization of the vimentin central rod domain. The data strongly suggest that within the vimentin dimer, the fundamental building block of vimentin IFs, the central rod domain is assembled into a symmetric, tripartite structure composed of amino coiled-coil domain, central parallel helices domain and carboxy coiled-coil domain. Based on existing nomenclature, we suggest the domains be labeled rod 1, parallel helices domain (PHD) and rod 2.

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Crosstalk between plectin-modulated vimentin network architecture and signaling pathways controlling focal adhesion dynamics and migration.

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Plectin is a highly versatile cytolinker protein that stabilizes cells and tissues mechanically, regulates cytoskeletal filament dynamics, and serves as a scaffolding platform in signaling. Besides cross-linking of vimentin filaments to each other and to other filament systems we have recently shown that plectin plays a crucial role in anchoring vimentin filaments to focal adhesion contacts (FACs). Here we investigated how plectin affects vimentin network architecture in fibroblasts and whether network alterations are connected to signaling pathways involved in cell migration. Using electron microscopy we show in plectin-null cells that individual vimentin filaments have increased diameter and that the whole vimentin network organization is altered in these cells including extension of filaments to the very perimeter of cells, increased filament tangling, and loosening of network compactness. Networks of this type were more susceptible to heat stress-induced disruption and okadaic acid-induced retraction from the cell periphery. Altered cytoarchitecture of plectin-deficient fibroblasts, particularly the absence of vimentin filament anchorage at FACs, led to reduced adhesion of cells on various substrates in comparison to their wild-type counterparts. Consequently, we observed integrin dependent reduction in focal adhesion kinase (FAK) and Src kinase activities, and up-regulation of the small GTPase RhoA in plectin-deficient cells. Impaired signaling was paralleled by compromised focal adhesion dynamics, decreased PDGF-dependent migration potential and directionality as assessed in collective and single-cell migration assays. Thus, by modulating vimentin anchorage to FACs, plectin reinforces integrin dependent cell adhesion, positively affecting FAK and Src kinase activities and thereby exerting a negative feed-back loop on the small GTPase RhoA. These signals are further translated into an increase in focal adhesion turn-over and migration.

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Vimentin serves as a signaling scaffold at focal adhesion sites to regulate cell adhesion.*L. S. Havel¹, A. Marcus¹; ¹Hematology and Medical Oncology, Emory University, Atlanta, GA*

Vimentin is a dynamic type III intermediate filament that is overexpressed in various cancers including non-small cell lung cancer (NSCLC). It is a classic biomarker for highly invasive tumors including those of NSCLC and is associated with a higher likelihood of metastasis and lower survival rate, suggesting a possible role in cancer metastasis. Vimentin exists in 3 forms: particles, squiggles and mature filaments. A population of vimentin filaments localize to focal adhesions, where they assemble and disassemble to allow cell migration. Focal adhesions attach to the extracellular matrix and are composed of a complex of proteins including focal adhesion kinase (FAK) and Src. Since our preliminary work shows that vimentin depletion reduces cell adhesion and other evidence shows that both FAK and vimentin localize to the focal adhesions, we hypothesize that vimentin acts through FAK to regulate cell adhesion and metastasis. We verified this finding by immunocytochemistry, which shows that vimentin filaments directly enter the pFAK and pSrc positive focal adhesion sites. We also show that upon vimentin depletion, pFAK, pSrc and total FAK levels decrease indicating that vimentin regulates FAK expression and activation. To explore the mechanism by which vimentin regulates FAK, we performed a screen for molecules whose phosphorylation status is altered upon vimentin depletion. One of the identified proteins was a guanine nucleotide exchange factor (GEF). We hypothesize that this GEF may be linked to vimentin and FAK mediated cell adhesion. This work provides insight into the mechanism by which vimentin affects cell adhesion and potentially metastasis of NSCLC tumors.

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Vimentin movement in vivo is driven by acto-myosin activity.*A. J. Ehrlicher¹, M. Guo¹, D. Weitz¹; ¹SEAS, Harvard University, Cambridge, MA*

The mechanics of the cytoskeleton in vivo are incredibly complex, making it difficult to determine the activity and roles of its components. While actin is a common biopolymer in virtually all cells, intermediate filaments are believed to contribute biochemically and mechanically. Nevertheless, many fundamental aspects of their function are unclear; how do intermediate filaments associate with the rest of the cytoskeleton, how are they transported, and how rapidly do they turn-over? Here using confocal microscopy and Dendra2 tagged vimentin, Fluorescence Recovery After Photobleaching (FRAP), and simultaneous Fluorescence Loss After photoConversion (FLAC), we measure both the time for individual filaments to move within the cell, and the turnover time of these filaments. By selectively inhibiting actin polymerization and myosin contraction, we find that vimentin movement is not driven by its polymerization, but by actin flows. Fluorescence data also demonstrate that vimentin polymerization/depolymerization is orders of magnitude slower than that of actin filaments. These data taken together demonstrate that vimentin provides mechanical strength, yet does not treadmill. Thus vimentin does not have the high ATP consumption of actin filaments, making it an efficient mechanical element of the composite cytoskeleton.

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Intermediate filament, vimentin, strengthens actin networks.*E. Morris¹; ¹Harvard University, Cambridge, MA*

The mechanics of the cytoskeleton in vivo are incredibly complex. Purified and reconstituted proteins have taught us a great deal about the mechanical properties of isolated cytoskeletal

components. Typically, however, these reconstituted systems may be composed of a single biopolymer, and while highly specific, fail to recreate the complex interactions in a composite material. Previous work in our lab has shown that when actin and microtubules (Brangwynne, 2008) or actin and myosin (Gardel, 2006, Koenderink, 2009) are combined that new and unexpected features emerge. Here we combine the intermediate filament vimentin with actin, and study their composite rheology using novel fiber tracking algorithms. By tracking individual actin filaments as opposed to beads, we visualize the actual network strain field under a given magnetic tweezing stress. When mixed with even small amounts of vimentin, we find that the deformation of the actin network is altered, suggesting that *in vivo* a small amount of vimentin can strengthen the actin network, without ATP costs of treadmilling or motor activity. Thus the two biopolymers are highly mechanically interconnected and form a composite network, indicating the structural importance of vimentin in cytoskeletal networks.

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Elastic behavior of bundled vimentin networks.

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A cell's response to mechanical stress is closely linked to the structure and elasticity of its cytoskeleton, which is comprised primarily of actin, microtubule, and intermediate filament (IF) networks. Vimentin is an IF found in mesenchymal cells that plays a role in anchoring organelles and contributes to overall cellular elasticity. Previous research has shown that vimentin networks behave like softly crosslinked gels in the presence of divalent cations. The linear elastic modulus, a measure of stiffness and resistance to elastic deformation, of the network is related to the degree of crosslinking, which is itself controlled by the cation concentration. Increasing the concentration of the divalent cations further results in the formation of bundles within the network, but this bundling behavior is not well understood. Here we investigate the response of *in vitro* reconstituted vimentin networks to applied shear in the presence of several divalent species to obtain a better description of how they work together (or in competition) as they would in a living cell.

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Recombinant synemin-L caps growing and severs mature intermediate filaments of the desmin- and vimentin-type.

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Synemin is supposed to co-assemble with the intermediate filament (IF) proteins desmin and vimentin, as it colocalizes with them at the Z-discs of skeletal muscle of vertebrates. Correspondingly, synemin is found together with desmin in sarcoplasmic aggregates of myocytes from patients with desmin-related myopathy. Therefore, we wanted to characterize how synemin interacts with desmin and vimentin.

In particular, we wanted to reveal the co-assembly mechanism and the *in vitro* assembly kinetics of desmin and vimentin in the presence of synemin. For this investigation, we have produced and purified recombinant desmin, vimentin and the smallest splice form of synemin, i.e. synemin-L. We renatured the proteins, either alone or in pairs, by dialyses from 8 M urea to 5 mM Tris-HCl, pH 8.4 ("tetramer buffer"). The resulting complexes were analyzed by analytical ultracentrifugation experiments. As revealed by velocity centrifugation runs, synemin-L sedimented with an s-value of 3.0 S, independent of the presence of desmin or vimentin during

renaturation. This behavior strongly indicates that it does not form a hetero-coiled coil with desmin and vimentin. Also, when synemin-L was renatured individually and then with desmin and vimentin mixed in “tetramer buffer”, no interaction occurred. Moreover, by sedimentation equilibrium centrifugation it was determined that synemin-L forms a monomer in “tetramer buffer”. When we then subjected synemin-L to “standard” assembly conditions, i.e. 50 mM NaCl, 22.5 mM Tris-HCl, pH 7.5 (“assembly buffer”), it formed small, uniform and round aggregates of about 35 nm diameter.

In a next step, we investigated how synemin-L influences the assembly process of vimentin and desmin. Notably, when synemin-L was present during the initiation of assembly with vimentin, it inhibited filament formation of vimentin completely, yielding short round, unit-length filament size aggregates similar to those seen with synemin-L alone. In contrast, desmin was able to “escape” the negative effect of synemin-L to some extent, but formed - even after extended assembly periods - short filaments only that were, however, terminated by round ends as revealed by electron microscopy. This result apparently reflects the fact that desmin assembles 5 times faster than vimentin, however, synemin-L eventually causes a “dead-end” situation for desmin assembly too. Very much to our surprise, the same globular structures at the filament ends were observed, when synemin-L was added to mature vimentin and desmin IFs, indicating it “cuts” IFs.

Together, these data suggest that synemin-L acts as a capping and severing factor of IFs *in vitro*. Overexpression experiments with synemin-L in authentic myoblasts are under way.

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Cell stiffness correlates with cell volume.

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Cells usually have a certain size, and their size has been shown to be correlated to gene expression across species. There are careful studies about standard cell sizes of different species, as well as cell size changes during stages of the cell cycle. Furthermore, people have shown that cells change their spreading area, stiffness, gene expression and even stem cell fate when grown on substrates of varying stiffness. However, whether cell size changes for the same cell type under varying micromechanical environments, and whether this is related to the observed phenomenological changes is not known. Here we show that height and volume of single adherent cells decrease when grown on a stiffer 2D substrate, while spreading area increases. When cell spreading area is confined by micropatterning on stiff substrate, we find cell volume is dependent inversely on their spreading area. We further measure cell stiffness with optical magnetic twisting cytometry. By controlling their volume in three different ways – varying substrate stiffness, cell spreading areas, and osmotic pressure in the medium – we find that cell stiffness correlates with cell volume but not substrate stiffness. Cells can be soft on a stiff substrate by changing only the spreading area; they can be stiff on a soft substrate by increasing osmotic pressure. Furthermore, we show that vimentin may be involved in cellular volume regulation.

1281B566

Geometric Control of Cytoskeletal Elements: Impact on Vimentin Intermediate Filaments (IF).*M. M. Cleland¹, S. H. Shabbir¹, M. Mrksich¹, R. D. Goldman¹; ¹Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, IL*

Significant efforts have been directed to study the role of vimentin IF in cell motility, cell shape, adhesion and its connections to other cytoskeletal elements like microfilaments and microtubules. By using micropatterning we show how these cytoskeletal elements are dependent on each other. By confining mouse fibroblasts in 8 different types of micropatterns of a constant size, we find that unlike microfilaments, vimentin IF are globally controlled. For example, both square and circle-shaped cells have a similar vimentin IF distribution while microfilament distribution in these two shapes is quite different. Furthermore, in asymmetric and polarized shaped cells vimentin IF avoid the sharp edges where microfilaments are highly localized. We have carried out a comparative study between wild-type and vimentin null mouse embryonic fibroblasts (MEF's) using 2 polarized patterns. The results show that in vimentin null MEF's microtubules are less dense and slightly disorganized at the curved edge of the shape. This indicates that the absence of vimentin IF affects the microtubules organization in the cells. Next, we analyzed cell polarity in teardrop shaped cells, both in wild-type and vimentin null MEF's and found that the absence of vimentin IF interferes with cell polarity in the teardrop shaped cell, which has been used as a model of a migrating cell. This phenotype can be reversed by over expression of wild-type vimentin IF whereas a non-filamentous mutant form of vimentin IF cannot rescue this phenotype, thus indicating filamentous vimentin IF is essential for microtubule organization. Supported by NIH/NHLBI training grant T32HL076139, NIGMS Intermediate Filament Program Project Grant 5P01GM096971-02 and by the Chicago Biomedical Consortium with support from the Searle Funds at The Chicago Community Trust.

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Vimentin organization is mediated by its active transport along microtubules and its binding to actin microfilaments.*C. Hookway¹, L. Ding², A. Robert¹, M. Lakonishok¹, G. Danuser², V. I. Gelfand¹; ¹Department of Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, IL, ²Department of Cell Biology, Harvard Medical School, Boston, MA*

Vimentin is organized in cells by microtubules and actin microfilaments, but the molecular mechanisms that mediate vimentin distribution by these systems is largely unknown. Here, we use live-cell imaging of photoactivatable forms of vimentin intermediate filaments (VIFs) and their unit-length filament (ULF) precursors to study the role of microtubules and actin filaments in VIF dynamics. We demonstrate that both VIF and ULF are rapidly transported in cells in anterograde and retrograde directions. We show that this motility is dependent on microtubules, as it is prevented by microtubule depolymerization, and strongly affected by actin microfilaments, as fast motility is dramatically increased by actin depolymerization with latrunculin B. We also see that ULF heavily decorate actin stress fibers and focal adhesions. Further, we demonstrate that vimentin transport does not occur passively with actin retrograde flow, nor is it mediated by microtubule dynamics. Therefore, we have investigated how VIF and ULF transport depends on active mechanisms by exploring the dependence of transport on motor proteins. We find that RNAi-mediated knockdown of microtubule motors results in VIF redistribution, and we show the effect of microtubule and actin microfilament motor knockdown or inhibition on transport. Thus, VIF organization in cells is established by its active transport along microtubules and by binding to actin filaments. Supported by NIGMS grant P01GM096971 to V.I.G. and G.D.

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Vimentin IFs are involved in regulation of mitochondria potential.

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Almost all functions of mitochondria that determine multiple cellular processes depend on their membrane potential. So the maintenance of potential level is very important for cell physiology. Our recent data demonstrate that vimentin intermediate filaments (vimIFs) control mitochondria motility and membrane potential. The effects of vimIFs on mitochondria are mediated by the mitochondria binding site of vimentin molecule located between 45 and 70 amino acid residues. This site contains several serine residues that are the phosphorylation sites of known protein kinases and could be potential spots where mitochondria-IF interactions are regulated. One of the sites is Ser55 which is phosphorylated by p-21 activating kinase-1 (PAK-1) an effector of the small GTPase Rac1. We performed the experiments to investigate the possible role of Rac1 protein in regulation of mitochondria membrane potential and their interaction with vimIFs. Using constitutively active mutant of Rac1 we verified that this regulatory protein really increases mitochondria motility and attenuates their membrane potential. Then we have found that protein kinase PAK-1 is indeed a downstream effector of Rac1 that is responsible for the effect on mitochondria. Our data demonstrate that activation of Rac1 does not affect mitochondria in the presence of dominant negative mutant of PAK-1 or when the point mutation S55A is introduced in vimentin. So we conclude that interaction of mitochondria with vimIFs is controlled by small GTPase Rac1 through its effector PAK-1 that modifies the putative mitochondria binding site in vimentin N-terminus.

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Ablation of keratin 8 ser74 phosphorylation predisposes to colon injury in transgenic mice.

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Enterocytes express cytoskeletal keratin 8 (K8) as a major intermediate filament protein. A significant physiological function of K8 is to protect intestinal epithelia from colitis as shown in study of K8-null mice that develop chronic spontaneous colitis. K8 ser74 phosphorylation occurs under various stresses and represents a physiological stress marker for simple epithelia. We study the role of K8 ser74 phosphorylation in intestinal epithelia by using the mouse colitis model of dextran sulfate sodium (DSS). Human K8 ser74-to-Ala mutant (hK8 S74A) overexpressing mice are compared with hK8 WT mice after DSS administration. K8 S74A mice are significantly more susceptible to DSS-induced colon injury as compared with K18WT mice (94% and 17% lethality; $p < 0.0001$). It is comparable sensitivity of K8-null mice vs nontransgenic FVB/n mice (83% and 15% lethality; $p < 0.0001$). However, hK8 G62C mutant, partially blocked K8 S74 phosphorylation, overexpressing mice show similar sensitivity to DSS-induced colon injury as compared with control mice (6% and 17% lethality; $p > 0.38$). The mortality data are confirmed by histopathological analysis that reveals more severe denudation of epithelial layer and mucodepletion of glands in S74A colon as compared with K8 WT and G62C colons. Notably, activation of p44/42 MAPK, related to cell proliferation, is upregulated, whereas expression of hsp70, well-established to play a cytoprotective role, is severely downregulated in S74A colon after DSS administration. In addition, 11% (4/37) of S74A mice develop anorectal prolapse at the age about 1 year but none of K8 WT and G62C mice suffer from the intestinal disorder. Therefore, K8 S74 phosphorylation plays a critical role in protection from intestinal injury.

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Predisposition to apoptosis in keratin 8-null liver is related to inactivation of NF- κ B and SAPKs but not decreased c-Flip.*J. Lee¹, K-H. Jang¹, H. Kim¹, Y. Lim¹, S. Kim¹, H-N. Yoon¹, I. Chung¹, J. Roth¹, N-O. Ku¹;**¹Yonsei University, Seoul, Korea*

Keratin 8 and 18 (K8/K18) are major intermediate filament proteins of liver hepatocytes. They provide mechanical stability, thereby protecting cells from stress. Hence, K8-null mice are highly sensitive to Fas-mediated liver cell apoptosis. However, the role of c-Flip protein in K8-null related susceptibility to liver injury is controversial. Here we analyzed c-Flip protein expression in various K8 or K18 null/mutant transgenic livers and show that they are similar in all analyzed transgenic livers and that previously reported c-Flip protein changes are due to antibody cross-reaction with mouse K18. Furthermore, analysis of various apoptosis- or cell survival-related proteins demonstrated that inhibition of phosphorylation of NF- κ B and of various protein kinases such as p38 MAPK, p44/42 MAPK and JNK1/2 is related to the higher sensitivity of K8-null mice hepatocytes to Fas-mediated apoptosis. Notably, we found that NF- κ B and the studied protein kinases are associated with the K8/K18 complex and are released upon phosphorylation. Therefore, interaction of keratins with cell survival-related protein kinases and transcription factors is important for hepatocyte survival.

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Computational analysis of keratin network dynamics.*M. Moch¹, G. Herberich², T. Aach², R. Windoffer¹, R. Leube¹; ¹Institute of Molecular and Cellular Anatomy, RWTH Aachen University, Aachen, Germany, ²Institute of Imaging and Computer Vision, RWTH Aachen University, Aachen, Germany*

Keratin intermediate filaments are major components of the epithelial cytoskeleton, which protects epithelial cells against different forms of stress. The keratin network is therefore quite adaptable and may either be rather static or highly dynamic to meet the challenges at hand. At present, however, it is difficult to describe keratin dynamics in quantitative terms.

We therefore developed a workflow to measure the movement and turnover rate of fluorescently tagged keratin filaments in living cells. We used A431 vulva carcinoma cells stably transfected with green fluorescent protein-tagged keratin 13. Time-lapse fluorescence images were recorded of single cells using standardized conditions. Based on a maximum-a-posteriori motion estimation algorithm motion vector fields were generated from the image data. Taking the fluorescence intensity into account we also calculated keratin flow fields to measure keratin assembly and disassembly in different situations and subcellular topologies. For comparison, fluorescence recovery after photobleaching (FRAP) experiments were performed to determine keratin filament turnover in a different way in independent experiments. Finally, the results were transformed into a unit cell model to account for cell shape changes.

Using these methods we were able to show that increased time after splitting inversely correlates with keratin motility and turnover and that, on the other hand, treatment with epidermal growth factor (EGF) increases keratin motility and turnover. Taken together, the data further support the model of a continuous keratin turnover cycle that is under regulatory control.

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Caspase cleavage-resistant keratin 18 mutation protects liver from apoptosis in transgenic mice.*H-N. Yoon¹, N-O. Ku¹; ¹Yonsei University, Seoul, Korea*

Cytoskeletal keratin 8 and 18 (K8/K18) proteins provide the structural integrity of simple epithelial cells including liver hepatocytes. Caspase cleavage of K18, but not K8, occurs during apoptosis and triggers the dramatic filament disassembly. We study the significance of K18 caspase cleavage by generating transgenic mice that overexpress human (h) K18 caspase cleavage-resistant mutant, K18 D238/397E. hK18 overexpression is comparable in hK18 WT and hK18 D238/397E mice, and caspase cleavage-resistance of hK18 is confirmed biochemically in D238/397E mouse tissues. There is no effect on viability, organ histology or keratin filament organization under basal conditions in D238/397E mice. We compare them to liver injury with hK18 WT overexpressing mice or nontransgenic mice. The D238/397E mice show similar sensitivity to Fas-induced liver injury as compared with nontransgenic or WT mice [44%, 42% and 53% (nontransgenic, WT and D238/397E) mice lethality; $p>0.58$]. This result may be due to intact endogenous mouse (m) K18 in hK18 overexpressing mice that has caspase cleavage site. We then generate K18 WT and K18 D238/397E mice without mouse K18 by breeding with K18-null mice, and compare them to Fas-mediated injury. Absent mK18 is confirmed biochemically in hK18WT;mK18-null or hK18D238/397E;mK18-null mouse tissues. hK18D238/397E;mK18-null mice are significantly more resistant to Fas-induced injury as compared with hK18WT;mK18-null mice (23% and 57% lethality, respectively; $p<0.001$). The mortality data are supported by less haemorrhage and apoptosis in hK18D238/397E;mK18-null livers. The alleviated apoptosis in hK18D238/397E;mK18-null livers involves in the sustained phosphorylation/activation of cell-survival related protein kinases (such as Akt, p38 MAPK, p44/42 MAPK, JNK1/2 and pRSK90) and transcription factor (NF- κ B) up to 4–6 hrs after Fas administration. However, the phosphorylation/activation of the kinases and transcription factor in hK18WT;mK18-null livers is detected up to 4 hrs, but hardly detected 6 hrs after Fas injection with accompanying less amount of the protein levels, likely due to enhanced apoptosis. Hence, caspase cleavage-resistant keratin 18 mutation protects transgenic liver from apoptosis.

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Assembly perturbations in mutant desmin filaments bound to nebulin in desminopathy.*D. A. Hernandez¹, M. A. Caragea¹, J. M. Hord¹, H. Herrmann², G. M. Conover¹; ¹Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX, ²Division of Molecular Genetics German Cancer Research Center, Heidelberg, Germany*

Desminopathy is a genetic human disease caused by mutations in desmin, an abundantly expressed intermediate filament muscle protein. Its symptoms are heterogeneous; although most cases present respiratory insufficiency, cardiomyopathy and painful skeletal muscle weakness. Intermediate filaments are flexible apolar polymers that provide cells with a dynamic cytoskeleton and nucleoskeleton. Their assembly proceeds in vitro from soluble mixtures of dimer and tetramers to long mature filaments under high ionic strength. To gain insight into the molecular mechanisms responsible for desminopathy, our study aims to understand structurally how desmin incorporates mutant desmin into the filament, and if its assembly is influenced by association to thin filament actin-binding muscle protein nebulin. The effect of nebulin binding on desmin length distribution was evaluated early during assembly using atomic force microscopy. Our length measurements show that a statistically significant shorter mutant desmin forms when associated to nebulin. Viscometry experiments evaluated the bulk behavior of the sheet of desmin filaments upon nebulin binding. Our results showed that homozygous mutant desmin

dramatically increases bulk sheet viscosity, while a 3-fold lower viscosity was found for either WT or heterozygous protein. Remarkably, we found that nebulin binding to heterozygous desmin dramatically decreased bulk sheet viscosity, while little changes were detected when nebulin bound to WT desmin. By total internal fluorescent reflection microscopy, single molecule experiments evaluated in real-time how fluorescently labeled WT desmin bound to mutant desmin during late stages of filament elongation. The co-assembly of WT desmin and mutant desmin primarily assembled via intercalary subunit exchange forming heteropolymers. Ongoing cell fractionation and immunofluorescence studies in myocytes are probing the role of phosphorylation in the organization of desmin filaments and their association to nebulin. Our combined biochemical and biophysical approach offers a powerful strategy to kinetically track how complex cellular cytoskeletal networks respond to normal or abnormal interactions that occur in desminopathy. Our working model is that desmin provides multiple attachment sites for Z-disc protein assemblies to stabilize the actin thin filaments and that nebulin plays a central role in opening the heterozygous intermediate filament mesh surrounding the sarcomere Z-discs.

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Role of the pre-coil domain of desmin and its partnership with muscle proteins.

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The intermediate filament (IF) family of cytoskeletal proteins shares a tripartite structure: a non-alpha-helical flexible head, and tail end domains flanked by a conserved central alpha-helical coiled-coil rod domain. Previous work showed that removal of the vimentin head domain yields soluble molecules that failed to associate laterally into stable tetramers. To further understand the function of a 20-residue segment (87-108) preceding the rod domain, termed pre-coil domain (PCD), which is unique to vimentin-like type-III IF proteins, we produced a desmin delta PCD (108-470) truncated protein and characterized its biophysical and biochemical properties. Desmin, a type-III IF protein is abundant in striated muscle, assembles in vitro from soluble tetramers to make long mature filaments by lateral association of unit-length filaments that elongate making ≈ 11 nm average width filaments. Desmin delta PCD protein failed to make elongated filaments as observed by electron microscopy. After 10 min of assembly, we measured filament length in atomic force micrographs and found that delta PCD proteins made very short filaments ($\approx 0.06 \mu\text{m}$), while headless desmin made ($\approx 0.2 \mu\text{m}$) and WT desmin made ($\approx 0.5 \mu\text{m}$) average length filaments. Consistent with these results, PCD failed to precipitate and remained in the soluble fraction while most of the WT desmin formed filaments and was recovered from the pellet fraction after high-speed co-sedimentation assays. Analytical sedimentation velocity analysis comparing headless and delta pre-coil desmin proteins yielded s-values (4.1 to 4.0) under low salt, while both proteins had undistinguishable s-values of 4.5 under conditions that favored filament assembly. Subtle changes in sedimentation profiles were detected in presence of nebulin. Delta PCD bound with a ≈ 2 -fold higher binding affinity to actin-binding muscle protein nebulin as compared to WT desmin as determined by ELISA. Expression studies in cardiomyocytes showed that GFP-head is unable to integrate efficiently at the Z-discs. All together, our data suggest that headless and PCD are soluble proteins that arrest assembly as tetramers of similar size. We present a model by which accessibility for binding partners to the central coiled-coil region of desmin is increased when head and PCD domains are absent, indicating that these domains participate in desmin filament-partner protein interactions by regulating their contact.

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Gigaxonin Regulates the Degradation of Intermediate Filament Proteins: Insights into Giant Axonal Neuropathy.

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Giant Axonal Neuropathy (GAN) is a rare neurological disorder caused by mutations in GAN, the gene that encodes for gigaxonin. Patients experience progressive muscle weakness and impaired sensation, develop brain and spinal cord lesions, and eventually suffer mental retardation, dysmetria, seizures and death by 20-30 years. The patient's nerve cells are characterized by large axonal swellings that are filled with neuronal intermediate filaments (IF); interestingly their skin fibroblasts also contain large aggregates of vimentin IF. Based on sequence homology, gigaxonin is predicted to be an adaptor for Cullin-3 ubiquitin ligase and thus may be involved in protein degradation. To begin to understand its cellular function, Wt-gigaxonin was over-expressed in fibroblasts from GAN patients, normal controls and GAN-/- mice. In all cases, over-expression of Wt-gigaxonin leads to the disappearance or clearance of vimentin IF from cells, as determined both biochemically and immunocytochemically. Similarly, over-expression of gigaxonin in neuronal cells resulted in the clearance of neuronal IF (both peripherin and neurofilament light-chain). In all cases, the microtubule and actin filament systems were unaltered. Immunoprecipitation studies revealed an interaction between gigaxonin and vimentin, and truncation mutations suggested that binding was via vimentin's central rod domain. Addition of the proteasomal inhibitor MG-132 reversed the clearance of IF caused by gigaxonin over-expression, suggesting that the proteasomal pathway is involved. Gigaxonin is the first factor identified to play a specific role in the degradation of IF proteins and these findings provide insight into the mechanisms responsible for the turnover of a major class of cellular proteins which comprise the most stable cytoskeletal system in cells. These discoveries may shed light on the causes of more common neurodegenerative diseases that are also characterized by large accumulations of IF proteins, including Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies, Charcot-Marie-Tooth disease (CMT), neuronal intermediate filament inclusion disease (NIFID) and diabetic neuropathy.

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Epidermolysis Bullosa Simplex (EBS): Effect of shear stress level on EBS keratinocyte morphology and necrosis.

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Epidermolysis bullosa simplex (EBS) is a genetic disorder characterized by recurrent skin blister formation from cytolysis of basal epidermal keratinocytes in response to minor injury or friction. Most cases of EBS are associated with mutations in the genes encoding keratin proteins K5 and K14 that form a filamentous cytoskeletal network in basal keratinocytes. In spite of the tight correlation between EBS phenotypes and mutations in keratin (and keratin associated) genes, the exact mechanisms by which these mutations lead to cell fragility in EBS patients are unknown. We recently showed that cells expressing a mutant EBS K14 protein are not more fragile than WT cells when subjected to extreme uniaxial stretches. Here we tested the hypothesis that EBS cells are more fragile when exposed to shear stress than WT cells. We

subjected a wild type keratinocyte line (NEB-1), a mutant K14-R125P EBS cell line (KEB-7), and a K14-null keratinocyte cell line (KEB-11) to shear stresses within microfluidic devices and found that mutants displayed more shear-induced cell necrosis than WT. Moreover, the preliminary cell morphology analysis showed that mutant cells deform less than WT cells when subjected to high shear stress. We also subjected NEB-1, KEB-7 and KEB-11 cell lines to large scale stretching and found that NEB-1 and KEB-7 cells exhibited almost no fragility, whereas KEB-11 cells displayed significant amounts of necrosis. This is the first study to demonstrate the fragility of EBS cell monolayers in culture and also the first to demonstrate that EBS cells are particularly fragile when loaded in shear.

Connexins

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Intrinsically disordered proteins aggregate at fungal cell-to-cell channels and regulate intercellular connectivity.

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Multicellular fungi, like plants and animals, possess cell-to-cell channels known as septal pores. However, not much is known about the fungal septal pores. Electron micrographs show an electron dense proteinaceous structure at the septal pore, but the identity of these proteins is not known. Several septal pore associated organelles have been described in filamentous fungi, like the peroxisome-derived Woronin body in the Pezizomycotina that plugs septal pores in injured hyphae to minimize cytoplasmic bleeding. Here, using a combination of mass spectrometry of Woronin body associated proteins and a bioinformatics approach that identifies related proteins based on composition and character, we identify 17 septal pore associated (SPA) proteins that localize to the septal pore in rings and in a pore-centered foci. SPA proteins are not homologous at the sequence level, but share similar physical characteristics with intrinsically disordered proteins. Some SPA proteins form aggregates at the septal pore and in vitro assembly assays of these purified proteins suggest a non-amyloid aggregation mechanism driven by alpha-helical and disordered structures. Some SPA proteins appear to be involved in membrane resealing process and compartmentalized cell death. However deletions in all but three SPA proteins and multiple deletions do not show obvious phenotype, which suggests functional redundancy of some SPA proteins. Three SPA deletions show different phenotypes, which include excessive septation, degeneration of septal pores, and uncontrolled Woronin body activation. Taken together, we have identified the septal pore as a complex subcellular compartment and focal point for the assembly of intrinsically disordered proteins that control diverse aspects of intercellular connectivity.

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Is the β_2 subunit of the Na⁺pump a self-adhesion molecule?

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The membrane enzyme Na⁺,K⁺-ATPase is distributed in a polarized manner in epithelial cells. We have previously shown that Na⁺,K⁺-ATPase polarity depends on the β subunits of Na⁺,K⁺-

ATPases located on neighboring cells (Shoshani et al. 2005). Then we demonstrated by protein-protein interaction methods (FRET, co-immunoprecipitation and pull down) that the extra-cellular domain of β_1 subunit associates in a specific and direct manner with another β_1 subunit localized on adjacent cell (Padilla-Benavides et al. 2010). Now we wonder if the β_2 isoform, known as the adhesion molecule on glia (AMOG) is able to associate with itself or with the β_1 subunit? To answer this question we expressed the human β_2 subunit fused to YFP in MDCK and CHO cells. In both stable cell lines, as observed by fluorescence microscopy, the recombinant protein is localized to the plasma membrane and concentrates in cell-cell contacts. When MDCK β_2 -YFP cells are co-cultured with NRK cells (epithelial from rat kidney) the β_2 -YFP is expressed both in homotypic and heterotypic borders suggesting probable interaction with the β_1 subunit localized on adjacent NRK cell. Therefore we first analyzed β_2/β_1 interaction by pull-down and Co-immunoprecipitation assays. We detect positive interactions when using dog β_1 subunit as bait and human β_2 -YFP as prey in pull down experiments. Also, when we IP the β_2 isoform from rat brain the endogenous brain β_1 subunit is co-precipitated. These findings are indicating a probable interaction *in vitro* and *in vivo* between the two isoforms.

We then looked for β_2/β_2 interaction in aggregation assay analyzed by FACS. We found that CHO β_2 -YFP cells make significantly more cell aggregates than CHO wt, indicating that the expression of the β_2 isoform confers to the CHO fibroblasts adhesiveness, that could be explained by β_2/β_2 interactions. Therefore, we assayed for this possible interaction using endogenous β_2 from rat brain and from human RPE cell line (ARPE-19). Pull down experiments, using β_2 -YFP as bait and rat brain or human RPE extracts as prey did not reveal any β_2/β_2 interaction suggesting that at least under these experimental conditions, they do not associate. Overall, our preliminary results suggest that β_2 isoform participates in heterotypic but not homotypic interactions.

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Cx45 can functionally replace Cx43 in activation of TGF- β to support mural cell differentiation from mesenchymal precursors.

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During blood vessel formation, smooth muscle development involves signaling from endothelial cells (EC) that mediates the recruitment of mesenchymal cell (MC) precursors and promotes their differentiation toward a mural cell fate. Previously, we demonstrated that gap junction channel formation between EC and MC is required for EC-induced TGF- β activation and mural cell differentiation. Furthermore, Cx43-comprised gap junctions mediate these processes. However, Cx43^{-/-} embryos survive to birth with relatively normal vascular smooth muscle development suggesting involvement of another connexin in this process during embryogenesis. Developmental studies suggest that Cx45 is required for mural cell development, although its exact role was not delineated. We show herein that Cx43^{-/-} MC transfected to stably express Cx45 in the absence of Cx43 form functional heterocellular gap junctions when co-cultured with EC. Furthermore, heterocellular Cx45-containing channels (like Cx43 channels) enabled TGF- β activation, which then promoted the upregulation of mural cell-specific proteins in the mesenchymal precursors. Thus, these studies reveal a critical role for Cx45 in the regulation of EC-induced mural cell differentiation. Our findings are consistent with the phenotype of Cx45-deficient embryos, which exhibit dysregulated TGF- β and lack mural cell development.

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Identification of intracellular toxic signals required for bystander killing through gap junctions from HIV infected astrocytes to uninfected astrocytes.

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HIV entry into the central nervous system (CNS) is an early event after infection, resulting in neurological dysfunction in a significant number of individuals. As people with AIDS live longer, the prevalence of cognitive impairment is increasing, despite antiretroviral therapy. The mechanisms that mediate CNS dysfunction are still not well understood, but are postulated to be a combination of inflammation, and viral infection and/or replication. In addition to those mechanisms, we recently demonstrated that HIV infection of astrocytes mediated survival of HIV infected cells and bystander killing of surrounding uninfected cells by a mechanism that is gap junction dependent. We now characterize the mechanisms of HIV mediated protection of infected astrocytes with an emphasis on mitochondrial dysregulation and identification of the intracellular factors that mediate bystander killing of uninfected cells. Our findings describe a novel mechanism by which HIV maintains survival of HIV infected astrocytes and we identify IP3, calcium and Cytochrome C as key signals involved in bystander killing of uninfected cells in contact with HIV infected astrocytes by a gap junction dependent mechanism. Thus, our data provide novel mechanisms of HIV survival and toxicity in the current NeuroAIDS era, where viral replication is not a major component due to effective antiretroviral treatments. Our findings identify new potential therapeutic targets to reduce the devastating consequences of NeuroAIDS.

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Structure and functional studies of Cx43 mutants linked to human diseases- Oculodentodigital Dysplasia (ODDD).

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Connexins are gap junction proteins which form intercellular channels between the vast majorities of cell types in the human body. A wide variety of diseases ranging from developmental disorders to cancer occur when the genes encoding the 21 member connexin family become mutated or dysregulated. Oculodentodigital dysplasia (ODDD) is a developmental disorder caused by predominantly autosomal dominant mutations that occur in the GJA1 gene that encodes Cx43. Patients that harbor these mutations suffer from developmental defects in the hands, feet, eyes, teeth and occasionally in a plethora of other organs. Collectively, we and others have classified the over two dozen characterized mutants to: a) be gap junction functionally dead; b) have residual gap junction channel function; or c) exhibit gain-of-hemichannel function. Furthermore, most, but not all, of the mutants tested are dominant to co-expressed Cx43. In the current study, we focus on the structure and functional changes linked to missense mutations in the N-terminus of Cx43. We found that all missense mutants formed non-functional gap junction-like plaques and exhibited dominant-negative effects on co-expressed endogenous Cx43. NMR protein structure determination of the N-terminal 23 amino acid polypeptides of wild-type and G2V mutant of Cx43 revealed that both polypeptides folded into kinked α -helical structures. These findings predicted that the tryptophan (W) at the 4th position may be critically important in intra- and inter- molecular interactions. Thus we engineered and characterized a W4A mutant and found that this mutant formed a

regular, non-kinked α -helix but did not form functional gap junctions. As predicted from NMR studies, the G2S mutant stabilized the N-terminal helix and form functional gap junctions. Collectively, these studies suggest that elongated α -helical structure at the N-terminus of Cx43 is likely essential for Cx43 channel formation and function similar to Cx26. Furthermore, the nature of the mutation conveys loss-of-Cx43 function by distinctly different mechanisms that are rooted in the structure of the N-terminal region. Funded by the CIHR to DWL, DB and GSS.

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Mammary gland defects as revealed by genetically-modified mice harboring an oculodentodigital dysplasia-linked Cx43 mutant.

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Genetically-modified mice mimicking oculodentodigital dysplasia (ODDD), a disease characterized by reduced Cx43-mediated gap junctional intercellular communication, represent an *in vivo* model to assess the role of Cx43 in mammary gland development and function. We previously reported that severely compromised-Cx43 function delayed mammary gland development and impaired milk ejection in mice that harboured a G60S Cx43 mutant. Surprisingly, there are no definitive reports of lactation defects in ODDD patients. To address this further, we obtained a second mouse model of ODDD expressing an I130T Cx43 mutant to assess if a Cx43 mutant with residual gap junction channel activity would rescue mammary gland development and function. Our findings show that virgin Cx43^{I130T/+} mice, distinct from Cx43^{G60S/+} mice, develop with similar body weights compared to control, despite having a reduction in the highly phosphorylated species of Cx43 and reduced Cx43 gap junctional plaques. In addition, virgin Cx43^{I130T/+} mice exhibit a delay in ductal elongation at four weeks that is not observed by seven weeks. Cx43^{I130T/+} mice develop smaller mammary glands at parturition due to reduced proliferation despite similar overall gland architecture. Distinct from Cx43^{G60S/+} mice, Cx43^{I130T/+} mice adequately produce and deliver milk to pups suggesting milk ejection is unaffected. Thus, these studies suggest that loss-of-function mutants of Cx43 with residual gap junction channel activity can rescue functional defects in the mammary gland and helps to explain the lack of lactation defects associated with ODDD patients. Supported by the Canadian Breast Cancer Foundation.

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Autosomal Recessive Cx43 Gene Mutations Cause Oculodentodigital Dysplasia by Distinct Mechanisms.

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Oculodentodigital dysplasia (ODDD) is mainly an autosomal dominant human disease caused by mutations in the GJA1 gene encoding the gap junction protein connexin43 (Cx43). Surprisingly, there have been two autosomal recessive mutations reported that cause ODDD; a single amino acid substitution (R76H) and a premature truncation mutation (R33X) that results in only 32 amino acids of Cx43 being encoded. Intriguingly, patients with the R33X mutation are essentially equivalent to a Cx43 knockout, but unlike mice, patients do survive at birth, albeit with severe ODDD. In the present study, we characterized the effect of these mutants on Cx43 channel formation and their potential ability to effect co-expressed members of the connexin family. When expressed in either gap junctional intercellular communication (GJIC)-deficient HeLa cells or Cx43-expressing NRK cells, the R76H mutant trafficked to the cell membrane to

form gap junction-like plaques while the R33X mutant remained diffusely localized to the cytosol and nucleus. As expected, the R33X failed to form functional channels. Dye transfer studies in HeLa cells revealed that the R76H mutant could form functional channels that passed Alex350 dye more readily than Lucifer yellow and electrical conductance analysis in GJIC-deficient N2A cells revealed that both the macroscopic conductance and single channel conductance were smaller than those of wild-type Cx43. To evaluate if either the R76H or R33X mutants could act as trans-dominant on other members of the connexin family, we co-expressed the mutants in HeLa cells that transiently expressed Cx40 or stably expressed Cx37, Cx26 or Cx32. Alex350 dye transfer studies revealed that the R76H mutant had no detectable negative effect on the function of any of the other connexins tested while the R33X mutant exhibited significant trans-dominant effects on Cx40 and Cx37, but not Cx26 or Cx32. Taken together, our results suggest that trans-dominant effect of R33X together with its complete inability to form a functional channel may explain why patients harboring this autosomal recessive R33X mutant exhibit greater disease burden than patients harboring the R76H mutant.

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Connexin30 mutants may cause skin disease through the induction of cell death pathways.

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Gap junctional intercellular communication occurs when connexin protein complexes connect adjacent cells to mediate the passage of ions and various small hydrophilic cellular metabolites and secondary messengers. Connexin30 (Cx30) is a member of the large gap junction protein family, and autosomal dominant mutations in the gene that encodes this connexin, GJB6, are linked to non-syndromic deafness and various skin diseases. The mechanisms responsible for Cx30 mutants exhibiting distinct pathologies is not clear but our previous studies have suggested that the Clouston Syndrome-linked V37E and A88V mutants induce cell death, while the T5M mutant linked to non-syndromic deafness does not. More recently, patients harboring a newly discovered G59R mutant present with symptoms similar to those of Vohwinkel's and Bart-Pumphrey Syndromes. In our study, when HeLa cells were engineered to express wild-type Cx30 or the various mutants, only Cx30 and the T5M mutant were able to pass Alexa 350 suggesting that all mutants, except T5M, are loss of gap junction function mutants. Next, when we expressed the Cx30 mutants in rat epidermal keratinocytes (REKs), TUNEL assays performed 24 hours later revealed that the V37E and A88V mutants induced cell apoptosis. Confocal microscopy revealed that wild-type Cx30 and the T5M mutant readily assembled into gap junction-like plaques while all skin disease-linked mutants were primarily found within intracellular compartments, where they frequently co-localized with resident molecules of the endoplasmic reticulum (ER). Interestingly, a population of the A88V mutant was detected in gap junction-like plaques at 12 hours after transfection prior to the onset of cell death. Currently we are investigating the hypothesis that select Cx30 mutants are retained primarily in the ER, resulting in ER stress, which subsequently triggers the unfolded protein response and premature apoptotic cell death. Collectively, these findings are beginning to provide insight into the mechanisms of how select mutants may cause only non-syndromic deafness while others are syndromic in nature resulting in severe skin diseases. Funded by the CIHR to DWL.

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Mefloquine inhibits connexin26 hemichannel mutants linked to keratitis-ichthyosis-deafness syndrome.

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Connexin (Cx) proteins form intercellular gap junction channels to facilitate the exchange of ions and small molecules between adjacent cells. Mutations in connexins cause several human diseases by interrupting normal cell-to-cell communication and coupling. Connexins also form functional hemichannels in nonjunctional membranes that have an unclear role in normal physiology but have altered activity in certain pathological states. Mutations in *GJB2*, the gene encoding connexin26 (Cx26), are linked to sensorineural hearing loss as well as syndromic deafness associated with skin disorders. In contrast to Cx26 mutations causing nonsyndromic deafness, those that also cause skin disease are exclusively gain-of-function single amino acid changes with autosomal dominant inheritance patterns. At least 9 missense mutations near the N-terminus and first extracellular loop of Cx26 have been linked to keratitis-ichthyosis-deafness (KID) syndrome, which can consist of life threatening skin phenotypes. Functional changes related to hemichannel open probability and permselectivity have been shown for 7 of the 9 KID-causing Cx26 mutations but the specific pathophysiologic mechanism leading to skin disease is presently unknown. We hypothesize that hemichannel dysregulation, downstream of a causative Cx26 mutation, interferes with keratinocyte proliferation and differentiation in the epidermis. To further enable evaluation of the deleterious consequences of aberrant Cx26 hemichannel activity, we have identified and characterized a series of quinine-family small molecule inhibitors. A *Xenopus laevis* oocyte expression assay combined with single-cell voltage clamp electrophysiology was used to measure mutant Cx26 hemichannel current in the presence and absence of blocking candidates. Our data show that distinct Cx26 mutant variants respond to pharmacologic agents differently, highlighting the specificity of inhibition. The highest efficacy for Cx26 blockade was achieved by mefloquine, an FDA-approved drug with minimal untoward effects reported in the skin. Mefloquine was tested at 10, 30, and 100 μ M and showed a dose response for G45E, D50N, A40V, D50A, and G12R mutants with average maximal hemichannel current suppression of 89.6%, 75.4%, 69.8%, 60.1%, and 78.8% respectively. The A88V and N14K mutants did not significantly respond to mefloquine at these concentrations. This work will pave the way for *in vivo* studies with a transgenic mouse model of KID syndrome by tissue-specific and inducible expression of Cx26-G45E, having the potential to establish a basis for pharmacologic prevention or intervention strategies.

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Mast cell degranulation induced by amyloid β peptide is mediated by pannexin 1 hemichannels.

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Mast cells are resident immune cells of vascularized tissues. In granules they store an array of pro-inflammatory mediators, which are rapidly released to the extracellular milieu upon activation.

Objective: Since prolonged exposure to the amyloid A β_{25-35} peptide *in vitro* leads to neurotoxicity mediated by activation of a hemichannel-dependent cascade of glial cells and promotes mast

cell degranulation, we evaluated if acute treatment with A β ₂₅₋₃₅ peptide promotes mast cell degranulation via pannexin hemichannel activation.

Methods: Primary naïve mast cells were derived from bone marrow precursors of wild type (WT) and Panx1 K.O. (Panx1^{-/-}) mice using WEHI3 cells conditioned medium. Panxs 1, 2 and 3 mRNAs were evaluated by RT-PCR analyses. Bone marrow derived mast cells were stimulated with A β ₂₅₋₃₅ peptide. The hemichannel activity was assessed by 4',6-diamidino-2-phenylindole (DAPI) uptake assays and membrane currents were evaluated by whole cell patch clamp, while degranulation was evaluated by quantification of extracellular histamine and release of toluidine blue (TB) from TB preloaded mast cells.

Results: Only Panx1 mRNA was detected in bone marrow derived WT mast cells. Stimulation with A β ₃₅₋₄₂, but not A β ₄₂₋₃₅ peptide, induced histamine and TB release and also enhanced DAPI uptake and total membrane currents in WT mast cells. These responses were drastically reduced in WT mast cells pretreated with 10 μ M carbenoxolone, which blocks Panx hemichannels, and was absent in mast cells derived from Panx1^{-/-} animals.

Conclusion: Bone marrow derived mast cells express functional Panx1 hemichannels, which are essential for the degranulation response induced by A β ₃₅₋₄₂ peptide. Thus, inhibition of Panx1 hemichannels might avoid the spread of inflammatory responses coordinated by mast cells in Alzheimer's disease.

1302

The channel protein Pannexin1 regulates early events in skin development.

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Pannexins are a family of channel-forming glycoproteins found at the cell surface of many mammalian cells. Panx1 and Panx3 channels have been reported as conduits for ATP release, and play an important cellular role in the differentiation of keratinocytes, chondrocytes and osteoblasts. We previously reported the expression of Panx1 and Panx3 in mouse dorsal skin as early as embryonic day 13.5. We also found that ectopic expression of Panx1 in organotypically grown keratinocyte cultures resulted in disrupted architecture of the epidermis. Using a Panx1 knockout mouse model we set out to evaluate the role of Panx1 in skin differentiation and development. Although there were no overt skin abnormalities in Panx1 null mice, histological staining of newborn skin sections revealed a significant reduction in skin thickness compared to controls. Also, in Panx1 null mice, as early as four days after birth, there was an apparent increase in hypodermal adipose tissue that persisted into adulthood. To more thoroughly characterize the cellular constituents of the epidermis and dermis, we isolated primary keratinocytes and dermal fibroblasts from both knockout and control neonatal mice. Both primary cell types from control mice expressed high levels of Panx1 and were capable of sulforhodamine B dye uptake after mechanical drip stimulation, while dye uptake in Panx1 null keratinocytes and fibroblasts was significantly less. Scratch wound experiments and proliferation assays revealed that Panx1 null primary keratinocytes were more proliferative and migratory while Panx1 null dermal fibroblasts were more proliferative than controls. Western blots of differentiating primary keratinocytes from control mice in the presence of calcium revealed a progressive decrease in the levels of Panx1 as the markers of keratinocyte differentiation increased. Thus far we can conclude that Panx1 is present at early stages of murine skin development and appears to regulate the cellular properties of resident keratinocyte, fibroblast and adipocytes of the skin. We are beginning to assess the response of

the Panx1 null mouse skin to wounding and UV irradiation to determine if the lack of Panx1 also affects normal skin function after injury. Supported by the Canadian Institutes of Health Research.

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Denervation induces functional expression of connexin hemichannels followed by an inflammatory state in fast skeletal muscles.

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Denervation of skeletal muscles induces several responses, between them are included reduction of the resting membrane potential and increase of total intracellular Ca²⁺ levels. However, the molecular mechanism that leads to these changes remain poorly understood. In addition, it occurs reexpression of proteins that were present during muscular ontogeny, such as embryonary acetylcholine receptor. Connexins (Cxs) 39, 43 and 45 are proteins expressed during muscular development but not during adulthood. In this work, we studied if Cxs 39, 43 and 45 and functional hemichannels formed by these Cxs (Cx HCs) are reexpressed in denervated fast skeletal muscles. In denervated EDL and FDB muscles of Sprague-Dawley rats, levels of Cxs and activity of Cx HCs increased *in vivo* (Evans blue dye uptake) and *in vitro* (ethidium uptake). Both responses were inhibited by Cx HC and P2X₇ receptor blockers. In addition, denervated muscles did not show rhodamine-dextran (10 kDa) uptake *in vivo*, indicating that the cell membranes were permeabilized to Evans blue dye but not irreversibly damaged. Also, denervated myofibers showed activation of nuclear factor-kappaB (Phosphorylated-NFκB), and increased levels of IL-6 and IL-1β mRNA. In addition, the expression of P2X₇ and the TRPV2 receptors was increased. It is proposed that Cx HCs together with P2X₇ and TRPV2 receptors, all permeable to ions, including Ca²⁺, not expressed in normal muscles, might explain the reduction in resting membrane potential and increase of total intracellular Ca²⁺ levels observed in denervated skeletal muscles.

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Dexamethasone induces inflammation through expression of functional connexin hemichannels in skeletal muscle fibers.

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Glucocorticoids are used in clinic as anti-inflammatory agents. However, prolonged exposure to synthetic glucocorticoids leads to skeletal muscular atrophy, among others undesired changes. We studied whether dexamethasone affects the expression of connexin hemichannels (Cx HCs), which are absent in adult skeletal muscles. Wild type and specific skeletal muscle K.O. for Cx43/Cx45 (Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre) mice were acutely treated with dexamethasone (300 nM, i.p.). After 5 h treatment, myofibers were dissociated from flexor digitorum brevis (FDB) muscles, where the Cx HC activity (ethidium uptake) was tested. Cxs 39, 43 and 45 were detected in muscles of wild type mice treated with dexamethasone but not in control animals. The Cx HC activity increased ~3.5-fold and was blocked by La³⁺, a Cx HC inhibitor. This response was absent in myofibers from skeletal muscle Cx43/Cx45 K.O. mice. Furthermore, the

resting membrane potential of myofibers decreased from -78 mV (control) to -64 mV (dexamethasone). This response was absent in myofibers from skeletal muscle Cx43/Cx45 K.O. mice. Additionally, muscles of wild type mice treated with dexamethasone showed numerous positive nuclei for phosphorylated NF- κ B (P-NF κ B) (absent in control animals), and elevated mRNA levels of IL-6 and IL-1 β (RT-PCR). Therefore, we propose that atrophy induced by dexamethasone is, in part, due to early Cx HC expression that are permeable to ions including Ca²⁺ and thus, can mediate activation of the inflammasome.

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Molecular mechanisms underlying the Connexin43-dependent amplification of Fibroblast Growth Factor-2 signaling in MC3T3 osteoblasts.

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The gap junction protein connexin43 (Cx43) plays a fundamental role in osteoblasts/osteocytes function and bone formation. In addition, fibroblast growth factor-2 (FGF2), an important modulator of skeletal tissue, activates several signaling cascades, including phospholipase C γ 1 (PLC γ 1), to affect osteoblast function. We have previously shown in MC3T3 osteoblasts that overexpression of Cx43 potentiated the FGF2-induced transcriptional activity of Runx2, a key regulator of osteoblast differentiation, in an ERK and PKC δ -dependent manner. The current study aimed to define the molecular mediators of this Cx43-dependent potentiation of osteoblast signaling and transcription, thus identifying second messengers generated downstream of PLC γ 1 that may be communicated by Cx43 to affect osteoblast gene expression. Modulation of transcriptional activities was assessed by luciferase assays using the p6xOSE2-Luc construct, which contains six repeats of a Runx2 binding cognate upstream of a minimal promoter, in MC3T3 cells transiently overexpressing Cx43. Additionally, pharmacological inhibitors or siRNA directed against various mediators of the PLC γ /inositol polyphosphates cascade were tested. Following the knockdown of expression and/or inhibition of function of PLC γ 1, the Cx43-mediated potentiation of the FGF2 response was abrogated, indicating that PLC γ 1 is needed for the Cx43-dependent amplification of FGF2 stimulation of Runx2. Surprisingly, diacylglycerol lipase or inositol triphosphate receptor inhibitors failed to affect the Cx43/FGF2 synergistic effect on Runx2. Accordingly, we examined other inositol phosphate mediators. Knockdown and/or inhibition of inositol polyphosphate multikinase (IPMK), and inositol hexakisphosphate kinase 1 (IP6K1), which generates inositol poly and pyrophosphates respectively, prevented the ability of Cx43 to potentiate FGF2-induced signaling through Runx2. Disruption of the IP6K pathway blocked the nuclear accumulation of PKC δ and the FGF2-dependent interaction of PKC δ and Runx2, reducing Runx2 transcriptional activity. In total, our data show that activation of the PLC γ 1/IPMK/IP6K1/PKC δ signaling cascade is required for the potentiation of FGF2-induced Runx2 transcriptional activity by Cx43. Also, our findings implicate the water-soluble inositol polyphosphates as mediators of the Cx43-dependent amplification of the osteoblast response to FGF2, and suggest that these low molecular weight second messengers may be biologically relevant mediators of osteoblast function that are communicated by Cx43-gap junctions.

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Utilizing hTERT-immortalized primary mouse osteoblasts to assess the role of Cx43 in osteoblast signaling pathways.*A. M. Buo¹, J. P. Stains¹; ¹Orthopaedics, University of Maryland School of Medicine, Baltimore, MD*

The coupling of bone-forming osteoblasts by connexin43 (Cx43)-comprised gap junctions is critical for osteoblast differentiation and optimal bone formation. We have begun to define the molecular details that enable Cx43 to regulate osteoblast differentiation and osteoblast function. Previously, using an MC3T3-E1 osteoblast-like cell line, we identified several signaling cascades that are affected by overexpression or knockdown of Cx43. Further, alterations of these signaling cascades modulate the activity of the essential osteogenic transcription factors Runx2 and Osterix. In this preliminary study, we used a recently reported human telomerase reverse transcriptase (hTERT)-immortalized primary mouse osteoblast cell line isolated from Cx43-null mice (MOB-Cx43KO) and a paired wild type control cell line (MOB-WT) (M. Thi et al., *Am J Phys Cell Phys.* 2010) to determine if the effects observed in MC3T3-E1 cells translated into this more sophisticated cell system. We examined the hypothesis that the osteogenic defects characterized in Cx43-deficient cells are due to reduced signaling resulting in impaired Runx2 and Osterix activation. Confluent cultures of MOB-Cx43KO and MOB-WT cells were examined by western blotting for levels of Cx43, Runx2, Osterix, and key signaling molecules. To test the effects of growth factor signaling, cells were serum starved overnight and then treated with FGF2 for 0, 5, or 30 minutes and analyzed for phospho-extracellular signal-regulated kinase (pERK) and phospho-protein kinase C delta (pPKC δ) levels. Under standard culture conditions, we found that MOB-Cx43KO cells had reduced pERK and pPKC δ levels compared to MOB-WT cells. Runx2 abundance was unaffected by loss of Cx43. Conversely, Osterix expression was diminished in MOB-Cx43KO compared to MOB-WT cells. Additionally, MOB-Cx43KO cells displayed reduced pERK and pPKC δ levels relative to MOB-WT cells when treated with FGF2. These findings are consistent with our results in mouse MC3T3-E1 osteoblasts that implicate ERK and PKC δ as downstream mediators of the Cx43-dependent effects on Runx2 and Osterix activity. Future studies will examine Runx2 and Osterix recruitment to target osteoblast promoters by chromatin immunoprecipitation assays. In summary, our data support the hypothesis that the presence of Cx43 is needed for efficient basal and growth factor induced activation of ERK and PKC δ and highlight that this model (MOB-Cx43KO cells) is appropriate for more detailed mechanistic studies of Cx43 function in osteoblasts.

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The Role of the Connexin43 C-Terminal Tail in the Potentiation of FGF2 Signaling in Osteoblast-like Cells.*C. Hebert¹, J. P. Stains¹; ¹Orthopaedics, University of Maryland School of Medicine, Baltimore, MD*

Gap junctions play an important role in the differentiation and function of bone forming osteoblasts, yet little is known about the molecular mechanisms by which they affect bone cell function. Connexin43 (Cx43) is the most abundant gap junction protein expressed in these cells. In this study, we examine the structure-function relationship of Cx43 with signaling in osteoblasts. The cytoplasmic, C-terminal tail of Cx43 interacts with numerous signaling complexes. We hypothesize that signal complex docking to the Cx43 C-terminal tail (CT) is required to propagate the molecules being shared by gap junctions. We have previously shown in osteoblasts that Cx43 impacts the responsiveness to FGF2 in a PKC δ - and ERK-dependent manner. In this study, we mapped the interaction domain of Cx43 and PKC δ to amino acids

243-302 of the Cx43 CT by GST pulldown assay. Using luciferase reporter assays, a Cx43 Δ CT construct lacking amino acids 244 to 382 failed to support the Cx43-dependent potentiation of transcription following FGF2 treatment in MC3T3 osteoblast-like cells. Overexpression of just the CT of Cx43 (amino acids 236 to 382) was unable to substitute for the ability of full-length Cx43 expression to potentiate transcription following FGF2 treatment, indicating that the potentiation of signaling and transcription by Cx43 is not independent of gap junction function. Notably, in the presence of full-length Cx43, overexpression of the Cx43 CT dose-dependently inhibited the potentiation of the transcriptional response to FGF2 and blocked FGF2 activated signaling via PKC δ and ERK. Conversely, this inhibition did not occur in an osteoblastic cell line (UMR-106) that lacks endogenous Cx43, suggesting that overexpression of the Cx43 CT blocks endogenous Cx43 function through direct or indirect modulation of the full length Cx43 function. In summary, the data support a model in which an intact Cx43 is required for both signal propagation/permeability and local recruitment of signaling complexes to the CT in order to mediate its cellular effects.

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Cx30 and Cx43 exhibit distinct partitioning and dynamics when co-expressed in the same cell.

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Connexins oligomerize into pore forming hexamers called connexons. Typically, two compatible connexons dock to form a gap junction channel that allows for the direct intercellular exchange of small molecules. Channels, in turn, proceed to cluster into aggregates known as gap junction plaques. In keratinocytes, any number of 9 connexins may be co-expressed and mutations in connexins such as Cx43 and Cx30 cause severe skin disease. In the present study we investigate how cells co-expressing Cx43 and Cx30 assemble these connexins and whether these two connexins have similar turnover kinetics. To further investigate a previously reported suggestion that Cx30 traffics via a Golgi-independent pathway, HeLa cells stably expressing Cx43 or Cx30 were treated with the ER-Golgi transport inhibitor Brefeldin A (BFA) for 8 hours before patch-clamping and assessing neurobiotin transfer. While Cx43 expressing cells exhibited a 60% decrease in neurobiotin spread, Cx30 expressing cells exhibited no change in comparison to controls. However, fluorescence recovery after photobleaching analysis of Cx30-GFP containing gap junction plaques in the presence and absence of BFA revealed that BFA delayed the recovery of the fluorescent plaque, suggesting that new delivery of Cx30 to the cell surface via ER-Golgi transport was necessary to renew the gap junction. Furthermore, when keratinocytes and NRK cells expressing both Cx30 and Cx43 were treated with BFA for 8-18 hours, Cx30 gap junctions were largely retained at the cell surface while Cx43 gap junctions were generally lost. However, in an aggressive mammary tumor cell line where the half-life of Cx43 is particularly short (1.5 hours), both Cx43 and Cx30 cleared from the cell surface in the presence of BFA within 4 hours. Finally, co-expression of Cx43-RFP and Cx30-GFP revealed that, while these connexins were found in the same gap junctions, they segregated into distinct domains suggesting their assembly was governed by different mechanisms. Collectively our data supports the notion that Cx30 traffics through the cell in a Golgi-dependent pathway, but segregates from Cx43 in the same plaque and acquires a more stable state at the cell surface in some cell types. The differential kinetics and assembly of these connexins may be of critical importance in understanding the skin pathology of patients that harbor mutations in either of the genes encoding these connexins. Supported by the CIHR to DWL, Royal Society to DJJ and DRUK to JJK.

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Analysis of Annular Gap Junction Vesicle Fission.

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Gap junction plaque disassembly involves an internalization process which results in the formation of annular gap junction vesicles. These annular gap junction vesicles undergo fissions, however, the molecular machinery or the benefit of such a process remains to be elucidated. Dynamin, a large mechanoenzyme, has been previously demonstrated to play a pivotal role in gap junction plaque internalization. Specifically, dynamin scissors the invaginated gap junction membrane from the cell surface. To investigate the role of dynamin in annular gap junction vesicle fissions, dynamin was inhibited in a SW-13 adrenocortical human cell population, either by siRNA knockdown or treatment with a dynamin GTPase inhibitor, dynasore. Immunocytochemical localization of connexin and dynamin protein antigen, time-lapse photography, transmission electron microscopy, and computer-assisted imaging with the tracking component of the Imaris software program were used to analyze annular gap junction vesicle fissions. Dynamin co-localized with annular gap junction vesicles as well as with gap junction plaques. With 3D-rotation the accumulation of the dynamin at or near the equatorial region of the annular gap junction vesicle was demonstrated. In addition, the number of annular gap junction vesicle fissions per hour was reduced in dynamin inhibited populations compared to the number of fission events in controls. Fewer annular gap junction vesicles were observed to form in the dynamin inhibited populations and those that formed were larger than in control populations. This corresponded to an increase in the number and size (area) of gap junction "buds" suspended from the gap junction plaques in dynamin inhibited populations. In control populations such buds, were frequently observed to be released from the parent membrane to form annular gap junction vesicles. We believe this to be the first report describing the details of annular gap junction vesicle fissions and demonstrating a role of dynamin in not only annular gap junction formation but in the fission process. This information is critical to understanding gap junction protein trafficking and turnover. This research was supported by NSF grants # MCB-1023144 and NIH 5T36GM008622.

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The cataract related mutation of human Connexin46 D3Y and N188T affect the voltage sensitivity and the formation of gap junction channels.

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Xenopus oocytes and HeLa cells were used as expression systems to analyze how the cataract related mutations in connexin46 affect gap junctional intercellular communication. In Xenopus oocytes, we observed that hCx46N188T formed voltage gated hemichannels similar to the wild type. The channels were activated at a depolarization voltage above -50 mV. In contrast, hCx46D3Y was not able to form voltage gated hemichannels. In HeLa cells, we found that 38%, 37% and 12% of cells expressing hCx46wt, hCx46D3Y and hCx46N188T respectively formed gap junction plaques. Lucifer Yellow transfer experiments showed that 35%, 62% and 15% of the cell pairs expressing hCx46wt, hCx46D3Y and hCx46N188T formed dye coupled gap junctions. It is noteworthy that in non-transfected HeLa cells, 4.7% of the cell pairs were dye coupled. Since the D3Y mutation replaced a negative charged residue by a neutral residue, and the N188T replaced a residue with an amide group by a residue with a hydroxyl group, we generated a D3E mutation which does not change the charge at the third position and hCx46N188Q which contains also an amide group. The reintroduction of a negative charge at

the third position (hCx46D3E) could restore the formation of voltage gated hemichannels in the *Xenopus* oocytes but the reintroduction of an amide group at the position 188 failed to restore the capacity to form gap junction plaques and dye coupled gap junction channels when expressed in HeLa cells. The results indicate that the voltage sensitivity might be related to the presence of a negatively charged residue at the third position. The role of the asparagine residue at position 188 in formation of gap junction channels seems to be related to this specific residue and cannot be overtaken by a structurally similar amino acid residue.

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Oligomeric structure and functional characterization of *C. elegans* innexin-6 gap junction channels.

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Invertebrates possess gap junction channels composed of innexins that evolved independently from vertebrate connexins. The important role of innexins in electrical activity in neurons and muscles has been confirmed in *Drosophila* and *Caenorhabditis elegans* (*C. elegans*), but only limited information is available on the innexin channel structure. Here we successfully expressed and purified *C. elegans* innexin-6 (INX-6, 45 kDa) gap junction channels, and characterized the structural properties and channel permeability using electron microscopy and microinjection of fluorescent dye tracers, respectively. The *C. elegans* INX-6 channels formed gap junction plaques with a loosely packed hexagonal lattice. Channel distance of INX-6, based on the reflection spots of gap junction plaques, was longer than those of connexin-26 (Cx26, 26 kDa) and connexin-43-GFP (Cx43-GFP, 70 kDa). Thin-section electron microscopy revealed that the INX-6 junction was thicker than the Cx26 and Cx43-GFP junctions. The purified INX-6 channels in dodecylmaltoside solution generally ran slower than Cx26 channels and exhibited the elution peak at ~400 kDa in gel filtration analysis, suggesting that the purified INX-6 channels were mostly hemichannels, which was supported by electron microscopy and native gel electrophoresis. Dye transfer experiments showed that 3-kDa dextran-Texas red and 10-kDa dextran-Texas red passed through INX-6-GFP-His channels at rates of approximately 50% and 10%, respectively, while no significant amounts passed through Cx43-GFP channels. These results indicate the larger dimensions of overall structure and pore diameter of INX-6 channels compared with those of most connexin channels.

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The role of connexins in melanoma tumorigenesis as revealed in 2D and 3D microenvironments.

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Gap junctions formed from connexins (Cx) have been reported to regulate cell proliferation, differentiation and apoptosis through the formation of intercellular channels between apposing cells. Interestingly, Cxs have been identified as tumor suppressors and enhancers, a distinction that appears to be dependent on the type of tumor and stage of disease. In melanomas, however, the role of Cxs and their status during cancer onset and progression remains poorly understood. In the current study, we hypothesize that the exogenous expression of Cx26 and/or Cx43 in BL6 mouse melanomas will decrease their tumorigenic properties when grown in homocellular cultures and/or within the 3D milieu of organotypic epidermis. Western blots, immunofluorescence and Alexa350 dye coupling studies revealed that mouse melanoma BL6

cells express very low levels of Cx26 and Cx43 and are poorly coupled. When cells were engineered to express GFP-tagged Cx26 or Cx43, dye transfer studies revealed that both Cx26- and Cx43-expressing cells were highly coupled, similar to keratinocyte controls. Interestingly, when these connexin-rich melanomas were co-cultured in 2D with Cx43-rich keratinocytes, preliminary dye transfer studies revealed that the melanoma microtumors that formed remained functionally isolated and failed to couple to surrounding keratinocytes suggesting a lack of heterocellular gap junctional intercellular communication. To assess the tumorigenic properties of the newly generated melanoma cell lines, wild type, empty vector and connexin-expressing cells were subjected to proliferation, migration and anchorage-independent growth assays. Growth curve studies performed over 6 days revealed that exogenous Cx43, but not Cx26, significantly reduced total cell numbers by approximately 2.5-fold while cell migration was statistically unaltered by the expression of either connexin. Furthermore, preliminary studies suggest that Cx43 significantly reduces anchorage-independent growth by approximately 4-fold compared to controls. Overall, our results to date would suggest that Cx43, but not Cx26, affects several in vitro melanoma tumorigenic properties despite the fact that both connexins greatly enhance homocellular gap junctional intercellular communication. Currently we are assessing the growth of melanoma microtumors in the context of keratinocyte organotypic cultures to determine the influence of the microenvironment on the progression of tumors rich in connexin content. Supported by the Canadian Institute of Health Research to DWL.

Cadherins and Cell-Cell Interactions

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Making a link between polar cell growth and speciation: a molecular and cellular perspective to understand the barrier to cross-pollination between maize and teosinte.

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Even with the increasingly available genomic, genetic, cellular and molecular tools, mechanisms of how a crossing barrier forms between two populations that were previously interbreeding is still elusive. This study aims to understand the crossing barrier between maize and some strains of teosinte, which is governed by a single locus *tcb1* (*teosinte cross barrier1*). The *Tcb1-s* haplotype present in these strains encodes a female factor that blocks maize pollen (haplotype *tcb1*) and a male factor that overcomes that block. *In vivo* assays showed that maize pollen tubes are arrested in *Tcb1-s* silks. Out of a mapping population of 16,451 chromosomes, the male factor, *Tcb1-m*, and female factor, *Tcb1-f*, have been separated by three recombination events. *Tcb1-m* is located within an interval flanked by markers that are 43,691 base pairs apart in the B73 reference genome. Only one protein-coding gene and a portion of the promoter of a second gene are in this region in the maize B73 reference genome. Gene expression analysis showed that the second gene is highly expressed in mature pollen grains and expression level in *Tcb1-s* is significantly higher, which makes it a promising candidate. This gene has been cloned and is being transformed into maize to test its *Tcb1-m* function by pollinating *Tcb1-s* females. By screening for loss-of-function mutant of *Tcb1-s*, one line with loss-of-function of *Tcb1-f* while retaining function of *Tcb1-m* has been obtained; an additional seven putative *Tcb1-f* loss-of-function mutants are being retested. To determine whether or not there are novel genes absent from the maize reference genome but present in the *Tcb1-s* haplotype that encode *Tcb1-s* function, we have constructed a BAC library from plants of a *Tcb1-s* containing maize line. BACs that cover this region were fished out and sequenced. Candidate genes are to be cloned and transformed into maize to confirm their function. Identification of the male and female genes that produce a reproductive barrier will provide novel insights into the processes

of speciation, polar cell growth and cell-cell communication. More broadly, this understanding may lead to novel applications for the control of cross-pollination and the restriction of gene flow.

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A Genome-Wide RNAi Screen and Analysis of the Cadherin-Mediated Cell-Cell Adhesion Pathway.

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The adherens junction plays critical roles in cell-cell recognition, initiation of cell-cell adhesion and directing structural and functional polarization of cells in tissues. Defects in adherens junctions are linked to many diseases and cancers. A few proteins are known to function at adherens junctions, but a comprehensive analysis of protein function at cell-cell adhesion remains to be established. We completed a genome-wide (~14000 gene) RNAi screen based on short term, Ca⁺⁺-dependent formation of large cell aggregates by DE-cadherin *Drosophila* S2 cells in suspension culture, which eliminated Ca⁺⁺-independent cell-cell adhesion, integrin-ECM based adhesion and cell spreading, and cell migration. Stringent screen and validation strategies were used: 1). Screen DE-cadherin S2 cells against the genome-wide RNAi collection (~1200 hits), and filter/ rescreen those that inhibited cell aggregation against new RNAis (~400 hits); 2). Analyze the 100 strongest hits for DE-cadherin/catenin levels by western blotting; 3). Perform cell-cell adhesion assays with siRNA-treated MDCK cells in suspension culture, and analyze E-cadherin-catenin levels by western blotting and immunofluorescence microscopy; and, 4). Target germ-line RNAi in *Drosophila* using TRiP vectors and score for defects in DE-cadherin-dependent oocyte positioning in the *Drosophila* germarium. These hits highlight several integral pathways (actin cytoskeleton organization, protein trafficking and turnover, signaling, gene transcription) that function to co-ordinate cadherin-mediated cell-cell adhesion. RNAi of several of these mutants had defects in oocyte positioning in *Drosophila* oogenesis and mammalian MDCK cell hanging drop assays suggesting they have conserved roles in cell-cell adhesion.

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Alpha-catenin's role in cell-cell adhesion of DLD1 cells.

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Alpha-catenin is an actin-binding protein that we hypothesize to function as part of a cadherin-actin linkage in the protein complex at the adherent junction of adhesion-competent cells. To test this assumption we need a catenin positive and a catenin negative cell lines. The latter are commercially available. To obtain the former, we first subjected the heterogeneous DLD1 parental cell line to an aggregation assay, collecting the aggregation competent clusters. Those cells were dissociated, cultured to near confluence, and subjected to the same assay a second time. The doubly-selected population was then subcloned by limiting dilution. From the resulting colonies a few were selected that formed isolated islands of tightly adherent cells. We tested the colonies meeting those criteria by dot blot analysis using anti-alpha-catenin antibody (Ab 6299, ABCam) to select putative alpha-catenin positive clones. The presence of a catenin was verified by the appearance of a 102K mw band in Western blots of 10% SDS gels developed using the same antibody. To permit estimation of a catenin expression levels in the various clones, gels were loaded at constant total protein per lane (Lowry protein assays) verified by anti-tubulin staining (Thermo scientific). Trizol extracted RNA from a-catenin positive and

negative cells was verified by nanodrop and screened using a human genomic chip (details) for genes displaying up- or down-regulation coincident with states of α -catenin expression.

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The involvement of talin in cadherin-mediated cell-cell adhesions.

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Talin is an essential cytoskeletal protein that is known to act as a scaffold for integrin-mediated cell-matrix adhesions. Until our recent publication, little was known about the role of talin in cadherin-mediated cell-cell adhesions, which underlie the formation of multicellular organisms. We found that through calpain-2 proteolysis and posttranslational arginylation, a C-terminal fragment of talin contains vinculin- and actin- binding domains and a dimerizing domain (therefore referred to as the VAD fragment) that is recruited to cadherin-mediated adhesion sites and involved in promoting the polymerization of actin near cadherin. Here we found that the generation of the VAD fragment and its recruitment to the cadherin adhesions sites are highly dynamic processes mediated by multiple factors. Our data also suggest that the generation of the VAD fragment is likely associated with the force-induced conformational change of the talin molecule, and likely a direct result of the competition between the integrin- and cadherin-mediated adhesions in the cells.

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Myosin 1c regulates the morphogenesis of E-cadherin-based cell-cell contacts in polarized epithelial cells.

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Cooperation between cadherins and the actin cytoskeleton controls the formation of cell-cell adhesions during morphogenesis of polarized epithelia. We find that epithelial cells in which myosin 1c (myo1c) expression is knocked down (KD) are spread out and flatter with more stress fibers than control cells; moreover, E-cadherin localization is disorganized and rather than being vertical, lateral membranes have convoluted edges. After developing polarized monolayers, myo1c KD cells are more sensitive than control cells to reduced calcium concentrations, which cause cell-cell detachment. Myo1c sediments in the same fractions as E-cadherin following fractionation of plasma membranes on iodixanol gradients, and myo1c KD causes a significant reduction in E-cadherin recovery in one of the peak fractions. Using Myo1c point mutants or mutants in which specific domains are deleted, we find that the tail domain, especially the PIP₂ binding sites, are necessary for its localization to cell-cell adhesion sites. FRAP assays with GFP-myosin1c mutants reveal that motor function is also important for myo1c dynamics at cell-cell adhesions. Actin recovery at cell-cell junctions is unaffected by myo1c KD, and jasplakinolide treatment has no effect on GFP-myosin1c mobility. Furthermore, only the expression of wild-type GFP-myosin1c (but not motor-dead or PIP₂ mutants) restores the epithelial phenotype in myo1c KD cells. We conclude that myo1c is critical for the formation and maintenance of adherens junctions in polarized epithelial cells.

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Evidence for monomeric α -catenin as a physical linker between cadherin and the actin cytoskeleton.*U. Tepass¹, R. Patel¹, R. Sarpa¹, M. Pellikka¹, N. Ishiyama^{1,2}, M. Ikura^{1,2}; ¹University of Toronto, Toronto, ON, Canada, ²Ontario Cancer Institute, Toronto, ON, Canada*

The linkage of adherens junctions (AJs) to the actin cytoskeleton is essential for effective cell adhesion. How the cadherin-catenin complex contributes to the AJ-actin interaction remains a subject of intense investigation that centers on the function of α -catenin, which binds to cadherin via β -catenin and can bind either directly or indirectly to F-actin. We have delineated a number of regions within *Drosophila* α -Catenin (α -Cat) that are important for AJ performance in static epithelia and dynamic morphogenetic processes. Moreover, we address two prevailing questions concerning the mechanism of α -catenin function in order to distinguish between a physical linkage model and an allosteric regulation model of α -catenin function. First, is persistent α -catenin-mediated physical linkage between cadherin and F-actin crucial for cell adhesion? Second, what functions do α -catenin monomers and dimers have at AJs? Our data support the view that monomeric α -catenin acts as an essential physical linker between the cadherin- β -catenin complex and the actin cytoskeleton whereas α -catenin dimers are cytoplasmic and form an equilibrium with monomeric junctional α -catenin.

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Flotillins are new cadherins' partners that control cadherin stabilization at cell-cell contacts through interaction with the actin cytoskeleton.*C. Gauthier-Rouviere¹, S. Bodin¹, E. Guillaume¹, F. Comunale¹; ¹CRBM - CNRS, Montpellier, France*

Cadherins assemble into large macromolecular complexes at the cell-cell contact sites and form adherens junctions. These major adhesive structures are involved in many fundamental processes during development and adult life. Moreover, adherens junction assembly and function is perturbed in many diseases. Thus, identification of new regulators of cadherin complexes assembly is a very important issue.

Here we identify flotillin 1 and 2 as new partners of many cadherins (N, E, M, P, R and 11) that are recruited at cell-cell contacts in a cadherin-dependent manner and required for their stabilization at the cell-cell contacts. Flotillin 1 (Flot 1 ou reggie-2) and flotillin 2 (Flot 2 ou reggie-1) are ubiquitous and highly conserved proteins, located at the cytoplasmic face of the plasma membrane (PM), involved in the scaffolding of large complexes that signal across the PM thanks to their ability to form hetero-oligomeric complexes. Knockdown of flotillins dramatically affected N- and E-cadherin recruitment at cell-cell contacts in both mesenchymal and epithelial cell types. Mechanistically, we show that, although flotillins and cadherins are constitutively associated at the PM, flotillins stabilize cadherins exclusively at cell-cell contacts. Super-resolution structured illumination microscopy revealed that cadherins/flotillins complexes at cell-cell contacts are associated with actin stress fibers. Finally, we demonstrate that flotillin interaction with the F-actin cytoskeleton is required for cadherin stabilization at cell-cell contacts. In conclusion, we report here for the first time, that flotillins are major regulators of cadherin assembly and stabilization at cell-cell contacts and are thus essential for the formation of adherens junctions.

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Nectin and cadherin junctions are independent but coordinated by actin cytoskeleton.*I. Indra¹, R. Troyanovsky¹, S. Hong¹, S. M. Troyanovsky¹; ¹Dermatology, Northwestern University, Chicago, IL*

Two transmembrane adhesive receptors, nectin and cadherin, establish two interrelated cell-cell adhesion systems. How the function and assembly of these two adhesive systems are coordinated is not completely understood. We show here that both systems can be assembled independently. Assembly of both systems requires the integrity of the actin cytoskeleton. Upon co-expression, nectin and cadherin are co-recruited into the same adherens junctions. However, their intrajunctional distributions are different. Furthermore, their relative amounts in any one junction can significantly fluctuate over the time. Taken together, our data suggest that cadherin clustering results in local remodeling of actin filaments that, in turn, become a scaffold for nectin recruitment.

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N-cadherin limits the recruitment and dynamics of microtubules at cell-cell contact during stable cell-cell contact formation.*R-M. Mege¹, P-O. Strale¹, C. Plestant¹; ¹Inserm U839, Institut du Fer à Moulin, Paris, France*

Engagement of cadherin at cell-cell contacts either drive the re-enforcement of these contacts leading to the formation of stable intercellular junctions or maintain cell contact instability during cell-cell intercalation and cell migration. The mechanisms leading to these opposite cell responses are not fully understood but involve coordination between plasma membrane adhesion to the surrounding environment and cytoskeleton remodeling. Well-known actors of this process are cadherins and catenins that are part of a multi-molecular complex linking the cell membrane to the underlying microfilaments. Another element of this plasticity may be the microtubule networks (MTs) working in functional interaction with cadherins and actin. However, the relationship between cadherins and MTs is still unclear and subject to controversy. We investigate here the relationship between N-cadherin and MTs during stable cell-cell contact formation. We use spreading of myogenic C2C12 cells on recombinant N-cadherin to analyze the effect of cadherin engagement on MTs distribution and dynamics. Newly formed MTs were redirected toward adhesion sites by their +TIPs shortly after cell contact initiation. However, these MTs neither aligned nor colocalized with cadherin adhesions. A quantification analysis revealed a dramatic inhibition of the MT penetration in cadherin adhesions compared to fibronectin (FN) adhesions, an inhibition that was alleviated by the expression of a dominant negative form of N-cadherin. This inhibition paralleled a significant reduction of MT +ends forward movement. In addition we observed that N-cadherin engagement induced a two-fold stimulation of actin tread milling, suggesting that forward actin movements may relay the inhibition of MT penetration and dynamics elicited by N-cadherin engagement. We thus investigated the implication of actin filaments in the response of MTs to N-cadherin engagement. Treatment with cytochalasin D increased the speed of MT +ends forward movement. In contrast, inhibition of myosin II activity with blebbistatin, which blocks the actin tread-milling, induced an increased inhibition of MT +TIPs mean velocity, suggesting that the two processes are not directly linked. Interestingly, most of the MTs of cells spread on N-cadherin turned tangentially and did not pass through the acto-myosin belt present at the rear of the adhesion zone. In accordance, the destabilization of the actin network by cytochalasins or blebbistatin triggered an increased penetration of MTs in cadherin adhesion areas, likely unraveling an obstructive effect of tangential actin bundles on MTs penetration independently of MT dynamics. In turn, we observed that N-cadherin mediated contacts form independently of MTs but that the blockade of MT dynamics by taxol induced a strengthening of cadherin

adhesions. Altogether, these results establish a complex functional cross-talk between N-cadherin adhesion and MTs organization during cell-cell contact reinforcement.

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The Regulation of β -catenin and N-cadherin by CD82.

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Tetraspanins are regulatory membrane proteins that are integral for cell-cell and cell-matrix interactions. CD82 is a member of the tetraspanin family and has been demonstrated to regulate multiple cellular processes including cellular adhesion, signal transduction, cancer progression, and exosome trafficking. CD82 has the unique ability to interact with multiple cell surface molecules and cytosolic signaling components, which results in the various described cellular functions. Our previous work has implicated a role for CD82 in cellular communication between stem cells and their niche, more specifically in the processes of homing, engraftment and adhesion. A major signaling component in stem cells and in cell-cell interactions is the β -catenin signal transduction pathway. In this study we investigate the potential regulatory role of CD82 in these processes. We have found that the knock down of endogenous levels of CD82 with shRNA led to a significant loss of β -catenin compared to wild type Kg1a cells (CD34+ hematopoietic progenitor-like cell line). The decrease of β -catenin is not at the mRNA level but at the protein level observed by western blot analysis and immunofluorescence. Blocking the proteasome with MG115 in the CD82 KD cells led to the recovery of β -catenin back to wild type protein levels. While the cellular localization of β -catenin in Kg1a cells is membrane associated, it does not have the same cellular distribution as CD82 suggesting the involvement of another component. Since β -catenin and CD82 have been implicated in cadherin regulation, we sought out to determine whether cadherins were affected. We found that N-cadherin has similar distribution patterns to β -catenin in Kg1a cells. Interestingly, upon knock down of CD82, we observe that N-cadherin expression is markedly decreased and qRT-PCR data indicate a loss of message. As such, our data suggest a role for CD82 in regulating and stabilizing β -catenin intracellular pools and N-cadherin expression. We also observe that the main regulatory component of β -catenin, glycogen synthase kinase 3 β , is found in the phosphorylated state which correlates with our observed changes in cellular metabolism. The CD82-mediated perturbations in metabolism and down stream signaling will be discussed. In combination these data suggest that CD82 has an intricate role in maintaining cell-cell interactions and therefore may serve as a potential therapeutic target for improving stem cell transplantation.

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Regulation of mammary epithelial dissemination.

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Epithelia are fundamental tissues composed of polarized cells connected by numerous cell-cell junctions. Most solid tumors originate from epithelial cells, and their metastasis represents the major cause of death among cancer patients. Thus, a fundamental question in cancer biology is: how might cells escape from an epithelial-derived tumor?

A major paradigm in our current understanding of metastasis is loss of the adherens junction protein E-cadherin. However, most studies have tested the effect of downregulating E-cad in

cells already containing multiple genetic perturbations. The specific consequences of E-cad loss in otherwise normal cells and its sufficiency for metastasis remain unclear.

Mature mammary epithelium forms a bilayered tube separated from the surrounding ECM by basement membrane. We aimed to elucidate how an epithelial cell within such a highly organized tissue could acquire the ability to detach and individually migrate. With primary mammary tissue as a starting point, we sought to define the molecular perturbations sufficient for dissemination.

We developed methods for gene deletion and inducible gene expression, with coupling to fluorescent reporters that allow real-time observation of the cell behavioral consequences of genetic manipulations. We have demonstrated that deletion of *E-cadherin* inhibits branching morphogenesis and causes cells to lose polarity and invade locally beyond basement membrane. However, E-cad null cells surprisingly remain non-protrusive, adhere to one another, and rarely disseminate. *E-cadherin* deletion was not sufficient for dissemination in 3D culture or in vivo. We therefore hypothesized that dissemination requires additional changes to the fundamental characteristics of epithelial cells. We looked for insight in the epithelial-mesenchymal transition (EMT), a developmental transcriptional program thought to be co-opted by metastasizing cancer cells.

We next chose to test the effect of directly overexpressing the bHLH transcription factor Twist1, an EMT activator often overexpressed in invasive breast cancer. When we induced *Twist1* overexpression in all mammary epithelial cells, we observed not only inhibition of normal branching but also rapid migration of cells away from the tissue. Twist1's major recognized effect is E-cad repression, but our results suggest that a model for metastasis driven chiefly by E-cad loss is incomplete. We are now using *Twist1* overexpression as a platform for isolating the minimum molecular requirements for dissemination through (1) structure-function analysis of Twist1 mutants and (2) deep sequencing of early Twist1-induced transcriptional changes.

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Tetraspanin18 is a FoxD3-repressed antagonist of neural crest epithelial to mesenchymal transition that stabilizes Cadherin6B.

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Unlike typical neuroepithelial cells in the developing central nervous system, neural crest cells undergo an epithelial to mesenchymal transition (EMT), detach from the neural tube, and migrate to give rise to diverse structures, such as the peripheral nervous system and craniofacial skeleton. Despite the fundamental importance of the neural crest to the developing embryo, and the crucial role of EMT in diverse biological processes, the mechanisms that regulate neural crest EMT remain incompletely understood. Tetraspanin18 (Tspan18) is a member of the tetraspanin family of transmembrane proteins that have been implicated in cell signaling, motility and adhesion. Our objective was to determine whether Tspan18, which is expressed in chick premigratory neural crest cells and downregulated prior to migration, negatively regulates neural crest EMT. Strikingly, when chick neural crest precursors are electroporated with a Tspan18 expression construct to sustain its expression, migration is inhibited. Furthermore, neural crest cells that fail to migrate maintain epithelial Cadherin6B (Cad6B) protein despite temporally normal downregulation of Cad6B mRNA, suggesting Tspan18 antagonizes EMT by stabilizing Cad6B protein and promoting cell adhesion. In support of this, electroporation of a Tspan18 morpholino leads to premature loss of Cad6B protein from

the neural folds. Nevertheless, neural crest migration occurs on time in the majority of embryos, at least in part because basement membrane breakdown and mesenchymal marker expression, which are later steps in EMT, are normal. Curiously, Tspan18 knockdown also represses Cad6B transcription in some cases. This effect on Cad6B mRNA is independent of the Cad6B repressor Snail2, but correlates with increased nuclear β -catenin, perhaps as a consequence of its release from Cad6B-dependent adherens junctions. Finally, consistent with the fact that the neural crest transcription factor FoxD3 regulates neural crest cell adhesion and promotes migration, FoxD3 negatively regulates Tspan18 expression: FoxD3 overexpression represses Tspan18 mRNA expression, while FoxD3 knockdown leads to sustained Tspan18 mRNA expression and inhibits neural crest migration. Taken together, our data show that Tspan18 stabilizes adherens junctions to post-translationally antagonize neural crest EMT, and suggests that FoxD3 represses Tspan18 expression to promote neural crest migration. Supported by NIH F31 GM087951 and a U of MN Grant in Aid.

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Up-regulation of semaphorin 4A expression in retinal pigment epithelial cell by neural cells.

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Development of multicellular organisms requires interactions between cells and their local environment and between neighboring cells. We recently established an in vitro model based on the coculture of human corneal epithelial cells, fibroblasts, or neural cells separated by a collagen membrane.

We have now examined the role of neural cells in retinal homeostasis, and effects of neural cell to the RPE (retinal pigment epithelial cell). As a result, we showed that presence of neural cells (PC12) resulted in upregulation of neural guidance protein, semaphorin 4A in retinal pigment epithelial cells (ARPE19). This effect of neural cells was apparent at both the mRNA and protein levels, and was mimicked by exposure of ARPE19 to neuropeptides (substance P and CGRP). Furthermore, depletion of substance P and CGRP by antagonist in coculture media, largely abolished their stimulatory effect on semaphorin 4A expression in ARPE19. In multiplex-assay, the amounts of inflammatory cytokines, interleukin-6 (IL-6) was decreased by the presence of neural cells, and stimulation antagonist of substance P, or CGRP inhibited its effects.

Therefore, our findings indicated that neuropeptides, substance P and (,or) CGRP released from neural cells may play an important role in the regulation of intercellular communication between retinal pigment epithelial cells as well as in the maintenance of retina structure and function. In the future, these findings provide a useful model for understanding cell-cell interaction that occur between RPE and optic nerve cells, or photoreceptors.

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Role of Rho1 during adherens junction remodeling.

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Proper maintenance of cell-cell junctions is critical for the integrity of epithelial tissues during development, adult homeostasis, and in adult pathologies such as cancer and inflammation this maintenance is disrupted. The Rho family of GTPases is appreciated as key regulators of epithelial junction formation, but their precise role in the maintenance and remodeling of formed junctions is less well understood. Previous work in our lab using mosaic analysis in *Drosophila* pupal eyes revealed that Rho1 GTPase was required for the maintenance of adherens junctions (AJs) independent of Rho1's regulation of actomyosin contraction (Warner and Longmore,

2009). Loss of Rho1 function resulted in increased Cdc42 activity that promoted E-cadherin endocytosis, thereby disrupting AJs between adjacent mutant cells but not those between a wild type and mutant cell. We conducted a candidate gene suppressor screen in vivo in *Drosophila* pupal eyes to determine whether other genes implicated in E-cadherin endocytosis or vesicular trafficking rescued the AJ defect between Rho1-deficient pupal epithelial cells. This screen revealed that Rho1 influenced Rab11-mediated recycling endosome formation, independent of its effects upon Cdc42 and endocytosis. Overexpression of Rab11, but not Rab8 or Rab14, restored AJs in cells lacking Rho1. An in vivo endocytosis assay tracking the trafficking of E-cadherin showed that in the absence of Rho1 E-cadherin arrested in Rab5- and Rab11-positive common recycling endosomes (CRE) suggesting that Rho1 activity influences the progression of E-cadherin from the CRE to recycling endosomes. This occurred as a result of a localized reduction in Rab11 staining in the AJ/apical region, without affecting total cellular protein level. These effects were independent of Rho1's downstream actin modulating effectors Rok, Dia, and Pkn and the Cdc42/aPKC/Par6 complex. Instead, Rho1 functioned through the Rab11-FIP Nuf, but not the related FIP dRip11 or the exocyst complex, to restore the distribution of E-cadherin to recycling endosomes and thus rescue AJs. These data reveal that Rho1 remodels E-cadherin AJs in epithelia by regulating Cdc42-dependent endocytosis and, independently, Nuf/Rab11 recycling endosomes.

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The Rho GEF Bcr regulates cytoskeletal architecture of keratinocytes and controls epidermal differentiation via the desmosomal cadherin Desmoglein-1.

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Desmoglein 1 (Dsg1) is a desmosomal cadherin that is essential for maintaining cell-cell adhesion in the suprabasal epidermis. In addition to its important structural functions, we recently showed that Dsg1 participates in a signaling pathway required for normal epidermal differentiation and morphogenesis. Other studies have identified Rho GTPases as key players in controlling the three-dimensional architecture and stratification of keratinocytes. However, while signaling factors downstream of Rho (e.g. Rho Kinase) are known to contribute to the process of epidermal differentiation, little is known about which upstream regulatory proteins (guanine nucleotide exchange factors, GEFs; or GTPase activating proteins, GAPs) are involved in coordinating Rho signaling, or how Rho-related signaling mediates the keratinocyte differentiation program. Here we have identified the Rho GEF breakpoint cluster region (Bcr) as being a major regulator of RhoA activity in epidermal keratinocytes. Knockdown of Bcr in these cells significantly reduces RhoA activity, concomitant with a decrease in the formation of stress fibers and focal adhesions. Further, in both two and three dimensional models of human epidermal morphogenesis, loss of Bcr reduces the expression of multiple different markers of differentiation, such as Desmoglein-1 (Dsg1) and Desmocollin-1 (Dsc1), as well as Keratin-1, Keratin-10 and Loricrin. We further demonstrate that loss of Bcr, SRF, or the co-factor MAL reduces levels of Dsg1 mRNA transcript in human keratinocytes. Importantly, defects in differentiation seen upon loss of MAL/SRF signaling are rescued by ectopic expression of Dsg1, suggesting that Dsg1 expression induced by Rho/SRF signaling is important for the proper differentiation of keratinocytes. Taken together, these data identify the Rho GEF Bcr as a regulator of Rho signaling in human keratinocytes, as well as the process of differentiation by a mechanism involving the desmosomal cadherin Dsg1.

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The guanine-nucleotide exchange factor Trio regulates endothelial adherens junction formation through the activation of the small GTPase Rac1.

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Adherens junctions serve to maintain a restrictive endothelial barrier, but are dynamically regulated during inflammation and angiogenesis. The assembly of adherens junctions can be affected by activities of Rho GTPases. Conversely, cadherins can signal in an outside-in fashion to Rho GTPases, in particular Rac1, thereby relaying information from adhesion to the organization of the actin cytoskeleton. It is still unclear which GEF functions in endothelial cells to locally activate Rac1 during formation of VE-cadherin-mediated cell-cell adhesion. We show that the Rho-GEF Trio localizes at endothelial cell-cell junctions, where it interacts with the VE-cadherin complex. Trio silencing by shRNA results in more punctate junctions, accompanied by a decrease in the resistance of the endothelial monolayer. This decrease in endothelial monolayer resistance upon Trio depletion is rescued by expression of the N-terminal GEF-D1 domain of Trio, but not by the C-terminal GEF-D2 domain, indicating that Trio-induced Rac1 or RhoG activation is involved. Expression of the N-terminal Trio domain results in a morphological conversion from punctate to more linear junctions, similar to the effect seen by expression of a constitutively active mutant of Rac1 but not RhoG. In addition, Trio localizes primarily at newly forming contacts, suggesting that Trio, like Rac1, is involved in the initiation of novel cell-cell contacts. Finally, using beads coated with the ectodomain of VE-cadherin to mimic nascent junction formation, we demonstrated that VE-cadherin-dependent cell-cell adhesion activates Rac1, but not RhoG. Interestingly, Trio silencing blocked the increase in Rac1 GTPase activity observed after VE-cadherin-dependent contact formation. Collectively, we show that Trio is important for VE-cadherin-dependent Rac1 activation upon cell-cell contact formation. Our data suggest that Trio-mediated signals that are induced upon novel cell-cell contact formation promote VE-cadherin-dependent endothelial cell-cell adhesion and hence junction stabilization.

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p120-catenin binding masks an endocytic signal conserved in classical cadherins.

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Classical cadherins assemble into adherens junctions and mediate adhesive interactions that are essential for tissue architecture and integrity. During development, wound healing, and other dynamic morphogenic events, cadherin-based adhesive interactions display remarkable plasticity that is thought to be essential for tissue patterning. The regulated trafficking of cadherins to and from the plasma membrane is a critical cellular mechanism that modulates cadherin cell surface levels. However, the molecular machinery that determines cadherin fate after delivery to the plasma membrane is not fully understood. p120-catenin (p120) is an armadillo family protein which binds to the cadherin juxtamembrane domain and inhibits cadherin endocytosis. In the absence of p120 binding, cadherins are rapidly internalized and degraded. Here, we demonstrate that the core p120-binding site on classical cadherins contains a previously unidentified endocytic motif and that p120 binding physically occupies this motif to prevent clathrin-mediated endocytosis of cadherins. Using a gain of function approach

in which cadherin tail sequences were fused to the IL-2 receptor extracellular domain, we found that the core p120-binding regions of VE-, E-, N- and *Drosophila* DE-cadherins mediate endocytosis. Mutagenesis of VE-cadherin revealed that a three amino-acid acidic motif (DEE) is critical for both p120 binding and cadherin endocytosis, resulting in a cadherin that was both uncoupled from p120 and, paradoxically, stable at the cell surface. Mutation of three amino-acids (GGG) directly adjacent to this endocytic motif also uncoupled p120 binding, but this mutant was internalized normally from the cell surface. The p120-uncoupled mutant cadherins both assembled into cell junctions and recruited beta-catenin and alpha-catenin, but not p120, to cell borders. Using these mutants, we examined the effects of p120 binding and cadherin endocytosis on cell migration. Expression of wild-type and GGG-mutant VE-cadherin had no effect on endothelial cell migration in response to the angiogenic growth factor VEGF. In contrast, expression of DEE-mutant VE-cadherin, which was unable to undergo normal endocytic cycling, strongly suppressed cell migration in scratch wound assays but not in single-cell migration assays. Similar results were obtained when these mutants were expressed in epithelial cell lines. Collectively, these findings demonstrate that p120 binds to a novel endocytic motif present in the juxtamembrane domain of classical cadherins to prevent clathrin-mediated endocytosis. Furthermore, cadherin endocytosis is a critical feature of intercellular junction plasticity and is essential for endothelial and epithelial migration.

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The phospho-regulation of cadherin-based cell-cell adhesion.

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Multi-cellular organisms require cadherin/catenin-based adhesion for cellular differentiation as well as tissue architecture and integrity. Classical cadherins, such as E-cadherin, mediate calcium-dependent homophilic interactions between cells at the adherens junction. Reduction or loss of E-cad is observed in human epithelial cancers and is considered a key rate-limiting step in tumor metastasis. The cytoplasmic domain of E-cadherin binds the dual function adhesion/transcriptional co-activator protein β -catenin (β -cat), which in turn binds the F-actin binding protein, α -catenin, coupling adhesion to the actin cytoskeleton. The cytoplasmic tail of E-cad is phosphorylated in the β -cat binding region, and this phosphorylation increases the affinity for β -cat *in vitro*. However, the function and regulation of E-cad phosphorylation *in vivo* remain poorly defined.

We mapped three serines (S686, 692, and 693) in the E-cad tail responsible for ³²P-incorporation, and binding to and stabilization of β -cat. These serines are required for efficient cell-cell adhesion. A phospho-deficient E-cad (3S>A) fails to restore cell-cell adhesion to the pan-cadherin null cell line A431D in contrast to while a WT and phospho-mimic E-cad (3S<D). Additionally, 3S>A E-cad accumulates in a lysosomal compartment, is less stable than WT or S>D E-cad, and its degradation is blocked by chloroquine. Preliminary cell surface biotinylation experiments show that S>A E-cad is internalized more rapidly than WT E-cad; conversely, S>D E-cad is recycled to the plasma membrane faster than WT. These findings suggest that the kinase that regulates E-cad phosphorylation may regulate cadherin endocytosis/recycling dynamics.

Using an antibody to E-cad phosphorylation at S684/686, we show that E-cad is phosphorylated *in vivo* in simple and stratified epithelial tissues, and in cultured epithelial monolayers. Monolayers "scratched" with a pipette tip show a down-regulation of phospho-E-cad at the migrating wound edge. Moreover, phosphorylation at S684/686 is sensitive to inhibitors of GSK-

3 β such as LiCl and constitutively active Akt. These data suggest that E-cad phosphorylation is regulatable *in vivo*. We hypothesize that S686 is phosphorylated by GSK, but because GSK phosphorylation requires C-terminal phospho-serines, it is dependent on a still unknown priming kinase. To identify the priming kinase, a dsRNA knock-down screen of all ~350 kinases in the *Drosophila* genome is underway.

Epithelial cells must regulate the amount of E-cad at the cell surface according to their morphogenic or adhesive requirements. Our data suggest that E-cad phosphorylation is an emerging mechanism for regulating adhesion in response to signaling events, such as epithelial migrations and EMTs.

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Self-contact induced membrane fusion depends on E-cadherin.

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Mutual, homophilic cell-cell adhesion between epithelial cells is required for proper maintenance of epithelial barrier function. While opposing membranes from neighboring cells rapidly assemble junctional complexes, self-contacting membranes do not, suggesting that cells have the ability to distinguish self from neighboring cells. Using a self-contact inducing micro-fabricated substrate, we show that self-contacts of normal epithelial cells are rapidly eliminated by membrane fusion between two opposing plasma membrane regions of a single cell. This auto-fusion is most frequently observed in E-cadherin expressing epithelial cells, but not in fibroblasts. The efficiency of auto-fusion depended on extracellular calcium concentration and E-cadherin expression, suggesting that E-cadherin, while not required, enhances auto-fusion efficiency by bringing opposing membranes into close apposition to one another. Additionally, ROCK inhibition decreased auto-fusion of epithelial cells, suggesting that auto-fusion may be mechanically regulated through the actin-myosin network. This is the first demonstration of self-contact induced membrane fusion in mammalian cells, and that membrane fusion may be a key feature of the cell self-recognition signaling pathway.

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Studying E-cadherin mediated cell-cell junction formation using supported lipid bilayers.

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E-cadherin mediated cell-cell junctions play an important role in the development and maintenance of tissue structure in multi-cellular organisms. Although the expression of E-cadherin is frequently used as a marker for epithelial to mesenchymal transition, the mechanism and dynamics of cell-cell junction assembly and disassembly is poorly understood. For a deeper understanding of cell-cell junctions, supported lipid bilayers were functionalized with the extracellular domain of E-cadherin. Fluorescence Recovery After Photobleaching (FRAP), Fluorescence Correlation Spectroscopy (FCS) and Photon Counting Histogram (PCH) analyses

were used to characterize the density, mobility, and oligomeric state of the membrane bound E-cadherin molecules. Human epithelial (MKN28) cells seeded on E-cadherin functionalized bilayers showed enrichment of E-cadherin molecules, indicating the formation of a hybrid E-cadherin-mediated junction. Interestingly, we found differences between fluid and non-fluid bilayers with respect to junction formation. These results will be discussed further.

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Adhesion Molecules in Cell Sheet Engineering for Corneal Epithelium Regeneration.

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Cell sheet engineering has been successfully used for cornea epithelium regeneration. This technology shows a great potential for other organ regeneration as it avoids the limitations of tissue reconstruction using biodegradable scaffolds or single cell suspension injection. The present study focuses on investigating the adhesive characteristics of epithelial cell sheet (ECS) engineered from isolated oral mucosal epithelial cells, cultured on UpCell® (CellSeed Inc.). E-cadherin is essential for the integrity of epithelial tissues and functions as a suppressor of cell migration. In many cell invasive diseases, E-cadherin expression is lost and is generally replaced by other cadherins such as N-cadherin, a marker for cell migration and invasion. Immunofluorescence analysis of ECS showed a positive staining of E-cadherin and a negative staining for N-cadherin. Western blot analysis of E-cadherin levels in the engineered cell sheet was found up regulated, while N-cadherin levels were down-regulated in ECS. E-cadherin positive expression is known to inhibit β -catenin signaling, and exerts a negative effect on phosphoinositide 3-kinase (PI3K)/Akt signaling activation, thus preventing uncontrolled cell proliferation. ECS did not show beta-catenin nuclear translocation, thus preventing de-regulated growth. E-cadherin affects the formation of tight junctions by interacting with ZO-1 through interaction with alpha-catenin. ECS stained positive for ZO-1, indicating a stabilized tight junction of ECS. ZO-1 interacts in epithelial cells tight junctions with the gap junctional protein connexin-43. It also stained positive in ECS, and showed an up regulation compared to the rabbit normal corneal epithelium. In conclusion, the levels of these adhesion molecules reflected a low level of cell migratory behavior, indicating that ECS has adhesiveness and epithelial integrity that would favor a successful transplantation to the corneal stromal bed. Supported by CellSeed Inc. and Emmaus Medical, Inc.

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Refinement of a monolayer cell-binding assay to quantify the time-dependent strengthening of cell-cell adhesion.

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Cell adhesion plays a critical role in early development (e.g., the sculpting of tissues and organs), in daily maintenance of the body (e.g., wound healing), as well as in a number of aberrant conditions (e.g., tumor metastasis). A thorough understanding of cellular adhesion is thus a theme of broad importance in biomedical research. We have developed a monolayer cell-binding assay that yields data comparable to those obtained with the dual pipette assay developed in the Dufour laboratory at Institut Curie, allowing us to study DLD1 cell adhesion at Colby-Sawyer College in a manner complementary to that done at Institut Curie. Currently, individual bound cells are counted in multiple photographic fields from the monolayer. Here we outline two approaches to improve the efficiency of the quantification. In the first, a single densitometric reading (iVision software, BioVision Technologies) summarizes the grayscale distribution of pixels across each photographic field, yielding a rapid assessment of bound

fluorescent cells. Variation across preparations is minimized by background correction. In the second, monolayers with bound cells are detergent-extracted and the clarified extracts read in a Synergy HT fluorimeter using KC4 software (BioTek, Inc.). We present data evaluating the sensitivity, linearity and reproducibility of both approaches.

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Single-molecule characterization of mechanical homeostasis at E-cadherin-mediated cell adhesions in live cells.

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Epithelial cells transmit mechanical forces through E-cadherin-mediated intercellular contacts, and these dynamic complexes undergo force-dependent remodeling. At present, however, fundamental aspects of how cells detect and generate mechanical forces at intercellular junctions remain poorly understood. Here, we describe a FRET-based molecular force sensor that reports the forces exerted via single E-cadherin complexes in living cells. We have created single-molecule tension sensors (SMTS) that replace the fluorescent proteins in a previously reported FRET-based force probe (Grashoff et al., *Nature* 2010) with organic fluorophores that can be observed at the single-molecule level. SMTS attach to glass coverslips, and present domains from protein A to immobilize the antibody Fc domain of a fusion protein containing the E-cadherin extracellular domain (Ecad-Fc). Madin-Darby Canine Kidney (MDCK) epithelial cells adhere to surfaces functionalized with SMTS and Ecad-Fc, but not to surfaces exposed to either SMTS or Ecad-Fc alone, showing that cell attachment occurs through the Ecad-Fc/SMTS complex. Ensemble FRET measurements reveal that the force per individual E-cadherin complex is approximately 2 pN, an order of magnitude lower than previously reported AFM measurements of E-cadherin homophilic bond rupture forces (Zhang et al., *Proc Natl Acad Sci USA* 2009). This difference may indicate that the maximal load supported by individual E-cadherins is considerably greater than what is generated at equilibrium. Consistent with this interpretation, the forces we measure agree with the single-pN tensions inferred from ensemble FRET measurements using a genetically-encoded E-cadherin force sensor (Borghi et al., *Proc Natl Acad Sci USA* 2012). SMTS allow us to image spatial and temporal variations in force generation by E-cadherin adhesions: in preliminary experiments, we find that regions of high force localize primarily near the cell periphery and around the nucleus of the cell. The ability of the SMTS described here to selectively recruit fusion proteins and antibodies via their Fc domains makes them a flexible and potentially powerful tool for studying cellular mechanotransduction.

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Traction forces exerted by multicellular clusters during stretch.

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Cells from different organs such as the heart, bladder, gut or lung are routinely subjected to stretch. In response to stretch, the cytoskeleton has been reported to reinforce and fluidize. A reinforcement response is characterized by a stiffening of the cytoskeleton and an increase in traction forces whereas a fluidization response is characterized by a decrease in cytoskeleton stiffness, a decrease in traction forces and an increase in macromolecular mobility. While these phenomena are now well established at the single cell level, their impact in collective cell dynamics remains unknown. Here we subjected micropatterned clusters of epithelial cells to pulses of stretch of different duration. Using traction force microscopy we measured forces exerted by the cells on their underlying substrate throughout the stretch-unstretch maneuver. In response to a 10% linear strain, we observed a prompt increase in tractions (+57%) followed by a relaxation toward values 19% higher than baseline. When stretch was suddenly released, traction forces dropped immediately (-29% from baseline) before recovering baseline values after a 2-3 minutes. Our data show that strain-stiffening and fluidization scale up to the multicellular level.

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Mechanical response of cadherin mediated adhesions to external forces.

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Cells have the ability to sense and respond to the mechanical stimuli of their microenvironment during tissue rearrangement, embryonic development and disease such as cancer. Cell-matrix and cell-cell adhesions appear as mechanosensitive structures used by the cells to transmit the forces exerted upon the cells by their environment and those exerted by cells on their neighboring cells and surrounding matrix¹. There is evidence that cell-cell junctions are heterogeneous in their molecular composition, adhesive strength and dynamics. In a previous work, we observed that cadherin adhesions respond to changes in the internal tension of the cell². Therefore, it results of great interest to study how forces are transmitted through the cell-cell junctions and the dynamics of the intercellular adhesions in response to external and local forces. To address this issue, in this work we combine microfabrication methods that allow to map forces applied to and/or developed by the cells and superresolution imaging to study the dynamics of the cell-cell junctions at a molecular level. We investigate how intercellular adhesions of epithelial cells seeded on fibronectin or E-cadherin coated elastomeric substrates respond to external mechanical forces. We also use structured illumination microscopy to study the dynamics of cadherin-cadherin contacts and other mechanosensory proteins involved in cell-cell adhesion such as vinculin.

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How do cell-cell junctions sense and transduce mechanical forces?

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How cells sense and respond to external mechanical forces is important in normal development and for the maintenance of cell and tissue homeostasis, but remains a poorly understood problem in biology. Mechano-transduction of external forces is mediated by cell-extracellular matrix adhesion and cell-cell contacts. While mechano-transduction through the cell-extracellular matrix is well-studied, signaling through cell-cell adhesion complexes in multicellular tissues is not.

We aim at understanding how cadherins, the major proteins of intercellular adhesions, transduce mechanical forces across groups of cells. I aim to develop molecular tools to investigate the role of cadherins and associated cytoskeleton-adaptor proteins, especially α -catenin, in mechano-transduction, as well as to design new assays to examine the effects of mechanical forces from the single cell to the multi-cellular level.

The originality of this approach is the combination of micro-fabrication, molecular and cell biology, biochemistry, and cell biophysics to study mechano-transduction at cell-cell junctions. Using these tools and advanced optical techniques, we will investigate the effects of forces and mechanical environments on the dynamics of cell-cell junctions in an epithelium, in correlation with the activity of cadherin-associated proteins and actin cytoskeleton.

Cell-Cell Junctions II

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Regulation of Coxsackie and Adenovirus Receptor (CAR) by Cytokines.

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Airway epithelial cells pose a formidable barrier for the entry of pathogenic viruses. The epithelial junctional proteins, which is largely composed of tight and adherens junction proteins, maintain the barrier integrity by sealing the space between the cells. Adenovirus, which commonly causes acute respiratory infections, uses CAR as its primary receptor for entry into the host cell. CAR, an adherens junction protein, is primarily expressed on the basolateral surface of polarized epithelial cells and thus is sequestered away from pathogen-exposed (apical) surface. However, despite the inaccessibility of CAR to invading adenoviruses, adenovirus infection is common. It is commonly believed that the mechanism of entry into airway epithelia is through a structural break in the epithelial barrier that exposes the basolaterally expressed CAR to the invading pathogen. However, we have recently shown that an eight exon splice form of CAR (CAR^{Ex8}) can localize at the apical surface of epithelial cells

and serve as adenoviral receptor (Excoffon et al., 2010). Interleukin-8 (IL-8) is a proinflammatory cytokine released by the epithelial cells and resident macrophages in the lung, during infection. We hypothesize that exposure of airway epithelial cells to the IL-8 increases apical adenoviral infection through increased expression and apical localization of CAR^{Ex8}. To test this hypothesis, the apical surface of polarized Calu-3 cells was treated with IL-8 for 4h and analyzed for CAR^{Ex8} expression and localization using Western blot analysis and apical surface biotinylation. Apical transduction with adenovirus carrying the β -galactosidase reporter gene was also evaluated. Our data indicate that exposure of airway epithelial cells to IL-8 increased both the expression and apical localization CAR^{Ex8}, as well as adenoviral infection. In summary, these results suggest that susceptibility to adenovirus infection in the lung might be modulated by IL-8.

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The Coxsackievirus and Adenovirus Receptor in cardiac remodeling.

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The Coxsackievirus and Adenovirus Receptor (CAR) is a type I transmembrane protein with critical functions in cell adhesion and virus uptake. Its extracellular IG-domains can form homodimers or mediate interaction with other transmembrane or extracellular matrix proteins. The cytoplasmic tail contains a motif that interacts with PDZ-domain proteins related to signal transduction and endocytosis. CAR is predominantly expressed in the developing heart and brain when cells divide and form new cell contacts. In human dilated cardiomyopathy and animal models of myocardial infarction CAR is re- induced. This suggests a role for CAR in the formation of a functional myocardium and cardiac remodelling.

We have generated a conventional CAR knockout (KO) that cannot form continuous myofilaments between adjacent cardiomyocytes. Interestingly the adult inducible knockout does not show this phenotype. Thus, we studied the differential maturation of cell-contacts and their role in cardiac remodelling on the subcellular level.

To visualize myofilament assembly and transition between cells we crossed our CAR KO animals with animals expressing fluorescently labelled myofilaments. ES cell derived cardiomyocytes and embryonic cardiomyocytes do not form continuous filaments between cells in the absence of CAR. In co-culture experiments we show proper attachment to WT cells independent on the CAR genotype. The unexpected connection between KO and WT cells implies a role for a CAR heterodimer rather than a homodimer in transitioning myofilaments between cells.

Here we provide molecular insights into the role of CAR in cardiac development and remodelling and describe a system to study cell grafting into the myocardium *in vivo*. Ultimately our approach might help improve cell-based therapy of myocardial infarction with proper mechanical and electrical coupling to the existing myocardium.

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Coordinated Remodeling of Cell-Matrix and Cell-Cell Adhesions in Developing and Diseased Cardiac Muscle.

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Cell-cell coupling between cardiac myocytes is essential for the heart to rapidly propagate electrical signals and contract synchronously. In the adult heart, cell-cell junctions form intercalated discs localized to longitudinal myocyte borders, while cell-matrix adhesions are segregated to transverse borders. The spatial organization of these two adhesion sites progressively evolves during tissue maturation and can become disrupted in different pathological states, forming an arrhythmogenic substrate. However, the cooperative remodeling of cell-matrix and cell-cell adhesions during development and the factors that disrupt their organization in disease are not well understood. We hypothesized that cell-matrix adhesions are important in cardiac development for guiding cell spreading and anchoring myocytes as they build their cytoskeleton and assemble intercalated discs with neighboring myocytes. As the intercalated disc matures, we reasoned that cell-matrix adhesions gradually disassemble as contractile stresses are increasingly transmitted intercellularly. Finally, we reasoned that increased microenvironmental stiffness secondary to fibrosis and disease increases force generation and excessively loads the intercalated disc, causing cell-matrix adhesions to re-assemble adjacent to the intercalated disc to stabilize the tissue. To test this, we engineered two-cell cardiac μ tissues consisting of neonatal rat ventricular myocytes on compliant substrates with tunable elasticity and measured the structural and functional maturation of the contractile cytoskeleton and cell-matrix and cell-cell adhesions in different conditions. We found that, over time, μ tissues increased their systolic force output while concurrently forming into an electromechanical syncytium by disassembling cell-matrix adhesions and assembling cell-cell adhesions to transmit contractile forces. When we increased substrate stiffness to mimic pathological microenvironments, force generation increased and cell-matrix adhesions formed adjacent to the cell-cell interface, potentially to reinforce the intercalated disc. μ tissues in stiff microenvironments showed further functional evidence of maladaptive remodeling, such as lower work efficiency, longer contraction cyclic duration, and weaker relationships between tissue architecture and force generation. Our results suggest that remodeling of cell-matrix and cell-cell adhesions in cardiac muscle is a cooperative process that is guided by a functional hierarchy during development and disrupted by pathological changes to the microenvironment in disease.

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Negative Intercalated Disc Remodeling with Age Impairs Drosophila Heart Function.

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Cardiac performance declines with age, but our understanding of the molecular origin of this decline has been stymied by either slow aging, geometric complexity of the heart, or genetic variation of animal models. Using new microscopy methods and the genetically malleable *Drosophila* model, we studied heart mechanics, physiology, and structure in high throughput across a variety of genotypes. Though the 3 fly strains studied universally experienced a decline

in fractional shortening with age, severity varied between strains from a 6% (*white*; *w1118*; $p < 0.05$) to an 18% reduction (*yellow-white*; *yw*; $p < 10^{-3}$). A similar continuum existed with arrhythmic index, i.e. the variation in beat frequency, as well as with heart tube stiffness; *yw* flies experienced a 111% increase in heart tube stiffness proximal to the intercalated discs with age (1.8 to 3.9 kPa; $p < 10^{-10}$). In contrast, *w1118* flies did not stiffen with age or position long the heart tube ($p = 0.1923$), and also undergo mechanical separation between the ventral and cardiomyocyte layers. Quantitative PCR revealed age-dependent increased expression of adhesion and intercalated molecules in *yw*, including vinculin, alpha-catenin, and cadherin; expression remained steady or declined in *w1118*. When vinculin was overexpressed in *w1118* myocardium specifically, the aging phenotype was restored with stiffness changing and no separation occurring between muscle layers. On the other hand, heterozygous knockout of integrin-linked kinase (ILK) showed the opposite effect where a degenerative aging phenotype was avoided in *white-Canton S* flies, a strain that normally exhibits 66% midline stiffening with age. These observations and mechanistic insight from genetic perturbations indicate that age-dependent decline in cardiac performance may be due in part to negative remodeling from cell-cell and cell-matrix connections. Moreover, these junctional genes have significant human homology, lending credence to their importance in age-related human cardiac decline.

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Analysis of the angulin family consisting of LSR, ILDR1 and ILDR2: tricellulin recruitment, epithelial barrier function and implication in deafness pathogenesis.

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Tricellular tight junctions (tTJs) seal the extracellular space at tricellular contacts (TCs), where the corners of three epithelial cells meet. To date, the transmembrane proteins tricellulin and lipolysis-stimulated lipoprotein receptor (LSR) are known to be molecular components of tTJs. LSR recruits tricellulin to tTJs, and both proteins are required for the full barrier function of epithelial cellular sheets. Here, we show that two LSR-related proteins, immunoglobulin-like domain-containing receptor (ILDR)1 and ILDR2, are also localized at TCs and recruit tricellulin. The expressions of LSR, ILDR1 and ILDR2 were complementary in various epithelial cell types, although LSR and ILDR1 were coexpressed in some epithelia. ILDR1 is required for a strong barrier establishment in the epithelium, similar to LSR, when introduced into cultured epithelial cells, while ILDR2 provided a much weaker barrier. We further analyzed human ILDR1, whose mutations cause a familial deafness, DFNB42, and found that most DFNB42-associated ILDR1 mutant proteins were defective in recruitment of tricellulin. We also found that tricellulin mutant proteins associated with another familial deafness, DFNB49, were not recruited to TCs by ILDR1. These findings show the heterogeneity of the molecular organization of tTJs in terms of the content of LSR, ILDR1 or ILDR2, and suggest that ILDR1-mediated recruitment of tricellulin to TCs is required for hearing.

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Spectrin is present at tubulobulbar complexes in the seminiferous epithelium.

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Here we show that spectrin is present in apical Sertoli cell regions containing tubulobulbar complexes. Tubulobulbar complexes are elongate actin-filament-related endocytic structures that form in regions of attachment between Sertoli cells and maturing spermatids. They function

to internalize intercellular junctions as part of the sperm release mechanism. In rat, tubulobulbar complexes form in two parallel rows adjacent to each spermatid head. Each tubulobulbar complex consists of a double membrane core formed from the plasma membranes of the two attached cells and is cuffed by a dendritic network of actin filaments. The entire structure is capped by a clathrin-coated pit. Fragments of epithelium, containing mature spermatids and adjacent Sertoli cell regions containing tubulobulbar complexes, from perfusion fixed rat testes, were labeled with probes for actin, spectrin and protein EPB 4.1. The material was imaged using conventional fluorescence or confocal microscopy. The immunological probes for spectrin and EPB 4.1 were co-distributed, but surprisingly, their signals did not overlap with the actin staining; rather their staining appeared to be concentrated between adjacent complexes or to surround the actin cuffs of the complexes. The spectrin antibody reacted with a single band on immunoblots of whole testis and seminiferous epithelium. We suspect that the spectrin network may stabilize the actin cuffs or perhaps function as spacers between adjacent complexes. Our observations add another level of complexity to the cytoskeletal organization of tubulobulbar complexes. Supported by an NSERC Discovery grant to AWW.

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Transforming Growth Factor- β 3-Mediated Regulation of Junctional Adhesion Molecule-B (JAM-B) in Testicular Cells.

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Junctional adhesion molecule-B (JAM-B) is found between Sertoli cells as well as between Sertoli and germ cells in the testis. The expression of JAM-B is highly regulated to facilitate the passage of developing germ cells across the blood-testis barrier as well as the release of mature spermatids. Transforming growth factor beta (TGF- β) family is implicated in the regulation of testicular cell junction dynamics during spermatogenesis. This study aims to investigate the influence of TGF- β 3 on the expression of JAM-B as well as the underlying mechanisms. TGF- β 3 (5 ng/ml) treatment coupled with RT-PCR and immunoblot analyses have shown that TGF- β 3 down-regulates JAM-B expression on mRNA and protein levels in a time-dependent manner in mouse Sertoli cell line, MSC-1 cells. Cycloheximide assay further indicates that the reduction of JAM-B protein by TGF- β 3 is mediated via post-translational modification. Moreover, the involvement of ubiquitin-proteasome pathway in TGF- β 3-mediated JAM-B protein destabilization was demonstrated by proteasome inhibitor, MG-132, treatment and ubiquitin siRNA knockdown assays. Furthermore, co-immunoprecipitation (Co-IP) assay has further confirmed that JAM-B protein is conjugated by a chain of ubiquitin upon TGF- β 3 stimulation in the presence of MG-132. TGF- β 3 also speeds up the degradation of JAM-B through Smad-dependent pathway. As knockdown of Smad3 and/or Smad4 effectively abolish TGF- β 3-mediated JAM-B degradation. Taken together, the involvement of both ubiquitin-proteasome pathway and Smad-dependent signalling are essential for TGF- β 3-mediated JAM-B regulation in mouse Sertoli cells. [This work was supported by Hong Kong Research Grants Council (HKU772009 and HKU773710) and CRCG Seed Funding for Basic Research.]

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Glioma cells induce neural stem cells to cross the blood brain barrier.

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The transit of human neural stem cells, ReNcell CX, through the blood brain barrier (BBB) was evaluated in an in vitro model of BBB. The model is based on rat brain microvascular endothelial cells (RBMECs) cultured on Transwell filters with 8 µm pores, placed in a multiwell plate containing, in the basal compartment, conditioned media (CM) from astrocytes or glioma C6 cells. Glioma CM induced a significant transmigration of ReNcells in comparison to astrocyte CM. When we analyzed the role of metalloproteinases (MMPs) and soluble factors secreted by glioma cells, we found that: 1) the same amount of pro-MMP 9 and 2 is present in astrocyte and glioma CM and 2) the addition of neutralizing antibodies against hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) to glioma CM, inhibits ReNcells transmigration. These results indicate that HGF and VEGF promote ReNcells transmigration. The quantitative analysis of cytokines and growth factors present in glioma and astrocyte CM revealed that 1) INF-α, TNF-α, IL-12p70, IL-1β, IL-6, IL-8 and IL-10 are present in equal amounts in both CM; 2) VEGF is abundant in glioma CM and is absent from astrocyte CM; 3) glioma CM contain 20% less prostaglandin E2 (PGE2) than astrocyte CM and 4) epidermal growth factor (EGF) is 2.6 times more abundant in astrocyte CM than in glioma CM. Addition of HGF or EGF to DMEM increases the transendothelial electrical resistance (TEER), and in accordance, the addition of neutralizing antibodies against EGF to astrocyte CM, decreases TEER. In contrast, the addition of neutralizing antibodies against VEGF, of the metalloproteinase inhibitor GM6001, or the Cox-2 inhibitor NS398, revert the deleterious effect of glioma CM on TEER. The detrimental effect of PGE2 on TEER is known to be counteracted by the tight junction (TJ) sealing effect of EGF. Therefore in astrocyte CM, the abundance of EGF presumably neutralizes the effect of PGE2 on TEER, whereas in glioma CM the scarce presence of EGF allows PGE2 to decrease TEER. Taken together, these results indicate that in glioma CM, the high concentration of HGF and VEGF, together with the presence of PGE2, favors ReNcells transmigration across the BBB. To cross the BBB, the transmigrating cells need to express adhesion molecules capable of establishing cell-cell contacts with their counterparts in endothelial cells. We observe that ReNcells express the TJ proteins occludin and claudins 1, 3 and 4, and the cell adhesion molecule CRTAM, while RBMECs express occludin, claudins 1 and 5 and CRTAM. Therefore, we propose that the transmigration of ReNcells across RBMECs monolayers involves contacts between CRTAM, occludin and claudins 1, 3 and 4 present in ReNcells, with the corresponding CRTAM, occludin and claudin 5 present on RBMECs. Finally, we observe that the addition of soluble CRTAM to the apical Transwell compartment containing the ReNcells placed on top of the monolayer of RBMECs, produces a significant decrease in ReNcells transmigration, thus indicating that cell-cell adhesion mediated by CRTAM plays an important role in ReNcells transmigration across the BBB.

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Gap junction coupling of vascular cells is a target for dipyridamole: the role of the cAMP/PKA dependent pathway.

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Gap junction dependent cell to cell communication has been proposed as a target for the treatment of cardiovascular impairments. We applied scrape loading/dye transfer technique on vascular endothelial and smooth muscle cells to investigate the effect of the antithrombotic drug dipyridamole. At concentrations between 5 and 50 μ M, the drug increased the gap junction coupling of vascular cells in a time and concentration dependent manner. The increase could be inhibited by application of PKA inhibitors such as H-89 or Rp-cAMPS. The application of forskolin or 8-Bromo-cAMP mimicked the dipyridamole induced effect, thus indicating that dipyridamole affected gap junction coupling by activation of the cAMP/PKA dependent pathway. This pathway is similar to that used to explain the inhibitory effect of dipyridamole on platelet aggregation, namely the inhibition of nucleoside transporters that increases external concentration of nucleosides such as adenosine, which leads to intracellular elevation of cAMP via activation of adenosine receptors and adenylyl cyclase. As a drug which prevents arterial thrombosis and leads to vasodilatation, dipyridamole is used in treatment of cardiovascular infarction and stroke as well as in secondary prevention of vascular occlusions. Our results show that dipyridamole could develop an additional benefit effect by its action on the vascular cell system, especially on the gap junction dependent cell to cell communication.

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Rap1 GTPase activation and barrier enhancement in RPE inhibits choroidal neovascularization *in vivo*.

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Inappropriate regulation or loss of barrier integrity precedes the development of many pathological situations such as tumor metastasis, inflammatory disorders, and blood-retinal-barrier breakdown characteristic of neovascular age-related macular degeneration (AMD), a leading cause of blindness worldwide. Rap1 GTPase is involved in regulating both endothelial and epithelial cell junctions; the role of Rap1A vs. Rap1B isoforms is less clear. Compromise of retinal pigment epithelium (RPE) barrier function is a contributing factor to the development of AMD. We used shRNA of Rap1 isoforms in cultured human RPE cells, and knockout mouse models to test the role of Rap1A and Rap1B on RPE barrier properties, with emphasis on the dynamic junctional regulation that is triggered when RPE cell-cell adhesion is challenged. Silencing of Rap1A *in vitro* reduced barrier integrity under steady state conditions, whereas loss of Rap1B enhanced EGTA-induced junctional breakdown, and caused a delay in reassembly. We also tested the role of Rap1 isoforms in resisting laser-induced CNV in a mouse model of AMD. Rap1b^{-/-} mice exhibited larger CNV volumes compared to wild-type or Rap1a^{-/-}; lesion width was increased in both Rap1a^{-/-} and Rap1b^{-/-} compared to wild-type. Rap1 activation in RPE of mice by intravitreal injection of a cAMP analog (8CPT-2'-O-Me-cAMP) reduced laser-induced CNV size. Rap1 activation increased recruitment of junctional proteins and F-actin to cell-cell contacts, increasing the linearity of junctions *in vitro* and in cells surrounding laser-induced lesions *in vivo*. We conclude that both Rap1 isoforms are important in resisting CNV size and for RPE barrier integrity - Rap1A in steady-state barrier integrity and Rap1B in reassembly following junction disruption. Furthermore, our data suggest that *in vivo* treatment

with 8CPT-2'-O-Me-cAMP may be a viable pharmacological means to strengthen the RPE barrier against the pathological choroidal endothelial cell invasion that occurs in AMD.

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The role of Estrogen Receptors on microvasculature in mouse skin.

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Objective: The goal of this study was to investigate the role of ER alpha and/or ER beta on microvasculature in the skin using unique genetic models: knockout ER alpha (ER alpha ^{-/-}) and ERbeta (ER beta ^{-/-}) female mice.

Methods: We first visualized and analyzed microvasculature in the skin of ER alpha ^{-/-}, ER beta ^{-/-} and control mice by injecting FITC-dextran into the heart of mice. Next, the microvessels were visualized by immunostaining with pericyte specific antibodies such as alpha-smooth muscle actin and desmin. To determine whether vessel stabilization is impaired in the knockout mice and leakage take place we performed in vivo permeability assay. Furthermore, we isolated mouse microvascular endothelial cell from ER alpha ^{-/-}, ER beta ^{-/-} mice followed by determination of genes important in stabilization of neovessels.

Results: In both ER alpha and ER beta knockout mice the vascular network was disorganized and the diameter of blood vessels was irregular as compared to the wild type animals. The expression levels of alpha-SMA and desmin were significantly decreased in the skin vessels of ER alpha ^{-/-} and to a lesser degree in ER beta ^{-/-} mice as compared to WT mice. To confirm that vascular leakage take place in these mice we performed in vivo permeability assay. The extraction of Evan's blue was significantly increased in the skin of ER alpha ^{-/-}, ER beta ^{-/-} mice as compared to WT animals. Interestingly, the extraction of Evans blue was also increased in the lung, heart and brain of these mice. Furthermore, we performed an array analysis of the skin taken from WT, ER beta ^{-/-}, ER alpha ^{-/-} mice. Several genes such as desmin, JAM-B, C, connexin 40, VE-cadherin, PDGFB were diminished in the tissue of knockout mice as compared to WT animals. Since the vascular changes were observed in dermal microvessels of ER alpha and ER beta knockout mice we determined the genes important in stabilization of neovessels in cultured endothelial cells isolated from ER beta ^{-/-}, ER alpha ^{-/-} and control mice. To examine mRNA levels of PDGFB, VE-cadherin and N-cadherin qPCR analysis was performed. Expression level of the following genes was downregulated: PDGFB (~15 folds), VE-cadherin (~28 folds), N-cadherin (~1.6 folds). This data may suggest that vessel stabilization is impaired in the skin of ER beta ^{-/-} mice. Similar results were obtained in cultured endothelial cells isolated from ER alpha ^{-/-} mice.

Conclusions: Our data indicate that ER alpha and ER beta play an important role in the process of neovascularization. Specifically, our results suggest that both receptors regulate the genes implicated in the vessel stabilization.

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Abl-related gene (Arg) is a novel mediator of endothelial barrier dysfunction.

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Introduction

Recently, we demonstrated that the tyrosine kinase inhibitor imatinib protected against inflammatory endothelial barrier dysfunction and vascular hyper-permeability. We identified the tyrosine kinase Abl-Related Gene (Arg / Abl2) as the target of imatinib. Here, we aimed to elucidate the role of Arg in the regulation of endothelial cell-cell and cell-matrix interactions.

Methods & Results

The barrier-disruptive agents thrombin [1U/mL], histamine [10⁻⁵M] and VEGF [10ng/mL] all transiently activated endothelial Arg as evidenced by phosphorylation of its downstream target CrkL at Tyr207. Depletion of Arg completely abrogated CrkL phosphorylation under basal and stimulated conditions. Activation of Arg involved a Src-family kinase, as the Src-inhibitor PP2 blunted Arg activity.

Genetic depletion or pharmacological inhibition of Arg attenuated thrombin-induced endothelial barrier dysfunction, as evidenced by a reduced drop in the trans-endothelial electrical resistance. Inhibition of Arg did not affect RhoA/Rho kinase activity or calcium-dependent signaling, known as main determinants of actomyosin contraction. Resolving the endothelial resistance into separate components reflecting cell-cell contact and cell-matrix interaction displayed that Arg predominantly mediated the decrease in cell-matrix interaction.

To evaluate how Arg contributes to this decrease in cell-matrix interaction, events downstream of Arg activation were analyzed. Inhibition of Arg resulted in enhanced Rap1 activity and affected cell-matrix interaction by activating beta1-integrins. Arg-depleted endothelial cells showed peripheral redistribution of beta1-integrin, yielding a broad band of beta1-integrin at the cell periphery, which colocalized with a thick cortical actin rim. To test whether this beta-1 integrin relocalization affects cell-matrix adhesion strength, cell adhesion and detachment assays were performed. Arg-depleted cells showed a more efficient cell adhesion, as evidenced by a higher number of adhering cells as well as faster cell spreading. Moreover, cell detachment by a combination of mild trypsinization and orbital shaking, revealed that Arg-depleted cells had a delay in rounding up and a reduced detachment rate.

To address the relevance of Arg signaling during endothelial barrier dysfunction in vivo, we measured vascular leakage in a murine model of sepsis (cecal ligation & puncture). Septic mice in which Arg activity was blocked by imatinib (50mg/kg, administered 6h and 18h after the induction of sepsis) showed significantly less vascular leakage in the lungs, the kidneys and the liver.

Conclusion

These data indicate that Arg is an important mediator of endothelial barrier dysfunction by reducing cell-matrix adhesion in a CrkL/Rap1/beta1-integrin dependent manner. Inhibition of Arg activation prevents disassembly of peripheral FAs. This improves the cell-matrix interaction, stabilizes the endothelial barrier by supporting cell-cell contacts, and is an effective means to reduce vascular leakage.

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Regulation of retinal vascular leakage by insulin: Implications for patients with diabetic retinopathy.

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Diabetes mellitus is a global disease with considerable morbidity and mortality. Leakage from the retinal vasculature contributes to the pathogenesis of diabetic macular edema (DME), an important cause of vision loss in patients with type 2-diabetes. While epidemiological studies and randomized clinical trials have suggested that glycemic control plays a major role in the development of vascular complications of diabetes, insulin therapies for control of glucose metabolism cannot prevent long-term retinal complications.

Several clinical studies have reported that intensive insulin therapy may cause a transient worsening of retinopathy. In prospective studies on non-insulin dependent (Type 2) diabetes

mellitus patients, a change in treatment from oral drugs to insulin was associated with a significant increased risk of retinopathy progression and visual impairment. In addition, it has been reported that patients who undergo total pancreatectomy for cancer, develop severe diabetes because of the complete absence of insulin, but rarely if ever develop proliferative diabetic retinopathy, even when they survive for more than 10-20 years. Re-analysis of the WESDR data determined that there was a correlation between insulin treatment and diabetic macular edema. These results suggested that insulin treatment maybe a predictor of the onset of DME in patients with diabetes. Based on these findings we modeled and evaluated the pathophysiological effects of insulin on the retinal vasculature in mice and obtained surprising results that suggest that insulin treatment of diabetic mice results in increased retinal vascular leakage.

In this study we report results that suggest that insulin treatment exacerbates retinal vascular leakage in STZ-induced diabetes in mice. We demonstrate that insulin can disrupt tight junctions in RPE cells, up-regulate ADAM10 and subsequently soluble active betacellulin (Btc). That the soluble Btc plays a role in the disruption of tight junctions, was determined by the ability of Btc and ADAM10 siRNA to prevent insulin-induced disruption of tight junctions. We also report that in STZ-induced diabetic mice, insulin-mediated increase in vascular permeability can be inhibited by EGF receptor inhibitors further confirming the potential cross talk between insulin and Btc in the regulation of retinal vascular permeability.

These studies suggest that a combination treatment of insulin and EGF inhibition might be a useful therapeutic combination to prevent macular edema.

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Drebrin preserves endothelial integrity by stabilizing nectin at adherens junctions.

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Regulation of cell-cell contacts is essential for the integrity of the vascular endothelium. Here, a critical role of the F-actin binder drebrin in maintaining endothelial integrity is revealed under conditions mimicking vascular flow. Knockdown of drebrin leads to pronounced weakening of cell-cell contacts, characterized by loss of nectin from adherens junctions and concomitant proteasomal degradation. Immunoprecipitation, pulldown, FRAP and mitochondrial retargeting experiments show that nectin stabilization occurs through a chain of molecular interactions. These involve drebrin binding to F-actin, drebrin and afadin interacting through their polyproline and PR1-2 regions, and recruitment of nectin through afadin's PDZ region. Key elements are drebrin's modules that confer binding to afadin and F-actin. Evidence is provided by minimal constructs containing afadin's PDZ region coupled to drebrin's F-actin binding region or to Lifeact, which restore junctional nectin even under knockdown of both drebrin and afadin. Drebrin, containing binding sites for both afadin and F-actin, is thus uniquely equipped to stabilize nectin at endothelial junctions.

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Role of two Rap1 Isoforms, Rap1A and Rap1B, in regulation of vascular permeability and endothelial cell barrier.

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Dynamic regulation of vascular permeability is critical for normal vessel function as evidenced in pathological conditions such as edema or inflammation; yet underlying mechanisms are not fully understood. Small G protein Rap1 has been implicated in promoting endothelial cell-cell (EC)

junction formation upon elevation of cellular cAMP but also in promoting angiogenic responses to VEGF.

However, what role Rap1 plays in regulation of vascular permeability in vivo is not known. The project's objective was to investigate the function of two Rap1 isoforms, Rap1A and 1B, in the formation and maintenance of adherens junctions (AJ) in ECs and in regulation of vessel permeability in vivo. To accomplish this, we used Cre-lox P method and Tie2 promoter to generate endothelial-specific knockout mice deficient in Rap1A (EC-Rap1A KO), Rap1B (EC-Rap1B KO) or partially depleted of both isoforms (EC-Rap1A KO; Rap1B^{+/-}). We found that vessel leakage of Evans blue dye was significantly increased only in lungs from EC-Rap1A KO and EC-Rap1A KO; Rap1B^{+/-} mice but not from EC-Rap1B KO mice suggesting that Rap1A is the main isoform promoting vessel barrier. Using bone marrow chimeras we showed that vessel barrier protection by Rap1 is EC-autonomous. In vitro, confocal microscopy revealed that Rap1A-deficiency led to altered organization of AJs in EC monolayers, with dispersed β -catenin distribution across AJs, in contrast to narrow distribution in control or Rap1B KO ECs, suggesting that Rap1A may regulate endothelial barrier properties by spatially organizing AJ proteins. In contrast, dissolution of AJs in response to VEGF was impaired in Rap1B-, but not in Rap1A-deficient ECs. Further, VEGF-induced permeability was reduced in Rap1B-KO EC monolayers. In vivo, VEGF-induced vascular permeability in the skin, as measured by Miles assay, appeared decreased in EC-Rap1B KO but no in EC-Rap1A KO mice.

On the other hand, deficiency of either Rap1 isoform in ECs led to a defect in de-novo AJ formation, as evidenced by slower VE-cadherin fluorescence recovery at AJs after junction disruption with EGTA and by a defect in adhesion to VE-cadherin. Overall, these results suggest that in addition to common functions, the two Rap1 isoforms play distinct roles; Rap1A is involved in vascular barrier maintenance and Rap1B – in regulation of VEGF-mediated permeability.

Muscle Structure, Function, and Disease

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Heart Failure on a Chip: Recapitulating Maladaptive, Multi-Scale Remodeling of the Failing Heart In Vitro.

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Tissue-specific biomimetic in vitro microsystems engineered to recapitulate in vivo human physiological responses, known as “organs on chips,” are currently being developed to potentially replace animal testing in the pharmaceutical industry. In addition to building microscale mimics of healthy organs, there is also a need to engineer “organs on chips” with pathological phenotypes to test the efficacy and toxicity of therapeutics in patients with specific diseases, such as pathological hypertrophy. In the heart, volume overload excessively stretches the ventricle and induces dilated cardiomyopathy, which often progresses to heart failure. We hypothesized that we could exploit this response to build and validate a “heart failure on a chip” microsystem by chronically stretching engineered ventricular tissues to stimulate maladaptive genetic, structural, and functional remodeling. To these aims, we applied chronic cyclic stretch to engineered neonatal rat ventricular tissues cultured on elastomeric silicone membranes and quantified remodeling on spatial scales ranging from the gene to the tissue. Chronic cyclic stretch initiated early in tissue assembly induced myocytes to align parallel to the loading direction. Gene expression in stretched tissues was characteristic of dilated cardiomyopathy,

including decreased α - to β -myosin heavy chain ratios. In tissues aligned with microcontact printing, transverse cyclic stretch decreased sarcomere alignment and longitudinal cyclic stretch increased cellular aspect ratio. Cyclic stretch applied in either direction extended the time to peak intracellular calcium, which has similarly been observed in failing hearts. We adapted the muscular thin film assay to our stretchable silicone membranes to perform quantitative stress measurements in our engineered tissues and found that cyclic stretch in either direction decreased systolic stress generation, which is consistent with maladaptive remodeling. Our results show that chronic cyclic stretch induces pathological genetic, structural, and functional remodeling, indicating that mechanical stretch can be used as a stimulus for engineering “heart failure on a chip” microsystems with potential applications as drug testing platforms.

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Transglutaminase as a critical mediator of phenotypic instability in vascular smooth muscle.

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Phenotypic instability of vascular smooth muscle underlies various vascular diseases including atherosclerosis and calcification of the blood vessels, myocardium and cardiac valves. The latter is a common complication associated with diabetes, chronic renal insufficiency, hypercholesterolemia, osteoporosis and ageing, and has been correlated with increased morbidity and mortality. Using in vitro analysis we demonstrate that both osteoblastic and adipogenic transformation in VSM is critically dependent on the novel signaling conduit involving activation of the canonical β -catenin by enzyme transglutaminase TG2. In contrast, other signaling pathways implicated in osteoblast differentiation including BMP, PKA, Notch, NF κ B and ERK play a limited role. These data suggest that inhibition of TG2 may represent a novel therapeutic target to prevent vascular disease in vivo. This hypothesis was tested using rat model of aortic elastocalcinosis which involves osteoblastic transformation in VSM. TG2 activity is increased in calcified aortas and here we employed two inhibitors to counteract this induction, including the TG2-specific small molecule inhibitor KCC-009 and bioflavonoid quercetin, which we have characterized as a novel inhibitor of TG2. Both treatments efficiently prevented β -catenin activation and deposition of calcified matrix in the arterial media. Re-activation of β -catenin in cultured VSMC by GSK inhibitor LiCl in the presence of quercetin restored calcium accumulation, confirming that quercetin acts via inhibition of the β -catenin pathway. These data provide new insights into molecular control of phenotypic instability in vascular smooth muscle.

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The impact of each domain of myosin heavy chain molecule on forming thick filaments in muscle and non-muscle cells.

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In skeletal muscle cells, myofibrillar proteins are highly organized into the sarcomere, in which thick filaments are interdigitated with thin filaments to generate contractile force. Thick filaments are composed of myosin heavy chains (MHCs) and their associated molecules. Importantly, the size of each thick filament is strictly regulated, *i.e.* 1.6 micrometer in length. However, little is known about the mechanisms which control the formation of thick filaments. Here, we demonstrated how MHC molecule functions in the assembly of thick filaments. First, to examine

the role of each domain of MHC to form thick filaments, Flag-tagged myosin fragments were exogenously expressed in cultured mouse skeletal muscle cells, and then visualized by double staining with anti-Flag, antibodies against myofibrillar proteins, and/or rhodamine-labeled phalloidin. We observed that exogenously expressed full length MHCs were assembled into thick filaments in myotubes. They formed 1.6 micrometer thick filaments in sarcomeres under light microscopy. Fragments of the light meromyosin (LMM) as well as the subfragment 2 plus LMM (S2+LMM) showed an ability to be incorporated into thick filaments with low efficiency. In contrast to those, exogenously expressed the S2 fragment was distributed diffusely throughout the cytoplasm of myotubes, suggesting that the LMM domain may facilitate to form myosin filaments in myotubes. Second, we examined whether or not myosin fragments were capable of forming thick filaments in non-muscle cells, such as COS cells. Exogenously expressed full length MHCs as well as the LMMs fragments formed filaments but their sizes were not uniform. Although expressed S2 fragments did not form any filament structures, the S2+LMM produced filaments and filamentous aggregates. Taken together, the LMM domain of MHC is essential to form filaments in both skeletal muscle and COS cells. Our results also showed that 1.6 micrometer thick filaments were generated by exogenous expression of MHCs in myotubes but not in COS cells, indicating that MHC molecules by themselves are not capable of controlling the size of polymerized filaments and that muscle cytoplasmic environments including myofibrils are indispensable for proper assembly of thick filaments.

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Myotonia Congenita Mutation Disrupts the Biosynthesis of CLC-1 Channels.

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Myotonia congenita is a hereditary muscle disorder caused by mutations in the human voltage-gated Cl⁻ channel CLC-1. We have studied a myotonia mutation that has not been functionally characterized. Despite a gating property similar to that of wild-type channels, the mutant CLC-1 channel displayed a diminished whole-cell current density and a reduction in the total protein expression level. Our biochemical analyses further demonstrated that the reduced expression of the mutant channel can be largely attributed to an enhanced proteasomal degradation as well as a defect in protein trafficking to surface membranes. Our data suggest that the molecular pathophysiology of myotonia congenita may involve a disruption of the balance between the synthesis and degradation of the CLC-1 channel protein.

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Differential phosphorylation of titin domains acts complementary to increase passive stiffness in failing human myocardium.

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Aims: Titin-based passive stiffness is an important determinant of myocardial diastolic function, and is modulated by two main mechanisms: i) alterations in the relative expression levels of the cardiac titin isoforms, N2B (3.0 MDa) and N2BA (> 3.2 MDa), and ii) phosphorylation of elastic I-band domains of titin. Importantly, phosphorylation of the N2-B unique sequence (N2-Bus) by cGMP-dependent protein kinase (PKG) or cAMP-dependent protein kinase (PKA) decreases titin stiffness in human heart, whereas phosphorylation of the titin PEVK domain by PKC

increases it. Here, we analyzed whether changes in the titin phosphorylation status affect passive myocardial stiffness in human failing hearts.

Methods and Results: Titin isoform composition was determined by 2% SDS-PAGE, and a set of human donor and failing hearts with similar isoform composition was selected for further analyses. Total titin phosphorylation was determined using ProQ-diamond/Sypro-Ruby stain and was slightly reduced in failing hearts compared to controls. To study specific modifications of titin domains phosphosite-directed antibodies were generated against pSer4185 in the N2-Bus and pSer11878 in the PEVK region, and relative phosphorylation of the titin domains was determined using Western blot analyses. In failing hearts, relative N2-Bus phosphorylation was significantly reduced by up to 28%, and relative PEVK phosphorylation was increased by up to 23% compared to donor hearts. Passive myocardial stiffness was analyzed using skinned fiber preparations, and the length-tension relationship was increased in fibers from failing hearts compared to controls. Because titin isoform composition was unchanged in the failing heart samples the observed increase in fiber stiffness is likely caused by the altered phosphorylation status of titin. Furthermore, using mass spectrometry we identified novel PKG- and PKA-dependent phosphorylation motifs in human and rat titin N2-Bus, which may explain the reduced phosphorylation status of total titin.

Conclusion: We conclude from our data that differential phosphorylation of the N2B and PEVK region of titin can act complementary to increase passive tension in the myocardium, and is an important physiological mechanism to fine-tune passive myocardial stiffness, and thus diastolic function of the heart.

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Insulin as a modulator of cardiac titin.

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The sarcomeric protein titin is the largest protein known to date, and one of the main molecular components that contribute to the passive stiffness of striated muscle tissue. In mammalian heart, titin-based myofilament stiffness is an important determinant of myocardial distensibility and diastolic function. The passive mechanical properties of cardiac titin depend on the expression ratio of the isoforms N2BA (~3.2 – 3.7 MDa, compliant) and N2B (~3.0 MDa, stiffer), and can be dynamically modified by phosphorylation through PKA, PKG and PKC. Importantly, phosphorylation by PKA and PKG reduces titin-based myofilament stiffness, whereas PKC increases it.

Here we studied the influence of insulin on titin isoform composition and phosphorylation using cultured cardiomyocytes and diabetic human heart tissue. Titin isoform composition and phosphorylation was analyzed using 1.8% SDS PAGE stained with either Coomassie, or the phosphoprotein stain ProQ-Diamond in combination with SYPRO-Ruby as well as antibody based western blot analysis. In embryonic rat cardiomyocytes (CMs) insulin treatment for 7 days significantly increased the relative expression of stiff N2B-titin by >12% compared to untreated cells. Moreover, in embryonic and adult rat CMs insulin caused a major increase in the relative titin phosphorylation within 15 minutes of treatment. This effect was blocked by inhibition of the NO/cGMP- pathway or the PI3K/AKT-pathway (LY294002), suggesting insulin-mediated phosphorylation of titin by either PKG, or PKC. Western blot analyses using phospho-specific antibodies showed that insulin treatment induced phosphorylation of the PKC-sensitive site S11878 (UniProtKB Acc. #Q8WZ42) in the PEVK region of titin. In addition, we investigated the influence of altered insulin homeostasis on titin isoform ratio and titin phosphorylation using samples from right atria of diabetic and non diabetic patients that underwent cardiac surgery

due to coronary artery disease. Diabetic heart samples showed a major increase in titin N2BA expression that was most pronounced in patients with a high HbA1c (> 7.0 %). First analyses also demonstrate a hypophosphorylation of total titin from diabetic hearts, probably caused by reduced phosphorylation of the PEVK element of titin, as indicated by Western blot analyses. We conclude that insulin is a potent modulator of cardiac titin, and may therefore play an important role in modifying myocardial stiffness in human hearts.

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An R441Q mutation of the WWP1 gene causes WWP1 degradation in skeletal muscle of chicken muscular dystrophy.

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A missense mutation in a gene encoding WWP1 was identified as the cause of chicken muscular dystrophy by genetic linkage analysis. WWP1 is a HECT-type E3 ubiquitin protein ligase, composed of 922 amino acids, containing four tandem WW domains that interact with proline-rich peptide motifs of target proteins. The missense mutation changes arginine at amino acid 441 into glutamine (R441Q) located within WW domains. The R441Q mutation would influence a property of WWP1 molecule, but little is known about it. In this study, we describe the effect of a single amino acid substitution on the expression of WWP1 protein in skeletal muscle cells. Immunoblot analysis of chicken skeletal muscle homogenate using specific antibody against WWP1 detected small fragment signals, at around 90 kDa, in addition to a 120 kDa full-length WWP1. The 90-kDa protein bands relatively increased in dystrophic chicken muscle while, in contrast, the full-length WWP1 tended to decrease. We also examine the WWP1 degradation using skeletal muscle of R441Q WWP1 transgenic mice and obtained the result compatible with that from dystrophic chicken. Moreover, we performed *in vitro* expression analysis by transfection using myogenic cell line, C2C12. In overexpression of R441Q and normal WWP1 in proliferating C2C12 cells, both of them showed marked increase of 120-kDa full-length WWP1. However, in differentiation stage of the cells, full-length WWP1 was remarkably down-regulated especially in the transfectant of mutant WWP1. This down-regulation of WWP1 was prevented to some extent by addition of cell-permeable proteasome inhibitor (MG132) into culture medium. These results suggested that R441Q missense mutation induced degradation of WWP1 during muscle differentiation, which was carried out at least in part through ubiquitin-proteasome system.

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Myosin dynamics in cultured skeletal muscle cells.

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Myosin is one of the major myofibrillar components in striated muscles. More than 300 myosin molecules assemble into a bipolar thick filament in the sarcomere. However, it is still unclear how myosin molecules in thick filaments are replaced by de novo synthesized myosin molecules without affecting the structural integrity and functions of thick filaments. In this study, we investigated the dynamics of myosin molecules in living cultured skeletal muscle cells using fluorescence recovery after photobleaching (FRAP). We observed that exogenously expressed GFP-tagged myosin heavy chain 3 (GFP-Myh3) showed the striations, which were precisely located in the A-band of the sarcomeres in myotubes. After photobleaching, the fluorescence

intensity of GFP-Myh3 gradually increased and then the striations were recovered at 6-8 hours post-photobleach. To examine whether de novo synthesized myosin molecules are required for the recovery, cycloheximide (CX), an inhibitor of protein biosynthesis, was added to the medium before photobleaching. The addition of CX did not interfere with the fluorescence recovery of GFP-Myh3, suggesting that de novo synthesized myosin molecules are dispensable for GFP-Myh3 replacement in thick filaments. Next, we compared the mobility of myosin molecules in the shafts of myotubes with that at the spreading ends (growth tips), in which sarcomeres are newly synthesized and added to the existing myofibrils. In the growth tips, the relative fluorescence intensity of GFP-Myh3 was recovered to about 90% of the initial value at two hours post-photobleach, while, in the shafts, it was recovered to 30-50% of the initial value at 10 hours post-photobleach. This result suggested that the mobility of myosin was faster in the growth tips than in the shafts of myotubes. Collectively, our FRAP results demonstrated that myosin molecules in thick filaments were actively replaced and traveled within the sarcomere, and their mobility was different between the shafts and the growth tips of myotubes.

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Treatment for inherited muscular diseases by antibiotics against nonsense mutations.

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Translational readthrough of a premature termination codon is a promising therapeutic method in more than 2,400 distinctly inherited human diseases caused by respective single genes. In order to investigate potent readthrough inducer with fewer toxicity than known readthrough-inducing drug such as gentamicin, we screened from kanamycin-related antibiotics using the novel transgenic mouse strain, named READ (Readthrough Evaluation and Assessment by Dual reporter). In consequence, we discovered that the arbekacin induced the in vivo nonsense suppression dose-dependently in READ mice for detection of readthrough activity. We found that arbekacin promoted the accumulation of dystrophin, the reduction of serum creatine kinase activity and the improvement of contractile function in mdx mice which carried nonsense mutation in dystrophin gene. Moreover, arbekacin exhibited the restoration of dystrophin expression on human muscle cells obtained by biopsies from Duchenne muscular dystrophy (DMD) patients with nonsense mutation of dystrophin gene. These results demonstrate that arbekacin induces readthrough of the premature termination codon, resulting in the partial restoration of dystrophin protein, in the reduction of creatine kinase activity, and in restoration of isometric force in mdx mice. Furthermore, arbekacin coaxes cells from DMD patients to ignore nonsense mutations and to promote dystrophin production. Compelling evidence is now available that arbekacin can mask the genetic defect and generate full-length functional proteins. Arbekacin is a breakthrough readthrough-inducing drug for muscular dystrophy patients harboring nonsense mutations, and can be use as gOff-label use h. Now, we are preparing "investigator-initiated clinical trial" supported by the Japan Medical Association Center of Clinical Trials. It is our hope that arbekacin will contribute towards the teatment for DMD.

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Roles of ADAM8 in skeletal muscle regeneration.

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During skeletal muscle regeneration, damaged muscle fibers are replaced by newly formed myotubes. Muscle satellite cells, muscle-specific stem cells, proliferate and fuse with each other to form these new myotubes. In addition to skeletal muscle lineage cells, inflammatory cells are required for proper muscle regeneration. Neutrophils and macrophages promote cell damage by producing super-oxide and remove cell debris by phagocytosis. Macrophages regulate myogenesis by secreting cytokine or ECM. Members of ADAM (a disintegrin and metalloprotease) family proteins encode transmembrane metalloproteases, and are involved in the ectodomain shedding of various cell adhesion molecules, transmembrane signaling molecules, and ECM proteins (Reiss and Saftig., *Semin Cell Dev Biol.*, 2009). Among the ADAMs, ADAM8 is highly expressed in leukocytes including macrophages, neutrophils or granulocytes, and its expression increases under the inflammatory conditions such as cancer, asthma or tissue injury. Whereas ADAM8^{-/-} embryos have no obvious developmental defects (Kelly et al., *Dev Dyn.*, 2005), evidence suggests that ADAM8 regulates inflammatory reactions and/or mediates ectodomain shedding of inflammation associated proteins such as TGF alpha, TNFR1, PSGL1, and L-selectin. However, whether ADAM8 is involved in muscle regeneration and whether loss of ADAM8 would cause pathological changes in muscular dystrophy remain unknown.

Here, we investigated roles of ADAM8 in muscle regeneration. To examine loss of ADAM8 would affect muscle regeneration, we utilized two muscle regeneration models; (1) dystrophin-null mice, models for Duchenne muscular dystrophy and (2) cardiotoxin (CTX) induced skeletal muscle injury. Firstly, we generated dystrophin-null; ADAM8^{-/-} mice, and found loss of ADAM8 exacerbated the muscle dystrophic phenotype. Histological analysis indicated enhanced mineralization and increased inflammatory cells in the Dystrophin-null; ADAM8^{-/-} mice. Next, we examine whether muscle regeneration process was impaired in the ADAM8^{-/-} mice by injecting cardiotoxin. We found that macrophages infiltrated into damaged muscle fibers were fewer in the ADAM8^{-/-} mice. These results suggest that clearance of dead cells during skeletal muscle regeneration was affected in the ADAM8^{-/-} mice. We previously showed that ADAM8 mediates the onset of blood circulation in zebrafish through triggering release of erythroblasts from the intravascular surface (Iida et al., *Curr Biol.*, 2010). ADAM8 could be required for the detachment of inflammatory cells from capillaries, the basement membrane surrounding muscle fibers, or interstitial cells in muscle regeneration. We are currently exploring mechanisms through which ADAM8 regulates behaviors of macrophages in regenerating muscles.

Defining Therapeutic Targets and New Therapeutics I

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A new method for the quantification of angiotensin converting enzyme in dried blood spots as a tool for the follow up of patients with Gaucher disease in Colombia: preliminary results.

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Gaucher disease is a Lysosomal Storage Disorder (LSD) caused by the deficiency of the enzyme beta acid glucosidase which leads to the accumulation of the metabolite glucocerebrosidase in the organism. There is treatment available for the disease, but, because there are more than 300 mutations described for Gaucher, it is difficult for clinicians to generalize a dose and monitor the response of each patient to the treatment.

Biomarkers are molecules that serve as indicators for diseases. Although specific biomarkers for Gaucher disease are not known, angiotensin converting enzyme (ACE) is commonly used for the follow up of patients. ACE is also a biomarker for Fabry disease (another LSD) and hypertension, because it is directly related with the renin-angiotensin system.

The aim of the present study is to evaluate ACE using a more economic and accessible sample: the dried blood spot (DBS). In Colombia the analyses for LSDs are carried out in big cities, making the transportation difficult and expensive. As this sample doesn't require refrigeration or big quantities of blood, is ideal for the analysis and follow up of patients.

To demonstrate that DBS is as accurate as traditional serum samples, we compared serum and DBS samples from 16 healthy individuals from 19 to 55 years old and samples from 11 patients with Gaucher disease with ages between 2 and 72 years old. ACE activity was determined by a colorimetric assay both in serum and in DBS using Furylacryloyl Phenilalanine Glycylglycine (FAPGG) as a substrate in a microplate assay.

We analyzed the kinetics of the enzyme to find out at which point there was a larger difference between healthy and Gaucher individuals. At 60 minutes the activities of both groups were the most different. We haven't found significant differences between serum and DBS samples ($p=0.5499$). Also we have seen that Gaucher patients are well discriminated both in serum ($p=0.0052$) and DBS ($p=0.066$) and the difference ratio is maintained between the two samples analyzed. The activity of ACE in patients with Gaucher disease was considerably greater compared to the control group ($p=5.5e-05$).

In conclusion, DBS is a good, inexpensive sample and can be used as a tool for the follow up of patients with Gaucher disease. These results show that the quantification of biomarkers in DBS is as accurate as in serum samples, making DBS sampling reliable for laboratory analyses.

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Proapoptotic role of TGF- β 1 and VEGF in the kidney of Fabry disease mouse model

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Fabry disease is a lysosomal storage disorder caused by deficiency of α -galactosidase A (α -gal A), resulting in deposition of globotriaosylceramide (Gb3; also known as ceramide trihexoside)

in the vascular endothelium of many organs. A gradual accumulation of Gb3 leads to cardiovascular, cerebrovascular, and renal dysfunction. Endothelial cell dysfunction leads to renal complications, one of the main symptoms of Fabry disease. However, the pathological mechanisms by which endothelial dysfunction occurs in Fabry disease are poorly characterized. The purpose of this study was to investigate whether the expression of transforming growth factor- β 1 (TGF- β 1) and vascular endothelial growth factor (VEGF) is associated with the renal pathogenesis of Fabry disease. We found that the protein expression levels of renal thrombospondin-1 (TSP-1), TGF- β 1, and VEGF were higher in the kidneys from Fabry mice than from wild-type mice. The expression levels of VEGF receptor 2 (VEGFR2), fibroblast growth factor-2 (FGF-2), and phospho-p38 (P-p38) were also higher in the kidneys from Fabry mice compared with wild-type mice. Activities of cysteine aspartic acid protease (caspase)-6 and caspase-9 were higher in kidneys from Fabry than from the wild type. These results suggest that overexpression of TGF- β 1 and VEGF in the Fabry mouse kidney might contribute to Fabry disease nephropathy by inducing apoptosis. To test whether Gb3 accumulation can induce apoptosis, we incubated bovine aortic endothelial cells with Gb3 and found increased expression of TGF- β 1, VEGFR2, VEGF, FGF-2, and P-p38. The combination of increased expression of TGF- β 1 and VEGF caused by Gb3 accumulation may allow upregulation of FGF-2, VEGFR2, and P-p38 expression, and these changes might be associated with Fabry disease nephropathy by inducing apoptosis.

Key words: Fabry disease, mouse, globotriaosylceramide, kidney, endothelial cell, TGF- β 1, VEGF

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Species-Specific differences in Locked Nucleic Acid Antisense Oligonucleotide Uptake *in vitro* Correlates with *in vivo* Renal Damage.

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Antisense oligonucleotides (ASOs) are RNA targeting therapeutics that can be designed to modulate RNA function through a diverse set of post-binding mechanisms by either a) binding to the RNA and interfering with its function without causing RNA degradation or b) by promoting degradation via nuclease recruitment or by acting directly to degrade the target (i.e. ribozymes, DNazymes). One active area of ASO therapeutic research has been in fully phosphorothioated single stranded locked nucleic acid (LNA) 3-8-3 (LNA-DNA-LNA) gapmers. Bolus administration of LNA gapmers results in rapid accumulation in the liver and kidney, allowing for modulation of targets expressed in these tissues. An LNA ASO approach has been used to reduce glucocorticoid receptor (GR) mRNA levels in liver for the treatment of type two diabetes mellitus. Lead LNA ASOs were chosen based on enhanced glucose tolerance and acceptable tolerability in mouse studies. Exploratory toxicity studies were completed for each compound in rats at all doses (2mg/kg, 20mg/kg, 60mg/kg, 2X/week for 3 weeks) where severe renal tubular degeneration with inflammation was observed. Interestingly, this was not observed in mouse studies where animals were dosed 2X/week for two weeks with 25 mg/kg. Hepatic necrosis was observed in rats at doses above 20 mg/kg, while these effects were generally not observed in mice. *In vitro* investigations were subsequently initiated employing two LNA ASOs (PF-05203021, PF-05201595), one of which was hepatotoxic in mice whereas the other was not with the purpose of identifying and understanding mechanistic differences in toxicity between species.

To further elucidate the mechanism of the observed species-specific renal toxicity, primary epithelial cells from rat and mouse renal proximal tubules were used to evaluate LNA cytotoxicity, uptake, as well as GR mRNA knockdown. In conjunction with these efforts, immortalized renal tubular epithelial cells from rat and mouse were used to evaluate the same endpoints. Target knockdown in immortalized and primary cell lines were significant ($p < 0.001$) after 24 hours of each LNA ASO, with the single exception being with LNA ASO PF-021 in rat cells where the oligo sequence contains a single base mismatch to the target. In immortalized kidney cell lines, one LNA ASO (PF-021) was cytotoxic to mouse and rat cell lines after 32 hours of exposure, while the second (PF-595) was not significantly cytotoxic. These results in mouse cells correlate well with *in vivo* studies (i.e. PF-021 caused hepatic necrosis in mice). Conversely, limited primary cell toxicity is observed, with the rat cells being least sensitive to LNA ASO toxicity. Additionally, with constant LNA exposure, ASO accumulates in all cell lines over 48 hours in a time dependent manner. Rat primary proximal tubule epithelial cells, however, were more efficient (~2-3x) in LNA uptake, regardless of sequence. It is possible from these results that the sensitivity observed in the rat toxicity study is associated with a greater accumulation of LNA in the proximal tubule epithelium. However, the mechanism of subsequent damage is unclear and thus is still a part of ongoing investigations.

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Characterization of infiltrating M1/M2 macrophages in animal models of non-alcoholic steatohepatitis.

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Non-alcoholic fatty liver disease includes steatosis, steatosis with inflammation, and steatohepatitis with necroinflammation (NASH) with or without fibrosis. Macrophages, which are great contributors to the disease, are classified into M1 (pro-inflammatory) and M2 (anti-inflammatory, pro-fibrotic) macrophages. We hypothesized that the macrophage profile changes during the development and progression of NASH. To test our hypothesis, we used animal models of steatosis (4-weeks high fat diet [HFD] or ob/ob mice) and steatohepatitis (9-months HFD or methionine-choline-deficient [MCD] diet for 1,2,5 or 8 weeks). M1 (TNF- α , IL-1 β , IP-10) and M2 (CD206, CD163, Arginase-1) macrophage markers were measured in the liver using qPCR and flow cytometry. M2 polarizing factors (IL-33, IL-10) and fibrosis markers (TGF- β , α -SMA, collagen, TIMP1) were assessed by qPCR, Western blot and ELISA. As a result, M1 markers were up-regulated in HFD induced steatohepatitis but not in steatosis. Among the M2 markers, only CD206 increased in HFD induced steatohepatitis. In MCD-steatohepatitis, M1 markers were up-regulated throughout the progression of the disease. All M2 markers were increased in early MCD-steatohepatitis (week 1), and CD206 remained elevated at all time-points. IL-10 and IL-33 mRNA were up-regulated in early MCD-steatohepatitis (week 1, 2) and in HF and MCD diet-induced steatohepatitis at all weeks, respectively. Fibrosis markers were also increased during the HF and MCD diet induced steatohepatitis at mRNA level. Serum TIMP1 protein showed an increase at 8 weeks of MCD diet feeding. In summary, our data confirms the change in M1 and M2 markers resulting in the dominance of pro-inflammatory markers in NASH. The decrease of some M2-markers might suggest exhaustion of the anti-inflammatory mechanisms while the CD206+ population might contribute to fibrosis in NASH.

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Size matters to a syncytiotrophoblast: alpha-Fe₂O₃ nanoparticles exhibit diameter-dependent effects.

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Ferric oxide nanoparticles (NPs) stand at the cornerstone of molecular imaging, water remediation, and the semiconductor industry due to their unique physico-chemical properties on the nanoscale (10⁻⁹m) compared to their bulk form. Indeed, ferric oxide nanoparticles offer a unique opportunity to exploit their capacity to target individual cells at the organismal level selectively through functionalization of the nanoparticle surface. However, increased use highlights the need to assess the potential toxic effects of these nanomaterials. Here, we report the size-dependent effects of alpha-Fe₂O₃ nanoparticles of three different diameters (i.e. 15-, 50-, and 80nm in diameter) after exposure to an *in vitro* model of the human placenta (BeWo B30 epithelium). In contrast to the conventional notion that smaller-size NPs exhibit greater toxicity, we show that the smaller-sized particles (e.g. 15nm) resulted in less toxic effects compared to larger alpha-Fe₂O₃ NPs after they were applied at the same mass concentration. Transepithelial electrical resistance (TEER) demonstrated a loss of epithelial integrity for 50-, and 80nm NPs, but not for 15nm NPs compared to untreated epithelia after a single 100µg/mL exposure to the nanoparticles. Live/dead analysis showed an increase in cell death in the larger-diameter particles (50-, and 80nm) from 1 to 5 days post treatment. Moreover, analysis of reactive oxygen species (ROS) indicates a marked increase in cells positive for ROS for the 50- and 80nm NPs. To assess the route of entry in BeWo cells we employed transmission electron microscopy and observed that these NPs likely entered the cells through endocytosis after 4 hours in culture as inhibiting endocytosis resulted in a dramatic decrease in the number of cells with internalized NPs. When the epithelia integrity was disrupted as measured by TEER for the large diameter NPs we show a large number of NPs localize in the extracellular space between cells at the putative level of the cellular junctions at 8 hours, but not in untreated epithelia or when small-diameter NPs (i.e. 15nm) were applied. Moreover, scanning electron microscopy demonstrated a significant (p<0.0001) disruption of the apical brush-border. Exposure to 80nm particles at a concentration of 100µg/mL results in fewer microvilli per unit area on the apical surface compared to untreated epithelia at each time point assessed. These data indicate that different diameters of alpha-Fe₂O₃ NPs result in different effects on the epithelium, emphasizing the need to further investigate the mechanisms by which these different sizes elicit different effects on the epithelium. </sup>

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Suppression of HIV-1 Tat-induced expression of ICAM-1/VCAM-1 and monocyte adhesiveness by valproic acid in human astrocytes.

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Extracellular HIV-1 Tat plays a critical role in the expression of inflammatory genes including adhesion molecules such as ICAM-1 and VCAM-1 in the central nervous system. Previous studies suggested that valproic acid (VPA), an anti-epileptic drug, exhibits anti-inflammatory properties. This study investigated whether VPA has a regulatory effect on the HIV-1 Tat-mediated up-regulation of ICAM-1/VCAM-1 expression and monocyte adhesion in human astrocytes. VPA significantly decreased HIV-1 Tat-induced ICAM-1/VCAM-1 expression in human astrocytes. In addition, VPA treatment significantly decreased HIV-1 Tat-induced

monocyte adhesion in human astrocytes. VPA inhibited HIV-1 Tat-induced increase of NF- κ B promoter activity without altering p65 phosphorylation and I κ B degradation. Furthermore, VPA suppressed HIV-1 Tat-induced expression of NF- κ B dependent pro-inflammatory genes such as CXCL10, MCP-1, but not IL-8. These data indicate that VPA can modulate the HIV-1 Tat-mediated expression of adhesion molecules and subsequent adhesiveness of monocytes in human astrocytes and might be useful for controlling HIV-1 Tat-mediated neuroinflammation.

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Exploration of the relationship between breast cancer progression and aggression, extra-cellular matrix stiffness and tissue inflammation.

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Tumor progression is influenced by the dynamic interplay between the genetically-modified epithelium and the associated microenvironment. We previously showed that the extra-cellular matrix (ECM) progressively stiffens as mammary tumors evolve, and that stiffening the ECM promotes malignant transformation while inhibiting ECM stiffening reduces tumor progression (Levental et al., 2009; reviewed in Butcher et al., 2009). Yet breast cancers also exhibit elevated influx of inflammatory cells and tissue inflammation promotes tumor progression by fostering angiogenesis and enhancing tumor cell growth and motility (Ruffell et al., 2011). Tumor-associated macrophages additionally stimulate stromal fibroblasts, implying that tissue inflammation could promote malignancy by stiffening the ECM. To address this possibility, we explored the relationship between ECM tension and macrophages in human breast cancer and during mammary tumor development in transgenic mice. Using fresh and archived tissue samples, we quantified a progressive increase in ECM remodeling and stiffening as human breast tumors and mouse mammary cancers developed. We found that the ECM associated with triple negative breast cancers was almost twice as stiff as the ECM adjacent to ER/PR+ve tumors and that ECM tension correlated significantly with increased numbers of infiltrating activated macrophages. Consistently, we established a positive correlation between tumor progression, elevated mechanosignaling, ECM stiffness and tissue inflammation in both human and mouse tissue. However surprisingly, when macrophages were depleted from the mammary glands of MMTV-PyMT tumors, ECM tension did not change. Instead we found that inhibiting lysyl oxidase activity to decrease collagen cross-linking and ECM stiffness reduced the activation state of the macrophages within the mammary glands of 14-week old mice. Consistently, in vitro studies showed that ECM tension potentiates the expression of pro-inflammatory chemokines and increases levels of phosphoStat3 and that ECM tension directly modifies macrophage polarity. These findings suggest that ECM tension may promote tumor progression and aggression either by directly regulating macrophage activity or indirectly through enhancing expression of pro-inflammatory chemokines. Further studies are now underway to explore these findings and to clarify their impact on tumor progression and response to therapy. Supported by: NCI SPORE P50 CA058207 to VMW, CP, & SH; U01 ES019458-02 to ZW; NCI R01 CA138818-01A1 to VMW; U54 CA143836-01 to VMW & JL; W81XWH-10-BCRP-EOHS-EXP to LMC; KG110560 and KG111084 Susan G Komen Breast Cancer Foundation Research Grant to SH and LMC and P0047389 Postdoctoral Fellowship to IA.

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The inhibition of HMG-CoA reductase pathway protects airway epithelial cells against bacterial pore-forming toxins.

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Pore-forming toxins are the single largest class of bacterial virulence factors. We have developed a novel host-directed strategy defending against some bacterial pore-forming toxins. Pneumolysin and α -hemolysin are the main pore-forming toxins shown to play critical roles in bacterial pathogenesis respectively in *Streptococcus pneumoniae* and *Staphylococcus aureus*, two common causes of bacterial infections. Airway epithelia are the initial and primary sites of many bacterial contacts and their barrier and immunity functions are critical to infectious diseases. Using primary normal human bronchial epithelial (NHBE) cells and an immortalized normal bronchial epithelial cell line, HBE1, we here show that the prior exposure to statins confers significant cellular resistance to the cytotoxicity of pneumolysin and α -hemolysin. We further found that the protection requires protein synthesis and is calcium dependent. With siRNA gene silencing, pharmacological inhibitors, immuno fluorescence microscopy and biochemical approaches, we have delineated the statin-mediated cellular protection mechanisms in airway epithelial cells. Our results suggest that the inhibition of HMG-CoA-reductase pathways and certain downstream lipid metabolism may increase airway epithelium resistance to pore-forming toxins. The ultimate goal of this study is to elucidate the interactions of pore-forming toxins and the host cells at the molecular level and this might be potentially developed as an adjuvant therapy in pore-forming toxin-related bacterial infections.

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Creation of a novel claudin binder by using *Clostridium perfringens* enterotoxin and baculoviral display.

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Objective: Claudins (CLs) are a family of tetra-transmembrane proteins comprising over 24 members. CLs play a crucial role in sealing intercellular spaces in epithelium and endothelium; they are frequently over-expressed in malignant tumors. CL binders are promising lead ligands for CL-targeted drug development; however, because the preparation of recombinant CL proteins is extremely difficult, CL ligands have never been fully developed.

The C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), which binds to CL-3 and -4, is the most characterized CL ligand. Previously, we prepared a C-CPE mutant-displaying phage library, and developed a screening system for CL ligands using this C-CPE mutant library and CL-displaying baculovirus (CL-BV). Here, we created and evaluated novel CL-1 ligand m19 as a candidate molecule for modulating the mucosal absorption of drugs.

Methods and results: The functional residues of C-CPE were randomly substituted with other amino acids to create a C-CPE mutant-displaying phage library. Recombinant CL-1-BV was prepared by using the Bac-to-Bac expression system. CL-1-BV-coated immunotubes were used to screen for CL-binding phages. CL binding was evaluated with ELISA by using CL-BV-coated immunoplates, and FACS analysis by using CL-expressing cells. Modulation of epithelial barrier was examined in Caco-2 monolayer cell sheets. Jejunal absorption-enhancing effects were investigated by in situ loop assay. The C-CPE mutant library was added to immunotubes coated

with CL-1-BV. CL-1-BV-bound phages were recovered and amplified. The resultant phages were again added to immunotubes and the procedure repeated. The panning procedure was repeated three times and the C-CPE mutants expressed by the CL-1-BV-bound phages were sequenced. Recombinant mutant C-CPE proteins were prepared, and their binding to CL-1-BV was investigated. We identified a CL-1-binding mutant, m19. m19 bound to CL-1-expressing cells and showed more epithelial barrier-modulating activity than C-CPE. m19 also enhanced jejunal absorption of dextran more than C-CPE did.

Conclusion: The C-CPE mutant library and CL-BV works as a CL-binder screening system. m19 may be a promising lead for modulating the mucosal absorption of drugs.

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Pathological function of transformation of growth form of *Candida albicans* by N-acetylglucosamine.

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The amino sugar N-acetylglucosamine (GlcNAc) is known to induce the hyphal mode of growth of *Candida albicans*. *C. albicans* is commensal yeast in healthy mammalian intestinal tracts, but according to the environmental condition, it transforms the growth form to hyphae. This process is considered to contribute for pathogenesis of the mucosal infection of *C. albicans*. Here we investigated whether ingestion of GlcNAc has an effect on the *in vivo* growth of *C. albicans* or the pathogenesis of a *C. albicans* infection. Using a murine model of oral candidiasis, we have found that oral administration of GlcNAc, but not glucose, increased oral symptoms of candidiasis and fungal burden. There was a dose dependent relationship between GlcNAc dosage and the severity of oral symptoms. Mice given the highest dose of GlcNAc 45 mM, also showed a significant increase in fungal burden, and increased histological evidence of infection compared to a control given water alone. GlcNAc is also a commonly used nutritional supplement for the self-treatment of conditions such as arthritis. We propose that ingestion of GlcNAc, as a nutritional supplement, may have an impact on oral health in people susceptible to oral or gastrointestinal candidiasis.

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Functional characterisation and gene expression analysis of trachoma conjunctival fibroblasts.

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Trachoma is a major cause of blindness worldwide, and number one infectious cause of vision impairments. The initiation of the disease is caused by *Chlamydia trachomatis* and after an initial phase of infection and chronic inflammation (active trachoma), scarring develops and the eyelashes turn inwards (trichiasis), rubbing on the cornea and causing visual loss. Because trichiasis occurs even after eradication of the bacterial infection, we hypothesized that trachoma scarring is due to functional alterations of the conjunctival fibroblasts. We isolated conjunctival fibroblasts from patients following trichiasis surgery as well as from matching control biopsies from Tanzanian individuals. The conjunctival fibroblasts were examined for collagen contraction in control conditions and after the addition of cytokines and growth factors expressed *in vivo*; cell force measurements; matrix metalloproteinase (MMP) expression profile; myofibroblast differentiation and whole genome expression analysis. We show that trichiasis-derived fibroblasts display increased collagen gel contraction compared to healthy controls both in

serum-free medium and in the presence of serum or platelet-derived growth factor (PDGF). However, both cell and matrix force were similar, suggesting the mechanical properties of diseased cells are not affected. Differences in MMP expression during three-dimensional cultures and collagen production were also observed, demonstrating a phenotype similar to the in vivo observations. Our findings indicate that trachoma conjunctival fibroblasts exhibit altered functional and molecular characteristics that are maintained in vitro.

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Urine of AKI patients promotes metanephric cell growth and recovery of renal function after ischemia-reperfusion injury in mouse.

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Chronic kidney disease causes renal death and recovery of the lost renal function is critical to prevent it. Chronic kidney injury with fibrosis is shown to recover significantly by mesenchymal stem cell transplantation probably through paracrine mechanism in mice, which may also be shared by the recovery from acute kidney injury (AKI) in patients. When renal tubular cells are injured in AKI, various molecules produced in the lesion leak into urine. Thus, we collected urine from AKI patients and examined its activities to promote mouse metanephroi growth including mesenchymal cells and recovery from the injury.

Fresh urine samples from 5 AKI patients were collected at their recovery stage. By centrifugation using tubes with molecular sieve-membrane, the samples were separated to the high molecular weight fraction (HMWF, MW>10 kD) and the low molecular weight fraction (LMWF, MW<10 kD). Each fraction was examined for promotive activities on cell-proliferation in metanephroi by organ culture and recovery from ischemia-reperfusion AKI in mice. Each fraction from urine containing 0.1 mg creatinine was added to 1 ml of culture medium and 4 metanephroi at E.S.12 were submerged for 3 days in it followed by protein measurement of cultured tissues. The ratio of measured protein contents in the tissue cultured in HMWF containing medium vs. that cultured in LMWF containing medium was used as a mitotic activity index for metanephric cells by the HMWF. HMWF from two patients promoted metanephric cell proliferation with the index of 1.6 and 1.9, respectively. HMWF with the highest mitotic activity was administered to 5 AKI mice intra-peritoneally with 25 mg creatinine-equivalent doses at 10 min after inducing the injury. Thereafter recovery of AKI was traced 3 days by measuring blood-urea-nitrogen (BUN) levels and kidneys harvested after the 3 days were examined histologically and by immunostaining for PCNA. BUN level increased at 1 day but decreased at 2 days after inducing injury and it was lower at 2 days in HMWF administered mice (26.3 ± 3.6 mg/dL, n=5) than in control (PBS administered) mice (33.8 ± 5.0 mg/dL, n=5) ($p \leq 0.05$). Histologically no difference was observed between HMWF-administered and control mice. but more PCNA-positive tubular cells were seen in the former than the latter.

In conclusion, HMWF of urine from AKI patients has promotive activities not only on metanephric cell proliferation but also on recovery from AKI in mice. These activities found in the urine may contribute to the recovery from AKI in patients.

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Methylxanthine-induced Neuroprotection in Neural Stem Cells via the System Xc pathway.

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Methylxanthines are being widely studied to understand their neuroprotective role in disease. Methylxanthines are also an attractive agent for neuroprotection in the neonatal population. Theophylline, a member of the methylxanthine family, is commonly used in neonates for the treatment of apnea of prematurity. Methylxanthines' mechanism involves inhibition of the adenosine receptor and as phosphodiesterase inhibitors. Other potential mechanisms or pathways of action for methylxanthines are still not clearly understood. The main aim of this study is to understand the methylxanthine theophylline's role in the regulation of the cystine glutamate exchanger- a known neuroprotective pathway. In this study, we use neural stem cells to examine whether the cystine glutamate exchanger is regulated by theophylline administration. Theophylline has a dose and time dependent effect on System Xc expression. Glutamate-induced apoptosis was attenuated by theophylline administration and there was a concomitant increase in System Xc expression. Cell survival also increased with theophylline administration. MAP Kinase inhibitor (PD98059) blocked the increase seen in System Xc expression in a dose-dependent manner. Adenosine did not have a significant effect on System Xc expression. In conclusion, theophylline administration regulates System Xc expression and activity leading to neuroprotection.

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Chronic Smoking Causes Decrease in Plasma Levels of Gamma-amino butyric acid (GABA) and Glutamate in the African American Population When Compared to Non-smokers.

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Smoking is a risk factor for various types of cancers and heart diseases. The result of the study, to assess if chronic cigarette smoking alters brain concentrations of the excitatory amino acids Glutamate and the inhibitory amino acid GABA, is presented here. Nicotine is a major substance inhaled by smokers and it is known to modulate various neurotransmitters that affect neural and cardiac function. Nicotine has been recognized as an important mediator of the actions of amino acids viz., GABA and Glutamate. However to our knowledge, no studies have assessed if chronic cigarette smoking in humans affects regional concentrations of these important amino acids. Glutamate is known to play a role in stimulating the activity of other neurons. On the contrary, GABA often slows other neurons down. GABA is a major neurotransmitter in the mammalian brain and controls neuronal excitability. It has been implicated in the addictive and withdrawal process of nicotine dependence. The production of GABA is dependent on Glutamate decarboxylase, the enzyme that catalyzes the decarboxylation of Glutamate to GABA. This ultimately affects healing of tissues by impairing the release of factors that stimulate the function of the cells responsible for the healing of tissues. GABA and Glutamate are two important neurotransmitters that are responsible for inhibition and excitation, respectively, and control the vertebrate nervous system. **Methods:** Blood samples were collected from smokers and non-smokers in a population of African Americans and plasma were prepared. Levels of GABA and Glutamate were measured by using ELISA. **Result:** Plasma level of GABA is decreased in male smokers (81.2 ± 2.4 ng/ml as against 122.2 ± 2.8 ng/ml) but not in female smokers (129.4 ± 4.0 ng/ml as against 136.5 ± 2.0 ng/ml), while Glutamate is decreased in both males (10.5 ± 5.0 ng/ml as against 19.4 ± 2.9

ng/ml) and females (13.7 ± 3.3 ng/ml as against 19.3 ± 3.1 ng/ml) when compared to non-smoking volunteers. **Conclusion:** In African Americans, levels of GABA and Glutamate are modulated by smoking and this plays a major role in altering neurotransmitter control on the nervous system. These findings provide a biological foundation for alteration of neurotransmitter levels among habitual smokers in the African-American population. This study was supported by James and Esther King Biomedical Research Program of Florida Department of Health Grant HBC-01.

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Influence of Antioxidants on Cellular Migration and Protection from Oxidative Stress.

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Resveratrol is a strong antioxidant and natural phytoalexin often found in red grapes and wine. It has been seen to have positive effects on metabolism and anti-cancer activity. Currently, our lab has been studying the regulation of cadherins, important proteins for cellular adhesion, in normal fibroblasts and fibrosarcoma cells. It is also being investigated whether antioxidants, such as resveratrol and N-acetylcysteine (NAC), can influence the control of cadherins, proliferation, or migration. Dishes of HT-1080 fibrosarcoma cells were treated with 10, 25, and 50 μ M resveratrol, resulting in inhibited cellular proliferation. While H_2O_2 inhibited both proliferation and cell migration, causing cells to senesce, NAC showed not to have such an inhibitory effect. NAC treatment also did not protect the cells against H_2O_2 -inhibition. Currently, we are testing if treatment with resveratrol can provide protection against H_2O_2 -inhibition of proliferation/migration or alter expression of cellular signaling proteins, including cadherins.

Lipids and Membrane Microdomains II

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Growth and lipid production in the haptophyte *Chrysochromulina* sp. under salinity stress: a potential role for stress response proteins.

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Lipid bodies serve as the cellular organelle for sequestering newly synthesized neutral lipids. Though lipid body formation represents the keystone of cellular lipid production in algae, little is known concerning the biogenesis of this organelle. Until recently, lipid bodies were thought to be static, intracellular vesicles that served as inert storage sites. New research has dramatically transformed this perception. Now lipid bodies are considered to be true organelles of complex structure that play an indispensable role in the maintenance of cellular homeostasis. The haptophyte *Chrysochromulina* sp. is an excellent model for understanding lipid body biogenesis and lipid production in algae. *Chrysochromulina* sp. has a high growth rate, two lipid bodies that are easily detected within the cell, and a genome (57 MB) that has been fully sequenced. However, limited data are available for the cell and molecular biology of this organism with respect to lipid body biogenesis. To date, studies have shown that most algal species grown under various stress conditions increase the number and/or size of lipid bodies within the cell as well as the production of lipids. The current study examines cell growth, lipid body biogenesis and lipid production in *Chrysochromulina* sp. using flow cytometry, BODIPY 505/515 staining with fluorescent microscopy and GC-MS, respectively, when placed under salinity stress. High salinity growth media (32mM NaCl) appears to delay the entry of *Chrysochromulina* sp. into the exponential growth phase and causes an increase in production of lipids when compared to the

control (8mM NaCl). Additionally, a classic eukaryotic stress response appears to be present in *Chrysochromulina* sp. Upon examination of the genome, we have found multiple sequences coding for highly conserved stress response genes including heat shock proteins (HSPs) 70 and 90. The proteins encoded by these genes have also been identified in *Chrysochromulina* sp. using anti-HSP70 and HSP90 antibodies. Proteomic analysis of proteins obtained from isolated lipid bodies shows that these HSP proteins may be associated with this organelle. We are concurrently investigating how proteins involved in stress response (HSPs) are regulated as well as their potential association with the lipid bodies using antigenic techniques.

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***Chrysochromulina* sp. a model alga for analyzing lipid body biogenesis.**

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Microscopic analysis has documented the presence of lipid inclusions in many algal species; however, lipid body inclusions have only recently been recognized as true sub-cellular organelles. Understanding the ability of algae to modulate cellular homeostasis through the regulation of lipid production is critical as these organisms are essential in sequestering global CO₂, serve an ecological role as the primary producers in marine and fresh water food webs, and there is increased commercial interest in these organisms as a source of biofuel and pharmaceutical co-products. A broad investigational strategy is being implemented to probe algal lipid body biogenesis. *Chrysochromulina* sp. (Haptophyte) has been chosen as a model system for probing the life history, lipid and protein composition of this dynamic organelle in chromalveolates. This small (~4µm) unicellular, oleaginous alga is experimentally tractable for it contains a simple complement of two large lipid bodies, lacks a cell wall which aids in lipid body recovery, sequesters a uniquely wide range of neutral lipids within the lipid bodies, can be synchronized by photoperiod and has a fully sequenced genome (57MB). Flow cytometric and quantitative lipid GC/MS analysis documents a diurnal correlation between lipid body size, lipid profile and lipid amount for this model system. The association of lipid bodies with chloroplasts and mitochondria as well as the co-localization with the endoplasmic reticulum is assessed using transmission electron microscopy as well as organelle-fluorescent probes. A newly devised lipid body isolation method, has allowed both the lipidomic and proteomic compliments of *Chrysochromulina* sp. lipid bodies to be assessed. LC-MS/MS revealed peptides with homology to proteins involved in lipid metabolic pathways in diatoms, including sulfolipid biosynthesis, lipoamide dehydrogenase and phospholipid scramblase. GC/MS lipid analysis of a lipid body fraction demonstrates a suite of fatty acids, ranging from C14:0 - C22:5, with elevated levels of C16:0.

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Spatial regulation UBXD8 and p97/VCP: at the intersection between ER protein quality control and fat storage.

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Lipid droplets (LDs) are endoplasmic reticulum (ER)-derived organelles composed of a neutral lipid core surrounded by a phospholipid monolayer, which is decorated with integral and peripherally-associated proteins. How proteins are sorted and delivered from their site of synthesis in the ER to LDs and the mechanisms underlying LD formation and turnover remain mostly unknown. To address these questions we investigated the mechanism by which UBXD8,

a hairpin-anchored protein implicated in ER-associated degradation, partitions between the ER and LDs. Our results identify the rhomboid pseudoprotease UBAC2 as a UBXD8 receptor that anchors UBXD8 to the ER and links it to upstream ubiquitination machinery. UBAC2 prevents UBXD8 trafficking to LDs and dominantly represses LD-targeting signals encoded in the UBXD8 hairpin structure or induced by lipogenic stimuli. Proteomic analysis of UBXD8 complexes affinity-purified from ER or LD biochemical fractions indicate extensive interactions with ubiquitination machinery and lipid metabolism enzymes, placing UBXD8 in a central position to globally coordinate neutral lipid metabolism in both organelles. Moreover, we found that UBXD8 recruitment of the AAA ATPase p97/VCP to LDs lead to a striking increase in LD abundance by impairing the rate of LD turnover. The most direct mechanism by which UBXD8 could suppress LD turnover would be to inhibit ATGL, the lipase that catalyzes the rate-limiting step in lipolysis, and an interaction between UBXD8 and ATGL detected in our proteomics studies was confirmed by endogenous co-immunoprecipitation. Given the established role for UBXD8 in protein degradation, we were surprised that although ATGL undergoes metabolically-regulated proteasomal degradation, its steady-state level, half-life, and LD association were unaffected by overexpression of UBXD8 or UBAC2. Instead, we found that ATGL is present in mutually exclusive complexes with either UBXD8 or its endogenous activator CGI-58, and co-immunoprecipitation and bimolecular fluorescence complementation experiments revealed that UBXD8 tonically represses the allosteric activation ATGL by inhibiting the association of ATGL and CGI-58. Together, our data identify a fundamental mechanism by which the unique compositions of the ER and LD proteomes are preserved/established despite sharing a common membrane of origin, and uncover an unrecognized regulatory role for UBXD8, UBAC2, p97/VCP and ubiquitin in cellular fat metabolism.

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Live Cell Analysis of Lipid Droplet Biogenesis Using the Hepatitis C Virus NS5A Protein.

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Lipid droplets (LDs) are the principal organelles for the storage of neutral lipids and cholesterol esters in eukaryotes. Considered for a long time as mere inert deposits, LDs are now conceived as dynamic organelles implicated in numerous biological processes in health and disease. Several RNA viruses have been shown to use host LDs at different steps of the viral life cycle. Particularly, important discoveries indicate an essential role for lipid droplets in the Hepatitis C Virus (HCV) replication and assembly. Despite the progress in lipid-droplets research, many fundamental questions are still not resolved. The molecular mechanisms that underlie LD formation, growth and movement as well as interaction with other organelles remain for the most part unclear. The most widely accepted model for LD formation postulates the synthesis and accumulation of neutral lipids between the leaflets of the endoplasmic reticulum (ER) membrane. A nascent lipid droplet emerges that is enclosed in a monolayer originating from the cytosolic leaflet. Using the fluorescently tagged, LD-binding non-structural protein 5A (NS5A) of HCV, we visualized LD biogenesis and maturation for the first time in living intact cells. NS5A binds ER membranes and partitions to the surface of triglyceride accumulations that constitute LD precursors. The putative dynamic LD precursor elements were identified as worm-like structures moving back and forth to the cell periphery. At the ER periphery, LDs bud and subsequently move slowly towards the cell center. Nascent LDs go through recurring homotypic fusions that lead to formation of mature LDs. We are currently assessing the role of Rab1 and its GAP TBC1D20 in LD biogenesis. These proteins interact with NS5A and are part of the HCV-host interface.

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Lipidated ApoB degradation and the ER-LD juncture.

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In the hepatocyte, lipids stored in LDs are utilized preferentially to synthesize very low-density lipoproteins (VLDL). Apolipoprotein B (ApoB), the principal protein of VLDL is lipidated cotranslationally by the microsomal triglyceride transfer protein (MTP) activity in the ER. When the lipidation process is perturbed, nascent ApoB is subjected to the ER-associated degradation (ERAD). On the other hand, lipidated ApoB becomes mature VLDL by acquiring additional lipids in the secretory compartment, but it is degraded in the ER and/or post-ER compartments under certain conditions. Macroautophagy has been reported as a possible post-ER degradation mechanism, but other mechanisms are also likely to be involved.

We previously reported that ubiquitylated ApoB accumulated in lipid droplets (LDs) when proteasomes and/or autophagy were suppressed, and proposed that LDs function as a platform of ApoB degradation. A further study showed that ApoB in LDs was lipidated and that the ApoB-laden LDs and the ER cistern formed an amalgamation structure called gApoB-crescent. The result raised the possibility that the ApoB-crescent is related to ERAD of lipidated ApoB.

To elucidate the molecular mechanism of ApoB degradation around LDs, we searched for proteins that are engaged in the process and identified UBXD8, p97 and Derlin-1. In Huh7 cells, UBXD8 mainly localizes in LDs, whereas Derlin-1 is a putative dislocon in the ER. Nonetheless they bound each other in the vicinity of LDs. Knockdown of UBXD8 reduced p97 recruitment to LDs, increased ApoB-crescents, and caused accumulation of ubiquitylated ApoB in LDs. Knockdown of Derlin-1 also increased ApoB-crescents, and suppressed the interaction between ApoB and UBXD8. However, the amount of ApoB cross-linked with ADRP was increased by UBXD8 knockdown, whereas it was decreased by Derlin-1 knockdown. These results suggested that lipidated ApoB is dislocated from the ER lumen to the LD surface for proteasomal degradation and that Derlin-1 and UBXD8 are engaged in the pre- and post-dislocation step, respectively.

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A conserved ER-membrane protein complex facilitates phospholipid exchange between the ER and mitochondria.

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Mitochondria are critical cellular components that are needed for energy production, lipid metabolism, and apoptosis. Mitochondrial membrane biogenesis requires the import of proteins and lipids. Most lipids are synthesized in the endoplasmic reticulum (ER) and transferred to different cellular compartments by vesicular trafficking. Interestingly lipid trafficking from the ER to mitochondria has been found to be non-vesicular as it is energy independent and does not require any cytosolic protein in vitro. It has long been thought that lipids are exchanged between the ER and mitochondria at regions of close contact between these organelles. However, the mechanism of transport and the proteins responsible for tethering the ER and mitochondria have not been determined. Here, we devised a novel genetic screen in *S. cerevisiae* to identify proteins needed for lipid exchange between the ER and mitochondria. Among the proteins we found were all six members of an evolutionarily conserved complex named ER-membrane protein complex (EMC). Deletion of multiple EMC genes resulted in a significant reduction in the transport of phosphatidylserine (PS) from the ER to mitochondria. The EMC-mutants showed growth retardation and loss of mitochondrial function, as they were unable to grow on a nonfermentable carbon source. Cells lacking EMC proteins and a protein that is a member of a

recently described complex that tethers ER and mitochondria (the ER-mitochondria encounter structure, ERMES complex) were not viable. Moreover in this strain, for the first time, we observed ER to mitochondria PS transfer to be almost completely abolished. The PS transport defect along with the growth defect could be rescued by the expression of an artificial ER-mitochondrial tethering protein. Together, these findings suggest that the EMC-proteins along with the EREMS-complex mediate ER to mitochondria PS transport by promoting membrane tethering between these two organelles. They also conclusively establish that lipid transport from the ER to mitochondria is an essential cellular process. Whether the EMC and ERMES complexes directly facilitate lipid exchange between the ER and mitochondria or simply tether these organelles remains a topic for future investigation.

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Erlins are part of an ER macromolecular assembly regulating cellular cholesterol levels.

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Cellular levels of cholesterol, a key component of biological membranes, are controlled by cholesterol sensing machinery in the ER. This involves the sterol binding protein Scap, which associates with the inactive precursor forms of sterol regulatory element binding protein transcription factors (SREBPs). Under cholesterol sufficiency, the SREBP/Scap complex is retained in the ER by binding to Insig. Below a critical ER cholesterol threshold, SREBP/Scap dissociates from Insig, allowing subsequent proteolytic activation of SREBPs in the Golgi.

Erlin-1 and -2 are heteromultimeric proteins of the ER that have been previously implicated in the proteasomal degradation of several ER proteins. These include 3-hydroxy-3-methylglutaryl-CoA reductase, the rate limiting enzyme of the sterol biosynthesis pathway. However, whether erlins directly affect cellular lipid homeostasis is not known. Here we show that erlin-1 and -2 are directly involved in cellular cholesterol regulation. We found that downregulation of erlins aberrantly activated SREBPs and their target genes in cells grown under sterol sufficiency. This was accompanied by substantial accumulation of cellular cholesterol. Using immunoprecipitation, in vivo crosslinking and colocalization studies we determined that erlins are physically associated with the SREBP machinery. Furthermore, recombinant erlins bound cholesterol selectively and with high cooperativity. FRAP analysis and sucrose gradient centrifugation showed that the diffusional mobility and size of the erlin complex are modulated by changes in ER cholesterol levels, suggesting that the erlin-cholesterol interaction is relevant in a cellular context.

Together our results indicate that erlins are cholesterol responsive proteins that directly regulate the SREBP machinery. Our data supports the notion that erlins help to specify a cholesterol-enriched domain in the ER that constitutes a regulatory environment for the cholesterol sensing machinery. The cooperative binding of cholesterol to erlins could contribute fundamentally to the previously observed switch-like behavior of the SREBP machinery in response to changes in ER cholesterol.

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Dynamic regulation of endoplasmic reticulum-plasma membrane junctions monitored by a genetically-encoded fluorescent marker.

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Many cellular functions, including secretion, cell migration and differentiation, depend on store-operated calcium entry (SOCE). A decrease of endoplasmic reticulum (ER) calcium and the subsequent activation of an ER calcium sensor, STIM1, initiate SOCE. Reduced ER calcium induces STIM1 to oligomerize and translocate to the ER-plasma membrane (PM) junctions

where the two membranes are closely apposed to each other within 20nm. The calcium channel Orai1 in the PM is then activated, which enables SOCE. Although ER-PM junctions have been observed by electron microscopy (EM) in many cell types, little is known about their formation and regulation. To help understand the dynamic regulation of ER-PM junctions, we first generated a genetically-encoded fluorescent marker of ER-PM junctions based on the translocation mechanism of STIM1. We have confirmed that this marker selectively localized to ER-PM junctions using confocal microscopy and EM, and that it did not perturb STIM1 and Orai1 translocation to the junctions nor disrupt SOCE. This marker has enabled us to visualize and track ER-PM junctions using live-cell total internal reflection fluorescence (TIRF) microscopy. We found that approximately two hundred stable ER-PM junctions exist in the adhesion surface of a single HeLa cell at resting state. During SOCE, a significant up-regulation of ER-PM junctions was observed. We further employed a cytosolic calcium chelator and a cytosolic caged-calcium reagent to demonstrate that the up-regulation of ER-PM junctions required an increase of cytosolic calcium but not a decrease of ER calcium. In summary, we have generated a marker enabling us to track the dynamic change of single ER-PM junctions in live cells and discovered cytosolic calcium-dependent regulation of ER-PM junctions.

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Ceramide synthase 2 down-regulation affects Golgi membranes and leads to G2 cell cycle arrest.

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Ceramide synthase 2 (CerS2), an endoplasmic reticulum resident, is one of the six mammalian ceramide synthases and it is responsible for the synthesis of very-long chain (VLC) ceramides (C22-C26). Following our previous finding that CerS2 down-regulation leads to G2/M cell cycle arrest, we aimed at understanding the underlying mechanism. Aphidicolin, a reversible DNA polymerase inhibitor, was used to synchronize the cells and study their cell cycle progression in relation to CerS2 down-regulation. First, we tested whether CerS2 expression or VLC sphingolipid levels changed when cells re-assumed cell cycle progression after synchronization with aphidicolin. The data showed that there was no significant change in CerS2 expression, but VLC sphingolipids increased significantly after cells re-assumed cell cycle progression. In addition, our flow cytometry results showed that CerS2 depleted cells re-assumed cell cycle progression after an aphidicolin cell cycle block at a slower rate compared to control cells. Our previous work has shown that CerS2 down-regulation results in not only a decrease in VLC sphingolipids, but also in a compensatory increase in long-chain sphingolipids (C14-C20) leading to aberrant membrane composition. Next, we tested whether altered membrane composition in CerS2 depleted cells affects membrane organization and dynamics during cell division. Membrane organization and dynamics, especially Golgi fragmentation, are important for proper execution of mitosis. Our confocal and electron microscopy data revealed that CerS2 depleted cells showed intracellular membrane disorganization and aberrant mitotic fragmentation of Golgi. In addition, in order to distinguish whether the cell cycle defect was in G2 or M phase we treated CerS2 depleted cells with paclitaxel, a known M phase cell cycle blocker. Our results showed that there was no difference in the cell cycle profile of the cells treated with CerS2 siRNA alone or in combination of paclitaxel, suggesting that CerS2 down-regulation resulted in cell cycle interruption preceding paclitaxel cell cycle block in M phase. In conclusion, our results suggest that CerS2 depletion leads to G2 cell cycle arrest likely because of affecting Golgi organization and membrane dynamics.

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***Drosophila* wound healing: A localized process of regeneration**

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An epidermal wound provides signals that initiate a variety of localized responses, some of which act to regenerate and repair the breach in the epidermal barrier. The *Drosophila melanogaster* embryonic epidermis provides an excellent system to discover new genes that regulate a conserved, wound-healing processes. Using fluorescent epidermal "wound" reporters that are locally activated around wound sites, we have screened almost 5,000 *Drosophila* mutants for functions required to activate or delimit wound-induced transcriptional responses to a local zone of epidermal cells. Among the seven new genes required to delimit the spread of wound responses are *Flotillin-2* and *Src42A*. These two genes are also sufficient, when overexpressed at high levels, to inhibit wound-induced transcription in epidermal cells. One new gene required to activate epidermal wound reporters encodes Dual oxidase, an enzyme that produces hydrogen peroxide. We also find that four biochemical treatments (a serine protease, a Src kinase inhibitor, methyl- β -cyclodextrin, and hydrogen peroxide) are sufficient to globally activate epidermal wound response genes in *Drosophila* embryos. Several of the biochemical treatments regulate the wound reporters in a complementary manner that parallels the phenotypes observed in the mutants identified from the genetic screen. We explore the epistatic relationships among the factors that induce or delimit the spread of epidermal wound signals. Our results define new genetic functions, and the interactions among them, which regulate the local transcriptional response to puncture wounds to mount a transcriptional response, mediating local repair and regeneration. Many stages of *Drosophila* development are suitable to assay regeneration. To gain more insight into the biological role of inhibiting wound response gene activation, we are testing new wound assays at later stages during *Drosophila* development. Additional data from our genetic screen highlights the function vesicle transport in promoting the localization of epidermal wound reporters. Preliminary data with a lipid raft stain suggests that lipid raft dynamics contribute to a localized wound response. The balance between activator and inhibitor signals may depend on the function of a *Flotillin-2*, *Src42A*, and *Duox* associated microdomain.

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Oligomerization and targeting to the epithelial basolateral surface of occludin is mediated by the MARVEL transmembrane motif.

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The MARVEL motif is an oligomerization and membrane lipid interacting module comprised of four transmembrane helices. MARVEL proteins are associated with the localization to or formation of diverse membrane subdomains via interaction with the proximal lipid environment. The functions of the Ocln MARVEL motif were never addressed. Bioinformatics sequence analyses and sequence based in-silico-structural analysis demonstrated that the MARVEL domain of Ocln protein family has distinct evolutionarily conserved features that are consistent with its basolateral membrane localization. Live cell microscopy, fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC) were used to analyze the intracellular distribution and self-association of fluorescent protein-tagged full-length human Ocln or the Ocln MARVEL motif excluding the cytosolic C and N termini (amino acids 60–269, FP-MARVEL-Ocln). FP-MARVEL-Ocln efficiently arrived at the plasma membrane (PM) and was sorted to the basolateral PM in filter-grown polarized MDCK cells. An Ocln chimera with its

MARVEL replaced by the apically targeted MAL proteolipid was redirected to the apical surface and disrupted epithelial polarity. A series of conserved aromatic amino acids within the MARVEL domain were found to be associated with Ocln dimerization and prevention of aggregate formation using BiFC. FP-MARVEL-Ocln inhibited membrane pore growth during Triton-X-100 – induced solubilization and was shown to increase the membrane ordered state using the laurdan lipid dye. These data demonstrate that the Ocln MARVEL domain mediates self-association and correct sorting to the basolateral membrane.

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Transmembrane potential modulates microdomain plasma membrane organization.

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Stability of highly ordered, sphingolipid-rich gel microdomains recently described in the plasma membrane of *S. cerevisiae* [1] was studied in response to dissipation of the plasma membrane potential. Fluorescent trans-Parinaric Acid (tPA) partitioning preferentially to ordered domains was used as a sensitive reporter of highly ordered lipid microenvironment [2]. 1,6-Diphenyl-1,3,5-Hexatriene (DPH) served for monitoring of overall membrane order. An effect of various transmembrane-potential modulation techniques was studied on cells in logarithmic and stationary growth phases. Significant drops of long lifetime component in tPA fluorescence, indicating decreased fraction of ordered plasma membrane microdomains, were detected following depolarization treatments of living BY4742 cells in suspension. Homogenization of the lipid microenvironment in the depolarized membrane did not significantly affect overall membrane order reported by DPH fluorescence anisotropy. We conclude that decrease of transmembrane potential correlates with decreased amount of gel sphingolipid microdomains in the yeast plasma membrane. This conclusion accents the role of transmembrane potential in microdomain organization of plasma membrane and complements our earlier observation of membrane depolarization-gated decomposition of ergosterol-rich MCC (Membrane Compartment of Can1) microdomains [3].

[1] Aresta-Branco et al., 2011, *J Biol. Chem.* 286(7):50043-54

[2] Sklar et al., 1977, *Biochemistry*, 16(5): 819-28

[3] Grossmann et al., 2008, *J. Cell Biol.* 183(6):1075-88

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Synthesis of Lipid Rafts-Like Microdomains is Important for Giardial Encystation.

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Giardiasis, caused by *Giardia lamblia*, is widespread throughout the world. This intestinal parasite, exists in two morphologic forms—trophozoites and cysts. While trophozoites colonize in the small intestine of humans and produce infections, cysts transmit the disease via contaminated water and food. Encystation (or stage-specific differentiation into cysts) is a critical step in the giardial life cycle that allows this parasite to survive in the environment before infecting a new host. Despite all of this information, the mechanism of encystation is yet to be elucidated. For example, it is not known how encystation-specific signals (ESSs) are generated and transduced across the membranes that trigger the cyst formation. Here, we report that *Giardia* synthesizes lipid raft (LR)-like membrane clusters, which could serve as platforms to

transmit ESSs generated in the small intestine. The LR synthesis in different stages of giardial life cycles was monitored by labeling Giardia with fluorescent-conjugated cholera toxin that binds to GM1 ganglioside present in the LR. Nystatin (27 μ M) and filipin III (7.6 μ M), two potent LR inhibitors, blocked the giardial LR synthesis completely. The effects of LR inhibitors on the biogenesis of encystation-specific vesicles (ESVs) and cyst production were evaluated by labeling the cells with anti-trophozoite and anti-cyst antibodies. We found that LR inhibitors significantly interrupted the ESV biogenesis and cyst production. Giardia cultured in a medium containing dialyzed fetal bovine serum (DFBS) with reduced cholesterol also interfered with encystation, and this could be reversed by adding cholesterol to the culture medium. Taken together, our results suggest that Giardia, although considered a primitive eukaryote, has the ability to synthesize raft-like domains, and that these microdomains are essential for encystation and cyst production. We propose that the lipid rafts in Giardia could serve as an ideal target for developing anti-giardial therapy.

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Transient GPI-anchored protein homodimers are units for raft organization and function.

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The dynamic organization of raft-associated glycosylphosphatidylinositol-anchored protein (GPI-APs) and their stimulation-induced changes in the plasma membrane were investigated, by applying advanced single-molecule fluorescent imaging methods, including single-molecule FRET, simultaneous, dual-color single-molecule tracking, and bimolecular fluorescence complementation. In resting cells, almost all of the GPI-APs investigated in the present research (CD59, DAF, and Thy1) exhibited rapid diffusion in the plasma membrane, and at the same time they continually form transient (~200 ms) homodimers through ectodomain protein interactions, stabilized by the presence of the GPI-anchoring chain and cholesterol (termed GPI-AP homodimer rafts or just homodimer raft for short). Prolonged heterodimers did not form, suggesting a fundamental role for the specific ectodomain protein interaction. In other words, raft-lipid interaction alone, without protein-protein interaction cannot induce 200-ms prolonged homodimer rafts. Since such homodimer rafts were found for three GPI-APs, i.e., CD59, DAF and Thy-1, it is concluded that the dynamic homodimers may be general features of GPI-APs. Because there are no sequence homologies among these molecules, these results suggest that, during the course of evolution inducing various amino acid changes, the properties of forming homodimers rafts are preserved, inferring the critical role of homodimers rafts for the function of GPI-APs.

Under higher physiological expression conditions, homodimers coalesce to form hetero- and homo-GPI-AP oligomer rafts by raft-based lipid interactions. Upon ligation, CD59 homodimer rafts formed stable homotetramer rafts conjugated with its transmembrane ligand, membrane attack complex, which triggered intracellular Ca²⁺ responses. Therefore, naturally, this signaling process depended on GPI-anchorage and cholesterol. Transient homodimer rafts are likely one of the key basic units for organization and function of raft domains containing GPI-APs.

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Eisosomes coordinate the organization and composition of the yeast plasma membrane.

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Compartmentalization of the plasma membrane underlies spatiotemporal control of diverse signaling pathways. Eisosomes are prominent structures in the plasma membrane of yeast

cells, yet the function of eisosomes has remained mysterious. We find that fission yeast eisosomes are linear, filamentous structures comprised of the BAR domain protein Pil1 and the peripheral membrane protein Sle1. Pil1 forms the core of fission yeast eisosomes and self-assembles into filaments *in vitro*. Sle1 is required for proper assembly of Pil1 filaments in cells, and contains separable domains for targeting to eisosomes and the plasma membrane. We used this initial characterization to perform a series of genomic screens aimed at elucidating the function of eisosomes. Surprisingly, we find that both Pil1 and Sle1 function with a specific synaptojanin isoform to regulate phosphoinositide levels in cells. Our genetic dissection of this pathway indicates that eisosomes control both the composition and spatial organization of cellular membranes. Further, we show that eisosome-dependent organization of the plasma membrane provides spatiotemporal control of conserved signaling pathways such as TORC2. Our findings indicate that eisosomes are multiprotein structures that control signaling pathways by coordinating the organization and composition of cellular lipids.

Receptors, Transporters, and Channels

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Identify STIM1 Associated Proteins by Tandem Affinity Purification in Mammalian Cells.

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Store operated Ca²⁺ entry (SOCE) is well characterized as the key Ca²⁺ influx process in non-excitabile cells. STIM1 and Orai1 are the essential components enabling the reconstitution of Ca²⁺ release activated Ca²⁺ (CRAC) channels underlying SOCE activity. STIM1, as an ER Ca²⁺ sensor, translocates within ER membrane upon store depletion to ER-PM junction, where it could interact with channel pore subunit, Orai1. Here, we performed Flag and His tagged tandem affinity purification (TAP) to identify STIM1 associated proteins in HeLa cells. Among several hits identified by mass spectrometric analyses, 3 candidates, including S1, S2, and S3, were selected as target proteins for further characterization. S1 only existed in the Stim1 complexes when cells were treated with thapsigargin or histamine in the absence of extracellular Ca²⁺, while S2 and S3 only existed in the Stim1 complexes when cells were incubated in the presence of extracellular Ca²⁺. Subsequently, these 3 candidate genes were individually knocked-down in HeLa cells. In addition, stably cell line overexpressing myc tagged candidate genes were established. The interactions between STIM1 and candidate proteins were validated by coimmunoprecipitation. We are currently examining the function of candidate proteins on SOCE activity in HeLa and Jurkat cells.

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Poor Binding to Serum Proteins may Facilitate Ricin Cellular Toxicity.

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The plant protein ricin is one of the most toxic substances known and has no antidote. Toxicity occurs after binding to cell surface galactose and endocytosis. Blood contains high concentrations of galactosylated glycoproteins and thus potentially could compete with ricin binding to cells. It is hypothesized that ricin binds preferentially to cell surfaces rather than fluid phase blood serum glycoproteins. Binding to whole human serum and serum glycoproteins: transferrin, alpha2HSGP and IgG were tested for ricin, Ricinus communis agglutinin-I (RCA-I), Sambucus nigra agglutinin (SNA) and Concanavalin A (ConA) lectins. Staining of nitrocellulose

blots of these proteins revealed that ricin bound poorly to serum when compared with RCA-I, SNA and ConA. In microtiter plate binding assays, ricin also bound poorly to serum when compared with RCA-I. Experiments were done to optimize A549 cell-based microtiter plate binding assays for RCA-I for testing of competition of cell surface lectin binding by serum proteins. In conclusion, ricin binds poorly to serum proteins compared to other lectins including RCA-I which has been described as having similar sugar specificity. These observations are consistent with the idea that serum glycoproteins would not effectively modulate ricin toxicity by competing with its binding to cell surfaces.

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Pro-Inflammatory Cytokine Secretion is Suppressed by TMEM16A or CFTR Channel Activity in Human Cystic Fibrosis Bronchial Epithelia.

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Cystic fibrosis (CF) is caused by the functional expression defect of the CF transmembrane conductance regulator (CFTR), a cAMP-stimulated chloride channel at the apical plasma membrane. Impaired bacterial clearance of airway epithelia together with hyperactive innate immune response are hallmarks of the CF lung disease and the primary cause of morbidity and mortality, yet the existence of and mechanism accounting for the innate immune defect that occurs prior to infection remain controversial.

Here we show that inducible expression of either CFTR or the calcium-activated chloride channel TMEM16A attenuate the secretion of the pro-inflammatory cytokines IL-6, IL-8, CXCL1 and CXCL2 in two human respiratory epithelial models (CFBE41o- and NCI-H441) under air-liquid, but not liquid-liquid interface culture condition. Expression of wild-type but not the dysfunctional G551D-CFTR indicate that the secretion of neutrophil chemoattractant IL-8 was inversely proportional to the CFTR and TMEM16A channel activity in *cfr*^{ΔF508/ΔF508} immortalized and primary human bronchial epithelia. These results were confirmed using CFTR-specific small molecule inhibitors and activators. Likewise, direct but not P2Y receptor activated TMEM16A attenuated the IL-8 secretion in CFBE41o- epithelia. Thus augmented proinflammatory cytokine secretion caused by defective anion transport at the apical membrane contributes to the excessive and persistent lung inflammation in CF and, perhaps in other respiratory diseases associated with documented downregulation of CFTR (e.g. chronic obstructive pulmonary disease). Direct pharmacological activation of TMEM16A offers a potential therapeutic strategy to correct the hyperinflammatory phenotype of CF airway epithelia.

1397

Sphingolipid biosynthesis and inflammatory signaling in asthma.

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Single nucleotide polymorphisms (SNPs) located within the human chromosomal region 17q21 are strongly associated with the incidence of inherited (so-called early-onset or childhood) asthma. These SNPs lead to increased transcription of nearby genes, including ORMDL3. Studies in budding yeast *Saccharomyces cerevisiae* identified the yeast Ormdl3 orthologs, Orm1 and Orm2, as components of and negative regulators of the enzyme complex, serine:palmitoyl-CoA transferase (SPT), which catalyzes the first step in sphingolipid biosynthesis. Ormdl3 and its paralogs Ormdl1 and Ormdl2 likewise associate with and

negatively regulate mammalian SPT. Given its inhibitory function, higher Ormdl3 level (in the asthma patients carrying the implicated SNPs) should diminish sphingolipid biosynthesis, perhaps causing an imbalance in the sphingolipid content of the plasma membrane, as compared to its glycerolipid composition. Sphingolipid deficiency impairs endocytosis in yeast. If Ormdl3 over-production causes a deficiency in membrane sphingolipids and results in a similar defect in endocytic function, signaling from cell surface receptors of the innate and adaptive immune systems, such as TLRs and the cytokine receptors, that have been implicated in asthma pathogenesis may not be down-regulated efficiently. If so, this scenario could provide an underlying mechanistic explanation for the chronic inflammation of bronchial epithelial tissue that is a hallmark of asthma. As an initial test to this overall hypothesis, we found that treatment of RAW macrophages with myriocin, a small-molecule inhibitor of SPT, blocked LPS-induced internalization of TLR4, but had a significantly weaker effect on fluid-phase pinocytosis (as judged by the rate of uptake of fluorescently-labeled dextran), suggesting that sphingolipid deficiency has a more severe effect on receptor-mediated endocytosis. Analysis of total sphingoid base content by HPLC demonstrated that myriocin treatment was effective in lowering cellular sphingolipid content. Thus, these preliminary data suggest that a deficiency of membrane sphingolipids could potentially influence the function of antigen-sensing immune receptors. We have raised antibodies against mammalian Ormdl3 and its paralogous proteins and are using them to assess the efficiency of various strategies to over-produce Ormdl3 to directly test whether elevated Ormdl3 levels leads to any detectable reduction in sphingolipid content and to a measureable defect in endocytosis.

1398

Characterization of gene expression in retinal pigmented epithelium of Basigin null mice.

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The Basigin null mouse is characterized as blind from the time of eye opening with degeneration of the photoreceptor cells beginning at eight weeks of age and continuing until complete ablation of the cell layer by eight months. Numerous studies have targeted the neural retina of these animals and indicate that a metabolic deficiency underlies the retinal dystrophy of Basigin null mice. Specifically, it is thought that inappropriate expression of monocarboxylate transporter 1 (MCT1) by photoreceptor cells and Müller cells accounts for the lack of photoreceptor function. Although it has been established that MCT1 expression in the Basigin null retinal pigmented epithelium (RPE) is altered, little else is known about the biochemistry and physiology of those cells. Therefore, the purpose of this study was to evaluate the expression of several transporters and enzymes within normal and Basigin null RPE as a first step toward understanding the role played by RPE in the Basigin null retinal dystrophy. Immunohistochemistry using antibodies specific for Basigin, MCT1, carbonic anhydrase (CA) XIV, and the Na⁺/K⁺ ATPase were performed on frozen sections of normal and Basigin null eye cups. In accordance with previous studies, it was determined that neither Basigin nor MCT1 is expressed at the RPE plasma membrane of Basigin null mice. Expression of CA-XIV was unaltered in the tissue. Most notably, expression of Na⁺/K⁺ ATPase changed from apical-only to both apical and basal portions of the RPE. Although the significance of mislocalization of the Na⁺/K⁺ ATPase on Basigin null RPE cells is not yet fully understood, it can be inferred that the cellular environment of the neural retina of Basigin null mice is negatively affected by the change in expression of this transporter protein.

1399

Nuclear AT1/AT2 Receptors Mediate Angiotensin II Induced Fibrosis-related Gene Expression Expression Changes in Cardiac Fibroblasts.

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Background: Angiotensin II (Ang-II) is central to arrhythmia-promoting fibroblast (FB) remodeling. Classically, Ang-II action is attributed to cell membrane receptors and intracellular second messengers. Here, we examined whether nuclear-delimited AT1 (AT1R) and AT2 (AT2R) receptors influence fibrosis-associated gene expression in atrial FBs and assessed underlying mechanisms. Methods: Canine atrial FBs were isolated. AT1R/AT2R localization was assessed by immunofluorescence and Western blot. Intact nuclei were purified by differential centrifugation. Nitric oxide (NO) production was measured via DAF-2 fluorescence, de novo RNA production by transcription initiation assay, and mRNA expression by qPCR. The expression of approximately 25,000 transcripts was analyzed in isolated nuclei using Agilent Rat Whole Genome microarrays. Results: AT1 and AT2 receptors colocalized with Topro 3 and Lamin A/C, indicating presence in FB nuclei. Ang-II dose-dependently induced de novo RNA synthesis in isolated cardiac nuclei. AT1R (L-162,313) and AT2R (CGP 42112A) specific ligands enhanced transcription initiation in FB nuclei (219±15* cpm/ng DNA and 148±8* cpm/ng DNA vs 69±9 control; n=5/group, *P<0.05). IP3R1 Ca²⁺-channels and eNOS colocalized with AT1R/AT2R on the nucleus. Ang-II enhanced FB nuclear NO production, which was reduced 85%* by the AT2R blocker PD123177 and 81%* by the NO synthase inhibitor L-NAME, but not affected by AT1R blocker losartan. AT2R-induced transcription initiation was reduced 79%* by L-NAME. AT1R-mediated transcription initiation was reduced 55%* by the IP3R-channel blocker 2-APB (but not L-NAME). Ang-II exposure upregulated ECM-genes (collagen1 1.4-fold*, fibrillin1 2.0-fold*, laminin 1.8-fold*) in isolated atrial FB nuclei while others (MMP2 0.24-fold* and MMP9 0.59-fold*) were downregulated. Ang-II also increased the transcription of cell-signaling genes like PLC (3.8-fold*), IP3R1 (3.5-fold*), and PDGF (2.5-fold*). Conclusions: Our results indicate that Ang-II acts through nuclear receptors to regulate ECM and cell signaling genes central to FB remodeling. These nuclear-delimited actions may be important in arrhythmogenic substrate development and could provide novel pharmacological targets.

1400

Existence of secretory granules in atrial and ventricular cardiomyocytes: Electron microscopic study of rat heart.

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Secretory granules are signature organelle of all secretory cells. Hence, cells that contain secretory granules are naturally considered to be secretory cells. Deviating from the traditional view of cardiac cells as nonsecretory cells, atrial cardiomyocytes have been shown to contain secretory granules, whereby providing the concept of endocrine heart. Secretory granules from a variety of cell types contain not only the largest amounts of calcium in the cell but also function as the major IP₃-dependent intracellular Ca²⁺ store of secretory cells (reviewed Yoo, S.H., FASEB J. (2010) 24, 653). We have here investigated the distribution of secretory granules in both atrial and ventricular cardiomyocytes of rat heart using electron microscopy. Atrial secretory granules are not rare in the heart, which are often found clustered in limited regions of atrial cardiomyocytes. Similar to the secretory granules of traditional secretory cells atrial secretory granules also contain large concentrations of Ca²⁺. In contrast, secretory granules in ventricular cardiomyocytes are relatively rare although they also clearly exist in the cell; the

number of secretory granules in ventricular cardiomyocytes appears to be far less than that in atrial cells. Our results not only confirm the existence of secretory granules in atrial cardiomyocytes but also demonstrate the existence of secretory granules in ventricular cardiomyocytes with electron microscopy. These results strongly suggest that the endocrine heart is in close physiological tune with the rest of the organism through the signals detected in the form of the volume and pressure of and the molecules contained in the circulating blood.

1401

Uric acid crosses the placental barrier through paracellular route.

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High serum urate level is a good marker for pregnancy induced hypertension (PIH), which is a generic name of diseases that have hypertension and proteinuria in the late stage of pregnancy. However, how urate is handled in the placenta is still unknown. In this study, we examined serum urate levels in maternal blood and umbilical cord blood. We also investigated urate transport mechanisms using the placenta and a model cell culture system.

The maternal and umbilical cord blood samples and placentas were collected from patients undergoing caesarean section at Kyorin University Hospital after obtaining informed consents. Urate and creatinine concentrations were measured by enzyme method. Expressions of urate transporters were detected by RT-PCR and immunohistochemistry. Transcellular [¹⁴C]-urate uptake and paracellular [¹⁴C]-urate movement was measured in the monolayer of BeWo cells, a trophoblast-derived epithelial cancer cell line. Barrier function of tight junctions was judged by the transepithelial electrical resistance (TER) and permeability of 3000 dalton dextran.

There were no significant differences in serum urate levels between maternal blood and umbilical cord blood, suggesting that urate is freely moved in the placenta. The results of RT-PCR showed that mRNA of OAT4, OAT10, URATv1 and ABCG2 were expressed in the placenta. Immunohistochemistry revealed that ABCG2 was expressed at the maternal side of the syncytiotrophoblast, OAT4 was expressed at the embryonic side of syncytiotrophoblast, OAT10 was expressed at both sides of syncytiotrophoblast, URATv1-short isoform was expressed at the maternal side of the syncytiotrophoblast and endothelial cells, URATv1-long isoform was expressed in the endothelial cells, respectively. However, to our surprise, BeWo cells did not take up urate despite the fact that they expressed almost the same set of urate transporters as the syncytiotrophoblast. After confirming that BeWo cells formed functional tight junctions on the transwell based on the elevated TER and no permeability of dextran, urate transport between upper and lower transwell chambers was measured. Urate moved both directions through BeWo cell monolayers even when the cells were incubated at 4 °C, suggesting that urate is transported through paracellular route.

1402

Identification of a Novel Myristoylated Protein Required for the Proper Ciliary Localization of PKD-2.

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The transient receptor potential polycystin (TRPP) channel PKD-2 and the polycystin-1 receptor LOV-1 constitute a putative mechanosensory complex that is required for *C. elegans* male mating behaviors. PKD-2 and LOV-1 localize to cilia found at the end of dendrites of male-specific sensory neurons, which include the CEMs in the head and the RnB and HOB neurons in the tail. Mutations in PKD1 and PKD2, the human homologs of *lov-1* and *pkd-2*, cause autosomal dominant polycystic kidney disease (ADPKD). The mammalian TRPPs localize to

cilia on renal epithelial cells. Hence we use *C. elegans* to identify genes required for polycystin ciliary localization.

The *cil-7(my16)* mutant was identified in a genetic screen for PKD-2::GFP ciliary localization [Cil] defective animals, (Bae, Lyman-Gingerich et al. 2008). In *cil-7(my16)*, we observe an abnormal accumulation of PKD-2::GFP in cilia and distal dendrites. With SNP mapping, deficiency mapping, and Whole Genome Sequencing, we determined that *my16* could be a mutation in either one of two ORFs. A fosmid or a single gene construct containing the second ORF rescued the *my16* Cil phenotype. We conclude that *my16* is a missense mutation in ORF2, which we hereafter refer to as *cil-7*.

cil-7 encodes a protein that has orthologs in *Caenorhabditis* species, *S. kowakevskii*, *B. floridae*, and *D. melanogaster*. CIL-7 is predicted to have coiled-coil domains and an N-myristoylation pattern, where a myristoyl group would be added onto the second glycine. A large and/or hydrophobic residue in the myristoylation pattern is predicted to inhibit myristoylation (Wright, Heal et al. 2009). The missense mutation in the *cil-7(my16)* allele changes a Serine(3) into a Phenylalanine, most likely affecting the myristoylation state of CIL-7. We are exploring the role of myristoylation on CIL-7 localization and function.

CIL-7 is co-expressed and co-localized with PKD-2 in the CEM, HOB, and ray neurons. Additionally, *cil-7* is also expressed in the IL2 neurons of the core nervous system. The *cil-7* expression pattern is identical to the kinesin-3 KLP-6 that regulates intraflagellar transport in male-specific cilia (Morsci and Barr 2011). Consistent with a role in protein transport, functional CIL-7::GFP moves along dendrites. We are currently testing the hypothesis that CIL-7 regulates dendritic or ciliary protein trafficking, perhaps as an adaptor for KLP-6. We are interested in further characterizing the gene mutated in *my16* animals, and examining genetic interactions between *my16* and other components in the PKD pathway.

1403

Glycosylation determines sodium-calcium exchanger 3 subcellular distribution during the cell cycle.

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Background: Sodium-calcium exchanger (NCX), as a plasma membrane antiporter, plays an important role in maintenance of the intracellular Ca²⁺ homeostasis. Although NCXs have been extensively studied in mammalian systems, the subcellular localization of different NCXs is still undetermined besides the fact that they are destined to be plasma membrane proteins. In this study, we tested the hypothesis that the subcellular localization of the NCX3 protein varies with the cell cycle phases in mammalian cells.

Methods: Construction of plasmids and site-directed mutagenesis. The cDNA containing the entire open reading frame (ORF) of the human NCX3 was previously cloned into the pcDNA3.1 expression plasmid, and recombinant NCX3-GFP was attained by polymerase chain reaction (PCR). Site directed mutagenesis was carried out using a protocol based on the QuikChange[®] Site-Directed Mutagenesis Kit (Stratagene). **Indirect immunofluorescence and live cell imaging.** Leica TCS SP5 II confocal system (Leica) and INU2 incubation system for microscopes (TOKAI HIT) constituted the integrated live cell imaging system. 100% pure CO₂ was supplied by AGA.

Results: Subcellular localization of human NCX3. To better understand the variable subcellular localization of human NCX3, we overexpressed NCX3 in HEK 293T cells and found at least two patterns of intracellular protein distribution, i) plasma membrane and ii) perinuclear

region associated with endoplasmic reticulum and nuclear envelope. **Tracking the subcellular dynamics of NCX3 with live cell imaging.** To perform live cell imaging, we recombined NCX3 with green fluorescent protein (GFP) and overexpressed it in HEK 293T cells. We discovered that NCX3 subcellular distribution is a dynamic process, which is related to the cell cycle phases. **The human NCX3 protein is post-translationally modified by N-glycosylation.** The treatment of NCX3 expressing HEK 293T cells with tunicamycin (an inhibitor of N-glycosylation) revealed that human NCX3 is glycosylated via N-linked glycosylation and both N-glycosylated and non-glycosylated forms of NCX3 are present in transfected 293T cells. **N-glycosylation is important for targeting of NCX3 to the plasma membrane.** We tested the effect of N-glycosylation on the subcellular distribution of NCX3 by making an NCX3 N-glycosylation-deficient mutant. NCX3 wild-type and the mutant forms thereof were transiently expressed in HEK 293T cells, and their subcellular distribution was investigated by immunofluorescence analysis. It is evident that the N-glycosylation-deficient mutant is predominantly maintained in the cytosol, suggesting that N-glycosylation is required for targeting of NCX3 to the plasma membrane.

Conclusion: The subcellular localization of the NCX3 protein varies with the cell cycle phases in mammalian cells. We depict that NCX3 can be N-glycosylated via a single asparagine residue which is normally required for targeting of NCX3 to the plasma membrane.

1404

A C-terminus mutation of SLC7A9 amino acid transporter frequent in Japanese cystinuria patients retains substrate binding but diminishes the transport activity.

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Cystinuria (MIM 220100) is an inherited disorder caused by the defect of amino acid transport systems for cystine and dibasic amino acids in the apical membrane of renal proximal tubules. The failure of cystine reabsorption results in recurrent nephrolithiasis which leads to severe renal dysfunctions. Mutations in either *SLC7A9* or *SLC3A1* genes result in cystinuria. *SLC7A9* encodes a functional subunit b^{0,+} AT that possesses the amino acid transport activity, whereas *SLC3A1* encodes an accessory subunit called rBAT. These two subunits form a heterodimer. Several mutations in *SLC7A9* have been identified in patients of western countries and Japan. Different from western populations, the most frequent mutation found in Japanese patients was a missense mutation P482L of *SLC7A9* which causes severe loss of the amino acid absorption. Residue 482 is near the C-terminus end of the protein. Generally, C-terminus mutations of transporters are not important for the transport activity but alter amounts of expression and localization of proteins on the plasma membrane. However, by means of transfected cells, it is shown that P482L protein has similar expression level and localization to wild-type but loss of transport activity. Therefore, the reason for the loss of transport function by P482L mutation is still unknown.

To study the mutant further, we have generated a P482L knock-in (KI) mouse as a human-disease model. Urine from KI mice was collected for 24h and subjected to the analysis of cystine and dibasic amino acids. The excretion of cystine, arginine, lysine and ornithine in KI mice was between 70 and 100 fold higher than that in wild-type mice. It is similar to that of human cystinuria patients, indicating that the KI mouse can be used as the human cystinuria model. Immunohistochemistry and western blot analysis revealed that the P482L protein had the same expression level and localization as those of wild-type protein at the apical membrane of renal proximal tubules. To study the function of the mutant, the transport and substrate binding activity were measured by using brush border membrane vesicles (BBMV) isolated from

kidney cortex of the KI and wild-type mice. The transport activity of P482L mutant was much lower than that of wild-type, whereas the substrate binding activities of the mutant and wild-type were similar. Likelihood, the P482L mutant has the similar substrate binding ability to the wild-type transporter. Transporters change their conformation upon substrate transport. Disturbing the conformational change leads dysfunction of transporters. Our results indicate that P482L yet binds the substrate but do not change the conformation to transport substrates.

1405

Diseased mutants of SLC4a11 protein are ER-retained but are functional when rescued to cell surface.

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The human cornea is composed of five different layers: the outer epithelium, Bowman's layer, corneal stroma, Descemet's membrane and corneal endothelium. The endothelial cell layer, underlying the cornea normally reabsorbs water from the corneal stroma. Disruption of this process results in corneal edema and significant impairment of vision, leading to some posterior corneal dystrophies (PCDs). Many cases of the PCDs - congenital hereditary endothelial corneal dystrophy (CHED), Harboyan syndrome and Fuchs endothelial corneal dystrophy are caused by the mutations in the SLC4a11 gene. The majority of point mutations in SLC4a11 cause this integral membrane protein to be misfolded and retained in the endoplasmic reticulum (ER). As a result, the function of SLC4a11 protein, which normally localizes to the basolateral plasma membrane of corneal endothelium, is impaired. Here we explored whether ER-retained mutants of SLC4a11 can be rescued to the cell surface. If rescued SLC4a11 retains function, it suggests that techniques to drive ER-plasma membrane rescue could be a viable therapeutic strategy. We chose the CHED mutant, SLC4a11-E143K, as a representative mutant having an ER-retained phenotype when expressed in HEK293 cells. We also made use of the catalytically inactive mutant, SLC4a11-R125H, which processes to the plasma membrane at levels similar to WT, yet does not have functional activity. We previously showed that SLC4a11 forms dimers. Our rationale was thus that SLC4a11-R125H would heterodimerize with ER-retained SLC4a11-E143K to rescue it to the plasma membrane surface. Immunoprecipitation experiments showed that R125H and E143K mutants form heterodimers. Co-expression of catalytically inactive R125H in HEK293 cells rescued E143K, moving it from ER to the plasma membrane. We measured significant functional activity arising in SLC4a11-E143K/R125H co-expressing cells. Hence we conclude that the functional activity arises from the plasma membrane rescued form of SLC4a11-E143K. Thus, therapies aimed at increasing the cell surface processing of ER-retained mutants has potential to treat posterior corneal dystrophies arising from SLC4a11 mutations.

1406

Membrane trafficking of epithelial anion transporters NaS1 (SLC13A1) and Sat1 (SLC26A1).

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Sulfate is an essential anion involved in many cellular processes. Cells obtain sulfate via sulfate transporters located on their plasma membranes. Renal sulfate transporters (NaS1 and Sat1) control sulfate homeostasis. Despite their physiological roles, little is known about the structural identities of NaS1 and Sat1 and sorting mechanisms that regulate their cellular expression. NaS1 encodes a 595 amino acid 13 TMD protein localized to the apical (brush-border)

membranes of epithelial cells. Sat1 encodes a 704 amino acid 12 TMD protein located on the basolateral membrane of cells. The aims of this study were to biochemically characterise the NaS1 and Sat1 proteins, identify the plasma membrane sorting mechanisms and determine the functional roles of non-synonymous SNPs in these proteins. Transient transfection of EGFP/NaS1 in renal epithelial cells (OK, LLC-PK1 and MDCK) demonstrated apical membrane expression, which was not affected by tunicamycin. Transfection of the EGFP/NaSi-1 N591S glycosylation mutant still led to apical expression, suggesting that apical sorting was independent of the glycosylation of this site. Treatment with cholesterol depleting compounds (lovastatin and methyl- β -cyclodextrin) was unable to disrupt apical sorting, suggesting that NaS1 apical trafficking may be independent of membrane lipid rafts. NaS1-His proteins analyzed by BN-PAGE appeared as a single complex. Dissociation revealed one additional band, indicating a dimeric structure of the complex. This data demonstrates that NaS1 forms a dimeric protein which is glycosylated at N591, whose sorting to the apical membrane in renal epithelial cells is independent of lipid rafts and glycosylation. Transfection of the Sat1 protein into MDCK and LLC-PK1 cells led to basolateral membrane sorting, as observed in vivo. To identify possible sorting determinants, truncations of the Sat1 cytoplasmic C-terminus were generated and transiently transfected into MDCK cells. Confocal microscopy revealed the removal of the last three residues on the Sat1 C-terminus, a putative PDZ domain, had no effect on the basolateral sorting in MDCK cells. Removal of the last 30 residues led to an intracellular expression for the GFP fusion protein suggesting a possible sorting motif lies between the last 3 and 30 residues of the Sat1 C-terminus. Elimination of a dileucine motif at position 677/678 resulted in the loss of basolateral sorting, suggesting this motif is required for Sat1 targeting to the basolateral membrane in kidney cells. Several non-synonymous SNPs exist in the coding regions of human NaS1 and Sat1 genes. The functional relevance of these SNPs and their effects on membrane protein sorting will be described.

1407

Structural Studies of the FGFR3 Dimer.*E. Li¹, K. Mudumbi¹, B. Eichman¹; ¹Saint Joseph's University, Philadelphia, PA*

Fibroblast growth factor receptor 3 (FGFR3) is a member of the receptor tyrosine kinase family of proteins. These receptors are characterized by an extracellular ligand-binding domain, a transmembrane domain (TMD), and a cytosolic kinase domain. Ligand binding and dimerization of FGFR3 in the plasma membrane initiates a series of signaling pathways that regulate cell growth, proliferation and mobility. Previous work with the isolated FGFR3 TM domains has shown that it has the ability to self-dimerize. Furthermore, several mutations in the TMD have been associated with skeletal dysplasias and cancers. The sequence of the TMD contains two adjacent SmXXXSm motifs, where the Sm residues represent small amino acids. The SmXXXSm motif has been shown to play an important role in helix-helix interaction in other transmembrane proteins. Currently, we are using site directed mutagenesis and ToxR activity assays to analyze the importance of this sequence motif for the interaction between the helices, and thus, for FGFR3 dimerization. Our goal is to obtain information about the wild-type FGFR3 dimer structure, as well as the structure formed by FGFR3 carrying pathogenic mutations.

1408

The Role of the CQC motif in the dimerization of MUC1.

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MUC1 is a heterodimeric protein consisting of two subunits. The C-terminal subunit (MUC1-C) is made up of a cytoplasmic tail, a transmembrane domain (TMD) and an extracellular domain which interacts non-covalently with the N-terminal subunit (MUC1-N). The MUC1-C subunit has been shown to form homodimers. Homodimerization of MUC1 is required for its nuclear localization and subsequent interactions with various nuclear transcription factors. Such interactions are known to promote the production of proteins involved in cell proliferation and survival signals. Overexpression of MUC1 is shown to be involved in increased metastatic behavior of cancer cells and is considered a marker indicating poor prognosis in breast cancer patients. The cytosolic domain of MUC1-C contains a membrane proximal cysteine-glutamine-cysteine (CQC) motif that has been suggested to be involved in MUC1 dimerization. However, its role in dimerization is still unclear. Currently, we are evaluating the role of the CQC motif in the dimerization of the MUC1 TMD and MUC1-C. We are using the ToxR assay to study the dimerization of MUC1 TMD. In ToxR assays, TMD-TMD interactions are measured in bacterial membranes using a chimeric protein consisting of a periplasmic maltose binding domain, the TMD of interest, and the cytosolic ToxR domain from *Vibrio cholerae*. Thus, the assay allows us to compare levels of dimerization between MUC1 TMDs with and without the CQC motif. In addition, we are using site-directed mutagenesis and chemical crosslinkers (DSS and BS3) to evaluate the role of the CQC motif in the dimerization of MUC1-C in mammalian cells. Our goal is to obtain structural information regarding the MUC1 homodimer and the physical principles driving dimerization of MUC1 in the plasma membrane.

1409

A Role of TRPM7 in Trace Metal Homeostasis.

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TRPM7 is a ubiquitously expressed channel-kinase widely permeable to a range of divalent metal ions, with a permeability sequence of $Zn^{2+} \sim Ni^{2+} >> Ba^{2+} > Co^{2+} > Mg^{2+} \geq Mn^{2+} \geq Sr^{2+} \geq Cd^{2+} \geq Ca^{2+}$. TRPM7's unusual permeability profile raises the possibility that the channel may be regulating the cellular homeostasis of more than one of these essential metal ions. Study of TRPM7 has linked the channel to a number of physiological roles, including cell proliferation and cellular Mg^{2+} homeostasis. It has been shown that genetic ablation of TRPM7 in DT40 B-cell lymphocytes produces cell cycle arrest that can be rescued by overexpression of the Mg^{2+} transporter SLC41A2, or by overexpression of a constitutively active form of phosphatidylinositol 3-kinase (PI3K). Despite these advances, TRPM7's role in Mg^{2+} homeostasis as well as the mechanism(s) by which the channel-kinase influences cell proliferation remains controversial, as conditional knockout of TRPM7 in neural stem cells, thymocytes, and T cells failed to produce any changes in cell proliferation or in Mg^{2+} homeostasis. To better understand the role of the channel in metal ion homeostasis we've generated recombinant adenoviruses that express TRPM7 channel-dead (TRPM7-E1047K) and TRPM7 channel/kinase-dead (TRPM7-E1047K/G1618D) mutants. Expression of these mutants in mouse embryonic fibroblasts (MEFS) potently inhibits cell proliferation and arrests cells in the S and G2 phases of the cell cycle. In addition, expression of the mutants lowers cellular free Mg^{2+} and decreases Zn^{2+} uptake. Consistent with a role for TRPM7 in Mg^{2+} and Zn^{2+} homeostasis, overexpression of the Mg^{2+} transporter SLC41A2 or supplementation of the growth media with high Mg^{2+} or Zn^{2+} ,

partially rescues the growth of cells expressing the TRPM7 mutants. To confirm these results we employed mouse embryonic fibroblasts (MEFS) derived from the conditional channel-dead TRPM7-E1047K mouse (TRPM7-E1047K-MEFS), which expresses TRPM7-E1047K in Cre recombinase dependent manner. Viral transduction of Cre recombinase into TRPM7-E1047K-MEFS inhibited cell proliferation compared to wildtype MEFS transduced with Cre recombinase. In addition, supplementation of the growth medium with Mg^{2+} and Zn^{2+} suppressed the proliferation defect caused by expression of TRPM7-E1047K. These results support the hypothesis that TRPM7's plays a critical role in both Mg^{2+} and Zn^{2+} homeostasis and that TRPM7's regulation of these metal ions is vital to the channel's control of cell growth and proliferation.

1410

Iron deficiency regulates ZIP14 degradation through a proteasome-dependent pathway.

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ZIP14 is a newly identified iron-import protein. It mediates both transferrin-bound iron (TBI) and nontransferrin-bound iron (NTBI) uptakes into cells. Previously, we showed that ZIP14 is subject to posttranscriptional regulation by hereditary hemochromatosis protein, HFE. Lacking HFE leads to genetic iron-overload disorder and HFE expression downregulates cellular iron content. Overexpressing HFE in HepG2 cells downregulates ZIP14 by promoting its degradation. The exact mechanism is not clear. In the present study, we show that ZIP14 increases with iron-overload and decreases with iron-deficiency. This suggests that ZIP14 is an iron transporter responsible for constant cellular iron accumulation under high iron conditions, and that HFE may exert its effect on ZIP14 through lowered cellular iron levels. We further demonstrate that proteasomal inhibition prevents downregulation of ZIP14 induced by iron-deficiency, suggesting the involvement of proteasomes in ZIP14 degradation. Moreover, inhibition of the proteasomal pathway induces a rapid accumulation of the nonglycosylated ZIP14. Biotinylation studies suggest that ZIP14 traffics to the plasma membrane before it undergoes deglycosylation. Proteasomal inhibition studies indicate that the nonglycosylated ZIP14 is found primarily in the cytosol, not associated with membrane. We therefore propose that deglycosylation and membrane extraction are required before proteasomal processing of ZIP14.

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Functional analysis of GJB2 (Cx26) mutations causing palmoplantar keratoderma and deafness.

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Mutations in *GJB2*, the gene encoding Cx26, are responsible for causing both non-syndromic and syndromic deafness. Non-syndromic deafness occurs due to a loss in Cx26 function. Interestingly, patients with non-functional Cx26 do not display any adverse skin pathology. Based on this observation, we have hypothesized that Cx26 function is not required for the maintenance of epidermal homeostasis; however, some mutations in *GJB2* can produce a novel gain of function resulting in syndromic deafness associated with skin disease. In addition to hearing loss, patients with Cx26 mutations exhibit a variety of phenotypes such as palmoplantar keratoderma (PPK), pseudoainhum and leukonychia. Previous functional studies of syndromic deafness Cx26 mutations have shown that trans-dominant inhibition of other connexins found in the skin by mutant Cx26 may represent one possible gain of function mechanism. We have tested two additional Cx26 mutants, Cx26-S183F and Cx26-H73R, in an effort to determine

mechanisms contributing to palmoplantar keratoderma and hearing loss. Using a *Xenopus laevis* oocyte expression system, we found no functional activity in cells injected with Cx26-S183F mRNA, suggesting a lack of homomeric/homotypic gap junction formation. In assays where cells co-expressed wild-type (WT) Cx26 and Cx26-S183F mRNA, functional gap junctions were able to form. Remarkably, when cells co-expressed WT Cx43 and Cx26-S183F, there was a significant ($p < 0.01$) reduction in conductance, suggesting a trans-dominant inhibition of WT Cx43. The mean conductance of cell pairs expressing both Cx26-S183F and WT Cx43 ($0.56 \mu\text{S}$) was reduced by 89% when compared to cells co-expressing WT Cx26 and WT Cx43 ($5.26 \mu\text{S}$). In experiments where cells co-expressed WT Cx26 and Cx26-H73R mRNA, gap junctions were able to form. However, when cells co-expressed WT Cx43 and Cx26-H73R mRNA, trans-dominant inhibition of WT Cx43 was again shown by a 92% decrease in conductance when compared to cells co-expressing WT Cx26 and WT Cx43. Taken together, Cx26-S183F and Cx26-H73R fail to inhibit WT Cx26 conductance, however, both mutants were able to trans-dominantly inhibit WT Cx43 thereby suggesting a mechanistic gain of function which may contribute to palmoplantar keratoderma.

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Pannexin1 expression and interplay with P2X₇ receptors in hTERT immortalized human bladder urothelial cells.

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Urothelial cells respond to bladder distension with ATP release, and ATP signaling to the CNS and within the bladder wall has been proposed to relay the degree of bladder fullness and modulate detrusor activity, which are essential for proper micturition. There is evidence that more than one ATP release mechanism operates in urothelial cells, including TRPV, ACh and P2 receptors. In other cell types, Pannexin1 (Panx1) channels provide a pathway for mechanically-induced ATP efflux and ATP-induced ATP release through its interaction with P2X₇ receptors (P2X₇Rs). In this study we investigated whether Panx1 channels participate in urothelial ATP release and signaling. Panx1 expression and function in hTERT-immortalized human bladder urothelial cells were investigated by PCR, Western blotting, ATP release, YoPro dye (375 Da) uptake and Ca²⁺ imaging. PCR and Western blotting indicated that urothelial cells express both Panx1 and P2X₇R. Mechanical stimulation by hyposmotic shock induced YoPro uptake, which was inhibited by mefloquine (Panx1 blocker; MFQ 100nM) and also by A438079 (P2X₇R blocker; 10μM). Mechanical stimulation induced by changing the bathing solution triggered ATP release ($7.0 \pm 2.0 \text{ nM}/\mu\text{g}$ protein, N=3), which was 4 times higher ($26.1 \pm 5.4 \text{ nM}/\mu\text{g}$ protein, N=3) when cells were bathed in low divalent cation solution (LDPBS), a condition known to enhance P2X₇R activation. This higher ATP release in LDPBS was prevented by MFQ. ATP signaling between urothelial cells, evaluated as the extent of intercellular Ca²⁺ wave (ICW) spread induced by focal mechanical stimulation of single cells, was sensitive to exposure to LDPBS, apyrase (ATP scavenger, 50U/mL) and MFQ. Radius of ICW in LDPBS was 70% higher than in control conditions ($246.2 \pm 3.7 \mu\text{m}$ vs $145.8 \pm 7.5 \mu\text{m}$, N=6 fields; $P < 0.01$) and such potentiation was prevented by MFQ ($175.0 \pm 16.9 \mu\text{m}$, N=9 fields; $P < 0.05$). In apyrase, ATP signaling was restricted to cells closer to the mechanically stimulated cell in both control ($24.5 \pm 4.2 \mu\text{m}$, N=6 fields) and LDPBS ($49.1 \pm 9.5 \mu\text{m}$, N=4 fields) conditions. In LDPBS, MFQ combined with apyrase abrogated ATP signaling in 7 out of 12 fields analyzed. These findings indicate that Panx1 is expressed and interacts with P2X₇R in human urothelial cells, and that

these channels not only provide a pathway for mechanically-induced ATP release but also participate in urothelial autocrine/paracrine ATP signaling. (Support: NIH DK081435)

1413

Adenosine metabolism and physiological effects in podocytes.

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Introduction. A key feature of both type 1 and type 2 diabetes is an altered circulating insulin level and persistently high glucose levels. The deficit or the excess of insulin have been associated with proteinuria in both types of diabetes, independently of insulin resistance. This situation resembled the injurious effects on the filtration barrier and glomerular cells integrity when the doses of the VEGF are up or down regulated. Since we have shown that adenosine is a key regulator of VEGF production in the glomerulus, our aim will be to find out whether changes in the extracellular levels of the nucleoside are induced by insulin and consequently, unbalanced VEGF could explain the alterations on podocyte function.

Results. Primary cultured podocytes expressing the specific marker synaptopodin were obtained from glomeruli of rats. Exposure of podocytes to insulin decreased the production of VEGF in a doses dependent manner. The expression of the equilibrate nucleoside transporters -1 and -2 (ENT-1 and ENT-2) was recognized in this cell type. Exposure of podocytes to high glucose concentration (25mM) reduced the ENT-1 mRNA levels and triggered the extracellular increase of adenosine content. The ENT-2 mRNA level was not affected by the high glucose-containing medium but was significantly increased by treatment with insulin. Concomitantly, exposure to insulin restored the high glucose-mediated increase of adenosine levels. Signaling through adenosine A2B receptor subtype leads to the activation of RhoA and actin redistribution. Further, the production of VEGF was strongly induced.

Conclusions. The sodium-independent and facilitative transporters ENT-1 and ENT-2 play a major contribution on adenosine availability for signaling through adenosine receptor. While ENTs activity mediates adenosine uptake to be metabolized intracellularly, the nucleoside can become accumulated outside when the transport activity is reduced. In this way, increasing glucose concentrations and insulin deficiency in experimental and human diabetes could mediate the glomerulopathy with augmented adenosine levels. Further, adenosine causes podocyte dysfunction by inducing the VEGF production and rearrangement of the cytoskeleton, both effects probably relevant in affecting the integrity of the glomerular filtration barrier.

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Vasopressin regulated diffusion of AQP3 in MDCK cells.

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Fine tuning of urine concentration occurs in the kidney collecting duct, where it is regulated by vasopressin which controls the level of apical AQP2 and thus water influx across the epithelial cell layer. Basolateral aquaporins, AQP3 and AQP4, represent the exit pathways for water entered by AQP2 in the apical membrane of collecting duct principal cells. To maintain body water homeostasis, it is extremely important that these water channels are specifically positioned in the subcellular domains at the right quantity. AQP2 localization is regulated by

vasopressin within minutes of stimulation facilitating increased influx of water; however, the short-term effect of vasopressin on basolateral AQP3 and AQP4 is unknown. We hypothesize that the plasma membrane dynamics of AQP3 might be regulated by vasopressin to facilitate the efflux of water absorbed by AQP2. To study the effect of vasopressin on AQP3 in a cell culture model, we labeled individual EGFP-tagged AQP3 proteins extracellular with quantum dots and followed the mobility at the single protein level. We stimulated cells with forskolin to mimic vasopressin stimulation and also studied AQP3 mobility during disruption of different cytoskeletons. k-space image correlation spectroscopy (kICS) was used for quantitative analysis of the diffusing proteins. Within 15-30 minutes of forskolin stimulation, the AQP3 diffusion coefficient increased significantly. In addition, disruption of the actin cytoskeleton, but not microtubules, caused a slight increase in diffusion of AQP3. These kinetic data support our hypothesis that AQP3 is regulated alongside AQP2 upon vasopressin stimulation. The increased dynamics may aid in facilitating an increased water efflux, perhaps by preventing AQP3 endocytosis.

1415

Non-enzymatic modulation of AQP1 activity by CAII.

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Aquaporins are a family of membrane proteins that function as water channels, facilitating the movement of water molecules across the plasma membrane, down a concentration gradient. The first family member AQP1, is predominantly expressed in red blood cells and the kidney. The basic structural and functional unit of the kidney is the nephron where AQP1 is expressed in both the proximal tubule and thin descending limb. Its presence there is essential for the reabsorption of two thirds of the 180L of water filtered by the glomerulus daily. Carbonic anhydrase II (CAII) is ubiquitously expressed. It catalyzes the reversible hydration of carbon dioxide. Apart from playing a pivotal role in acid-base balance, CAII also modulates the activities of other proteins through direct protein-protein interaction. Specifically CAII binds to and enhances the activity of the sodium/hydrogen exchanger NHE1, sodium bicarbonate cotransporter NBC1, monocarboxylate transporter MCT1 and the chloride/bicarbonate exchanger AE1. Sequence analysis of human AQP1 revealed the presence of two C-terminal contiguous motifs that match the CAII binding sites found in AE1. Moreover, osmotic swelling assays showed that the water flux of membrane-enriched cortical kidney fractions from CAII^{-/-} mice was approximately 50% lower than wild-type mice. These results lead us to hypothesize that water flux through AQP1 was enhanced by an interaction with CAII. To test this possibility we performed osmotic swelling assays on *Xenopus laevis* oocytes and HEK293 cells transiently expressing different combinations of AQP1, CAII and the catalytically inactive CAII isoform, V143Y. We found that CAII enhances the water channel activity of AQP1 regardless of catalytic activity, in both model systems. Future studies are aimed at clearly delineating the site of interaction between CAII and AQP1. Regardless this interaction is likely highly relevant to both respiratory and renal physiology.

1416

The Role of ERp44 on the Maturation of Serotonin Transporter Protein.

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Serotonin transporter (SERT) is an oligomeric glycoprotein with three Cysteine residues on the external loops. Here, initially we investigated an association between SERT and ERp44, an

endoplasmic reticulum (ER) luminal chaperone protein; then we studied the role of their association in disulfide formation on SERT because ERp44 facilitates the maturation of disulfide-linked oligomeric proteins.

Mutation in the thioredoxin-like domain of ERp44 (C29S) hampers its association with SERT. Then, once the retention mechanism is waved, in ERp44 silenced cell lines, the plasma membrane expression levels of SERT was elevated while the serotonin (5-HT) uptake rates of ERp44 silenced cells were decreased. Next, MTSEA-biotin, a membrane impermeable compound which specifically labels the free Cys residues from the extracellular surface, was applied on control and ERp44-silenced cell lines. MTSEA-biotin had higher binding efficiency to SERT in ERp44 cells than the one in control cell lines indicating the presence of more accessible Cys residues on SERT in silenced cells to be labeled than in control cell lines. These data support our hypothesis that ERp44 plays an important role in the maturation of SERT molecules via facilitating the disulfide formation which become a prerequisite step for the assembly of SERT monomers in an oligomeric form.

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1417

Silencing Of Arachin Encoding mRNAs By Glutamate Dehydrogenase-Synthesized RNA.

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Peanut (*Arachis hypogaea* L) seeds are extremely rich in protein. However peanut allergy is one of the most frequent causes of death resulting from food allergy. The protein found in peanut seeds that is responsible for peanut allergy is known as arachin. Peanut allergen arachin 1 (68KDa) can cause severe type1 hypersensitive reaction such as anaphylaxis. *Arachis hypogaea* Virginia peanut were cultivated and treated with mineral nutrient in ten experimental plots at Prairie View A&M University field plots in Waller County. Peanut seeds were harvested at maturity and homogenized in phosphate buffer to extract the arachin. The mRNA synthesis of arachin for each sample was analyzed by Northern blot analysis. Mineral nutrients induce glutamate dehydrogenase (GDH) to isomerize and to synthesize RNAs. Arachin mRNAs are silenced by homologous GDH-synthesized RNAs. The mRNA bands for the allergenic protein arachin (2.3Kb) for Phosphorus and Potassium (PK) and NPKS treated peanuts were Silenced. The silencing of mRNA that encodes for the arachin protein resulted in the abolishment of allergenic protein on (sodium dodecyl sulfate polyacrylamide gel) SDS-PAG.

Nuclear Lamins

1418

O-GlcNAc regulates emerlin binding to BAF in a chromatin- and lamin B-enriched 'niche'.

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Emerlin, a membrane component of nuclear 'lamina' networks with lamins and barrier-to-autointegration factor (BAF), is highly O-GlcNAc-modified ('O-GlcNAcylated') in mammalian cells. Mass spectrometry analysis revealed eight sites including S53, S54, S87, S171 and S173. Emerlin O-GlcNAcylation was reduced ~50% by S53A or S54A mutation in vitro and in vivo; S173A reduced O-GlcNAcylation only in vivo. We separated two populations of emerlin, A-type

lamins and BAF: one solubilized easily; the other required sonication and included histones and B-type lamins. Emerin and BAF associated only in sonicated fractions. Comparing GFP-emerin (wildtype, mutant) association with endogenous partners *in vivo*, only BAF was affected: S173D reduced BAF association 63%, and also 'primed' GFP-emerin hyperphosphorylation. Thus, essential enzyme O-GlcNAc-transferase controls an S53/S54-dependent region near the LEM-domain, and controls a molecular switch in emerin defined by S173, O-GlcNAcylation of which promotes emerin-BAF association in the chromatin/lamin B 'niche'. These results reveal a mechanism for crosstalk between O-GlcNAcylation (mitosis, epigenetics, nutrient sensing) and conserved chromatin-tethering lamina components.

1419

Assembly properties of human disease associated lamin A mutants: An *in vitro* – *in vivo* comparison.

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We have synthesized human wild-type lamin A and various disease associated mutant lamin A variants. Firstly, we compared their assembly using standard protocols for the generation of filaments and paracrystals, respectively (Aebi, U. et al. (1986) Nature 323, 560-564). Assembly products obtained were visualized by transmission electron microscopy of negatively stained samples. Then, we followed their assembly by a new assembly regime that employs stepwise dialysis from high salt containing buffers into physiological conditions. Using this procedure, we were able to collect intermediates during different stages of assembly and thereby tracked structures not discernible any more in the – mostly rather large - end products.

These studies were followed in parallel at the cellular level. We transfected cultured cells with constructs coding for mCherry-tagged lamin A as well as the mutants analysed *in vitro*. In human U2OS cells, most of the mutants did not integrate into the nuclear envelope, very different from the wildtype lamin A but were also found in extranuclear structures. This property was in stark contrast for the rather regular assembly observed *in vitro*. The mutant S143F, for instance, was not found in the nuclear envelope in many cells but in small dots outside the nucleus. Moreover, it caused strong reorganization of both emerin and LBR. When lamin S143F was not located in the nuclear envelope, it was found in contact with extra-nuclear LBR. Similar results were observed for the mutant T10I. However, the *in vitro* organization for both proteins was completely different. Whereas T10I exhibited entirely normal paracrystal formation, S143F was missing the ordered 24.5-nm repeat pattern but still formed extended fibers. Such a difference was even more so observed for S143P, which was indeed "super-aggregating" into huge round, interconnected structures instead of the needle-like paracrystals or fibers seen with wild-type lamin A and S143F, respectively, under the same conditions. With these comparative studies we believe that we can help to distinguish those mutants that cause disturbances in lamin assembly from those that build normal polymers but cause a fatal molecular change in the binding epitope normally recognized by lamin interacting proteins.

1420

Divalent cations alter farnesylated lamin tail domain structure and are required for membrane association.

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Lamin proteins contribute to nuclear structure and function, primarily at the inner nuclear membrane. Posttranslational processing pathway of lamin A includes farnesylation of the C-terminus, likely to increase membrane association, and subsequent proteolytic cleavage of the C-terminus. Hutchinson Gilford progeria syndrome (HGPS) is a premature aging disorder wherein a mutant version of lamin A, $\Delta 50$ lamin A, retains its farnesylation. We examine purified tail domains of prelamin A and $\Delta 50$ lamin A to determine the role of the farnesylation in protein-protein and protein-membrane interactions. We find that divalent cations are required for association of farnesylated tail domains with tethered bilayer membranes. The farnesylated form of $\Delta 50$ lamin A tail domain binds the membrane interface only in the presence of Ca^{2+} or Mg^{2+} and also forms multiple layers on the membrane. Aggregation of prelamin A suggests stronger protein-protein association than protein-membrane association. Thermodynamic experiments and molecular dynamics simulations show that the farnesyl group is sequestered within a hydrophobic region of the tail domain in the absence of divalent cations for prelamin A and $\Delta 50$ lamin A. With calcium bound to the tail domain, the structure of the Ig-fold is altered, increasing the solvent accessibility of the C-terminus. This data collectively shows a divalent cation-induced conformational change in $\Delta 50$ lamin A, which may be important in disease pathogenesis. Also, divalent cations may provide a regulatory role in the post-translational processing of wild type lamin A.

1421

Expression of different nuclear lamins during *Xenopus laevis* development may contribute to nuclear scaling.

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Nuclear size differs in different organisms, cell types, and stages during development. Understanding how nuclear size is regulated is important because abnormal nuclear size and morphology are associated with some diseases state. Nuclear lamins have been demonstrated to regulate nuclear size, but how different lamin isoforms affect nuclear size is unknown. In order to address this question, we are using the frog *Xenopus laevis* as our model system. During *X. laevis* embryo development, the nuclear size decreases both prior to the midblastula transition (MBT) and post-MBT. It was shown that pre-MBT nuclear size is regulated in part by nuclear import. We focus on post-MBT nuclear scaling, when different lamin types and isoforms are expressed as development proceeds. Our approach is to microinject one-cell *X. laevis* embryos with different lamin mRNAs and quantify effects on nuclear size. Our data show that lamin A expression decreases nuclear size while lamin B1 increases nuclear size in post-MBT embryos, consistent with the developmentally regulated expression patterns of these two proteins. Interestingly, lamin B3 effects on nuclear size depend on the expression level. Higher levels of lamin B3 expression decrease nuclear size while lower concentrations increase nuclear size. These results indicate that different lamin isoforms differentially affect post-MBT nuclear size. Future experiments will address the role of lamin B2 in regulating nuclear size, how different lamins act together, and the contributions of other nuclear structural components.

1422

Hierarchical determination of nuclear deformability by lamin isoforms during adult hematopoiesis.*J-W. Shin¹, K. R. Spinler¹, J. Swift¹, D. E. Discher¹; ¹Biophysical Engineering Laboratory, University of Pennsylvania, Philadelphia, PA*

Bone is a porous tissue where marrow resident hematopoietic cells must migrate through. It has been known that different blood cells show distinct deformability, and mature blood cells become softer during differentiation from immature progenitors so that they can better traffic from marrow through the endothelial barrier and into the circulation. However, the molecular basis of this phenomenon remains to be understood. Cellular deformability is determined both by membrane and nuclear elasticity, the latter that is dynamically regulated by changes in gene expression and structural reorganization, which are driven by nuclear lamins. We developed a novel protein expression analysis algorithm, “mass spectrometry calibrated intracellular flow cytometry”, to comprehensively profile lamin isoform expression across different human blood cell types. This approach reveals the hematopoietic lineage map of nuclear deformability, which is verified by nuclear micropipette aspiration. Lymphoid and myeloid lineages show decreased total lamin intensity and pliable nuclei, consistent with their ability to transmigrate into blood. In contrast, megakaryocytes (MKs) remain in marrow because their polyploid nuclei are too large and rigid, indicated by high lamin intensity, but they do extend membrane projections into blood, where shear generates circulating platelets. Erythroid lineages share the same progenitor with MKs and migrate into blood as enucleated RBCs, likely because of high lamin A/C intensity relative to B in the progenitors and stiff chromatin. As predicted from the lineage map, lamin A/C overexpression increases MK and erythroid differentiation, while the knockdown increases migration through pores. Increasing the lamin A/C to B ratio by lamin B1 knockdown decreases nuclear deformability. Together, the study suggests that nuclear deformability is hierarchically programmed by differential expression of the nucleoskeletal lamin A/C and B isoforms during hematopoietic differentiation, which in turn influence the ability of blood cells to migrate through marrow in 3D.

1423

A-type lamins relocate the IL-2 locus and enhance T cell activation.*M. I. Robson¹, J. González-Granado², V. Andrés², E. C. Schirmer¹; ¹School of Biological Sciences, Wellcome Trust Center For Cell Biology, Edinburgh, United Kingdom, ²Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain*

A-type lamins are intermediate filaments of the nuclear envelope that provide mechanical stability to the nucleus and regulate a wide range of cellular functions, including higher-order chromatin organization, replication, transcription, DNA repair, proliferation, differentiation, and migration. Whilst A type lamins are found in most differentiated somatic cells they were believed to be largely absent from the immune system. Here we show that lamins A/C are in fact expressed in T-lymphocytes upon T-cell receptor (TCR)-mediated activation and enhance T-cell responses. A-type lamins augment mRNA expression and protein release of interleukin-2 (IL-2), a key factor in T-cell function and the regulation of the immune response. This increased activation of the IL-2 locus occurs concomitantly with relocation of the IL-2 locus from the nuclear periphery to the nucleoplasm. By contrast progerin, a lamin A mutant responsible for the premature aging disorder Hutchinson-Gilford progeria syndrome, is unable to mediate the same repositioning and inducing effect. Moreover, treatment of progerin cells with farnesyl transferase inhibitors rescues the normal repositioning phenotype further supporting that the repositioning and enhanced gene expression is mediated by lamin A. These results demonstrate that A-type lamins are needed for optimal T-cell activation.

1424

The nuclear mechanostat that scales with tissue stiffness and amplifies lineage: lamin-A,C.

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Tissue can be soft like brain, stiff like muscle, or rigid like bone. Proteomic profiling of human and mouse tissues and cells reveals that the nucleoskeletal protein lamin-A,C scales with various collagens and with tissue microelasticity, E . Among the many cell structure and nuclear components quantified here, lamin-A,C acts most clearly as a “mechanostat” in increasing as $\sim E^{0.7}$, whereas B-type lamins are nearly constant. Lamin-A,C dominates in stiff tissues and has been implicated in aging and diseases that impact muscle, bone, and fat but rarely brain or marrow, and nuclei in stiff tissue cells also prove much stiffer than nuclei from softer tissues. Mesenchymal stem cell differentiation in vitro further shows that lamin-A,C amplifies lineage signals from matrix, with low lamin-A,C favoring a soft tissue fate and high levels favoring stiff tissue. Regulation of lamin-A,C occurs at multiple levels, with conformational changes in isolated nuclei revealing its direct response to stress. Systematic relations thus exist between tissue stress and stiffness and the nucleus.

1425

Isolated nuclei stiffen in response to force on nesprin-1.

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Mechanical forces influence many aspects of cell behavior. Forces are detected and transduced into biochemical signals by force bearing molecular elements located at the cell surface, in adhesion complexes or in cytoskeletal structures. The nucleus is physically connected to the cell surface through the cytoskeleton and the linker of nucleoskeleton and cytoskeleton (LINC) complex, allowing rapid mechanical stress transmission from adhesions to the nucleus. Whereas it has been demonstrated that nuclei experience force, the direct effect of force on the nucleus is not known. Here we show that isolated nuclei are able to respond to force by adjusting their stiffness to resist the applied tension. Using magnetic tweezers, we found that applying force on nesprin-1 triggers nuclear stiffening that does not involve chromatin or nuclear actin, but requires an intact nuclear lamina and SUN proteins. Our results demonstrate that mechanotransduction is not restricted to cell surface receptors and adhesions but can occur within the nucleus, indicating that cellular organelles can behave as independent mechanosensitive structures contributing to an integrated mechanoresponse.

1426

How deeply cells feel: from soft matrices of controlled thickness to nuclear readouts.

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Lacking eyes to see and ears to hear, cells physically sense their microenvironment and feel into the depths of a matrix by actively deforming their surroundings. To study how deeply cells feel, mesenchymal stem cells, as prototypical but particularly sensitive adhesive cells, were cultured on collagen-coated gels-based microfilms of controlled elasticity (E) and thickness (h). Cellular morphologies and nuclear deformations were distinctively smaller on soft compared with soft but thin or stiff films. As indicated by the transition from small to large spreading, the

tactile length scale for mechanosensitivity was 6-10 microns. Transcriptional analysis and protein profiling revealed a set of four most malleable nuclear envelope genes in tissues and in a dish as related to matrix-induced nuclear deformations. Overexpression and siRNA knockdown of Lamin-A,C, which is widely implicated in cell differentiation and development, directed inside-out nuclear remodeling that involved the regulation of the other three mechano-malleable components. The dynamics of nuclear spreading on rigid glass was equivalent to steady-state morphologies on soft-to-stiff matrices, and was linked with protein-level nuclear remodeling. Lamin-A,C expression levels maintained proportionality with cellular contractility by myosin activity. Taken together, the interplay between nuclear remodeling, myosin activity and matrix are indicative of gene expression regulation by the physics of the microenvironment.

1427

Mutations in the nuclear envelope proteins lamin A/C and emerin modulate actin dynamics and impair MKL1/SRF transcriptional activity.

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Background: Mutations in the *LMNA* gene encoding the nuclear envelope protein lamins A and C cause Emery-Dreifuss muscular dystrophy (EDMD), dilated cardiomyopathy (DCM), and a broad spectrum of other diseases (laminopathies). The mechanisms by which the nearly ubiquitously expressed lamins A/C can lead to tissue-specific phenotypes remain incompletely understood, but the muscle-specific phenotypes suggests a mechanosensitive component. Myocardin-related transcription factor A (MRTF-A) is an important pathway in cellular mechanosensing that responds to dynamic changes in the actin cytoskeleton by translocating from the cytoplasm to the nucleus, where it acts as co-activators for serum response factor (SRF) to induce expression of cytoskeletal genes.

Objective: To test the hypothesis that lamin A mutations associated with muscular laminopathies can result in impaired MRTF-A signaling and thereby contribute to striated muscle dysfunction in EDMD and DCM.

Methods: We examined MRTF-signaling in cells and tissue sections from lamin A/C-null (*Lmna*^{-/-}) and *Lmna* N195K (*Lmna*^{N195K/N195K}) mice, which represent models of EDMD and DCM, as well as wild-type littermates for defects in MRTF-A translocation. We used GFP-fusion constructs, biochemical methods, and immunostaining to characterize the actin-MRTF-A-SRF signaling pathway in these cells. Activation of downstream genes was assessed by Real-Time PCR and luciferase reporter constructs.

Results: Nuclear translocation of MRTF-A in response to serum stimulation was almost completely abrogated in *Lmna*^{-/-} and *Lmna* N195K mouse embryonic fibroblasts (MEFs) and these cells had impaired activation of SRF-target genes. Skeletal and cardiac muscle sections confirmed impaired nuclear translocation of MRTF-A *in vivo*. The impaired nuclear translocation of MRTF-A in lamin mutant cells did not represent a general defect in nuclear import/export but resulted from altered actin dynamics in the mutant cells. Since emerin, a nuclear envelope protein, was recently shown to promote actin polymerization and is mislocalized in lamin A/C-mutant cells, we examined whether emerin could be involved in the observed defects in MRTF-A translocation defect by modulating nuclear or cytoskeletal actin dynamics. Emerin-null MEFs displayed similar defects in actin organization and MRTF-A translocation as the lamin mutant cells, and ectopic expression of emerin rescued the defects in MRTF-A translocation and actin dynamics in emerin-null and lamin mutant MEFs.

Conclusions: We identified a new mechanism by which lamins A and C, mediated by emerin, can modulate actin polymerization and thereby alter activity of MRTF-A and SRF-dependent genes, providing a possible explanation for the muscle-specific phenotype in several laminopathies.

The Nuclear Envelope and Nuclear Pore Complexes

1428

Dramatic changes in the shape and function of intestinal cell nuclei are enhanced by progerin expression.

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As we face an epidemic of obesity that has been debilitating the health and economies of Western societies, understanding the mechanisms through which humans acquire and process nutrients from food has never been more critical. Amazingly many fundamental aspects of nutritional lipid uptake and dispersal by the absorptive cells of the intestine, called enterocytes, are poorly understood. Using zebrafish, we are examining the impact of a high-fat diet on enterocyte morphology and function. We have determined that zebrafish enterocytes undergo numerous structural changes typical of mammalian enterocytes during feeding. These include increases in size, expansion of endoplasmic reticulum, fusion of mitochondria, and the formation of lipid droplets. Interestingly, we observed a novel phenomenon: the rapid and reversible distortion of the nuclear periphery upon feeding. This dramatic structural change of the nucleus is coupled with interesting transcriptional changes. Since the nuclear lamina helps regulate both the shape of the nucleus and the transcription of genes, we examined the role of nuclear lamins in these processes in enterocytes. Using zebrafish that express a mutant allele of lamin A, called progerin, we studied the impact of a disrupted nuclear lamina on the morphology of enterocytes and on their ability to absorb and process lipids.

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Analysis of factors involving three distinct stages during apoptotic nuclear condensation using a cell-free system.

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In apoptotic execution step, cells perform final processes, such as DNA cleavage and nuclear condensation before engulfment by other cells.

The molecular mechanisms for nuclear condensation are still unclear, while upstream cytoplasmic pathways are well characterized.

Earnshaw and his colleagues (Lazebnik et al. 1993) and others have been developed a cell-free system which allows isolated nuclei could undergo apoptosis in vitro as intact cells did. Using this system, we have previously demonstrated that nuclei exhibited consistent structural changes and apoptotic nuclear condensation could be classified into three distinct steps, stage 1 ring, stage 2 necklace and stage 3 nuclear collapse (Tone et al. 2007). Time-lapse imaging of individual nuclei showed all nuclei could follow a reproducible program of nuclear condensation, suggesting the existence of an ordered biochemical pathway. DNase activity was not required

for stage 1 ring condensation, which could occur in apoptotic extracts depleted of all detectable DNase activity. DNase(s) were however, essential for stage 2 necklace condensation. In the present study, we confirmed the necessity of caspase-6 activity for stage 3 nuclear collapse, because stage 3 was suppressed by preincubation of extracts with pan-caspase inhibitor, zVAD. In addition, incubation of stage 2 necklace nuclei with caspase-6 caused nuclear collapse. Finally, for the activity inducing stage 1 ring condensation, phosphorylation for histone 2B Ser 17 (H2BS17) could be involved, because an antibody specific for phospho H2BS17 could detect positive band not only in nuclei incubated with apoptotic extracts but also in ring stage nuclei made by DNase-depleted extracts. This suggests that kinase could phosphorylate H2B resulting chromatin remodeling, ring condensation. In conclusion, kinase activity in stage 1, DNase activity in stage 2, and caspase activities in stage 3, are essential for each condensation stages.

1430

XPC-centrin interactions in *Dictyostelium*.

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Dictyostelium discoideum Rad4 (DdRad4) is a DNA repair protein. The yeast and human homolog of DdRad4, XPC, has been shown to interact with centrin proteins but little is known about the possible interaction of the two proteins in *D. discoideum*. Past research has shown that a knockout of the centrin protein DdCenB in *D. discoideum* causes nuclear and mitotic defects. The amino acids that mediate the interaction between human XPC and centrin2 have been well characterized and are conserved in their respective *D. discoideum* homologs. This prompted us to directly examine the interaction between DdCenB and DdRad4. The c-terminal region of DdRad4 was expressed as a GST-tagged protein in bacterial cells and purified using glutathione-coated beads. These beads were subsequently mixed with cell lysates from GFP-DdCenB expressing cells and probed for the presence of DdCenB. GST-Rad4 beads were able to pull down GFP-DdCenB, whereas GST-beads alone were not. Because both DdCenB and DdRad4 localize to the nucleus, we next wanted to test if the known interaction of DdCenB with chromatin was DdRad4-dependent. Western blot analysis of cells expressing GFP-DdCenB, but lacking DdRad4 (DdRad4-KO), were used. If DdRad4 is required for DdCenB-chromatin interaction then we would expect to see no histone protein pulled down along with GFP-DdCenB from these lysates. This, however, was not the case and a significant histone band was indeed observed, confirming that the DdCenB interaction with chromatin is independent of DdRad4. Finally, we wanted to test if the nuclear localization of DdCenB was also independent of DdRad4. Confocal images of DdRad4-KO cells expressing GFP-DdCenB reveal that DdCenB does not require DdRad4 for its nuclear localization. Furthermore, DdRad4-KO cells were found to have an increased growth rate, as well as mitotic defects similar to that of DdCenB knockout cells. Novel data suggest that DdRad4 is involved in both mitosis and cytokinesis. Taken together these findings suggest that DdRad4 is a multifunctional protein involved in both the cellular response to DNA damage and the progression of the cell through both mitosis and cytokinesis.

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Human ASUNDER promotes dynein recruitment and centrosomal tethering to the nucleus at mitotic entry.

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Recruitment of dynein motors to the nuclear surface is an essential step for nucleus-centrosome coupling in prophase. In cultured human cells, this dynein pool is anchored to nuclear pore complexes through RanBP2-BICD2 and Nup133-CENP-F networks. We previously reported that the *asunder* (*asun*) gene is required in *Drosophila* spermatocytes for perinuclear dynein localization and nucleus-centrosome coupling at G2/M of male meiosis. We show herein that male germline expression of mammalian ASUNDER (ASUN) protein rescues *asun* flies, demonstrating evolutionary conservation of function. In cultured human cells, we find that ASUN downregulation causes reduction of perinuclear dynein in prophase of mitosis. Additional defects following loss of ASUN include nucleus-centrosome uncoupling, abnormal spindles, and multinucleation. Co-immunoprecipitation and overlapping localization patterns of ASUN and LIS1, a dynein adaptor, suggest that ASUN interacts with dynein in the cytoplasm via LIS1. Our data indicate that ASUN controls dynein localization via a mechanism distinct from that of either BICD2 or CENP-F. We present a model in which ASUN promotes perinuclear enrichment of dynein at G2/M that facilitates BICD2- and CENP-F-mediated anchoring of dynein to nuclear pore complexes.

1432

The nuclear envelope protein emerin functions with myosin IIB to organize actin flow and nuclear movement in polarizing cells.

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Nuclear positioning is a tightly regulated process and plays important roles in cellular and developmental events, including differentiation, cell division, fertilization, and cell migration. To study nuclear positioning during cell migration we utilize wounded monolayers of starved NIH 3T3 fibroblasts as a model system. In this system, nuclear movement occurs during centrosome orientation and is triggered by addition of the serum factor lysophosphatidic acid (LPA), which results in the Cdc42-dependent activation of myosin and generation of polarized, retrograde actin flow (Gomes et al., Cell 2005). A nuclear membrane structure composed of nesprin-2G and SUN2, termed transmembrane actin-associated nuclear lines (TAN-lines), attaches dorsal actin cables to the nucleus to move the nucleus rearward (Luxton et al., Science, 2010). Interestingly, we found that emerin, a nuclear protein that localizes to both the inner and outer nuclear membranes and interacts with nesprin and SUN proteins, was required for proper nuclear movement and accompanying centrosome orientation in NIH 3T3 fibroblasts. Knock down of emerin prevented LPA-induced nuclear movements in most cells, and when nuclei moved, they moved chaotically, rather than directionally rearward. Analysis of TAN line dynamics using GFP-mini-nesprin-2G revealed that TAN lines formed normally, but slipped over the nucleus rather than moving with it, similar to the phenotype in lamin A/C null cells (Folker et al., PNAS, 2011). Analysis of actin flow using LifeAct-mCherry revealed that dorsal actin cables formed normally, but their orientation was random, rather than parallel to the leading edge and their flow was nondirectional. Cells knocked down for myosin IIB, but not myosin IIA, showed

similar chaotic nuclear movements and actin cable flows, suggesting emerin may function with myosin IIB to control nuclear movement by organizing actin flow. Consistent with this, we found that emerin specifically interacted with myosin IIB and that myosin IIB accumulated near the nucleus in an emerin dependent fashion after LPA or V12-Cdc42 stimulation of nuclear movement. We conclude that emerin functions with myosin IIB to polarize the flow of actin cables and control the directionality of nuclear movement in cells. Our results suggest a novel function for the nuclear envelope in the organization of contractile actin flow in cells.

1433

UNC-84 is an inner nuclear membrane SUN protein that directly connects lamin in the nucleoplasm to KASH proteins in the perinuclear space.

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Positioning of the nucleus is essential for many cellular processes, including cell polarization, fertilization, cell motility, and cell division. In most eukaryotes, a nuclear envelope bridge consisting of an inner nuclear membrane SUN protein and an outer nuclear membrane KASH protein form the LINC complex (for LInker of the Nucleoskeleton to the Cytoskeleton) to link the cytoskeleton to the nucleoskeleton. In *C. elegans*, the inner nuclear membrane SUN protein UNC-84 recruits the KASH protein UNC-83 to the outer nuclear membrane. UNC-83 then recruits microtubule motors dynein and kinesin-1 to move nuclei. However, many questions remain regarding the structure of *C. elegans* nuclear envelope bridges and their ability to withstand forces generated in the cytoplasm and transferred to the nucleoskeleton. Recently, the structure of a human KASH-SUN pair was solved, allowing predictions to be made about the structure and function of LINC complexes in vivo. Here, we present a functional analysis of the nucleoplasmic, nuclear-envelope-luminal and SUN domains of UNC-84. We observed an interaction between the UNC-84 N-terminus and lamin in a yeast two-hybrid assay. This interaction was weakened by the P91S mutation previously found to partially disrupt nuclear migration. Preliminary data suggests that UNC-84 is part of an interaction network at the inner nuclear membrane that includes the NET5/Samp1/Ima1 homolog. In the perinuclear space, the majority of the linker domain between the trans-membrane domain and SUN domain is not required to facilitate nuclear migration, because a deletion of approximately 300 residues between the transmembrane domain and the SUN domain remains functional. A 50 amino acid span immediately upstream of the SUN domain, which we hypothesize to include a trimerization domain, is required for function. Additionally, mutations in conserved residues in the UNC-84 SUN domain predicted to facilitate KASH binding disrupt in vivo nuclear migration events. We propose that the N-terminus of UNC-84 directly binds lamin to transfer forces from the LINC complex to the nucleoskeleton. Furthermore, our model predicts that UNC-84 spans the perinuclear space before directly binding KASH proteins near the outer nuclear membrane.

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Architecture and function of the trypanosome nuclear envelope revealed using high resolution interactome mapping.

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The nuclear pore complex (NPC) is a macromolecular assembly embedded within the nuclear envelope (NE) that mediates transport between the nucleus and cytoplasm. Both the NPC and NE serve as structural platforms from which intranuclear compartments and the nuclear lamina are organized, playing a key regulatory role in gene expression through influencing nuclear architecture and acting as a point for the control for various nuclear functions. Each NPC is a large (~50 MDa in yeast) cylindrical octagonally symmetric complex comprised of ~480

components (termed nucleoporins or Nups) built from a common set of ~30 different proteins. Currently, detailed compositional and structural information of NPCs is only available in yeast and metazoa, which are closely related. We previously identified 22 Nups in *Trypanosoma brucei* (TbNups) using a proteomics based approach, and tagged each ORF genomically with GFP. Further understanding of NPC/NE interactions within the trypanosome genome, and hence uncovering precise functions, requires a detailed description of the NPC/NE protein interactome. Using GFP as an affinity handle, we are currently dissecting the interactions formed by these proteins in an effort to build a structural map of the NPC/NE. Using a novel cryogrinding approach, we have affinity isolated core scaffold sub-complexes that form the inner and outer ring of the NPC. We demonstrate a highly efficient method for isolation of trypanosome protein complexes, finding several novel nucleoporins that represent trypanosome-specific subunits. These results are already shedding new light on the evolution of the NPC and the nuclear architecture of the last common eukaryotic ancestor.

1435

The Molecular Architecture of Nuclear Transport.

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The nuclear pore complex (NPC) is one of the largest macromolecular machines in the cell, composed of hundreds of proteins, selectively controlling all traffic between the nucleus and the cytoplasm. The architecture of the NPC is central to understanding nuclear transport. However, due to its sheer size, its local environment and its dynamic nature, determining its structure at molecular resolution remains a challenge for conventional techniques. Cryo-electron tomography (cryo-ET) provides unprecedented insights into the 3-D macromolecular organization of cells in their native state, yet the thickness of most cells makes them inaccessible to cryo-ET. Focused ion beam (FIB) milling can be used to prepare 200-500 nm lamellae from intact cells, opening large windows into the cell's interior, enabling the use of cryo-ET to study it at molecular resolution. Combining FIB milling, cryo-ET, and image processing enables the study of the NPC in its native environment, free of the distortions caused by purification. This approach has not only revealed the NPC architecture at unparalleled resolution, but also captured different conformational states in action. I will discuss how the structure obtained by cryo-ET will serve as a scaffold upon which data from a wide range of techniques can be integrated, unveiling the molecular mechanisms of nuclear transport.

1436

The *Xenopus* embryonic protein XDppa2 couples chromatin-induced microtubule disassembly to nuclear re-formation.

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During mitosis, the metazoan nucleus is dismantled and must be reassembled at its conclusion to complete chromosome segregation and cell division. If this process is compromised, aberrant nuclear assembly and morphology may lead to altered gene expression, tissue pathology and developmental defects. *Xenopus sperm* are an ideal system for studying nuclear assembly and morphology regulation; upon exposure to egg cytoplasm, sperm nuclei undergo a dramatic shape transition from compact crescents to expansive spheres. Here we show that this process requires the embryonic protein XDppa2, which promotes microtubule disassembly in the vicinity

of chromosomes. In extracts depleted of XDppa2, chromosomes are associated with excess microtubules at mitotic exit, leading to a delay in nuclear expansion and aberrant nuclear morphology. These abnormal nuclei recruit less lamin B and fewer nuclear pore complexes, leading to defective genome replication. We show that nuclear assembly is exquisitely sensitive to the balance of microtubule assembly and disassembly, and is compromised both by excessive microtubule stabilization and in the complete absence of microtubules. Moreover, this sensitivity is only observed during the early stages of nuclear assembly and not during late nuclear assembly, highlighting the importance of temporally-regulated microtubule dynamics.

We will present recent work on the interactions between nuclear envelope components and microtubules that impinge on nuclear structure, as well as the mechanism by which XDppa2 is activated. Chromatin is essential for XDppa2 activation, and promotes multimerization and chromatin-dependent SUMOylation of XDppa2. Thus these mechanisms spatially and temporally regulate microtubule dynamics, ensuring the fidelity of nuclear assembly. XDppa2 and its homologues are essential for embryonic survival and only expressed in embryonic tissues, where maintenance of nuclear organization may be important for gene expression and subsequent developmental programmes. Moreover, like many embryonic genes human Dppa2 is overexpressed in certain cancers, and thus may contribute to common proliferative pathways between embryonic and cancerous cells.

1437

Engineered nuclear pore complexes unveil how FG domains form a functional permeability barrier.

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Nuclear pore complexes (NPCs) conduct and control traffic between the nucleus and the cytoplasm. They suppress fluxes of inert macromolecules and thus prevent intermixing of nuclear and cytoplasmic contents. Shuttling nuclear transport receptors (NTRs), however, cross the NPC- barrier in a highly facilitated manner and thereby supply nuclei with proteins and the cytoplasm with nuclear products like ribosomes.

FG repeat-containing nucleoporins (FG Nups) are critical for the barrier. They are anchored to the NPC scaffold and their FG motifs bind NTRs during facilitated translocation. While the crucial importance of FG domains is well established, currently discussed models differ widely in their explanation as to how FG domains confer transport selectivity. To test the predictions of various models we used *Xenopus laevis* egg extracts to assemble nuclei and analysed the embedded NPCs for active transport as well as passive exclusion of inert molecules. Depletion of the Nup54⋅58⋅62 and Nup62⋅88⋅214 complexes prior to nuclear assembly caused surprisingly mild functional defects in assembled nuclei. By contrast, NPCs additionally depleted of Nup98 lacked a passive permeability barrier and showed no active transport. These defects were rescued by bacterially expressed Nup98 derivatives that contained FG domains with high cohesiveness along their entire sequence. Nup98 derivatives containing non-cohesive FG domains did not rescue, even if they were fully proficient in NTR-binding.

We describe an experimental system that, for the first time, allows testing the functionality of FG domains in vitro within engineered NPCs, i.e. in their physiological context. Our data indicate that the NPC barrier critically depends on multivalent cohesion between FG domains and argue against models that do not accommodate cohesion into barrier function. Instead, our data support the assumption that the barrier is a sieve-like FG hydrogel as proposed by the "selective phase model". Similar reconstitution experiments will ultimately unveil all the requirements as to

how a functional NPC barrier is formed. We argue that the experimental system established will be pivotal to grasp the nature of the strikingly selective boundary between nucleus and cytoplasm.

1438

Depletion of Nucleoporins from HeLa Nuclear Pore Complexes to Facilitate the Production of Ghost Pores for *in vitro* Reconstitution.

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During cell division, Nuclear Pore Complexes (NPCs) are broken down into protein subcomplexes that are the basis for reassembly in daughter cells. This is the driving force for the establishment of an *in vitro* reconstitution system to study aspects of NPC reassembly. In this study, nuclear envelope (NE) was isolated from HeLa cells. NE was treated with increasing concentrations of heparin to extract nucleoporins (Nups) for the production of “ghost pores”, which are pores severely deficient in Nups, while still containing Pore Membrane proteins (POM) needed to anchor the NPC. Ghost pores have been subjected to incubation with previously stripped Nups and some re-binding has been shown to occur by western blot analysis. A portion of transport proteins like FG-Nups, Nup62 and Nup54 were found well embedded within the pore region, nevertheless we have shown affinity for re-attachment either to ghost pores or to one another for these proteins. Also nuclear basket FG-Nup Nup153 re-associates, presumably at the nuclear side of the NPC, perhaps as a foundation for re-formation of the nuclear basket. Several scaffold Nups from the Nup107-160 and Nup93 subcomplexes can re-associate to potentially restore the structural integrity of the NPC. Further investigations with ghost pores will lead to a better understanding of the process of NPC reassembly.

1439

The nuclear pore complex protein Ndc1 regulates the LINC complex to promote nuclear positioning in migrating fibroblasts.

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Positioning of the nucleus within cells has a role in different processes such as cell migration and muscle differentiation. In migrating fibroblasts, the nucleus moves rearwards prior to cell migration and this movement is dependent on the actin cytoskeleton and on LINC complex proteins Nesprin2G (N2G) and SUN2. In order to identify novel proteins involved in this process, we performed a live-imaging screen for inhibition of nuclear movement through short interfering RNA (siRNA) against a set of 230 potential/confirmed nuclear envelope proteins from published organellar proteomics studies. Ndc1, a conserved transmembrane protein of the nuclear pore complex (NPC) was one of our hits. Depletion of Ndc1 caused the inhibition of centrosome reorientation and nuclear positioning in a fixed cell assay. Furthermore, cell migration was also impaired in this condition, similar to what was observed for N2G siRNA treated cells. Although the full knock down of Ndc1 caused general disruptions of the nuclear envelope, potentially due to its role in nuclear pore assembly, a partial knockdown was sufficient to inhibit nuclear movement without affecting nuclear envelope proteins and structure. We therefore probed for a specific interaction between and LINC complex members that could explain the nuclear movement involvement. We found that the N-terminal region of Ndc1 interacted with the luminal region of SUN2. This region of Ndc1 is distinct from the proposed NPC interacting region localized on the conserved C-terminus. Expression of full length siRNA resistant Ndc1 or Ndc1 lacking the c-terminus were able to rescue centrosome reorientation in Ndc1 siRNA cells, suggesting that the interaction of Ndc1 with the nuclear pore is not required for nuclear

movement. Furthermore, SUN2 does not colocalize with nuclear pores and we found a population of nuclear envelope Ndc1 not at the NPC. We propose a model where a non-NPC population of Ndc1 is involved in the regulation of SUN2-N2G LINC complex during nuclear movement.

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Molecular mechanisms underlying extensive elongation and coiling of the *Marsilea* nucleus.

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The nuclei in spermatids of the male gametophyte of *Marsilea vestita* change dramatically from being spherical to an elongated coil with 9 gyres. The change is rapid and is completed in <3 hours. As the nucleus elongates, it becomes appressed against a large mitochondrion and a ribbon of crosslinked microtubules. On the dorsal face of the ribbon, there are 140 basal bodies, which had formed *de novo* two hours earlier in development. The chromatin becomes highly condensed and arranged against the nuclear envelope and nuclear pores become localized on the ventral side of the elongating nucleus. We are investigating the roles of the cytoskeleton, nuclear envelope proteins and the process of chromatin condensation in nuclear shaping during spermatid differentiation in this organism. Rapid gametophyte development relies mainly on the translation of stored transcripts. A transcriptome was constructed *de novo* from RNA isolates obtained from different time intervals during gametophyte development. Here, we focused on nuclear pore protein transcripts (e.g., nuclearporins, or nups), nuclear movement domain containing proteins (e.g., SUN, KASH) and transcripts of proteins affecting the microtubule cytoskeleton (e.g., alpha tubulin, tubulin-specific chaperone A and tubulin polyglutamylase). Among these transcripts was a group of mRNAs that was present at all time intervals and a second group that was only present during spermatid differentiation (nup GLE1, nup 188). RNAi experiments were combined with histology and immunohistochemistry to assess the roles of these transcripts in nuclear elongation and coiling. Analysis of the transcriptome revealed that there is extensive processing of stored transcripts. Transcript availability for translation involves several levels of regulation. A central regulator in RNA processing is a single RRM domain containing protein, named MvU620. Knockdowns of MvU620 inhibited nuclear shaping and blocked the formation of the microtubule ribbon. Deep sequencing of transcripts in MvU620-knockdowns showed some of the transcripts in the second group (e.g., tubulin polyglutamylase, tubulin specific chaperone A, nup GLE1) appear to be incompletely spliced, which is being further investigated using RT-PCR. These results show that a novel RNA-binding protein plays a central role in the regulation of RNA processing that is essential for nuclear shaping in a rapidly developing spermatid. Supported by NSF grant 0842525 to SMW.

Endocytic Trafficking II

1441

The functions of anionic phospholipids during endocytic site initiation and vesicle formation.

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Anionic phospholipids PI(4,5)P2 and phosphatidylserine (PS) are enriched in the cytosolic leaflet of the plasma membrane where endocytic sites form. In this study, we investigated the roles of PI(4,5)P2 and PS in endocytic site initiation and vesicle formation in *Saccharomyces*

cerevisiae. Live-cell imaging of endocytic protein dynamics in an *mss4ts* mutant, which has severely reduced PI(4,5)P₂ levels, revealed that PI(4,5)P₂ is required for endocytic membrane invagination but less important for endocytic site initiation. We also demonstrated that in various deletion mutants of genes encoding components of the Rcy1-Ypt31/32 GTPase pathway, endocytic proteins dynamically assemble not only on the plasma membrane but also on intracellular membrane compartments, which are likely derived from early endosomes. In *rcy1* knock-out cells, fluorescent biosensors indicated that PI(4,5)P₂ only localized to the plasma membranes while PS localized to both the plasma membrane and intracellular membranes. Furthermore, we found that polarized endocytic patch establishment is defective in the PS-deficient *cho1* knock-out mutant. We propose that PS is important for directing endocytic proteins to the plasma membrane and that PI(4,5)P₂ is required to facilitate endocytic membrane invagination.

1442

Ubiquitination of endocytic machinery regulates coat formation.

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Endocytosis in yeast requires the regulated recruitment and disassociation of over 60 proteins at discreet plasma membrane puncta. The post-translational modifications that act as signals in this highly processive and ordered process are of great interest. Ubiquitination of several endocytic proteins has been reported, but neither phenotypes nor functional consequences of these modifications are known. Here we report that ubiquitination of Ede1p, the yeast Eps15 homolog, affects the stability and disassembly of the endocytic coat. We identified the deubiquitinase Ubp7p as a late arriving endocytic protein, which likely removes the ubiquitin on Ede1p. Deletion of Ede1p does not fully suppress the deubiquitinase deletion phenotype, suggesting that Ede1p is not the only endocytic target whose deubiquitination affects coat formation.

1443

Molecular brightness analysis reveals phosphatidylinositol 4-kinase II β association with clathrin coated vesicles in living cells.

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Mammalian cells express two classes of phosphatidylinositol 4-kinase (PI4K), designated Types II and III, which phosphorylate phosphatidylinositol to generate PI4P. A number of studies indicate that these enzymes are important for Golgi trafficking and early as well as late stages of endocytosis. In this study, we focus on PI4KII β , a protein that is evenly distributed between membrane and soluble fractions and is believed to participate in stimulus-dependent phosphoinositide signaling. Using molecular brightness analysis, we found that EGFP-tagged PI4KII β exists as two distinct species in the cytoplasm, a soluble monomer and a high order complex enriched with multiple copies of PI4KII β . This observation is confirmed by autocorrelation analysis which identifies two species with distinct mobilities. We further demonstrate that the high order complex enriched with PI4KII β is sensitive to inhibition of palmitoylation, indicating that it is associated with membranes, very likely vesicles. Indeed, we show that the high order PI4KII β complex is sensitive to expression of dynamin 2-K44A, a dominant-negative inhibitor of endocytosis. We further directly detect that PI4KII β co-moves with clathrin light chain on vesicles using dual-color heterospecies partition analysis. This analysis allows us to isolate the co-mobile species in the presence of strong background contribution from the monomeric pool of PI4KII β . Our results strongly suggest that PI4KII β is

involved in an early stage of endocytosis, and associated with clathrin-coated vesicles. Moreover, we establish molecular brightness as a powerful tool to characterize cellular cytosolic vesicles that are otherwise difficult to characterize by other techniques. This work is supported by the National Institutes of Health (R01 GM64589) and the National Science Foundation (PHY-0346782).

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Crosslinking-Induced Endocytosis of Acetylcholine Receptors by Quantum Dots.

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In majority of patients with myasthenia gravis (MG), anti-acetylcholine receptor (AChR) antibodies are generated in their immune system, which target the postsynaptic AChR clusters and membrane integrity of neuromuscular junctions (NMJs) leading to muscle weakness. The antibody-induced endocytosis of AChRs in the postsynaptic membrane represents the initial step in MG pathogenesis; however the molecular mechanisms underlying AChR endocytosis remain largely unknown. Here, we used multivalent quantum dots (QDs) to mimic the pathogenic antibodies for inducing the crosslinking and internalization of AChR from the postsynaptic membrane. By labeling the muscle cells with biotin-conjugated α -bungarotoxin followed by QD-conjugated streptavidin, we were able to differentiate the surface versus internalized AChRs by comparing their size, fluorescence intensity, trajectory, and subcellular localization of the QD signals. These crosslinking-induced internalized AChRs were highly co-localized with an early endosomal marker. Furthermore, QD-induced AChR endocytosis was mediated via clathrin-dependent, and caveolin-independent, mechanisms. Nocodazole or cold temperature treatment, that largely disrupted microtubule structures, arrested the movement of QD-induced AChR vesicles inside the cells. Furthermore, activation of agrin/MuSK signaling pathway, by either agrin treatment or over-expression of MuSK and rapsyn, significantly suppressed QD-induced internalization of AChRs, suggesting synaptogenic signals increase the stability of surface AChRs. Lastly, QD-induced AChR crosslinking potentiated the dispersal of pre-patterned AChR clusters upon synaptic induction. Taken together, this study reports a novel approach to study the trafficking mechanisms of AChRs upon receptor crosslinking and endocytosis, and demonstrates the involvement of agrin-MuSK signaling in the crosslinking-induced AChR endocytosis.

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HK-ATPase trafficking and apical vacuolar membrane stability requires non-muscle myosin II in gastric parietal cell.

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The essential role for non-muscle myosin II (Myo II) network and dynamics in cytokinesis, cell migration, polarity, epithelial morphogenesis and nucleus positioning has been well documented. However, the impact of myosin II on the regulated membrane trafficking and recruitment of HK-ATPase H⁺ pump (HK) of gastric parietal cells from tubulovesicles in resting cells into the apical membrane of the stimulated cells has been elusive. Here, we use primary cultures of rabbit parietal cells to show that Myo II is required for proper positioning of apical

vacuole (AV's) and stimulus-dependent recruitment of HK-containing tubulovesicles into AV membranes. Myo II is localized to the AV membrane in non-stimulated cells, but relocates to cytoplasm and basal membrane when cells are stimulated to secrete acid by histamine. Inhibition of Myo II function by ML-7 (myosin light chain kinase inhibitor), but not Y-27632 (ROCK inhibitor) or blebbistatin (inhibitor of non-muscle myosin ATPase activity), destabilizes and relocates actin and ezrin, both of which are normally associated with AV membranes, to the basal membrane. Moreover, ML-7 completely prevented histamine-stimulated HK vesicle transport to the AV membrane. These results indicate that Myo II is critical for positioning and morphological configuration of AV membranes in resting parietal cells and for the proper trafficking of HK-containing tubulovesicles to the AV during histamine-stimulation.

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A Secretagogue-induced microtubule dynamics facilitate HK-ATPase vesicle transport in gastric parietal cell.

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Microtubule (MT) dynamics-based polarized vesicular transport is well documented in epithelial and neuronal cells, but how microtubule dynamics facilitate stimulus-associated HK-ATPase containing tubulovesicle (TV) transport to the apical vacuolar membrane (AV membrane) in the gastric parietal cell is not clear. Ultrastructure studies of high-pressure frozen parietal cells showed that microtubules were associated closely with tubulovesicles. These associations frequently included some electron dense material. These findings were confirmed by computer-assisted 3D electron tomography. Then we demonstrated by immunostaining that cytoplasmic MTs extend towards the periphery when parietal cells are stimulated by histamine, but in control cells they were more centrally located. Sub-cellular fractionation of gastric glands showed association of kinesin 1 and dynein (microtubule motor protein) in the TV-enriched fraction. This relatively close association was further confirmed by high salt and alkaline wash experiments. Immunoprecipitation followed by mass spec (LC/MS/MS) analysis showed that kinesin 1 binds with three kinesin light chains (KL1,2 and 3), and that dynein intermediate chain binds dynein and IQGAP but not Clip170 (Linker for IQGAP and molecular motors). Interestingly, interaction of dynein with IQGAP doesn't require Clip170. These data suggest that specific kinesin light chain and IQGAP might play a role to select cargo protein/vesicle. Surprisingly, nocodazole doesn't inhibit histamine stimulated HK-ATPase-containing TV transport to the AV membrane. Thus, MTs and molecular motor dynamics may facilitate long-distance transport of TV to AV membrane in gastric parietal cell to attain maximal acid secretion.

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Cell adhesion defines the topology of endocytosis and impacts signaling pathways.

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To understand which role cell adhesion plays in endocytosis we studied uptake of transferrin (Tfn) and epidermal growth factor (EGF) in cells whose adhesion geometry was defined by micropatterns that force cells to adopt a reproducible interaction with the extracellular matrix. We quantified the 3D organization of endocytosed molecules with probabilistic density maps that provide information about the behavior of the majority of endocytosed molecules. We found an unexpected asymmetry in clathrin -dependent and independent endocytosis of transferrin (Tfn) and epidermal growth factor (EGF), respectively: Tfn was enriched in adhesive areas on the top of adhesive patterns, whereas EGF was concentrated at the dorsal surface of the cell.

This spatial separation was not due to the distribution of corresponding receptors. Clathrin-dependent endocytosis of Tfn above the micropattern was due to the selective recruitment of AP2 and clathrin to adhesive areas. Consistently, clathrin-independent uptake of EGF at high concentrations (>100 ng/ml), used in this study for fluorescent visualization, was absent from adhesive areas. We found that the actin cytoskeleton played an important role in restricting uptake sites without being essential for continued operation of receptor-mediated endocytosis. Alterations in actin dynamics changed uptake topology of Tfn and EGF and diminished EGF-mediated downstream signaling revealing how cells may sense their environment and respond to stimulations in a polarized fashion. We propose that variations in cellular adhesion geometry convert receptor mediated endocytosis into spatial signal transduction.

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Specific α -arrestins Regulate GPCR Internalization in *Saccharomyces cerevisiae*.

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G-protein-coupled receptors (GPCRs) are the largest class of cell surface receptors and initiate cellular responses to a myriad of extracellular stimuli. Because prolonged GPCR-mediated signaling can be detrimental, post-agonist desensitization is exquisitely regulated. One mechanism for down-regulation of GPCR-initiated signaling, receptor endocytosis, proceeds through a series of characteristic steps, including GPCR phosphorylation, ubiquitinylation, recruitment to clathrin-coated pits, and internalization. In mammalian cells, members of a small family of dedicated trafficking adaptors, the β -arrestins, block signaling and reportedly mediate GPCR internalization. The *S. cerevisiae* genome lacks any obvious β -arrestin homologs, but encodes a more numerous class of related proteins, α -arrestins, which possess multiple copies of a motif (PPxY) required for interaction with the HECT domain-containing ubiquitin ligase Rsp5. It is well established in yeast that α -arrestins are required for efficient internalization of nutrient transporters. Given the lack of β -arrestins in yeast, we explored whether any of the α -arrestins are required for internalization of either of the two GPCRs that initiate the mating pheromone pathway — Ste2 on *MATa* haploids that binds α -factor and Ste3 on *MAT α* haploids that binds a-factor. We have found that specific α -arrestins regulate the mating pathway in a haploid-specific manner. *MATa* cells lacking two paralogous α -arrestins Rod1 and Rog3 (and/or the α -arrestin Ldb19) are detectably more sensitive to α -factor than wild-type cells, as judged by the standard halo bioassay for pheromone-induced G1 arrest. Similarly, *MAT α* haploids lacking two other paralogous α -arrestins, Aly1 and Aly2, as well as Ldb19, are detectably more sensitive to a-factor than otherwise wild-type cells. Given that the main difference between these two cell types is the GPCR itself, these findings suggest specific cargo-adaptor interaction between GPCRs and α -arrestins. Phenotypic rescue experiments in *MATa* haploids showed that removal of PPxY motifs in Rod1 and Ldb19 disrupts their ability to complement, indicating that interaction with Rsp5 is required for their function. As judged by fluorescence microscopy, the level of fluorescently tagged versions of Ste2 and Ste3 at the plasma membrane in *MATa rod1 Δ rog3 Δ* cells and *MAT α aly1 Δ aly2 Δ* , respectively, is elevated compared to wild-type controls. These findings indicate that, in yeast, efficient endocytosis of GPCRs is dependent on discrete members of the α -arrestin family.

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A calcineurin-dependent switch controls the trafficking function of α -arrestin Aly1/Art6.

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Protein endocytosis triggered by external stimuli is critical for cell growth and survival. In yeast, certain nutrients, when present in excess, induce endocytosis of the cognate permeases to prevent possible toxic accumulation of metabolites. Although permease internalization is regulated by the corresponding nutrient, the molecular basis of this effect has been elusive. Recent work revealed that α -arrestins, a family of trafficking adaptors, stimulate ubiquitin-dependent and clathrin-mediated endocytosis by interacting with both the target permease and the ubiquitin ligase Rsp5. Here we show that α -arrestin Aly1/Art6 is a phosphoprotein that specifically interacts with and is dephosphorylated by the Ca^{2+} - and calmodulin-dependent phosphoprotein phosphatase calcineurin/PP2B. Dephosphorylation of Aly1 at a subset of calcineurin-regulated phospho-sites is required for Aly1-mediated endocytosis of the aspartic acid and glutamic acid transporter Dip5, but does not alter ubiquitination or stability of Aly1. Dephosphorylation has no significant effect on the previously identified role for Aly1 in intracellular sorting of the general amino acid permease Gap1. These results suggest that phosphorylation of Aly1 inhibits its endocytic function and, conversely, that nutrient-induced activation of calcineurin serves as a regulatory switch by stimulating dephosphorylation of Aly1 to promote its endocytic function.

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Yeast alpha-arrestins play a cargo-selective role in Rho1-mediated clathrin-independent endocytosis.

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Although most cell types use both clathrin-mediated (CME) and clathrin-independent (CIE) endocytic pathways, yeast have been thought to rely solely upon CME for internalization of plasma membrane cargo. We recently uncovered a second endocytic pathway in yeast that acts independently of clathrin and CME machinery, but instead relies on the small GTPase Rho1 and its effector, the formin Bni1. While factors regulating cargo entry through CME have been identified, mechanisms for cargo delivery into the Rho1-dependent CIE pathway are currently unclear. A recent mass spectrometry screen identified Rom2, which is a guanine nucleotide exchange factor that acts upstream of Rho1, as a candidate binding partner for the α -arrestin Aly1. Since yeast α -arrestins are involved in cargo ubiquitination during CME by recruiting the E3 ligase Rsp5, we hypothesized that α -arrestins may additionally regulate cargo entry during CIE via interactions with components of the Rho1-dependent CIE pathway. We show that high-copy expression of the individual arrestins Aly1, Aly2 and Ldb19 promotes clathrin-independent internalization of the pheromone receptor Ste3. High-copy Ldb19 (but not Aly1 or Aly2) also promotes Mup1 internalization, suggesting that arrestins act in a cargo-specific manner during CIE. Surprisingly, mutation of the canonical Rsp5-binding sites on arrestins did not impair their ability to promote clathrin-independent internalization of Ste3 or Mup1. Thus, our findings suggest that α -arrestins are involved in yeast CIE through a mechanism that is distinct from their known role in CME.

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Ivy1 is a regulated interactor of the Rab7 GTPase Ypt7 that controls endosome-vacuole fusion.*J. Numrich¹, H. Artl¹, C. Ungermann¹; ¹University of Osnabrück, Osnabrück, Germany*

The yeast vacuole is a highly dynamic organelle, which undergoes fission and fusion during osmotic stress or vacuolar inheritance. The homotypic fusion of vacuoles is a well-studied process, which depends on a fusion machinery mainly consisting of SNAREs, the Rab7 homolog Ypt7 and its effector HOPS. Beside homotypic fusion events, heterotypic fusion events including the fusion of late endosomes and autophagosomes occur at the vacuole for the delivery of vacuolar hydrolases. All these fusion processes are also dependent on Ypt7 and its effector HOPS. Before fusion with the vacuole can occur, several steps are needed for maturation of these organelles. The processes that contribute to maturation are in the process of being understood but the question when exactly is an organelle ready for fusion is still unanswered.

A possible regulator interfering with fusion processes at the vacuole is Ivy1, which has been implicated as a Ypt7 and HOPS binding protein. I will present insights on the function of Ivy1 as an inhibitor of vacuole fusion in vivo and in vitro. The ability of recombinant Ivy1 to inhibit vacuole fusion in vitro strengthens our hypothesis that Ivy1 is a regulator of fusion processes at the vacuole. Furthermore, preliminary data suggest that the function of Ivy1 can be regulated by posttranslational modification.

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Role of ligand size and binding accessibility on clathrin- and caveolae-independent CAM-mediated endocytosis.*D. Serrano^{1,2}, R. Chadha³, C. Garnacho⁴, S. Muro^{4,5}; ¹Biological Sciences, University of Maryland, College Park, MD, ²Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, ³Department of Biology, University of Maryland, College Park, MD, ⁴Institute for Bioscience and Biotechnology Research, University of Maryland, College Park, MD, ⁵Fischell Department of Bioengineering, University of Maryland, College Park, MD*

Intercellular adhesion molecule 1 (ICAM-1) is a transmembrane glycoprotein overexpressed by endothelial cells particularly at sites of inflammation, making it a suitable candidate for targeted drug delivery. Drug carriers displaying multiple copies of an ICAM-1-targeting moiety (such as antibodies or peptides) bind multivalently to ICAM-1 and induce a pathway known as cell adhesion molecule (CAM)-mediated endocytosis, which differs from clathrin- and caveolae-dependent uptake. CAM-mediated endocytosis depends on enzymatic production of ceramide at sites of carrier engulfment, which leads to rearrangement of the actin cytoskeleton. This allows for uptake of carriers of different sizes, from 100 nm up to 5 µm in diameter. Here, we explored how carrier size affects CAM-mediated endocytosis, particularly in the context of using targeting moieties that have different lengths and, hence, different accessibility to ICAM-1. We coated 100 nm, 1 µm or 4.5 µm model polymer carriers with either a 150 kDa antibody or a 17-mer peptide, both of which specifically bind ICAM-1. We then determined binding and internalization levels of these carriers by endothelial cells using fluorescence microscopy. Carriers of 100 nm in diameter coated with anti-ICAM bound at levels 1.5-fold higher compared to peptide-coated counterparts. Increasing the size of anti-ICAM carriers to 1 µm or 4.5 µm led to a reduction in carrier binding levels, which was also observed with peptide-coated carriers but to a greater extent. This suggests that increasing carrier size affected ICAM-1 accessibility, but this effect was greater with peptides than with antibodies. In the case of all sizes, antibody-

coated carriers showed higher levels of internalization compared to peptide-coated counterparts. However, this effect was not due directly to lower binding levels because incubation of antibody carriers on cells expressing low levels of ICAM-1 (hence, exhibiting low levels of carrier binding) led to equally efficient internalization compared to conditions of high ICAM-1 expression. Thus, we asked if the differences in internalization might be due to differences in the signaling events associated in CAM-mediated uptake. We found that both antibody and peptide carriers elicited evident rearrangement of the actin cytoskeleton. However, only antibody-coated carriers were associated with detectable ceramide enrichment at sites of engulfment. Overall, our results indicate that coating short peptides instead of antibodies on carriers diminished binding accessibility to ICAM-1, and, independently, ICAM-1-associated signaling, and internalization of carriers. NIH R01-HL98416 and AHA 09BGIA2450014 (S.M.), NSF GRFP (D.S.).

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Trafficking of N-cadherin via macropinocytosis in migrating cells.

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N-cadherin is a calcium-dependent homophilic adhesion molecule important for cell-cell adhesion and migration. During cell migration, N-cadherin concentrates at leading edge and ruffles. These ruffles constantly fold back and form macropinosomes to regulate membrane reorganization and nutrient uptake. N-cadherin has been implicated in regulating the closure of macropinosomes via homophilic interactions.

To visualize the dynamics of surface N-cadherin in migrating cells, we monitored the behaviors of N-cadherin fusing with pHluorin [pHluorin-N-cadherin]. We have observed that the surface N-cadherin distributed evenly on the membrane ruffles and internalized by macropinocytosis judging by vesicle size and dextran uptake ability. When the fine localization of N-cadherin at leading edge was resolved by the photoactivated localization microscopy (PALM), rather than uniformly distributed, many N-cadherin molecules formed large yet discrete clusters. These large clusters have distinct behaviors than surrounding non-clustered N-cadherin proteins by fluorescence recovery after photobleaching (FRAP) assay. When in macropinosomes, N-cadherin precedes the arrival of Rab5 because of its presence on ruffles prior to the formation of macropinosomes. Of all macropinosomes arose from ruffles, all of them contained N-cadherin, and a few lack of Rab5 signals disappeared before reaching cell center. Interestingly, blockade of N-cadherin homophilic interaction using N-cadherin interfering antibody (GC-4) decreases the association of Rab5 with macropinosomes. This result suggests that N-cadherin is not only a passenger of macropinosomes, it also plays a role in regulating the stability and maturation of macropinosomes. Together, our results indicate a novel role for N-cadherin in macropinosome formation, and the role of macropinosome in cell migration will be discussed.

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Coronin 1B marks dynamic clathrin- and caveolin-independent endocytic sites in mammalian cells.

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Distinct endocytic pathways in mammalian cells have been classified based on the presence or absence of proteins such as clathrin, caveolin, dynamin and Rho GTPases. Although several

clathrin- and caveolin-independent pathways have been described, these pathways are largely defined by the absence of the proteins named above rather than by the presence of any specific, defining proteins. In the present study, we investigated the dynamics of coronin 1B at the plasma membrane and observed that it localizes to short-lived patches that lack clathrin and caveolin. Using live cell-imaging analysis, we observed that coronin 1B colocalizes with dynamin2 and the actin-related factors WIP, cortactin, Arp3 and cofilin in a specific temporal sequence. We also determined that these coronin 1B patches are directly involved in the internalization of the IL-2 receptor. We observed that the cortactin and coronin1b recruitment are dynamin dependent during IL-2R endocytosis. Interestingly, we also provide evidence for mutual dynamic coordination of cortactin and coronin 1B during the last step of the IL-2R endocytosis. Therefore, our results establish a strong dependency on the actin machinery during the clathrin- and caveolin-independent IL-2R endocytosis pathway. Importantly, this work provides direct evidence for a modular organization for the molecular machinery involved in this clathrin-and caveolin-independent endocytosis pathway and reveals similarities with yeast endocytosis.

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Oligomers of the ATPase EHD2 confine caveolae to the plasma membrane through association with actin.

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Caveolae are specialized domains present in the plasma membrane (PM) of most mammalian cell types. They function in signalling, membrane regulation, and endocytosis. We found that the Eps-15 homology domain-containing protein 2 (EHD2, an ATPase) associated with the static population of PM caveolae. Recruitment to the PM involved ATP binding, interaction with anionic lipids, and oligomerization into large complexes (60–75S) via interaction of the EH domains with intrinsic NPF/KPF motifs. Hydrolysis of ATP was essential for binding of EHD2 complexes to caveolae. EHD2 was found to undergo dynamic exchange at caveolae, a process that depended on a functional ATPase cycle. Depletion of EHD2 by siRNA or expression of a dominant-negative mutant dramatically increased the fraction of mobile caveolar vesicles coming from the PM. Overexpression of EHD2, in turn, caused confinement of cholera toxin B in caveolae. The confining role of EHD2 relied on its capacity to link caveolae to actin filaments. Thus, EHD2 likely plays a key role in adjusting the balance between PM functions of stationary caveolae and the role of caveolae as vesicular carriers.

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Cavin regulates caveolar endocytosis by forming "crescents" that girdle caveolae, stabilizes them in relatively flat conformations, and links them to cortical actin filaments.

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Caveolae are cave-like or flask-shaped invaginations of the plasmalemma that abound in three major cell-types: endothelia, fat, and muscle, but are found in virtually all cells (except neurons and immune-cells). Their function has remained entirely enigmatic for over 50 years, leading to many plausible theories, including endocytosis, transcytosis, fatty acid uptake, etc., but no definitive conclusions. The major 22kDa protein that coats them and stabilizes their structure by 'hairpin'-insertion into the plasmalemma was decades ago named "caveolin", but a second protein component has recently been discovered in them, variously called p60, PTEN, or more appropriately, "cavin." Recent studies claim that knockout of this "cavin" protein makes caveolae

disappear, and that exogenous expression of this protein in naturally cavin-null cells makes caveolae reappear, suggesting that this protein somehow acts in concert with caveolin to organize or stabilize caveolae. Possibly, cavin accomplishes this by linking caveolae to the cortical cytoskeleton under the plasmalemma. Here, we will demonstrate that the "deep-etch" technique of EM can confirm many of these recent claims about the protein cavin. Furthermore, it demonstrates that when one over-expresses cavin in the standard sort of cultured cell that already has caveolae and cavin, the structural consequences are dramatic. This not only greatly increases the abundance of caveolae over normal, but more importantly, exaggerates the prominence of one particular component of caveolae that has long been seen by "deep-etch" EM, but is not visible by other techniques: namely, the peripheral, crescent-shaped 'girdle' of granular proteins that commonly surrounds caveolae and links them to cortical actin filaments. In cavin-overexpressing cells, these "crescents" are much more frequent than normal, and are observed particularly frequently on a population of unusually flat caveolae that are relatively rare in control cells. In this poster, we will also demonstrate that these static 'deep-etch' EM observations can be confirmed and 'brought to life' in the light microscope by using fluorescent cavin-derivatives conjugated to GFP or to Dendra. Taken together, these EM and LM observations permit us to conclude that the new protein 'cavin' does indeed play an important role in caveolar formation and organization.

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Raft-dependent Endocytosis of Autocrine Motility Factor Regulates Gp78 Ubiquitin Ligase Activity via Rac1.

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Gp78 is a cell surface receptor that also functions as an E3 ubiquitin ligase in the endoplasmic reticulum-associated degradation (ERAD) pathway. The Gp78 ligand is the glycolytic enzyme phosphoglucose isomerase (also called autocrine motility factor or AMF), which functions as a cytokine upon secretion by tumor cells. AMF is internalized via a raft-dependent pathway to the smooth endoplasmic reticulum, however the relationship between AMF and Gp78 ubiquitin ligase activity remains unclear. AMF endocytosis is inhibited by both the dynamin inhibitor, dynasore, and the raft disrupting agent, m β CD, distinguishing the AMF endocytic route from the dynamin-independent CLIC/GEEC raft endocytosis of cholera toxin b subunit. Gp78 knockdown reduces AMF uptake and AMF stimulates downregulation of cell surface Gp78 that is prevented by dynamin inhibition of AMF endocytosis with dynasore. AMF uptake is not affected by overexpression of dominant-active or dominant-negative RhoA or Cdc42 but was found to be Rac1-dependent, inhibited by both expression of dominant-negative Rac1 and the Rac1 inhibitor NSC23766. AMF stimulated activation of Rac1 but not RhoA. AMF induced Rac1 activation was not observed in Gp78-knockdown cells and significantly reduced by dynasore treatment suggesting that AMF signaling to Rac1 requires Gp78-mediated endocytosis. Gp78 induces mitochondrial fission and degradation of the mitochondrial fusion proteins, Mitofusin 1 and 2. AMF prevented Gp78-induced mitochondrial fission and mitofusin degradation. The ability of AMF to inhibit Gp78 degradation of the mitofusins was prevented by dynasore, the Rac1-inhibitor NSC23766 and the PI3K inhibitor LY294002 that also inhibits AMF endocytosis. This defines a critical role for the raft-dependent endocytosis of AMF in its ability to signal through Gp78 to Rac1 and to regulate Gp78 ubiquitin ligase activity. It further identifies AMF as an extracellular regulator of Gp78 function in ERAD. Both AMF and Gp78 are closely associated with tumor cell motility and metastasis and the Rac1-dependent, raft-mediated endocytosis of AMF may represent an important regulator of Gp78 function in tumor progression. Supported by CIHR MT-15132.

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Cavins and Caveolins independently regulate the CLIC/GEEC endocytic pathway.

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Caveolins and cavins are crucial components of caveolae, which recently have been shown to have non-caveolar functions including regulation of cell polarization and clathrin independent endocytosis (CIE). However the mechanisms involved in these important cellular functions remains unknown. Here we report that specific members of the cavin family of peripherally-associated caveolar proteins, are potent inhibitors of CIE. Cavins act to inhibit endocytosis of CD44, a marker of the clathrin independent carriers endocytic pathway (CLIC/GEEC), in a process independent of caveola formation. CD44 endocytosis is upregulated in cells lacking, or with reduced expression, of cavin1 and cavin3. Ectopic expression of cavin1 and 3, but not cavin2 or cavin4, specifically inhibits the CLIC/GEEC pathway even in cells that lack caveolins and so cannot generate caveolae. Caveolin-1 and Caveolin-3 also inhibit the CLIC/GEEC pathway upon expression whereas the pathway is upregulated in Caveolin-1 deficient cells. Inhibition by caveolins is dependent on an intact scaffolding domain and is associated with inhibition of diffusion of lipid microdomain components; inhibition can be rescued by treatments known to increase plasma membrane diffusion of the components. In the absence of cavins (and caveolae) caveolin-1 is itself endocytosed preferentially through the CLIC pathway but the pathway is unpolarized in cavin1 deficient migrating cells. This work provides new insights into the regulation of non-caveolar clathrin-independent endocytosis by specific caveolar proteins illustrating the complex crosstalk between these pathways. We show for the first time a role for specific cavins in regulating the CLIC/GEEC pathway, providing a new tool to study this pathway and showing caveola-independent actions. We propose a novel mechanism for inhibition of the CLIC/GEEC pathway by caveolin.

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Possible Protein Interaction between Maspardin and ALDH18A1 in Mast Syndrome.

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Hereditary Spastic Paraplegias (HSPs) are a family of neurological disorders characterized by weakness and spasticity of the lower limbs and sensory impairment. Mast syndrome is a complicated, autosomal recessive form of HSP exhibiting symptoms of dementia, thinning of the corpus callosum, white matter abnormalities, and spastic paraparesis. Patients with Mast syndrome carry a mutation along their SPG21 gene producing a premature stop codon. Thus, Mast syndrome is likely due to loss of functional maspardin protein. Previous studies demonstrate maspardin interacts with ALDH16A1 and following maspardin deletion aldehyde dehydrogenase ALDH18A1, another member of the ALDH superfamily, was upregulated 1.66 fold suggesting an interaction between ALDH18A1 and maspardin may occur. Patients with a missense mutation, R84Q, occurring along ALDH18A1 gene exhibit progressive neurodegeneration and bilateral subcapsular cataracts. In recent studies, variants of ALDH18A1 were found to be significantly associated with dementia with Alzheimer's Disease in Down syndrome patients suggesting a link between ALDH18A1 and other neurodegeneration diseases. Interestingly, a new HSP has been found to be caused by a mutation within the ALDH18A1 gene and is being investigated. In vitro immunoprecipitation (IP) analysis in mouse embryonic fibroblast (MEFs) has suggested an interaction occurs. In vitro IP utilized Protein A (*S. aureus*) beads to physically isolate maspardin using α -maspardin antibody at the N terminus.

IP reaction was separated via 8% SDS-PAGE and blotted with α -ALDH18A1 primary antibody and α -goat secondary antibody for luminescence via western blot analysis. Reverse IP using α -ALDH18A1 antibody to concentrate ALDH18A1 will also be conducted as well as IPs in neurons. Confocal microscopy is currently being utilized to visualize potential ALDH18A1 and maspardin colocalization in MEFs and neurons.

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Exo84 Interacts with EHD1/3 for the Recycling of Cargos from Endosomes to the Plasma Membrane.

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Exo84 is a member of the exocyst complex implicated in the tethering of exocytic vesicles to the plasma membrane for exocytosis. Here we demonstrate that Exo84 interacts with Eps15-homology domain (EHD) proteins 1 and 3, which are important factors in endocytic trafficking pathways. Exo84 is localized to Rab8 and MICAL-L1 positive membrane tubules. EHD proteins co-localize with Exo84 on these endosome tubules, and are responsible for recruiting exocyst to these membrane domains. Exo84 is also responsible for efficient transport of endocytic cargos – a process that may also be regulated by the small GTPase RalA for which Exo84 is an effector. Our study demonstrates the cooperation between the vesicle fission machinery EHDs and the vesicle tethering exocyst complex for the recycling of proteins from endosomes to the plasma membrane.

Post-Golgi Trafficking

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Basolateral sorting machinery is recruited to the apical membrane infection site in epithelial cells infected by Enteropathogenic *E.coli*

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Epithelial polarity depends on correct protein trafficking to the apical and basolateral domains through coordinated sorting mechanisms. In order to understand basolateral protein sorting mechanisms we studied how Enteropathogenic *E.coli* (EPEC) infection of polarized epithelial cells alters cell polarity. EPEC is a foodborne pathogen that colonizes the epithelium of the small intestine by attaching to the apical side of epithelial cells. Infection leads to local disruption of cell polarity in the host cell by recruitment of basolateral proteins to the apical infection site and breakdown of cell-cell junctions.

To address the reversal of cell polarity we studied how EPEC directs basolateral proteins to the apical infection site. We found that two basolateral transmembrane proteins, AQP3 and E-cadherin, were recruited to the infection site. Both proteins were recruited in the presence of cycloheximide, indicating that newly synthesized proteins from the trans Golgi network were not the source of the recruited proteins. We also found that recruitment of AQP3 and E-cadherin took place during infection with a mutant EPEC strain that does not disrupt tight junctions suggesting that the recruitment is not achieved through membrane diffusion but rather via an intracellular route. We found that the exocyst component Sec8 is recruited to the bacterial attachment site in the apical membrane suggesting that vesicles from the TGN are redirected to

the infection site. In accord with this hypothesis, we found that Rab5, Rab7 and Rab9, markers of early and late endosomes, were all present at the infection site. Furthermore, VAMP-3, a t-SNARE, involved in docking and fusion of vesicles, was also located at the infection site suggesting that exocytosis occurs at the bacterial attachment site. Based on these results we propose that the major source of proteins recruited to the infection site is derived from the pool of existing proteins in the basolateral membrane by rerouting of endocytosed proteins.

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The zebrafish *kimble* mutation disrupts post-Golgi transport and is required for chondrocyte cell survival and endochondral ossification.

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Vesicular trafficking is a fundamental cellular process and is central to maintaining organization and function along the secretory pathway. Chondrocytes are especially dependent upon this process due to their high secretory activity in building and maintaining cartilage ECM. Using a forward genetics approach in zebrafish, we have identified a mutation in the exocytic pathway that leads to widespread chondrocyte cell death. Using electron microscopy (EM) and immunofluorescence we show that mutant chondrocytes initially secrete cartilage ECM proteins but then progressively accumulate vesicles at the cell cortex. TUNEL analysis indicates that these chondrocytes undergo apoptosis at later stages, resulting in cartilage elements devoid of functional chondrocytes. As a result, endochondral bones fail to form along mutant cartilage, although dermal bones do develop in *kimble* mutants. Additional EM analysis prior to onset of cell death shows that mutant chondrocytes assume a unique hourglass cell morphology in which the cell body and the nucleus are misshapen. Ultimately, dying chondrocytes accumulate condensed rough endoplasmic reticulum and multi-membrane vesicles, resembling autophagosomes. This process has been identified as a unique form of apoptosis previously termed chondroptosis. We are currently working to determine the mechanism by which this component of the exocytic pathway contributes to cell polarity and chondrocyte survival.

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Zebrafish *round* mutation reveals a novel component of post-Golgi trafficking machinery.

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Vesicular trafficking and secretion of extracellular matrix proteins are essential for cartilage formation and skeletal morphogenesis. Chondrocytes are highly secretory cell types that synthesize and transport a large number of diverse matrix proteins. In a large-scale mutagenesis screen for genes required for craniofacial morphogenesis, the zebrafish *round* mutations were identified. The mutation *round* (*rnd*^{m211}, *rnd*^{m641}, *rnd*^{m713}, *rnd*^{m715}) is an ENU induced, recessive mutation resulting in abnormal cartilage growth and differentiation. Using a positional cloning strategy, we have mapped the *round*^{m641} locus to a gene that is predicted to function as guanine nucleotide exchange factor (GEF) for Rab6, a small GTPase that directs targeting of secretory vesicles to plasma membrane. Histological and immunofluorescence analyses indicate that collagen secretion is disrupted in *round* mutant chondrocytes. Moreover, unlike other mutations in the pre-Golgi secretory pathway components, *round* mutation also leads to mislocalization of glycosylated plasma membrane proteins. These preliminary data strengthen the idea that *round* mutation may disrupt Rab6 activation, thereby impairing proper targeting of collagen carrying secretory vesicles to plasma membrane. We are using gene

network prediction and detailed phenotypic analysis to begin to construct a model for the developmental requirements of the round locus in extracellular matrix secretion.

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Protein sorting to dense core granules (mucocysts) in *Tetrahymena* depends on classical transmembrane receptors.

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Mucocysts in *Tetrahymena* are dense-core vesicles that dock at the plasma membrane and undergo rapid exocytosis to release their densely packed contents in response to extracellular stimulation. While the cargo proteins have been studied in detail, the basis for their sorting to mucocysts is unknown. We used an expression-based screen to detect genes that were co-regulated with genes encoding the known cargo proteins, and identified a small set of genes that are likely, based on the reported functions of their homologs in other organisms, to be involved in membrane trafficking. These genes include a family of four classical transmembrane receptors, called TSR1-4, which we have characterized by gene disruption and tagging. TSR3 is an essential gene and knockdown leads to gross perturbation of cellular endomembranes. TSR2 and 4, in contrast, appear to be specialized for mucocyst biogenesis. Cells that are individually disrupted in either TSR2 or TSR4 synthesize aberrant mucocysts that are incapable of undergoing efficient regulated exocytosis. TSR4 by itself is necessary for the trafficking to mucocysts of one class of cargo proteins, for which a sorting signal has been identified. In contrast, a second class of granule cargo proteins are sorted to mucocysts independently of these receptors. In addition, both TSR2 and TSR4 appear to serve as sorting receptors for proteases that function during mucocyst maturation in proprotein processing, for which strong candidates emerge from the expression-based screen. Live cell imaging of the GFP-tagged TSR4 product, expressed at the endogenous locus, also supports a model in which TSR receptors are essential for trafficking a subset of newly-synthesized mucocyst cargo. Since TSR receptors and other proteins identified in the screen have clear homologs in many eukaryotic lineages, the results also offer insight into the evolutionary relationship between regulated exocytic vesicles in ciliates and those in other organisms.

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AP-1A is required for normal secretory granule maturation in AtT-20 cells.

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Neuroendocrine cells synthesize, process, store and secrete peptide hormones. Upon exit from the trans-Golgi network, peptides and their processing enzymes are packaged into immature secretory granules (ISGs), which are not responsive to secretagogues. The maturation process through which they become secretagogue responsive remains unclear. Peptidylglycine- α -amidating monooxygenase 1 (PAM-1), a transmembrane protein spanning the membrane of SGs, can serve as an indicator of SG maturation. Upon entry into ISGs, PAM-1 is cleaved in the luminal domain generating a soluble protein, PHM, and a membrane protein, PALm; secretion of soluble PHM from mature SGs is secretagogue-responsive. The formation of specialized SGs such as the glue granules in *Drosophila*, Weibel-Palade bodies in endothelial cells and rhoptries in *Toxoplasma gondii* requires the adaptor protein 1A (AP-1A) complex. AP-1A is a heterotetrameric complex composed of two large subunits (β 1 and γ), a medium subunit (μ 1A)

and a small subunit (σ_1). AP-1A interacts with cargo proteins via μ 1A or γ/σ_1 and with clathrin via β 1 to transport proteins between the TGN and endosomes in clathrin-coated vesicles. In neuroendocrine cells, ISG maturation involves AP-1A mediated removal of non-granule proteins. To explore the role of AP-1A in the formation and maturation of SGs in neuroendocrine cells, AtT-20 corticotrope tumor cells expressing PAM-1 were stably infected with lentivirus expressing shRNA against μ 1A or a non-target shRNA control. Western blots revealed that the average level of μ 1A was reduced by 50% in fifteen clones when compared with control and non-infected cells. Cellular PHM and PALm was reduced compared to PAM-1 levels in μ 1A knock-down (μ 1A KD) cells suggesting a change in PAM-1 routing in the regulated secretory pathway. PHM secretion in response to stimulation by 2mM BaCl₂ was reduced to half in μ 1A KD cells. Pulse/chase metabolic labeling and immunoprecipitation revealed similar PAM-1 synthetic rates in control and μ 1A KD cells. After a 2 hour chase, secretion of newly synthesized PHM by control cells was stimulated by secretagogue; in contrast, secretion of newly synthesized PHM produced in μ 1A KD cells was not stimutable. These data indicate that AP-1A is required for the normal trafficking of PAM and the subsequent maturation of SGs in neuroendocrine cells.

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The relationship between post-translational processing of proglucagon and sorting to secretory granules in neuroendocrine cells.

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Introduction: Proglucagon is an endocrine prohormone that is processed into three major peptide hormones: glucagon, glucagon-like peptide 1 (GLP-1) and GLP-2. Proglucagon is processed in a tissue-dependent manner, to yield glucagon in pancreatic α -cells, and both GLP-1 and GLP-2 in intestinal L-cells and certain brainstem neurons. Regulated secretion depends upon both post-translational processing and sorting into secretory granules. Proglucagon is initially cleaved at the di-basic Lys70Arg71 site to yield glicentin and major-proglucagon fragment, and both are further processed to the major peptide hormones within granules. We hypothesize that cleavage at Lys70Arg71 is necessary for sorting into granules. We mutated the Lys70Arg71 cleavage site to neutral Gln70Gln71 to determine the requirement of post-translational processing of proglucagon on sorting into granules. Results: Sequences encoding either wild-type hamster proglucagon or the Gln70Gln71 mutant were fused to the N-terminus of EGFP and verified by DNA sequencing. Proglucagon-EGFP fusion proteins were expressed in the neuroendocrine cell line, PC12. EGFP alone was used as a negative expression control. Western blot analyses of EGFP in cell extracts revealed that WT proglucagon was cleaved at Lys70Arg71 while cleavage was blocked in the Gln70Gln71 mutant. Additional bands were consistent with cleavage at the C-terminal Lys159Lys160 mediated by CPE, and at Lys62Arg63 mediated by PC2. Detection of these cleavage products is consistent with proper sorting of WT and Gln70Gln71 mutant proglucagon into granules. Sorting of both the WT and mutant proglucagon into granules was seen by deconvolution immunofluorescence microscopy. Both WT proglucagon and the Gln70Gln71 mutant exhibited significant co-localization with the granule marker, chromogranin A, as measured by Pearson's correlation coefficient (PCC > 0.8). No significant differences (two-tailed t-test, p=0.282) between WT-proglucagon (n=19, PCC=0.84) and the Gln70Gln71 mutant (n=28, PCC=0.80) were observed. Conclusion: Our results indicate that proglucagon is efficiently sorted into granules independent of post-translational processing at Lys70Arg71.

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Pancreatic beta cells lack the GAD65-independent membrane anchoring pathway for GAD67.

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The two isoforms of the GABA-synthesizing enzyme glutamic acid decarboxylase, GAD65 and GAD67, are expressed in pancreatic beta cells and in GABA-ergic neurons. GAD65 is a target of autoimmunity associated with beta cell destruction and development of type 1 diabetes and in impairment of GABA-ergic neurotransmission in a rare neurological disorder stiff-man syndrome. In contrast, the highly homologous GAD67 isoform is not a target of autoimmunity. The two isoforms differ mainly in the N-terminal domain that in GAD65 harbors signals, including palmitoylation of cysteines 30 and 45, required for targeting to Golgi membranes and post-Golgi trafficking to cytosolic vesicles in non-neuronal cells and synaptic vesicles in neuroendocrine cells. In contrast, GAD67 protein remains hydrophilic but has been shown to acquire membrane association by two different mechanisms. One mechanism involves association of GAD67 with the hydrophobic GAD65 isoform in a heterodimeric conformation. The second mechanism is independent of GAD65 and may involve association with another membrane moiety. Each mechanism mediates robust membrane anchoring and targeting of GAD67 to Golgi membranes and cytosolic vesicles, similar to that of GAD65 itself. In this study, we have shown that while GAD67-GFP singly transfected into GAD65-negative MDCK, CHO, and COS-7 cells is targeted to the Golgi compartment and peripheral vesicles, it remains soluble and cytosolic in the insulinoma cell lines INS-1 and MIN-6. Furthermore, studies of transfected GAD67-GFP and endogenous GAD67 in GAD65-negative mouse islet single cells show that the protein is absent from Golgi membranes and vesicular compartment and does not co-localize with markers for ER, mitochondria, and insulin. These results suggest that insulinoma cell lines and mouse islet single cells do not harbor the GAD65-independent membrane anchoring machinery identified in neurons and several non-neuronal cell types. However, membrane targeting of GAD67-GFP in INS-1 and mouse islet single cells can be rescued by co-expression with GAD65-mCherry. Moreover, in rat islet single cells, which express both GAD65 and GAD67 isoforms, endogenous GAD67 co-localizes with GAD65 in the Golgi compartment and cytosolic vesicles.

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ADAP1 and Arf6 Regulate Neuronal Secretory Granule Trafficking.

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ADP ribosylation factor 6 (Arf6) is a member of the Arf family of small GTPases shown to function in vesicular membrane trafficking and cytoskeletal organization. Arf6 cycles between its GTP and GDP bound states, facilitated by GTP exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively. Our laboratory has investigated the neuronal functions of Arf6 and ADAP1, an Arf6 GAP expressed in neurons. We demonstrated that ADAP1 and Arf6 co-localize with secretory granules in neurons, and that siRNA-mediated knockdown of ADAP1 or Arf6 levels resulted in increased secretory granule size and an inhibition of the rate of trafficking of chromogranin B, a regulated secretory granule cargo. Recently, we investigated the role of ADAP1 and Arf6 in trafficking of brain derived neurotrophic factor (BDNF), a factor which has been shown to be involved in neuronal development and synaptic plasticity. In

neurons and neurosecretory cells, both ADAP1 and Arf6 co-localize with BDNF in secretory granules. Furthermore, knock down of ADAP1 or Arf6 leads to a significant increase in the colocalization of BDNF-GFP with furin, a peptidase localized to immature secretory granules, suggesting that ADAP1 and Arf6 participate in the maturation of BDNF secretory granules. Together our data indicate a role for ADAP1 and Arf6 in the trafficking of chromogranin B and BDNF-containing regulated secretory granules in neurons.

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Membrane trafficking of the D1 dopamine receptor to primary cilia.

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Spatial organization of signaling receptors on the cell surface and within the cell is critical to how cells execute and regulate signaling events. Signaling receptors must be targeted to the appropriate membrane domain through membrane trafficking events in order to carry out their function. The primary cilium, an antennae-like protrusion of the plasma membrane, is an example of a membrane domain with a high concentration of specific signaling receptors. One signaling receptor that localizes to the cilium is the D1 dopamine receptor (D1), which is highly concentrated on the cilia membrane relative to the rest of the plasma membrane. Although recent studies have identified several proteins that may be involved in trafficking signaling receptors to cilia, the basic process by which specific proteins are targeted to the cilium remains unclear. Photoactivation of a subset of the ciliary D1 indicates that D1 is highly mobile within the cilium, however, the photoactivated D1 fluorescence stays constant within the cilium over shorter time periods. These data support the idea of a diffusion barrier at the base of the cilium. Interestingly, D1 does not appear to be static in the cilium over longer time periods since more D1 can be photoactivated 30 minutes after exhaustive photoactivation of ciliary D1. My preliminary studies supports linear time dependence for the amount of new D1 in the cilium suggesting active transport to the cilium at a rate of about 1.5 percent new receptor delivered per minute. Comparison of the cell surface pool of D1 to the photoactivated D1 fluorescence before and after subsequent photoactivation indicates that new ciliary D1 traffics to the cilium from the plasma membrane as opposed to internal vesicles from the biosynthetic pathway. Future studies will further investigate the mechanism by which the D1 receptor concentrates in the cilium, contributing towards our understanding of how signaling receptors localize and function in primary cilia.

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Linking vesicle formation to vesicle targeting through multifunctional scaffold proteins.

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The delivery of the sensory receptor rhodopsin to the base of the cilium is mediated by traffic intermediates known as rhodopsin transport carriers (RTCs). Formation of RTCs is regulated by the protein complexes coordinated by Arf4 and the Arf GAP ASAP1. ASAP1 acts both as a scaffold for protein complex assembly that links the post-TGN carriers to the Rab11a-Rabin8-Rab8 ciliary targeting complex and a temporal regulator through GTP hydrolysis on Arf4. In this study we performed the Proximity Ligation Assay (PLA) in photoreceptor cells to reveal precise subcellular localization of rhodopsin interactions with its trafficking regulators in situ. Interactions of rhodopsin and ASAP1 with Arf4 were detected nearly exclusively at the Golgi/TGN, whereas rhodopsin-ASAP1-Rab11a-Rabin8-Rab8 interactions encompassed the TGN-derived nascent buds and the RTCs. Since ASAP1 recognizes the FR ciliary targeting motif of rhodopsin, we generated the [FR-AA]Rhodopsin-GFP-VxPx mutant and compared its localization in IMCD3 cells to that of Rhodopsin-GFP-VxPx. Ciliary localization of Rh-GFP-VxPx was completely

abolished in the FR-AA mutant. We compared the direct binding of Rh-GFP-VxPx, or the FR-AA mutant to ASAP1 by PLA in IMCD3 cells and found that the FR-AA mutant is defective in ASAP1 binding. Similar to ASAP1, Rab8 bound Rh-GFP-VxPx, but did not interact with the FR-AA mutant, which accumulated below the cilium. Our data indicate that the advancement of rhodopsin from the Golgi/TGN into post-TGN carriers proceeds in several orderly stages and that the assembly of the ASAP1-Rab11-Rabin8-Rab8 complex at the TGN is a crucial step in directing rhodopsin to the ciliary membrane. Since rhodopsin FR-AA mutant defective in ASAP1 binding also fails to interact with Rab8 and reach the primary cilium, our data further indicate that the vesicle formation at the TGN is tightly linked to the engagement of the targeting complex to ensure their proper delivery to the final destination.

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Shared Protein Complexes of Primary Cilia Link Craniofacial Disorders and Polycystic Kidney Disease.

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Primary cilia are specialized cellular organelles that are central to organ development and homeostasis based on their function as a signaling hub in the control of cell growth and differentiation. Craniofacial disorders and polycystic kidney disease frequently present together in human patients and are likely precipitated by defects in a common pathway affecting the primary cilium. How the soluble ciliary protein that underlies oral-facial-digital syndrome type 1 (OFD1) interconnects to the ciliary membrane proteins (polycystin-1 and polycystin-2) that cause polycystic kidney disease is unknown. To probe these interconnections, comparative evaluations of protein localization, trafficking and assembly were conducted on renal epithelia and oral cavity-derived odontoblasts. Direct PCR-based visualization of ciliary protein-protein interactions confirmed by co-immunoprecipitations identified a multimeric protein complex consisting of the polycystins, epidermal growth factor receptor, and OFD1. Ciliary protein complex assembly was dependent on the cholesterol membrane domain organizing, flotillin-1 and -2 proteins, demonstrating for the first time the spatial organization of key signaling proteins in specialized ciliary microdomains. Temporal analyses of membrane trafficking revealed polycystin-1 dependence on a C-terminal VxPx amino acid motif and the Arf4 GTPase for Golgi export and ciliary delivery. A conserved VxPx motif was also identified in the epidermal growth factor receptor and served to bind purified, active Arf4. The composite data support a conserved ciliary trafficking mechanism and assembly of a signaling protein complex in discrete microdomains in renal and odontoblast cells, defects in which may explain overlapping pathologies of kidney and craniofacial disorders.

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Tubulin detyrosination promotes monolayer formation and apical trafficking in epithelial cells.

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The role of posttranslational tubulin modifications in the development and maintenance of a polarized epithelium is not well understood. We studied the balance between detyrosinated (glu-) and tyrosinated (tyr-) tubulin in the formation of MDCK cell monolayers. Increased quantities of detyrosinated microtubules were detected during assembly into confluent cell sheets. These tubules were composed of alternating stretches of glu- and tyr-tubulin. Constant induction of tubulin tyrosination, which depleted the number of glu-tubulin by overexpression of tubulin

tyrosine ligase (TTL), disrupted monolayer establishment. Glu-tubulin depleted cells assembled into isolated islands and developed a prematurely polarized architecture. Thus, tubulin detyrosination is required for the morphological differentiation from non-polarized cells into an epithelial monolayer. Moreover, membrane trafficking, in particular to the apical domain, was slowed down in TTL-overexpressing cells. This effect could be reversed by TTL knockdown, which suggests that glu-tubulin-enriched microtubules serve as cytoskeletal tracks to guide membrane cargo in polarized MDCK cells.

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Regulation of vesicle transport to the apical membrane by kinesins in epithelial cells.

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Epithelial tissue function is required for maintaining homeostasis of the body, acting as a barrier to outside environments and allowing for the unidirectional transport of molecules. To establish this barrier, epithelial cells must polarize to create an apical domain and a basolateral domain. These domains differ in function by maintaining a unique lipid and protein composition. Previous work in the lab has characterized the transport of the p75 neurotrophin receptor (p75), which is targeted apically in polarized Madin-Darby Canine Kidney (MDCK) cells. Post-Golgi transport of p75 to the cell surface occurs along microtubules by different kinesin motor proteins in non-polarized and polarized cells. In non-polarized cells, the kinesin-3 family members Kif1A and Kif1B β mediate post-Golgi transport. In polarized cells, the kinesin-1 family member Kif5B mediates post-Golgi transport. These kinesins are expressed in both non-polarized and polarized cells, so this switch in kinesin use must be regulated. Currently, what regulates these kinesin-vesicle interactions is unknown. We hypothesize that these kinesin-vesicle interactions are regulated by three potential mechanisms: kinesin interaction with different Rab GTPases, kinesin interaction with different adaptor proteins, and kinesin autoregulation. To identify new trafficking regulators, we plan to compare the proteomes of different post-Golgi kinesin cargoes. We have begun by isolating post-Golgi vesicles from non-polarized and polarized cells. Cell homogenates are fractionated on a density gradient and identified by known organelle markers. We then isolate kinesin cargoes by using vesicle affinity for bead-immobilized Kif1A or Kif5B tails. p75-GFP containing vesicles act as a control for kinesin specificity. These isolates will be analyzed by mass spectrometry to identify differences between non-polarized and polarized kinesin-specific cargoes. Proteomes can then be compared for similarities and differences to identify potential trafficking regulators.

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The Rab GTPases Ypt31/32 and Sec4 may simultaneously associate with myosin V during secretory vesicle transport.

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Vesicle transport requires four steps; vesicle formation, movement, tethering and fusion. We recently found that in yeast, the myosin V, Myo2 is present at all steps. Through its cargo-binding domain, Myo2 interacts with several proteins that function in this pathway^{a,b}. Two Rab GTPases, Ypt31/32 are required for post-Golgi vesicle formation. A third Rab GTPase, Sec4, and the exocyst act in tethering and fusion of these vesicles with the plasma membrane. Myo2 binds directly to Ypt31/32, Sec4 and Sec15. These results suggest the testable hypothesis that Myo2 binds to each protein sequentially. Conventional biochemical approaches or static images of micrographs will not suffice to test the hypothesis, because most of the vesicles form and

complete their movement within a few seconds. Note that the average velocity of a Sec4-positive secretory vesicle is 3 $\mu\text{m/s}$ *in vivo*, and a yeast is approximately 5 μm .

As a first approach, we generated a yeast strain that expresses both GFP-Ypt31 and RFP-Sec4 expressed *via* their own promoters. We obtained the unexpected result that Ypt31 and Sec4 always co-localize on vesicles during vesicle movement. This finding raises the possibility that Myo2 simultaneously binds to Ypt31/32 and Sec4 throughout vesicle formation and fusion.

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^bLipatova, Z., Tokarev, A.A., Jin, Y., Mulholland, J., Weisman, L.S., and Segev, N. (2008). Direct interaction between a myosin V motor and the Rab GTPases Ypt31/32 is required for polarized secretion. *Mol Biol Cell* 19, 4177-4187.

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The Epac1-Rap1 pathway regulates Weibel-Palade body exocytosis from endothelial cells through the activation of Rac1 via PREX-1.

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Vascular endothelial cells provide a dynamic interface between circulating blood and underlying tissues that is critically involved in maintaining vascular integrity and homeostasis. Rapid release of bio-active components from intracellular storage pools serves a crucial role in maintaining vascular homeostasis. A significant number of haemostatic components and inflammatory mediators originate from endothelial cell-specific, cigar-shaped organelles called Weibel-Palade bodies (WPBs). WPBs function as storage vesicles for von Willebrand factor (VWF), a multimeric adhesive glycoprotein crucial for platelet plug formation, the leukocyte receptor P-selectin and a number of bioactive compounds that include the chemoattractants IL-8 and eotaxin-3. WPBs release their content following stimulation with agonists increasing intracellular Ca²⁺, like thrombin, or agonists increasing intracellular levels of cAMP, such as epinephrine. The physiological importance of the cAMP-mediated pathway is illustrated by the rise in VWF levels in patients with von Willebrand's disease and mild haemophilia A following administration of the vasopressin analogue desmopressin (DDAVP) or epinephrine.

Previously, we have shown that the exchange protein activated by cAMP, Epac1 and its substrate, the small GTPase Rap1 are involved in cAMP-mediated release of WPBs. In this study, we explored potential downstream-effectors of Rap1 in cAMP-mediated WPB release. Using a pulldown for putative downstream targets of activated Rap1 in endothelial cells we identified the PI3K-dependent Rac exchange factor 1 (PREX-1). We show that endothelial activation using epinephrine and forskolin, but also by the Epac1-specific cAMP-analogue 8-pCPT-2'-O-Me-cAMP-AM (Me-cAMP-AM) leads to the activation of the small GTPase Rac1. Furthermore, we show that RNAi-mediated downregulation of Epac1 or PREX-1 both result in a reduced activation of Rac1 in response to Me-cAMP-AM. However, to abolish activation of Rac1 in response to epinephrine preincubation with the PKA inhibitor PKI is also required suggesting that Rac1 can be activated by PKA- as well as Epac1-dependent mechanisms. Depletion of Rac1 by RNAi resulted in reduced epinephrine-induced VWF secretion. Also, the Rac1 inhibitor EHT1864 and PI3K inhibitor Ly294002 reduced epinephrine-induced WPB release. Taken together our findings suggest that Epac1-Rap1-mediated WPB release proceeds through activation of Rac1 by PREX-1, thereby regulating release of haemostatic, inflammatory and angiogenic components from WPBs.

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Roles of the Small G Protein Arl5b in Intracellular Membrane Transport.

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Many small G proteins, including Rabs and Arfs are involved in controlling the regulation of membrane transport in mammalian cells. A sub-class of Arfs, the Arf-like proteins or Arls, are less well defined. Arl1 is the most well characterised Arl, located at the Golgi. At the *trans*-Golgi network (TGN), activated Arl1 interacts with effector proteins called golgins, such as p230/golgin245 and golgin97 (Lu *et al* 2001, Lu *et al* 2003, Panic *et al* 2003). Arl1 is important for maintaining the Golgi structure and in the regulation of specific Golgi transport pathways. Here we have investigated other small G proteins and have identified Arl5b as a TGN localised protein (Houghton *et al* 2012). A stable cell line of HeLa over-expressing a GFP-tagged membrane-bound mutant of Arl5b (HeLaArl5b(Q70L)-GFP) was generated. The localisation of Arl5b(Q70L)-GFP using confocal and structured illumination microscopy (SIM) revealed the close association of Arl5b with specific TGN golgins at the TGN. Using a flow cytometric assay to assess the kinetics of anterograde transport, depletion of Arl5b by RNAi had no effect on the transport of the cargo E-cadherin to the cell surface. On the other hand, using 3D confocal microscopy, depletion of Arl5b significantly reduced the endosome-to-TGN transport of the membrane cargos TGN38 and Shiga toxin (STxB). The block in cargo transport to the Golgi was rescued with an siRNA-1-resistant Arl5b mutant. Enhanced transport rates of TGN38 were observed in cells overexpressing Arl5b-GFP. Collectively these results suggest that Arl5b is a TGN-localised small G protein that plays a key role in regulating transport along the endosome-TGN pathway. The influence of Arl5b in regulating the transport of a variety of cargos is currently under investigation.

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Examining the role of the yeast Lgl homolog, Sro7, as a Rab GTPase effector in polarized exocytosis.

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The tumor suppressor lethal giant larvae (Lgl) plays a crucial role in cell polarity and differentiation, however, the molecular mechanism by which Lgl carries out its function is unclear. Studies of the yeast Lgl homolog, Sro7, have demonstrated a function in polarized exocytosis that is linked to its regulated interaction with the SNARE protein, Sec9. Recent evidence suggests that Sro7 may also act directly in vesicle tethering as an effector of the Rab GTPase, Sec4, and has genetic properties consistent with it functioning downstream of Sec4 in a pathway parallel to that of the exocyst complex. However, the molecular details of how Sro7 acts as an effector of Sec4 and the importance of this physical interaction to both Sro7 and Sec4 function remain unclear. Using the recently determined crystal structure as a guide, we interrogated a set of 12 conserved surface patches on Sro7 to identify potential Sec4 docking sites. Proteins containing charge-reversal mutations at each of these sites were purified and examined for their ability to bind GTP-Sec4. While 10 of the 12 mutants had binding properties indistinguishable from wild-type Sro7, we identified two surface sites that when subjected to charge-reversal, showed a dramatic loss in binding to GTP-Sec4. These same mutant proteins bound normally to the t-SNARE, Sec9. Importantly, genetic analyses of the Sro7 charge-reversal mutants demonstrated a precise correlation between those mutants which had lost the ability to suppress temperature-sensitive growth of mutant exocyst strains and those mutants that had a severe defect in binding to GTP-Sec4. This provides the first direct evidence that

Sec4-GTP binding by Sro7 is likely to be critical for its function in vivo. Moreover, we are utilizing this structural data combined with computational docking studies to determine if these sites represent part of a binding pocket on Sro7 for Sec4-GTP. Understanding the structural, biochemical and genetic landscape of this interaction will reveal critical molecular insights into the function of an Lgl family protein in polarized trafficking.

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A Novel GTP-binding protein-adaptor protein complex responsible for export of Vangl2 from the trans Golgi network.

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Planar cell polarity (PCP) requires the asymmetric sorting of distinct signaling receptors to distal and proximal surfaces of polarized epithelial cells. We have examined the protein and sorting signal requirements for transport of one PCP signaling protein, Vangl2, from the trans Golgi network (TGN) in transfected mammalian cells. Using siRNA knockdown experiments, we find that the GTP-binding protein, Arfrp1, and the clathrin adaptor complex 1 (AP-1) are required for Vangl2 transport from the TGN. In contrast, Frizzled 6, which localizes to the opposing epithelial surface from Vangl2, does not depend on Arfrp1 or AP-1 for traffic to the cell surface. Mutagenesis studies identified a YYXXF sorting signal in the C-terminal cytosolic domain of Vangl2 that is required for Vangl2 traffic and interaction with the mu subunit of AP-1. We propose that Arfrp1 exposes a binding site on AP-1 that recognizes the Vangl2 sorting motif for capture into a transport vesicle destined for the proximal surface of a polarized epithelial cell.

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Atypical ubiquitination of CRN7 by Cul3-KLHL20 complex regulates post-Golgi carrier formation.

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Protein ubiquitination controls many aspects of membrane trafficking. KLHL20 is a BTB-kelch protein and functions as a substrate adaptor of Cul3-family ubiquitin ligases. Here, we show that KLHL20 is mainly localized on the Golgi apparatus, and is required for maintaining Golgi architecture and the actin-dependent post-Golgi transport of cargos. The Cul3-KLHL20 complex catalyzes a K33-linked polyubiquitination of coronin7 (CRN7), a protein shuttling between cytosol and Golgi and regulating post-Golgi transport. This ubiquitination event does not lead to CRN7 degradation but potentiates the recruitment of Crn7 to clathrin adaptor AP-1 complex. CRN7 regulates the assembly of F-actin foci at trans-Golgi network, thus facilitating the formation of carrier tubule. Our study reveals a crucial role of Cul3-KLHL20 in post-Golgi actin remodeling and carrier biogenesis through an atypical ubiquitination of CRN7.

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Transformation of Polarized Epithelial Cells by Apical Mistrafficking of Epiregulin.

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Establishment and maintenance of apico-basolateral trafficking pathways is critical to epithelial homeostasis. Although loss of polarity and trafficking fidelity is thought to occur as a

consequence of transformation, herein we show that selective mistrafficking of a growth factor from the basolateral to apical cell surface may drive transformation. The EGF receptor (EGFR) ligand epiregulin (EREG) is delivered preferentially to the basolateral cell surface of polarized MDCK cells. EREG basolateral trafficking is regulated by a conserved tyrosine residue within a YXX Φ motif (Y₁₅₆ERV) in its cytoplasmic domain. YXX Φ is an AP clathrin adaptor complex-dependent basolateral sorting motif whose function is impaired by phosphorylation of this tyrosine. Substitution of this tyrosine to alanine or phenylalanine redirects EREG to the apical cell surface. EREG basolateral trafficking is AP1B and AP2-independent and this tyrosine is phosphorylated. Moreover, apical mistrafficking of EREG has a distinctive phenotype. In contrast to transient EGFR activation after basolateral EREG stimulation, apical EREG leads to prolonged EGFR activation without negative regulatory Y1045 phosphorylation. MDCK cells stably expressing apically mistrafficked EREG form larger, poorly differentiated, invasive tumors in nude mice compared to wild-type EREG-expressing cells. This phenotype was recapitulated in Matrigel culture where apical mutant EREG increased the number of surviving luminal cells. We propose that apical mistrafficking of EREG crystallizes an apical EGFR signaling complex that is uncoupled from basolateral regulatory restraints.

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Actin-mediated traffic control: RhoA-mDia1 pathway involvement in regulating the secretion of monocyte-chemottractant protein 1.

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Formin family proteins promote actin polymerization by affecting both nucleation and elongation of actin filaments. Recently, RhoA-mDia1 pathway was shown to regulate Golgi architecture and dynamics [1]. Here, we have used chemokines as model exocytic cargo to elucidate the role of formins in Golgi to plasma membrane transport. Monocyte-chemottractant protein 1 (MCP-1/CCL2) is an important chemokine secreted during immune response. However, the role of actin, in general, and formins, in particular in regulating intracellular trafficking of this chemokine is poorly understood. We expressed fluorescently tagged CCL2 construct and observe its dynamics in transfected cells. Transfected CCL2 is localised to the Golgi and is transported in round vesicular carriers along microtubule tracks to the plasma membrane. Activation or RNAi silencing of the mDia1 formin, affects the speed and altered the morphology of the CCL2-positive carriers. Active RhoA or constitutively active mDia1 expression reduced the speed of CCL2-positive carriers while RNAi silencing of mDia1 apparently increase this speed. In cells overexpressing activated mDia1, an increase in intracellular CCL2 was detected in vesicular structures and these CCL2 positive vesicles were still localised on microtubule tracks but were unable to move towards the plasma membrane. Movements of CCL2 in cells expressing activated mDia1 were rescued by treatment with latrunculin A, an actin depolymerization agent, indicating the effect is due to actin polymerization. The changes in velocity coincide with the level of CCL2 secretion. In cells expressing activated mDia1, there was a 40% decrease in CCL2 secretion, whereas an increase of 50% in CCL2 secretion was observed in RNAi expressing cells. Notably, the drop in CCL2 secretion in cells overexpressing constitutive activated mDia1 was similar to that in control cells treated with 1 μ M of nocodazole, suggesting that mDia1 effect on the movement of CCL2 carriers along microtubules is responsible for the observed secretion changes. We hypothesized that RhoA-mDia1-driven activation of actin polymerization can be involved in physiological regulation of chemokine CCL2 secretion via controlling the microtubule based movement of exocytic carriers along microtubule track.

1. Zilberman Y, Alieva NO, Miserey-Lenkei S, Lichtenstein A, Kam Z, Sabanay H, Bershadsky A: Involvement of the Rho-mDia1 pathway in the regulation of Golgi complex architecture and dynamics. *Mol Biol Cell* 2011, 22(16):2900-2911.

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Tracking the role of endocytosis in the polarization of the Rho GTPase Cdc42.

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In budding yeast, the generation of a concentrated pool of Cdc42 on the plasma membrane is thought to be essential for its ability to control global cell polarity. Cdc42 establishes cell polarity by coordinating the organization of the actin cytoskeletal and membrane trafficking pathways towards a specific site on the plasma membrane. Consequently, the regulation of these two pathways by Cdc42 is tightly coupled with the maintenance of Cdc42's own polarization. However, much remains to be understood of the role of polarized exocytosis in the maintenance of the Cdc42 plasma membrane polarity patch. Exploiting a novel *in vivo* assay for secretory vesicle association, we have demonstrated that Cdc42 is associated with post-Golgi vesicles at concentrations that are comparable to, or greater than, its concentration at the polarity patch. This supports the possibility that fusion of vesicles carrying Cdc42 with specific sites on the plasma membrane can serve to promote, rather than dilute, the concentrated Cdc42 polarity patch. Previous studies have suggested that endocytosis may play an important role in recycling Cdc42 from the plasma membrane. Using the aforementioned assay, we screened through a large collection of endocytic genes and found that 1) disruption of several of these genes severely reduced Cdc42's association with post-Golgi vesicles and 2) this effect was independent of the cargo-specific adaptors yAP1801/1802 and the AP-2 complex. In contrast, we found that loss of the sole Rho GDI in yeast had no effect on vesicle association or concentration. These data support the notion that endocytic recycling of Cdc42 from the plasma membrane to post-Golgi vesicles may act distinctively from the GDI-mediated pathway in generating and maintaining Cdc42 polarity at the plasma membrane.

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Molecular dynamics of PCSK9 trafficking at the Golgi and its effect on LDLR degradation.

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Coronary heart diseases (CHD) are a leading cause of death in Western societies. Hypercholesterolemia is a major risk factor for CHD. It is characterized by high levels of circulating low-density lipoprotein cholesterol (LDL, also called "bad cholesterol"). The prolonged presence of LDL in the circulation increases the risk of the formation of atherosclerotic plaques, which can lead to obstruction of arteries and myocardial infarction. LDL is normally extracted from the blood by binding to the LDL receptor (LDLR) thereby mediating its endocytosis in the liver. Human genetic studies have identified PCSK9 as the third gene responsible of autosomal dominant hypercholesterolemia after LDLR and its ligand apolipoprotein B100. PCSK9 interacts with the LDLR and induces its degradation thereby causing plasma LDL levels to rise. PCSK9 gain-of-function (GOF) mutations are associated with elevated plasma LDL levels and premature CHD while PCSK9 loss-of-function (LOF) mutations reduce the risk of CHD by ~88% owing to a strong reduction of circulating LDL. Accordingly, PCSK9 is recognized as a major pharmacological target to lower the risk of CHD. PCSK9 binds the LDLR at the cell surface and/or in the Golgi apparatus of hepatocytes and causes its degradation in lysosomes by a mechanism not yet clearly understood. The goal of this study was to determine why some human PCSK9 mutations fail to induce LDLR degradation while

others increase it in lysosomes. Several PCSK9 LOF and GOF mutations were fused to the fluorescent protein mCherry to study their molecular mobility in living liver cells. Our quantitative analysis of fluorescence recovery after photobleaching (FRAP) showed that PCSK9 GOF mutations e.g., D129G, have a higher protein mobility (>35% compared to WT) at the trans-Golgi network (TGN). Their mobility was further increased (>90% compared to WT) in presence of a Golgi transmembrane protein that interacts with PCSK9. The LOF mutation R434W, which fail to accumulate in the TGN and to induce LDLR degradation, presented a much slower diffusion (<25% compared to WT). In addition, our confocal and electron microscopy analyses demonstrate for the first time that PCSK9 is localized in multivesicular bodies and concentrated in the TGN of human hepatocytes. Furthermore, our results demonstrate that PCSK9 localization in the TGN is mediated through its C-terminal cysteine-rich domain, which is essential for LDLR degradation. These results provide important new evidences on the mechanism of action of PCSK9 and may ultimately help in the development of inhibitors of the PCSK9-induced LDLR degradation.

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Testing the influence of a phospholipid flippase on membrane curvature in giant unilamellar vesicles.

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Vesicular transport of proteins between organelles is a process that is essential for cell health and viability. Integral membrane proteins, called phospholipid flippases, play important roles in the formation of these transport vesicles at sites of membrane budding. Flippases work by establishing and maintaining membrane asymmetry. This is achieved by translocation of specific phospholipids from the extracellular or luminal leaflet of the membrane bilayer to the cytosolic side. It is thought that the resulting imbalance in phospholipid number between the bilayers causes membrane bending, captured by accessory and coat proteins, and leads to the formation of mature cargo-containing transport vesicles. Drs2p is the founding member of these flippases (type IV P-type ATPases) in *Saccharomyces cerevisiae*, and is involved in clathrin-coated vesicle formation at the *trans*-Golgi network (TGN).

To elucidate the influence that flippase activity has on membrane morphology, we are reconstituting Drs2p in giant unilamellar vesicles (GUVs). Drs2p was purified using previously established protocols and was then reconstituted into proteoliposomes containing phosphatidylcholine (PC) and varying levels of phosphatidylserine (PS, the preferred substrate for Drs2p). These proteoliposomes were electroformed into GUVs that are 10-200µm in diameter and are visualized using fluorescence microscopy. Drs2p was activated by addition of ATP and imaged in real time to determine if there were any changes in membrane morphology. We observe that 20% PS-GUVs that contain active Drs2p tubulate after ATP addition. However, we continue to work on appropriate controls and improved imaging techniques to determine if the flippase activity of Drs2p is driving membrane tubulation. These studies should provide a better idea of the role that flippases play in the formation of transport vesicles.

Kinases and Phosphatases I

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Elucidating gsk-3 substrate and inhibitor binding sites as a tool for inhibitor refinement.

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a molecular understanding of substrate recognition of protein kinases provides an important basis for the development of substrate competitive inhibitors. here, we explored substrate recognition and competitive inhibition of glycogen synthase kinase (gsk)-3 β using molecular and computational tools. in previous work, we described gln89 and asn95 within gsk-3 β as important substrates binding sites. here, we show that the cavity bordered by loop 89-qdkrfkn-95, located in the vicinity of the gsk-3 β catalytic core, is a promiscuous substrate binding subsite. mutations within this segment highlighted phe93 as an additional essential contact residue for substrates' recognition. however, unlike gln89 and asn95, phe93 was also important for the binding of our previously described substrate competitive inhibitor, I803 [keappappqs(p)p], and its cell-permeable variant I803-mts. the effects of the substitution of charged or polar residues within I803 further suggested that binding to gsk-3 β is governed by hydrophobic interactions. our computational model of gsk-3 β bound to I803 was in agreement with the experimental data. it revealed I803 binding with a hydrophobic surface patch and identified interactions between pro8 (I803) and phe93 (gsk-3 β). computational modeling of new I803 variants predicted that inhibition would be strengthened by adding contacts with phe93 or by increasing the hydrophobic content of the peptide. indeed, the newly designed I803 variants showed improved inhibition. our study identified different and overlapping elements in gsk-3 β substrate and inhibitor recognition and provides a novel example for model-based rational design of substrate competitive inhibitors for gsk-3.

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GSK3 modulates tau phosphorylation in an age dependent manner.

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Alzheimer disease (AD) and related tauopathies are characterized by progressive neurodegeneration involving abnormal phosphorylation of the microtubule associated protein tau. Glycogen synthase kinase (GSK3) has been identified as one of the major candidates mediating tau hyperphosphorylation. In AD postmortem brain, tau phosphorylation has been found augmented at several epitopes modified by GSK3. GSK3 activity is modulated by the canonical Wnt signaling pathway. Moreover, the expression of the Wnt antagonist Dickkopf-1 (Dkk1) is increased in brains of AD patients and in AD transgenic mouse models, suggesting that dysfunction of Wnt signaling could contribute to AD pathology. However, the role of Wnt signaling dysregulation on GSK3 activity and its consequences on tau hyperphosphorylation during aging is still unknown. Thus, the aim of this study is to investigate the effects of the specific inhibitor of GSK3 (6BIO) and Dkk1 on tau phosphorylation in some sites relevant for AD (Ser199/202/214/396/404) in a model of metabolically active hippocampal slices from young and aged rats. We found that GSK3 inhibition significantly decreases tau phosphorylation on Ser199/202/396 and 404 in hippocampus from both, young and aged rats. These results suggest that the phosphorylation of those epitopes strongly depends of GSK3 activity. In contrast, phosphorylation of tau Ser214 was not affected by pharmacological inhibition of GSK3 or by DKK1, implicating that this epitope does not directly depend of GSK3 activity. While inhibition of Wnt signaling by Dkk1 increased tau phosphorylation in GSK3-dependent sites in

hippocampal slices from old rats, in young rats tau phosphorylation was not significantly modified. At present our results support the notion of a site and age dependent GSK3 regulation of tau phosphorylation.

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An Alzheimer's Associated Neurotoxic Lipid Remodels Phosphatidylinositol-4,5-bisphosphate and Inhibits Tor Signaling in *Saccharomyces cerevisiae*.

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Increasing evidence suggests that the alkylacylglycerophospholipid, PC(O-16:0/2:0), contributes to the neurotoxicity observed in Alzheimer's disease (AD). Our previous work revealed a role for phospholipase D (PLD) in buffering the toxic effects of PC(O-16:0/2:0) in yeast and human neurons but suggested other signaling pathways may also be required to buffer the toxic effects of PC(O-16:0/2:0). To directly address this we compared transcriptional changes between untreated and PC(O-16:0/2:0) treated *Saccharomyces cerevisiae*. This analysis revealed a significant enrichment in TOR-dependent transcripts including those involved in ribosomal biogenesis and the cellular stress response. Genetic evidence confirmed a role for TOR signalling in PC(O-16:0/2:0)-mediated toxicity as TOR2 temperature sensitive alleles exhibited increased sensitivity to the lipid. In agreement with these findings, biochemical assays revealed a pronounced reduction in Sch9 and Ypk1 phosphorylation, targets of TORC1 and TORC2 respectively, in PC(O-16:0/2:0) treated cells. The phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) binding proteins Slm1/2 play a critical role in regulating TORC2-mediated phosphorylation of Ypk1/2 therefore we investigated the effects of PC(O-16:0/2:0) treatment upon the distribution of PI(4,5)P₂ and the key metabolic enzyme Mss4. Interestingly, live cell imaging revealed that both PI(4,5)P₂ and GFP-tagged Mss4 were redistributed to distinct phosphatidylinositol enriched structures (PES) in the presence of PC(O-16:0/2:0) but not other lipids, chemicals or culture conditions. In addition, the effect of PC(O-16:0/2:0) appears to be specific to certain membrane components as we have not observed an effect upon many other membrane associated proteins. Congruent with our findings, Slm1-GFP and the TORC2 complex member Bit61-GFP, but not Ypk1, were found to relocalize to the PES suggesting that PC(O-16:0/2:0) may disrupt the recruitment of substrates, including Ypk1, to TORC2. We further report that remodelling of PI(4,5)P₂ and associated proteins is dependent upon PC(O-16:0/2:0)-induced changes in sphingolipid biosynthesis. In conclusion, our work identifies PC(O-16:0/2:0) as a novel mediator of PI(4,5)P₂ distribution and Tor signalling in yeast. Further work will be needed to determine whether AD associated changes in PC(O-16:0/2:0) metabolism are involved in precipitating the dysregulation of PI(4,5)P₂ metabolism which is also observed in patients with AD.

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Efficient traffic to the vacuole is necessary for TORC1 activity.

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The target of rapamycin (TOR) is a conserved Ser/Thr kinase that regulates cell growth and metabolism in response to environmental factors like nutrient availability and stress. The direct target of Tor complex 1 (TORC1) in the yeast *Saccharomyces cerevisiae* is the Sch9 protein, which contains six amino acids in the C terminus, that are directly phosphorylated by Tor1p.

Phosphorylation of Sch9p is lost when cells are treated with rapamycin, an inhibitor of Tor1. Tor1 inactivation also results in cell cycle arrest and activation of macroautophagy. A genome screening for vacuolar trafficking defects in our lab resulted in 13 mutants hypersensitive to hygromycin B (*hhy* mutants). All *HHY* genes are involved in vesicular traffic and all *hhy* mutants show Tor1 mislocalization in the presence of hygromycin. Among them, 9 exhibit even increased sensitivity to hygromycin and are referred to as super-hypersensitive to hygromycin (*shhy*) mutants. All *SHHY* gene functions converge at the trans Golgi and late endosome interface and *shhy* mutants exhibit increased sensitivity to rapamycin treatment, indicating compromised Tor signaling. Treatment of *shhy* mutants with hygromycin for four hours results in an inability to restart growth, further suggesting TORC1 signaling defect. In this study, we probe the state of TORC1 activity in *shhy* mutants in the presence and absence of hygromycin B. In order to study TORC1 activity, we monitored the phosphorylation of Sch9p. *shhy* mutants showed compromised Tor1 kinase signaling, which becomes more accentuated in the presence of hygromycin B. Since TORC1 inactivation results in activation of autophagy, we decided to test autophagy in those *shhy* mutants. All *shhy* mutants showed a defect in autophagy when tested in starvation plates with phloxine B. As Tor1 vacuolar localization is compromised in *shhy* mutants, these results suggest that physical Tor1 presence at the vacuole is necessary for TORC1 activity as well as onset of autophagy. Rapamycin and hygromycin are antibiotics produced by the same bacterium *Streptomyces hygroscopicus*, and although they use different mechanisms of action, they both affect the TORC1 pathway in yeast. Our results combined suggest that intact traffic at the trans Golgi and vacuole interface is necessary for Tor1 localization and function, and that hygromycin is targeting that interface in *shhy* mutants.

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Control of the *S. cerevisiae* Fps1 glycerol channel by its regulator Rgc2 and the MAPK Hog1.

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Under conditions of hyper-osmotic stress, many fungi maintain osmotic equilibrium by producing and retaining high concentrations of glycerol as a compatible solute. Intracellular glycerol concentration is regulated in *S. cerevisiae* by the plasma membrane aquaglyceroporin, Fps1. Increased external osmolarity induces Fps1 closure, whereas decreased osmolarity causes channel opening, both within seconds of the change in external osmolarity. This channel is required for survival of a hypo-osmotic shock when yeast cells must export glycerol rapidly to prevent bursting. Relative to other characterized aquaglyceroporins, Fps1 possesses N-terminal and C-terminal cytoplasmic extensions that are important for its regulation. The pathway responsible for Fps1 regulation in response to changes in osmolarity has not been delineated, but involves the MAPK Hog1 (High Osmolarity Glycerol response), which is known to bind to the N-terminal domain of Fps1. Fps1 activity is also controlled by a pair of positive regulators, Rgc1 and Rgc2 (for Regulators of the Glycerol Channel), which are phosphorylation targets of Hog1. Loss of either FPS1, or RGC1 and RGC2 function results in excess turgor pressure and consequent cell wall stress. Here we demonstrate how Hog1 modulates Fps1 channel activity through Rgc2.

We identified seven S/T-P phosphorylation sites in Rgc2 by mass spectrometry, some of which were demonstrated in a quantitative analysis to be Hog1-dependent and induced by hyper-osmotic shock. Mutation of these phospho-sites to Ala (Rgc2-7Ala) caused a constitutively

open Fps1 channel phenotype, supporting the conclusion that Hog1 drives Fps1 closure by phosphorylation of Rgc2. We found by co-immunoprecipitation experiments that, in the absence of osmotic stress, Rgc2 associates with the C-terminal domain of Fps1 through its Pleckstrin Homology (PH) domain. In response to hyper-osmotic shock, Rgc2 dissociated from Fps1 in a Hog1-dependent manner. Consistent with this result, the Rgc2-7Ala mutant failed to dissociate from Fps1 in response to hyper-osmotic shock, suggesting that Fps1 is maintained in the open state through its association with Rgc2. This conclusion was supported by the finding that a PH domain mutant of Rgc2, which fails to associate with Fps1, displayed a closed channel phenotype. Finally, an N-terminally truncated Fps1 mutant, which lacks its Hog1-binding site, was stably associated with Rgc2 in response to hyper-osmotic shock. This supports a model in which Hog1 must be poised on the N-terminus of Fps1 to respond rapidly to osmotic stress by phosphorylating Rgc2 bound to the C-terminus of Fps1, thereby driving release of Rgc2 and allowing Fps1 closure.

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Ectopic activation of cell wall integrity MAP kinase pathway from endosomal compartments in *Saccharomyces cerevisiae* upon phosphatidylinositol (4,5)-bisphosphate depletion.

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Class I phosphatidylinositol 3-kinase (PI3K) catalyzes the conversion of PtdIns(4,5)P₂ into PtdIns (3,4,5)P₃. In mammalian cells, this second messenger controls important functions, like cellular proliferation and inhibition of apoptosis. In consequence, hyperactivation of this protein is commonly observed in tumors. The model yeast *Saccharomyces cerevisiae* constitutively lacks class I PI3K activity. Heterologous expression of hyperactive versions of this protein in yeast leads to the growth inhibition due to the depletion of the essential plasma membrane pool of PtdIns (4,5)P₂. This yeast model has proven to be useful for applied purposes such as the screening of PI3K inhibitors, but also provides a tool to study the roles of PtdIns(4,5)P₂ in the yeast cell. A global transcriptomic analysis upon mammalian PI3K expression in *S. cerevisiae* lead to a pattern reminiscent of that of cell wall stress conditions. Consistently, PI3K but not a kinase-dead mutant version triggered the phosphorylation of the cell wall integrity (CWI) MAPK, as well as the expression of a typical CWI transcriptional reporter. Consistent with loss of PtdIns(4,5)P₂, depolarization of the actin cytoskeleton and a retard in the internalization of the vital endocytic marker FM4-64 were observed. Interestingly, instead of marking polarized sites at the cellular cortex, the upstream CWI pathway component Pkc1, which is recruited to sites of Rho1 activation, was abnormally located to intracellular compartments that were associated to Ypt31/32-containing endosomes. We propose that loss of essential cortical phosphoinositides triggers alternative activation of the CWI pathway from endosomal compartments.

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Independent pathways downstream of the Wnd/DLK MAPKKK regulate synaptic structure, injury signaling and axonal transport.

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MAP Kinase signaling cascades orchestrate diverse cellular activities with common molecular players. To achieve specific cellular outcomes in response to specific signals, scaffolding proteins play an important role. Here we investigate the role of the scaffolding protein JIP1 in neuronal signaling by a conserved axonal MAP Kinase Kinase Kinase (MAPKKK), known as

Walleranda (Wnd) in *Drosophila*, DLK in vertebrates and *C. elegans*. Recent studies in multiple model organisms suggest that Wnd/DLK regulates both regenerative and degenerative responses to axonal injury. Here we report a new role for Wnd in synaptic development, which implies that Wnd is also active in uninjured neurons. This synaptic role of Wnd can be functionally separated from Wnd's role in axonal regeneration and injury signaling by the requirement for the JIP1 scaffold and the p38b MAP kinase. JIP1 and p38b mediate the synaptic function of Wnd, but are not required for injury signaling or new axonal growth after injury. Previous studies indicate that JIP1 is transported to the presynaptic axon terminus by the kinesin-1 motor, and mutations in either *jip1* or *wnd* cause defects in axonal transport. Our genetic analysis indicates that JIP1's role in axonal transport can also be functionally separated from its signaling role in synaptic development. We conclude that Wnd regulates multiple independent pathways in *Drosophila* motoneurons, and that JIP1, which localizes to presynaptic axon terminals, scaffolds a specific downstream cascade required for the organization of presynaptic microtubules during synaptic development.

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Dephosphorylation Pattern of MAPK in Single Oocyte after Fertilization.

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The mitogen-activated protein kinase (MAPK) pathway is a highly conserved signal transduction cascade regulating cell proliferation, differentiation and apoptosis. In oocytes of many animals, the MAPK pathway is activated during meiosis, followed by inactivation after fertilization. The timing of MAPK dephosphorylation after fertilization is studied in single starfish oocytes for the first time. The starfish is an excellent model system for this study because large numbers of immature oocytes can be obtained at prophase of meiosis I. Maturation is initiated upon addition of 1-methyladenine and the oocytes become competent for fertilization within 40 minutes. Using the metric of fertilization envelope elevation, fertilization is tightly synchronized in an egg population and samples can be easily taken every few minutes. In this study, single starfish eggs or zygotes were taken from the larger population and snap frozen in liquid nitrogen for analysis of MAPK phosphorylation by western blotting. Compared with the gradual decrease in MAPK phosphorylation observed in groups of oocytes, the dephosphorylation appears more abrupt in single oocytes. In eight oocytes which were simultaneously taken at 20 minutes after fertilization, four of them show very weak but detectable signal MAPK activity, while the other four oocytes still display high level of MAPK activity. In the view of groups of oocytes, the percentage of the oocytes with MAPK dephosphorylation is increased as the time going after fertilization. In the first 10 minutes after fertilization, less than 10% of the oocytes show reduced MAPK phosphorylation. This increases to approximately 70% within at 40 minutes after fertilization. At the time of 60minutes after fertilization, 100% of the oocytes accomplish MAPK dephosphorylation. The single cell system applies a more accurate way to study the mechanisms of MAPK pathway regulation. It also helps to also hoped that this system will reveal the negative regulation pathway of the MAPK cascade following fertilization in the starfish.

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The Role of MAPKKs in Self-Renewal and Pluripotency of Mouse Embryonic Stem Cells.

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The ERKs1/2 MAP kinases (MAPKs) are involved in many cellular processes such as cell proliferation, cell differentiation, cell movement, and cell death. ERKs 1/2 are activated by MAPK kinases (MAPKKs), which in turn are activated by MAPKK kinases (MAPKKKs). Raf family, including A-Raf, B-Raf and C-Raf, are the MAPKKKs in activating ERKs1/2. Ying et al.

reported that the self-renewal of mouse embryonic stem (ES) cells is enabled by the elimination of differentiation-inducing signaling from MAPKs. Yet, the effects of MAPK inhibition on modulating self-renewal and pluripotency of mouse ES cells are still unknown. Here we individually knocked-down A-Raf, B-Raf and C-Raf in D3 mouse ES cell line. We found that the activation of ERKs1/2 in ES cells was not affected by these single knockdowns. Therefore, the A, B, C-Raf triple knockdown ES cells were generated, and the activation of ERKs1/2 was significantly down-regulated in the triple knockdown cells compared with that in control cells. We are currently generating the double knockdown of varied combinations of Rafs in ES cells and exploring the role of these MAPKKs on self-renewal and pluripotency of ES cells.

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1494

Combined transcriptome and proteome analysis of POPX2 related signaling pathways in breast cancer

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POPX2, a serine/threonine phosphatase belonging to the PP2C family, has been implicated in the regulation of breast cancer cell motility and invasiveness. The levels of POPX2 were high in the more invasive MDA-MB-231 cells compared with low invasive MCF7 breast cancer cells. Silencing POPX2 in the MDA-MB-231 cells resulted in lower motility and invasiveness. However, the underlying mechanism was not known. In order to find the POPX2 substrates and further map POPX2 related signaling pathways in breast cancer, a combined transcriptome and proteome study was carried out by using DNA microarray and SILAC (Stable Isotope Labeling of Amino acid in Cell culture)-based mass spectrometry, respectively. A combined KEGG pathway analysis was undertaken to determine complementary mechanisms of regulation between the transcriptome and proteome. A high percent of genes and proteins were distributed in pathways related to cancer, MAPK signaling, actin cytoskeleton regulation and the cell cycle. Focal adhesion regulation was found to be the only commonly enriched KEGG pathway. The role of POPX2 in focal adhesion and actin cytoskeleton regulation were further validated by immunostaining. Since the method we exploited for proteome fractionation also enriched the phosphopeptides, the phosphoproteome was analyzed. We found MAPK1 (ERK2) and MAPK3 (ERK1) to be regulated by POPX2 signaling. In addition, a MAPK1/3 sub-network was found to be over-represented through protein network analysis.

1495

The Nuclear Translocation of JNK and p38 MAPKs.

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Rapid and massive nuclear translocation of signaling proteins is an important step in the induction of transcription upon extracellular stimulation. Despite the importance of this process, the molecular mechanisms that govern this process have been elucidated only for few signaling components. In these cases, signaling proteins that utilize the classical nuclear localization

signal (NLS) interact with Imp α and Imp β , to facilitate their nuclear translocation. However, it is clear today that many other signaling proteins translocate to the nucleus upon stimulation using distinct, NLS-Imp α/β - independent mechanisms.

In a search for NLS-independent shuttling proteins, we have resorted to the MAPK family members JNK and p38. Unlike ERK1/2, the subcellular localization of JNKs and p38s has not been properly established so far. In this study we show that in resting cells, JNK1/2 and p38 α/β are localized mainly in the cytoplasm, and translocate to the nucleus upon stimuli, independent of their activation. We further found that despite the pronounced similarity between the MAPK family members, none of the JNK or p38 proteins contain the ERK1/2-NTS in their KID regions. Furthermore, mutations in the aligned residues of this region resulted in a marginal effect on the nuclear translocation of JNK1/2 and p38 α/β , indicating that the mechanism of the translocation is not only NLS- but also NTS- independent. We hypothesized that the nuclear translocation is still dependent on other, ill-defined, b-like importins. Therefore, we used Co-IP and SiRNA experiments with all these importins, and found that the translocation of JNK1/2 and p38 α/β is mediated through their interactions with either Imp7 or Imp9, which require further dimerization with Imp3. Thus, the stimuli-dependent nuclear translocation of these MAPKs is mediated by the dimerization of different b-like importins. As such, it consists an unexplored layer of transcriptional regulation.

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Engineered manipulation of signaling networks: Novel control of kinase activation and interactions dissects parallel Src pathways.

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Spatio-temporal control of signaling is orchestrated by a complex network of interactions between scaffolding and enzymatic components. Current tools typically cannot activate a specific protein and simultaneously cause it to interact with one specific downstream target, which would be of great value in dissecting the organization of signaling networks. Here we provide such a tool, and use it to determine the role of individual Src-mediated signaling pathways in stimulation of different cell morphological changes. Protrusive activity of a cell and its adhesion to extracellular matrix is regulated by Src via multiple interactions. Focal adhesion kinase (FAK) and p130Cas can bind and activate Src, propagating signals through parallel pathways. Localization of Src in the cytoplasm or at the plasma membrane also determines the function of Src. Thus, we focused specifically on activation of Src acting through FAK or through p130Cas, or at different locations (cytoplasm versus plasma membrane). An engineered, "insertable" FKBP12 protein (iFKBP) was introduced into the catalytic domain of Src, while FKBP12-rapamycin binding domain (FRB) was attached to the specific downstream effector. This rendered the kinase inactive until rapamycin, added to the extracellular medium, induced heterodimerization with FRB. Using this technology we restricted Src activation to the complex it formed with FRB-bearing downstream targets. Simple activation of Src, without targeting specific downstream molecules, led to cell spreading, reorganization of focal adhesions, and the production of filopodia and lamellipodial protrusions. Using the new approach, dubbed RapR-TAP, we showed that activation of Src specifically in complex with FAK led to focal adhesion rearrangement and only slow lamellipodial protrusion, while activation in complex with p130Cas led to rapid spreading and filopodia formation, but no apparent effects on focal adhesions. Comparing Src activation in the cytosol versus at the plasma membrane showed that membrane localization is necessary to stabilize protrusions, whereas cytoplasmic Src drives

rearrangement of focal adhesions. The novel method presented here isolates specific differences between parallel Src-induced signaling events, and demonstrates the feasibility of a broadly generalizable strategy to activate specific kinase-mediated signaling pathways in living cells.

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Investigation of Src regulation.

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Src activity is regulated by its autophosphorylation on Tyr416 and phosphorylation of the C-terminal Tyr527, though the efficiency of these reactions has never been examined. To study regulation of Src activity we have employed a recently published method by Karginov et al. (2010). By inserting FK506-binding protein 12 (FKBP) into the kinase domain (RapR-kinase), stability and thus kinase activity is diminished. Rapamycin treatment and heterodimerisation with FRB (FKBP12-rapamycin binding domain of mTOR) increases rigidity and re-establishes kinase activity, producing an engineered allosteric switch. The kinase activity was monitored via substrate phosphorylation, but the question about the phosphorylation state of Src kinase itself remained open. We wanted to investigate whether phosphorylation of the inhibitory C-terminal Tyr527 and activatory Tyr416 of RapR-Src changes when the kinase is turned on by addition of rapamycin and FRB. Using the allosteric switch we have examined the phosphorylation of Tyr527 and Tyr416 in RapR-Src^{WT} and RapR-Src^{D388R} (kinase-dead) in the destabilised and induced state. Against expectations the phosphorylation levels did not seem to change upon addition of rapamycin and FRB. Surprisingly the kinase-dead Src appeared to be only marginally phosphorylated on both Tyr416 and Tyr527. Tyr416 is usually autophosphorylated by Src in trans. Since RapR-Src^{KD} is not catalytically active, it can't autophosphorylate on Tyr416. Although Src^{KD} is still a substrate for endogenous Src and can be phosphorylated, endogenous levels are insufficient to phosphorylate much of the overexpressed RapR-Src^{KD}. Tyr527 is phosphorylated mainly by C-terminal Src kinase (Csk). Since the kinase-dead Src should still be available as a substrate, this lack of phosphorylation begs the question of whether its own activity might be needed. It has been postulated that Src family kinases exist in a complex with the membrane-bound Cbp/PAG (Csk binding protein) (Ingley et al, 2006). The active kinase phosphorylates Cbp at Tyr317, thus inducing recruitment of cytoplasmic Csk to the complex. Csk can then phosphorylate the C-terminal tyrosine of the kinase and inhibit its activity. I'm currently carrying out further experiments to investigate the recruitment mechanism of Csk in Src regulation.

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Critical Role for Histone Deacetylase 6 (HDAC6) in the Regulation of IL-6, and the JAK/STAT3 Signaling Cascade in Macrophages.

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Histone Deacetylases (HDACs) have divergent effects over the production of anti- and pro-inflammatory cytokines in antigen-presenting cells (APCs). We have previously shown that modulation of specific HDACs can alter the immunogenicity of APCs, either to an activating or tolerogenic phenotype. Recently, we identified HDAC6 as a positive regulatory factor in the production of IL-10. However, the participation of this HDAC in other immune related cellular processes remains unknown. In this work we present evidence of the important role of HDAC6 in the modulation of the JAK/STAT3 pathway through the IL-6 regulation. We generated knockdown cell lines of HDAC6 (HDAC6KD) and non-target (NT) cells as a control in RAW264.7 murine macrophages using lentiviral shRNA. Two HDAC6KD and two NT cell lines were treated with LPS or were left untreated and then analyzed by microarray. In HDAC6KD cells we found 1542 genes were down-regulated and 775 up-regulated in HDAC6KD cells. Their ontology distribution revealed significant changes in immune-related (632) and apoptosis/cell cycle control (47) genes. Most importantly, IL-6 was one of the most highly down-regulated genes in HDAC6KD cells. Significantly down or up regulated genes were analyzed by their ontology distribution and selected genes were validated by qRT-PCR, ELISA, or immunoblots. Our analysis revealed that the STAT3 and SP1 target genes were down regulated in HDAC6KD cells. Also, we observed by immunoprecipitation that the acetylation status of these proteins was diminished in HDAC6KD cells when compared against control (NT) cells which had been treated with LPS. Next, we analyzed the relevance of these findings by studying the tolerogenic JAK/STAT3 signaling pathway, which is known to be activated by IL-6 and critical in the final outcome of APCs in response to stimuli. Our observations included a complete abrogation in the phosphorylation of JAK2 and STAT3 proteins in HDAC6KD cells in response to LPS, which was reverted when these cells were treated with exogenous IL-6. Our final results demonstrate a critical role of HDAC6 in the modulation of important regulatory cytokines in macrophages, such as IL-6 and IL-10, and the potential role of HDAC6 in the regulation of the JAK/STAT3 pathway. These findings provide insight into the molecular mechanisms controlling the immunogenicity of APCs, supporting the use of HDAC6 inhibitors to enhance immune activation, positioning HDAC6 as a potential therapeutic target.

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In vitro reconstitution of T cell receptor signaling.

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The mechanism by which a T cell is activated by a foreign antigen is still poorly understood. The earliest signaling event after TCR-antigen binding is the phosphorylation of the cytosolic tails (termed ITAM motifs) of the TCR complex, which leads to the recruitment and activation of a cytosolic kinase ZAP70 and subsequent downstream signaling events. The phosphorylation state of the ITAMs is controlled by a complex network formed by the membrane associated Src family kinase (SFK) Lck, the transmembrane tyrosine phosphatase CD45 and the inhibitory kinase Csk. Antigen-TCR engagement perturbs this network leading to net phosphorylation. However, the quantitative behavior of the kinase/phosphatase network that controls ITAM

phosphorylation is still poorly understood, in part because of many complexities in the system including the allosteric regulatory mechanism of Lck, positive and negative roles of CD45, and the spatial distribution of these proteins in the cellular environment. In order to better understand the behavior of the kinase/phosphatase network that controls the phosphorylation of ITAMs, we have reconstituted purified kinases and phosphatase of the TCR signaling network onto artificial liposomes. To measure phosphorylation of membrane-bound TCR zeta chain, we have developed a fluorescence based assay. Using this assay, we show that kinase and phosphatase reactions occur ~700-fold faster on membranes than in solution. By measuring TCR zeta chain phosphorylation as a function of both kinase and phosphatase concentrations, we document the behavior of the TCR/kinase/phosphatase network and produce a phase diagram for TCR phosphorylation. We also have dissected the effect of mutations that alter the allosteric regulation of the Lck kinase, and demonstrate stimulation of TCR phosphorylation when TCR and the Lck are co-clustered on the artificial liposomes. These studies provide a framework for better understanding the biochemistry of TCR triggering.

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The relationship between expression of A-Kinase Anchoring Proteins and phosphorylation of Akt/PKB in neonatal rat Schwann cell proliferation.

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Development of the peripheral nervous system is dependent upon the proliferation of Schwann cells which rely on the heregulin/neuregulin family of growth factors secreted by neurons. In neonatal rat Schwann cell cultures heregulin stimulates robust cell division only if forskolin, an agent that elevates cAMP is also added. The molecular mechanism mediating this synergistic response is unknown. Earlier investigations have revealed that A-Kinase anchoring proteins (AKAPs) of the cAMP/PKA pathway play an important role in mediating Schwann cell growth. Results from previous studies have shown that transfection of Schwann cells with SiRNA oligos synthesized against AKAP150 and AKAP95 reveal a reduction in protein levels of both AKAPs, associated with a decline in cell proliferation and a decrease in expression of Akt/PKB. The goal of this study was to determine if there was an association between the expression of AKAPs and phosphorylation of Akt when Schwann cells were stimulated with heregulin and forskolin. To perform this study, neonatal rat Schwann cells were cultured with no mitogens (control) followed by incubation with heregulin, forskolin or heregulin and forskolin (H+F). Immunoblot analysis of heregulin-stimulated cells revealed an upregulation of AKAP95 (85.69% ± 10.07) in comparison to treatment with forskolin (74.37% ± 21.61) or H+F (61.08% ± 19.97). This upregulation was accompanied by an increase in phospho-Akt expression (88.87% ± 22.70) for heregulin-stimulated cells, in contrast to treatment with forskolin (61.45% ± 14.11) or H+F (65.48% ± 10.71). Alternatively, the expression of AKAP150 was significantly increased in forskolin-treated cells (74.37% ± 21.61) in comparison to heregulin (63.48 ± 15.56), or H+F (51.84% ± 16.16) stimulation. Forskolin-treated cells also showed a significant increase in Akt (114.49% ± 33.83) in comparison to heregulin (79.95% ± 20.46) and H+F treatment (86.48% ± 31.62). However, as the levels of AKAPs decreased, the percent expression of phospho-Akt over Akt in heregulin (115.72% ± 11.5) or H+F treatment (111.43% ± 20.59) increased, in contrast to forskolin-treated cells (73.38% ± 9.62). In summary, mitogen-stimulated Schwann cells exhibited a decrease in the expression of both AKAPs, but an increase in phosphorylation of Akt. These preliminary observations suggest that a relationship between expression of AKAP proteins and phosphorylation of Akt may be necessary to mediate Schwann cell proliferation when stimulated with heregulin and forskolin.

Post-Translational Modifications in Signaling

1501

Lysine Protein Acetylation During NGF-Stimulated Neuriteogenesis Differentiation Of PC12 Cells.

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Neuriteogenesis is a key cellular process necessary for proper development of the adult nervous system and neuronal regeneration. Neurite extension from the soma depends on precise cytoskeletal and adhesion dynamics induced by sensing of extracellular signals. This process is similar to directional pseudopodial formation involving regulated actin-mediated membrane protrusion and signal transduction processes. Neuronal differentiation relies on a complex signaling network. Reversible acetylation and deacetylation of certain proteins may play important role(s) in this regulation. Alterations in these pathways may result into deregulation of neuriteogenesis and neuroregeneration. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) catalyze the acetylation and deacetylation, respectively, of proteins at Lys (K) residues. HDAC inhibitors having neuroprotective, neurotrophic, and anti-inflammatory effects can correct these deficiencies and has emerged as a promising new strategy for therapeutic intervention in neurodegenerative disease. The interest of this work is to understand role of proteins acetylation/deacetylation functions, in neuronal differentiation, employing rat adrenal pheochromocytoma (PC 12) cell line. These cells resemble the phenotype of sympathetic ganglion neuron upon differentiation with NGF. PC 12 cell stop dividing and terminally differentiate when treated with nerve growth factors (NGF). These make PC 12 as a useful model system for neuronal differentiation. The NGF differentiated PC12 cells were less susceptible to cytotoxic effects of TSA, a pan inhibitor of Class 1 and 2 HDACs. TSA treatment did not affect the overall process of NGF-stimulated neuriteogenesis in PC12 cells. Longer TSA treatment durations of >24 hr did not show significant effect on status of protein acetylation as probed by anti-acetylated lysine antibody. During short treatment durations (1-8 hr) there was an increase in signals for acetylated proteins in cells treated either with TSA alone or with TSA and NGF compared with control and cells treated with NGF. Two low molecular weight proteins were found to show a significant increase in signals along with few other unknown protein bands. However, there was no significant change in the acetylating pattern in protein for cells treated with NGF compared to untreated undifferentiated control cells. There was no change in the expression level of HDAC2 a nuclear enzyme and HDAC6 a cytosolic enzyme during NGF stimulated neuronal differentiation and also in the presence of TSA. TSA treatment resulted in significant increase in numbers of neuronal nodes, a parameter measured for relative branching of neurons. The results indicate the role of lysine protein acetylation in neuriteogenesis. Characterization of hyperacetylated proteins, besides histones, will provide useful information on role of this post-translational signal transduction pathway in the process of neuronal differentiation.

1502

Evidence for the recycling of Porcupine: a Conserved Membrane-bound O-acyltransferase Involved in the Post-translational Lipid Modification of Wnts.

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Wnts are secreted signal molecules that are important for patterning of tissues during embryogenesis and cell homeostasis. Wnt secretion is dependent on the lipidation of one or two conserved residues. Lipidation of the conserved serine is facilitated by Porcupine (Porcn), a membrane-bound O-acyltransferase. To better understand the role of Porcn in Wnt secretion, we sought to characterize the subcellular localization of overexpressed and endogenous Porcn. Immunohistochemical studies from our lab and others have shown that tagged-Porcn variants are localized to the Endoplasmic Reticulum (ER), where it is thought to palmitoylate Wnt proteins. Interestingly, biochemical studies from our lab also show the presence of Porcn in lipid rafts and on the cell surface. These data led us to hypothesize that Porcn undergoes dynamic membrane trafficking in cells. To test this hypothesis, we first developed polyclonal rabbit antibodies against Porcn. Antibodies were then validated for specificity to Porcn using competitive assays with Porcn peptide in Western blot and immunohistochemical assays. We then used this antibody to define the subcellular localization of endogenous Porcn. After optimizing fixation and solubilization procedures, we immunostained COS7 cells and collected images via confocal microscopy. In contrast to overexpressed Porcn, the majority of endogenous Porcn was detected in a Golgi-like structure. Overexpressed GalT-GFP, which localizes to the Golgi, overlaps with endogenous Porcn; further supporting the presence of Porcn in the Golgi. Additionally, staining of cells treated with Brefeldin A, a compound that promotes the retrograde transport of Golgi proteins to the ER, shows a loss of Golgi specific Porcn staining and further confirms the localization of Porcn to the Golgi. To test whether endogenous Porcn is found in lipid rafts, we immunostained cells for Flotillin-1 (a lipid raft marker) and Porcn. Endogenous Porcn in cell lines partially co-localizes with Flotillin-1, thus, supporting the localization of Porcn to lipid rafts. Together, these findings suggest Porcn undergoes dynamic membrane trafficking in the Wnt secretion pathway and suggest the possibility that Porcn has functions that extend beyond its role in the ER.

1503

Identification of N-terminal residues of Sonic hedgehog important for palmitoylation by Hedgehog Acyltransferase.

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Sonic Hedgehog (Shh) is a secreted morphogen that regulates embryonic development. After removal of the signal peptide, Shh is processed to the mature, active form through autocleavage and a series of lipid modifications, including the attachment of palmitate. Covalent attachment of palmitate to the N-terminal cysteine of Shh is catalyzed by Hhat (Hedgehog acyltransferase) and is critical for proper signaling. The sequences within Shh that are responsible for palmitoylation by Hhat are not known. Here we show that the first six amino acids of mature Shh (CGPGRG) are sufficient for Hhat-mediated palmitoylation. Alanine scanning mutagenesis was used to determine the role of each amino acid and the positional sequence requirements in a cell-based Shh palmitoylation assay. Mutation of residues in the "GPGR" sequence to Ala had no effect on palmitoylation provided that a positively charged residue was present within the first 7 residues. A neighboring positively charged residue could compensate for the loss of the

other N-terminal positive residue. A similar requirement for N-terminal positively charged residues was also observed in Spitz, a second substrate of Rasp, the *Drosophila melanogaster* Hhat homolog. The N-terminal position exhibited a strong, but not exclusive requirement for Cys. Constructs with an N-terminal Ala were not palmitoylated. However, an N-terminal Ser served as a substrate for Hhat, but not Rasp, highlighting a critical difference between the mammalian and fly enzymes. These findings define residues and regions within Shh that are necessary for its recognition as a substrate for Hhat-mediated palmitoylation. Finally, we report the results of a bioinformatics screen to identify other potential Hhat substrates encoded in the human genome.

1504

Regulation of protease-activated receptor-1 signaling by extracellular loop 2 N-linked glycosylation: A possible bias signaling "switch"?

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Protease-activated receptor-1 (PAR1) is a G protein-coupled receptor (GPCR) irreversibly proteolytically activated by thrombin. Thrombin binds to and cleaves the N-terminus of PAR1 unmasking a new N-terminus that functions as a tethered ligand by binding intramolecularly to the extracellular surface of the receptor to initiate signaling. N-linked glycosylation is an enzymatic process that links glycans to asparagine residues within the NXS/T consensus sequence (X=any amino acid except proline). To study the effects of PAR1 extracellular loop 2 (ECL2) N-linked glycosylation in signaling we generated asparagine to alanine mutants. Here, we report that the PAR1 ECL2 domain is highly modified by N-linked glycosylation. Moreover, the PAR1 ECL2 glycosylation mutant displayed an enhanced efficacy in Gq mediated thrombin signaling. The increase in PAR1 ECL2 mutant signaling was not due to defects in desensitization, receptor cleavage kinetics, or recycling back to the cell surface after activation. In addition, there was only a modest affect on activated receptor internalization. Interestingly, in converse the PAR1 ECL2 mutant displayed a significant decrease in G12/13 mediated RhoA activation. We employed BRET to study possible differences in receptor interactions with various adaptor proteins. Our BRET data revealed differences in PAR1 wildtype versus ECL2 mutant G13 protein interaction, as well as beta-arrestin 2 associations. Thus, these findings suggest that PAR1 N-linked glycosylation at ECL2 affects the conformation of the receptor that results in differential coupling to signaling effectors and components of the endocytic machinery leading to the altered signaling and trafficking.

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1505

Ciliopathy proteins regulate broad paracrine signaling by context-specific proteasomal degradation of signaling mediators

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The ciliopathies are a group of disorders caused by structural or functional defects in cilia and their anchoring structures, the basal bodies. The proteins which are causally linked to ciliopathies are important for paracrine signal transduction. To understand the mechanism by which ciliopathy proteins regulate signaling transduction, we first examine the function of BBS1

and BBS4, two ciliopathy proteins causing Bardet-Biedl syndrome (BBS), in Notch signaling. Here we show that loss of BBS1 or BBS4 leads to persistent Notch signaling in zebrafish embryos and cells, resulting from the stabilization of the Notch receptor intracellular domain (NICD) and JAG1 ligand. These findings mirror the accumulation of β -catenin in the absence of BBS proteins, leading us to posit a broad proteasome-dependent role for these proteins. Consistent with this notion, we were able to ameliorate BBS-established Notch and Wnt signaling defects, either by overexpression of proteasome activator proteins, or by treating cells and embryos with the chemical proteasome agonist sulforaphane (SFN). These observations were not unique to BBS and Notch signaling. Similar accumulation of Sonic hedgehog components GLI2/3FL and SUFU was observed in neurons from *Odf1* (another ciliopathy protein) mutant mice and could be rescued by SFN. Moreover, we tested the effect of loss of basal body proteins on NF- κ B, which has not been associated previously with basal body dysfunction. Loss of BBS1, BBS4 or OFD1 leads to decreased NF- κ B activity and concomitant I κ B β accumulation, which can also be ameliorated with SFN. Finally, we show that both BBS4 and OFD1 interact with proteasomal subunits. Depletion of ciliopathy proteins disturbs proteasome function by changing the centrosomal distribution of proteasome regulatory subunits and its composition in whole 26S proteasome. Taken together, our data indicate that basal body-proteasome regulation is a common mechanism governing the regulation of paracrine signaling, and suggest that activation of the proteasome might be of clinical benefit to some ciliopathy patients.

1506

New insights into nicotinamide signaling associated with insulin production in pancreatic beta cells.

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Objective: Nicotinamide has pleiotropic effects on pancreatic beta cells such as protecting against cell damages and promoting functional differentiation. In this study, we tried to clarify the nicotinamide signaling networks in pancreatic beta cells.

Methods: We immobilized nicotinamide on magnetic nanobeads (Tamagawa Seiki), and then screened for nicotinamide-binding proteins from cell lysates of rat pancreatic beta cell line INS-1. The binding molecules were purified and identified using mass spectrometry and the identified molecules were analyzed their association with nicotinamide using specific antibodies and recombinant proteins. We also investigated gene expression profiles in INS-1 cells and mouse islets with or without nicotinamide treatment using quantitative real-time PCR and oligo microarrays (Affymetrix Rat 230 2.0 or Mouse 430 2.0, respectively).

Results: About 100 nicotinamide-binding factors including RNA- or DNA-binding proteins were identified, and the associations of some of the identified molecules with nicotinamide were confirmed *in vitro*. Microarray analysis revealed Nicotinamide-induced changes of the expression in a variety of genes, ranging from transcription factors, protein folding factors to anti-apoptotic factors, and quantitative PCR analysis characterized the fluctuations of the gene expression after nicotinamide treatment. Nicotinamide treatment increased the gene expressions of *Ins2* and *MafA*, which is a crucial transcription factor for insulin gene expression. In addition, insulin content in INS-1 cells was also increased by nicotinamide treatment.

Conclusion: We identified nicotinamide-binding factors and nicotinamide-regulated genes in pancreatic beta cells, which would provide important clues for nicotinamide signaling networks for beta cell functions, especially for insulin production in the present study.

1507

Nardilysin regulates the mammalian circadian clock via modulating PER2 stability.

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Circadian rhythms are regulated by endogenous molecular oscillators called circadian clocks. In mammals, the transcription factor heterodimer CLOCK:BMAL1 activates the expression of Period and Cryptochrome genes. PER and CRY proteins in turn heterodimerize and repress CLOCK:BMAL1 activity to inhibit their own expression. In addition to the core transcriptional feedback loop, posttranslational modifications of clock proteins are crucial for the correct operation of molecular clockworks. For example, phosphorylation of PER proteins by casein kinase I (CKI), which affects nuclear translocation and timely degradation via ubiquitin-proteasome pathway, is a vital part of clock regulation. However, it remains unclear how CKI are regulated. Here, we show that a metalloendopeptidase nardilysin (N-arginine dibasic convertase; NRDC) acts as a potent circadian clock regulator via modulating PER2 stability. Circadian period shortened in NRDC deficient (NRDC-KO) mice. In livers from NRDC-KO mice, nuclear PER2 proteins were retained longer than wild-type (WT) mice. When expressed in cells, NRDC formed a complex with PER2. Moreover, co-expression experiment showed that NRDC makes a complex with PER2 and CKI δ /CKI ϵ . These results suggest that NRDC may regulate PER2 stability by modulating CKI activity.

1508

Alterations in mTOR pathway signaling following status epilepticus in immature vs. mature rats.

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Chemoconvulsants such as pilocarpine are used to induce episodes of prolonged continuous seizure activity (status epilepticus, SE). In adult rats, this initial insult leads to the generation of unprovoked seizures (epilepsy). A similar insult in immature rats does not lead to spontaneous seizures later in life; however, the insult does result in long-lasting cellular and molecular changes that alter hippocampal plasticity in adulthood. While the effects of SE on mTOR signaling in the adult brain have been widely studied, relatively less is known about the effects of SE on mTOR signaling in the immature brain. We examined hippocampal mTOR pathway activation through the phosphorylation (P) of the downstream molecules ribosomal S6 protein, eukaryotic initiation factor binding protein 1 (4EBP1), and AKT, in developing and adult rats following pilocarpine-induced SE compared to age-matched shams. We performed a time course of mTOR activation over development to guide our choice of time points for the SE experiments. Western blotting revealed a significant decrease in hippocampal levels of P-S6, P-4EBP1 and P-AKT over postnatal development. However, at postnatal day (PD) 10 these levels remained significantly elevated compared with PD35 and adult (>6 months) (P14 days) increase in the levels of P-S6 and P-4EBP1, and a transient decrease in P-AKT (3 days) (n=5-8). In conclusion, the effects of SE on mTOR activation are different in immature vs. mature

hippocampus. While additional investigations are necessary to further characterize this effect, our findings may have implications for therapeutics in epilepsy.

1509

CDK1 cooperates with PLK1 to ensure the temporal regulation of kinetochore microtubule dynamics and spindle checkpoint.

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Chromosome segregation during mitosis involves a dynamic interaction between spindle microtubules and kinetochore, a macromolecular complex that localizes at the centromeres of mitotic chromosomes. Cdk1 controls many aspects of mitotic chromosome behavior and spindle microtubule dynamics to ensure accurate chromosome segregation. PLK1 is a highly conserved mitotic kinase and activation of PLK1 requires phosphorylation of the T-loop. Here we show that phosphorylation of PLK1 T-loop by CDK1 in conserved Thr214 is essential for temporal control of kinetochore microtubule dynamics and spindle checkpoint signaling. Phosphorylates PLK1 at Thr214 suppresses microtubule dynamics and promotes the stabilization of initial kinetochore-microtubule attachments in prometaphase. Using fluorescence resonance energy transfer-based reporter of PLK1 activity at the kinetochore and quantitative analysis of native PLK1 substrate phosphorylation, we show that constitutively phosphorylation of Thr214 reduces inter-kinetochore tension and turnover of kinetochore microtubules, resulting in increased microtubule attachment errors and a checkpoint dependent mitotic arrest. Therefore, we reason that phosphorylation of PLK1 by CDK1 is essential to ensure the temporal regulation of kinetochore microtubule dynamics and spindle checkpoint control.

1510

Redistribution of the fission yeast Clp1/Cdc14 phosphatase upon genotoxic stress is influenced by multiple protein kinases.

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The Cdc14 phosphatases, a proline directed protein phosphatase family, are important for mitotic exit and antagonize Cdk1 phosphorylation. In order to gain access to their substrates, Cdc14 phosphatases must be released from nucleolar sequestration during mitosis. Additionally, Clp1/Flp1, the *Schizosaccharomyces pombe* Cdc14 ortholog, and Cdc14B, a mammalian ortholog, are relieved of nucleolar sequestration in interphase upon DNA replication stress or damage, respectively. This suggests that Cdc14 phosphatases contribute to the cellular response elicited upon genotoxic insults. A complete mechanistic understanding of how Cdc14 phosphatases are released from their nucleolar sequestrations under these conditions is incomplete. We show that redistribution of Clp1 upon genotoxic stress is governed by complex phosphoregulation. We found that the cell wall integrity mitogen-activated protein kinase, Pmk1, the cell cycle kinase, Cdk1, and the Rad3 checkpoint effector kinases, Cds1 and/or Chk1, phosphorylate Clp1 directly promoting genotoxic stress-induced nucleoplasmic accumulation. More specifically, Pmk1 and Cdk1 phosphorylate Clp1 TP sites preferentially upon hydrogen peroxide treatment, while Cds1 and/or Chk1 preferentially phosphorylate RxxS sites upon hydroxyurea treatment. Abolishment of both Clp1 TP and RxxS phosphosites eliminates the redistribution of Clp1 upon genotoxic stress. Alternatively, preventing Clp1 autodephosphorylation at Clp1 TP sites results in constitutive nucleoplasmic localization. Also, we provide further mechanistic information and insight into cell cycle mediated nucleolar release

of Clp1. Our data elucidates a better understanding of pathways contributing to Clp1 localization, which may shed light on mechanisms controlling Cdc14B phosphatases in higher eukaryotes.

1511

4E-T Phosphorylation by JNK Promotes Stress-dependent P-body Assembly.

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Processing Bodies (P-bodies or PBs) are cytoplasmic granules involved in mRNA storage and degradation that participate in the regulation of gene expression. PBs concentrate non-translated mRNAs and several factors involved in mRNA decay and translational repression, including the eIF4E-transporter (4E-T). 4E-T is required for PB assembly, but little is known about the molecular mechanisms that regulate its function. Here, we demonstrate that oxidative stress promotes multisite 4E-T phosphorylation. We show that the c-Jun N-terminal kinase (JNK) is targeted to PBs in response to oxidative stress and promotes the phosphorylation of 4E-T. Quantitative mass spectrometry analysis reveals that JNK phosphorylates 4E-T on six proline-directed sites that are required for the formation of 4E-T complex upon stress. We have developed an image-based computational method to quantify the size, number and density of PBs in cells and we find that while 4E-T is required for steady state PB assembly, its phosphorylation facilitates the formation of larger PBs upon oxidative stress. Using polysomal mRNA profiling we assessed global and specific mRNA translation, but did not find that 4E-T phosphorylation impacts on translational control. Collectively, these data support a model whereby PB assembly is regulated by a two-step mechanism involving a 4E-T-dependent assembly stage in unstressed cells and a 4E-T phosphorylation-dependent aggregation stage in response to stress stimuli.

1512

Persistent ATF2 Signaling is a Potential Determinate of Cell Fate post Radiation Exposure.

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Oxygen derived radicals such as superoxides, hydrogen peroxide, singlet oxygen and hydroxyl radicals are generated as a byproduct of normal aerobic metabolism and are toxic to cells. These reactive oxygen species are also produced as a consequence of radiation exposure and have been implicated in the etiology of a wide range of human diseases, including cancer. Protein phosphorylation events are key to integrating the cellular communication post radiation exposure to the downstream biological response and essential to ensure normal cellular homeostasis. Persistent phosphorylation of certain signaling proteins has been correlated to prolonged oxidative stress. Interestingly, prolonged phosphorylation of several proteins involved in DNA damage response has been noted in both precancerous lesions and various tumors. The link between persistent phospho-signaling and cellular oxidative stress is unclear and could potentially shed light on the mechanisms that enhance cancer risk.

ATF2 (Activating Transcription factor), which is phosphorylated at Ser490-498 in response to DNA damage and at Thr69-71 during oxidative stress-mediated transcriptional response is a potential node in both these pathways. We have hypothesized that the nature of ATF2 signaling initiated and the magnitude of response will correspond to the complexity of radiation damage

and provide clues to a cell's sensitivity to oxidative stress and increased cancer susceptibility. Mammary epithelial and fibroblast cells derived from reduction mammoplasty from the same individual are being studied to better understand why certain cell types are more cancer susceptible. These cells were exposed to different radiation qualities and the profiles of pATF2 were examined at various times by flow based assays and fluorescent microscopy. To investigate the radiation quality dependence of cancer risk, we are studying a surrogate cancer endpoint of cancer risk, namely changes in telomere length, using a novel flow cytometry based assay. Our studies indicate that at late times post radiation exposure, a fraction of cells continue to show high amounts of residual ATF2 signaling. Our data has shown that cellular localization, degree of activation and persistent signaling of ATF2 and its site dependent phosphorylation is dependent on the nature and complexity of damage elicited by different radiation qualities. Studies reveal a dose-dependent shortening of telomere length in both mammary epithelial and fibroblast cells post radiation exposure, with shorter telomeres more evident with high LET exposures. We have also observed an interesting correlation between the persistent signaling of ATF2 and changes in telomere length. A compilation of the findings to date will be presented.

1513

Proline rich sequences of the ubiquitin ligase Itch bind SH3 domains with individual specificities.

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Itch is an ubiquitin ligase that belongs to the C2-WW-HECT (CWH) family containing well-characterized ligases such as Nedd4 in humans and Rsp5p in yeasts. In addition to the conserved domains common to all CWH proteins, Itch has a unique proline-rich region (PRD) that allows it to interact with SH3-domain containing proteins.

The PRD of Itch contains several minimal SH3 binding motifs that could potentially accommodate many substrates. Yet, only a few SH3 interacting partners are identified and little is known about their binding preferences towards Itch PRD.

We use bioluminescence resonance energy transfer (BRET) to evaluate Itch affinity with SH3-domain containing proteins. We confirm the previously reported interaction between Itch and endophilin, and identify novel SH3 partners: pacsin, amphiphysin as well as the isolated SH3 domains of intersectin. Pull-down experiments and far-Western blot further confirmed that pacsin and amphiphysin are indeed novel partners, but only weak interaction was found with the full length form of intersectin. As suggested by Itch ligase activity, we also show Itch-induced ubiquitylation of these potential substrates.

We next address the possibility that different SH3 proteins may bind different motifs inside Itch PRD. Using in vitro pull-down assays with different sub-sequences of the PRD, we were able to compare the affinity of different SH3 for these sequences. We found that endophilin and amphiphysin bind distinct yet overlapping motifs in the N-terminal region of the PRD, distinct from the preferred binding site reported for β -PIX (Janz et al, 2007).

Thus, Itch PRD contains several minimal SH3 binding motifs able to accommodate different SH3 partners. These proteins also have distinct binding preferences towards Itch PRD. These results will certainly help the prediction of novel SH3 substrates for Itch. They will also allow the design of Itch mutants abolishing specific SH3 interactions, thus helping to establish the biological significance of these interactions.

1514

USP7/HAUSP mediated stabilization of Foxp3 increases Treg suppressive capacity.

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Regulatory T cells (Treg) are a specific subset of T lymphocytes responsible for suppression of the immune response to maintain immunological self-tolerance. The Forkhead box (Fox) transcription factor Foxp3 is a lineage commitment factor for Treg, and stable Foxp3 expression levels are required for both their development and functional maintenance.

Here we demonstrate that Foxp3 protein levels are post-translationally regulated through poly-ubiquitination on multiple lysine residues, resulting in proteasome-mediated degradation. The deubiquitinase (DUB) USP7 was found to be upregulated and active in Treg and associated with Foxp3 in the nucleus. Ectopic expression of USP7 decreased poly-ubiquitination of Foxp3, thereby increasing both Foxp3 protein levels and transcriptional activity. Conversely, either treatment with a pan DUB inhibitor or USP7 knockdown decreased endogenous Foxp3 expression levels in Treg. Furthermore, USP7 knockdown significantly decreased Treg mediated suppression *in vitro*. In addition, in an induced colitis mouse model, either inhibition of DUB activity or USP7 knock down in Treg abrogated their ability to resolve inflammation *in vivo*. Taken together, our data provides a novel molecular mechanism in which rapid temporal control of Foxp3 levels in T cells can be regulated by USP7, thereby modulating Treg numbers and function.

1515

Deubiquitinating enzyme Ubp3 as a novel regulator for PKA signaling.

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The cAMP-dependent protein kinase (PKA) is a highly conserved key component in nutrient signaling pathway. In yeast, PKA responds to glucose and antagonistically interacts with TOR pathway to control cell growth. The molecular mechanisms that regulate PKA signaling remain to be fully understood. Here, we show that a deubiquitinating enzyme Ubp3 is a new regulator of PKA signaling. Disrupting the Ubp3 activity leads to hyper-activation of PKA signaling, as evidenced by increased phosphorylation of PKA substrates, decreased accumulation of glycogen, increased sensitivity to heat shock, and an enlarged cell size. The levels of intracellular cAMP and the active form of Ras proteins are also significantly elevated in the *ubp3Δ* mutants. Consistent with a model that the elevated level of cAMP may be responsible for the abnormal PKA signaling displayed by the *ubp3Δ* mutant, overexpression of *PDE2*, which encodes a phosphodiesterase that hydrolyses cAMP, significantly suppressed the hyper-activation of PKA signaling in *ubp3Δ* cells. Together, these data suggest that Ubp3 is a new negative regulator of PKA signaling and it acts likely at a step upstream of Ras.

1516

a modulating effect of il-32 α on transcriptional regulator plzf in pma treated thp-1 promonocytic cells.

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human il-32 α has been reported as a proinflammatory cytokine that expresses in the activated natural killer cells and t-cells. although several variants such as β and γ isoforms have been known to induce inflammation, the precise role of each isoforms remains elusive. in this study, we observed that il-32 α isoform interacted with promyelocytic leukemia zinc finger gene (plzf), a transcriptional regulator belonging to the poz/domain and kruppel zinc finger (pok). plzf has many functional properties: inhibits cell growth, concentrated into punctated nuclear subdomains and is a sequence-specific transcriptional regulator recruiting a histone deacetylase-repressing complex. we cotransfected hek293 cells with il-32 α and plzf, then treated these cells with phorbol 12-myristate 13-acetate (pma). the interaction between il-32 α and plzf was pma-dependent, which means that pkc may mediate the bindings of these factors. we further examined whether il-32 α would modulate the function of plzf transcriptional regulator in stable il-32 α - expressing thp-1 cells. we observed that cxcr10, ifit2 and plscr1, target genes of plzf, were down-regulated in thp-1-il-32 α stable cells compared with control thp-1 cells. plzf is modified by sumo or ubiquitin conjugation and these modifications regulate the biological function of plzf. we showed that il-32 α regulates these modifications of plzf in a pkc dependent manner. taken all these results, il-32 α modulates the transcriptional regulator activity of plzf via direct binding, ubiquitination and inhibiting sumoylation in pma-treated thp-1 cells.

Chromatin and Chromosome Organization

1517

An induced dicentric chromosome promotes genomic rearrangement.

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Chromosomal rearrangements are prevalent in cancer and can impact cellular function at many levels, including changes in gene expression and the production of unique fusion proteins with altered functions. Several mechanisms have been implicated in the induction of chromosome rearrangements, including the induction of 'break - bridge - fusion' cycles caused by telomere erosion, which lead to the end-to-end fusion of chromosomes. Such fused chromosomes contain are dicentric, and thus have two points of attachment to the microtubule spindle during mitosis. It is supposed that during mitosis, the dicentric chromosome is broken leading to additional translocation events and the propagation of genomic instability over multiple cell divisions. While this process has been observed cytologically in cancer cells, the mechanism by which a dicentric chromosome can lead to cancer causing genetic alterations remains unknown. We have developed an experimental system to transiently induce the conversion of a single defined chromosome to a dicentric chromosome with high temporal control. We find that the behavior of a dicentric chromosome is highly variable and cell type specific. An induced dicentric chromosome is often damaged and mis segregated during mitosis, and that this can

lead to its translocation with itself as well as other chromosomes. We are currently using this system to probe the role a single dicentric chromosome can play in cellular transformation.

1518

Visualizing Chromatin Ultrastructure by Combining EdU Labeling with Stochastic Optical Reconstruction Microscopy.

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Understanding chromatin packaging is essential to know how gene expression is spatially and temporally regulated. To visualize chromatin packaging, electron microscopy and fluorescence microscopy are the two most popular tools. Although electron microscopy has revealed many important chromatin structures such as the chromatin fiber, understanding the nature of these structures in the nucleus would need a less invasive and perturbation approach. Conventional optical microscopy can probe thick specimen with few perturbation, while its spatial resolution is only near 200 nm due to optical diffraction limitation. Recently developed super-resolution microscopy techniques, including Stochastic Optical Reconstruction Microscopy (STORM), have solved this resolution problem. STORM has been used to image DNA in the nucleus using fluorescent protein fusion of histones or in situ hybridization. Here, we demonstrate the visualization of chromatin ultrastructures by direct chemical labeling of the DNA backbone to improve labeling density and reduce perturbation to the DNA structures. By incorporating a nucleoside analogue 5-ethynyl-2'-deoxyuridine (EdU) to replace dTTPs during DNA replication, followed by click reaction with photoswitchable dyes, we achieved high density labeling of DNA backbones in insect cells. STORM images were capable of revealing newly replicated chromatin fibers. Moreover, facilitated by relaxed inhibition of replication origin, the re-initiation of specific origins in *S. cerevisiae* enabled us to label a specific stretch of DNA in the nucleus. Combining with other genetic tools, this method could be applied to the study of replication fork structure, heterochromatin packaging, and epigenetic regulation of gene expression in eukaryotes.

1519

Single Molecule dynamics of short nucleosome arrays.

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In the eukaryotic nucleus, chromatin forms a 3D structure that exhibits multiple levels of folding, and compacts the genome to an extent that is both site-specific and highly dynamic. Despite its central role in regulating DNA templated processes such as transcription or replication, we have limited understanding of the mechanisms that direct the folding of chromatin into a heterogeneous and modular landscape. While numerous biochemical determinants have been implicated in the regulation of chromatin compaction, there still lacks a unifying description that relates biochemical composition to structure, and connects local physical properties of the fiber (e.g. persistence length or self-interaction properties) to larger scale folding. Part of the challenge lies in the absence of a robust technique capable of measuring structural and dynamical information at the scale of a few nucleosome repeats (few tens of nm). We have developed novel fluorescence assays based on Single Molecule Tracking Spectroscopy (SMTS) that allow us to investigate the dynamics of short reconstituted nucleosome arrays. SMTS relies on a feedback-enabled confocal microscopy setup, where an optical modulation scheme senses the displacement of a freely diffusing fluorescently labeled array and a feedback loop actively cancels Brownian motion. Long observation periods (tens of seconds) are achieved and permit extraction of the diffusion coefficient of individual arrays. We have applied STSM to measure changes in condensation in response to changes in ionic environment. An advantage of STMS

over bulk diffusometry methods is the single-fiber resolution which enables probing of a population of arrays of different compactions. We next present a fluorescence correlation assay (tracking-FCS) that we are currently applying to measure the relative motion between the two ends of an array. Unlike in FRET, where the 2 dyes need to be separated by less than a few nm, the 2 dyes in tracking-FCS can be distant by several tens of nm permitting measurement of the internal dynamics of short arrays on μ s to s timescales. We finally present a method to characterize self-association between multiple fibers driven by divalent ions or chromatin binding proteins. In this assay, individual oligomers are tracked one-by-one and fluorescence quantification combined with diffusometry allows us to study self-associated fiber morphology.

1520

Live cell imaging of endogenous repetitive genomic loci using TALE proteins.

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The study of chromosome dynamics in living cells has provided important insight into the mechanisms of chromosome condensation, chromosome segregation and nuclear organization. The earliest methods for tracking the movements and organization of chromosomes used transmitted light microscopy to follow whole chromosome movement. Fusing fluorescent proteins to chromosome binding proteins made it possible to follow the dynamics of chromosomal domains that are bound by the fusion proteins using fluorescence microscopy. Chromosomal integration of tandem arrays of bacterial operator sequences (e.g. lacO) and expression of fluorescent protein fusions to bacterial repressor proteins enable the tracking of individual chromosomal loci in living cells. However, operator/repressor systems for labeling chromosomes require engineering the chromosome by integration of operator arrays. To follow an endogenous chromosomal locus in living cells without modifying the genome we have begun to develop methods to label chromosomal domains in live cells based solely upon the underlying DNA sequence. Transcription activator like effector (TALE) proteins contain 34 amino acid modules, each capable of recognizing a single nucleotide within a DNA molecule. By joining multiple modules, we have generated TALE proteins targeting specific genomic loci. We constructed TALE-YFP fusion proteins that recognize the DNA binding site for Centromere protein B (Cenp-B) and the telomeric repeat sequences. Expression of these engineered sequence recognition proteins in human cells labelled the centromeres and telomeres in living cells. We are currently using this method to follow the dynamics of repetitive genomic regions in live cells and expanding this approach to non-repetitive elements in the genome.

1521

The KNL-2 Myb domain directs epigenetic centromere specification via a conserved, structure based mechanism.

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Centromeres are chromosomal loci that direct kinetochore assembly in mitosis. Unlike genetic features, centromere DNA sequence is not conserved through phylogeny nor is it sufficient for centromere function. Therefore, it is commonly accepted that centromeres are epigenetically defined; a process mediated by the histone H3 variant CENtromere Protein-A (CENP-A). Extensive biochemical studies have identified most of the players involved in CENP-A loading at centromeres, however understanding the molecular mechanisms has to be achieved. Particularly, the first protein which binds centromeric chromatin for loading of newly synthesized

CENP-A to centromere has to be identified. Kinetochores NulL-2 (KNL-2) was identified in *C. elegans* by functional genomics as required for CENP-A localization at centromeres. Sequence analysis reveals a conserved MYB like DNA binding domain within KNL-2. In other well studied MYB proteins, this domain is known to bind DNA with high specificity via the 3rd of three helices. Thus, KNL-2 might bind centromeric chromatin via its Myb domain and subsequently recruit proteins important for CENP-A loading at centromeres. To test this model, we first solved the 3 dimensional structure of *C. elegans* KNL-2 Myb (CeMyb) domain using Nuclear Magnetic Resonance (NMR). This revealed an expected helix-loop-helix structure; which is the same for Human KNL-2 Myb (HsMyb) domain (available on pdb). With further biochemical approaches, we have found that the HsMyb and CeMyb domains are able to bind genomic DNA *in vitro*. Although those two domains do not bind any specific DNA sequence by Electrophoretic Mobility Shift Assay (EMSA), we show by Total Internal Reflection Fluorescence (TIRF) microscopy a specific binding of the domains to CENP-A chromatin. Therefore, the KNL-2 Myb domain is sufficient to recognize and bind a specific structure generated by the presence of CENP-A nucleosomes at centromeres. To characterize the binding of the Myb domains with DNA, a ^1H - ^{15}N Heteronuclear Single Quantum Coherence (HSQC) NMR spectra of the MYB domain in presence of centromeric DNA sequence (α -satellite sequence) revealed the amino acids that are potentially interacting with the DNA. For CeMyb domain, the identified residues surprisingly all reside within the first helix. The NMR results were further confirmed by EMSA showing a deletion of the first helix of the Myb domain that is not able to bind DNA. Although not conserved at the sequence level, the KNL-2 Myb domains display conserved activity for CENP-A chromatin binding. Thus, the mechanism controlling centromere specification may be ubiquitous in metazoans.

1522

DNA Topoisomerase II acts as a mitotic scaffold protein in chromosome assembly in *C. elegans*.

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Introduction: Assembly of mitotic chromosomes is a dynamic event, occurring rapidly during prophase of each cell cycle. Given the complexity of this process, genetic and biochemical approaches have identified surprisingly few factors involved in condensation, notably histone H1, the condensin complex, DNA TOPOisomerase II (TOPO II) and CENtrome-re-Protein A (CENP-A). TOPO II can cut and reseal DNA and has been hypothesized to act in a structural capacity, but how it acts in mitotic chromosome condensation remains unknown.

Methods and Results: To define the roles of known and novel proteins in chromosome condensation, we are performing high-resolution live imaging of the *C. elegans* zygote. Images of fluorescently-tagged core histones are thresholded and segmented to quantify the distribution of chromatin within the prophase nucleus over time. When TOPO II is depleted by RNAi, chromosomes collapse prematurely during prophase. When the catalytic activity of TOPO II is inhibited with Dexrazoxane or Etoposide, chromosomes do not collapse prematurely; rather, chromatin remains diffuse throughout prophase. These results indicate that independent of its catalytic activity, TOPO II acts to scaffold condensation. Indeed, TOPO II decatenation activity is required for primary chromatin organization. We are able to distinguish and define scaffolding and catalytic functions of TOPO II *in vivo* using our assays. Interestingly, TOPO II partial depletion reveals that the majority of TOPO II, 70%, functions as a scaffold whereas 30% functions as a catalytic enzyme. Fixed cell immunostaining of prometaphase chromosomes revealed that TOPO II, Condensin, and CENP-A form spatially independent chromosome axes. Co-depletion of TOPO II with Condensin or CENP-A does not rescue premature chromosome collapse, indicating that these three proteins have different and independent mechanisms of function.

Conclusion: We conclude that metaphase chromosomes are built by the distinct scaffold activities of three proteins: TOPO II, CENP-A and Condensin. Importantly, our analysis revealed that depletion of these candidate proteins results in quantitatively distinct phenotypes, indicating that they function in discrete events during mitotic chromosome assembly.

1523

Differences in chromatin accessibility modulated by different histone variants in *C. elegans*.

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The introduction of histone variants enables eukaryotic cells to alter chromatin structure and modulate cellular processes that include gene regulation, DNA damage and heterochromatin formation. However, how different histone variants function remains a mystery. Our study aims to characterize two *C. elegans* H2A variants, HTZ-1 and HTAS-1, with significant variation from H2A (59% and 51% identical, respectively). While HTZ-1 is ubiquitously expressed, HTAS-1 is found only in sperm, where DNA becomes tightly compacted and transcriptionally repressed for transit to the new embryo. The disparity these two proteins exhibit in localization is reflected structurally: HTZ-1 has an extended acidic patch within its docking domain while HTAS-1 has an extended N terminus. How do such structural differences enable these variants to function in distinct cell types?

To understand how HTZ-1 and HTAS-1 modulate chromatin structure, we expressed recombinant *C. elegans* H2A, HTZ-1, HTAS-1 in *E. coli*. We probed the secondary structure of H2A and its variants as monomers and heterodimers (H2A-H2B) using circular dichroism. HTAS-1 and HTZ differ in the amount of alpha helical content each histone possesses with HTAS-1 being significantly more structured while HTZ-1 is slightly less structured relative to canonical H2A. Consistent with previous studies, we have found Htz-1 structure is stabilized by its interaction with H2B. However, this is curiously not true for HTAS-1, which is more structured as a monomer.

Nucleosome core particles (NCPs) containing H2A, HTZ-1, and HTAS-1 were then formed by combining them with *C. elegans* H2B, H3, and H4. The stability of NCPs, which is inversely proportional to DNA accessibility, was measured as a function of ionic concentration using tandem FRET and electrophoresis. Initial analysis shows HTZ-1 decreases NCP stability. Another factor affecting chromatin accessibility, reversible nucleosome unwrapping, was monitored using restriction enzyme activity on a restriction site buried within the nucleosome. This analysis reveals that while HTZ-1 promotes reversible unwrapping of DNA relative to canonical H2A, HTAS-1 inhibits it.

Our study is the first *in vitro* study of the *C. elegans* NCP and provides a mechanistic basis for understanding genome wide studies of histone variants for this model organism. Thus far our results indicate HTZ-1 may act to destabilize NCPs and make DNA more accessible, thus enabling transcription by unfolding chromatin to binding factors. In contrast, HTAS-1 may inhibit accessibility, consistent with a function in chromatin compaction and gene repression observed during sperm development.

1524

The JIL-1 Kinase Does Not Phosphorylate H3S28 or Recruit 14-3-3 to Active Genes in *Drosophila*.

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JIL-1 is the major kinase controlling phosphorylation of histone H3S10 and has been demonstrated to function to counteract heterochromatinization and gene silencing (Wang et al., J. Cell Sci. 124:4309). However, an alternative model has been proposed in which JIL-1 is required for transcription to occur, additionally phosphorylates H3S28, and recruits 14-3-3 to active genes (Ivaldi et al., Genes Dev. 21:2818; Kellner et al., Genome Res. 22:1081; Karam et al., PLoS Genetics: e1000975). Since these findings are incompatible with the results of Cai et al. (Development 135:2917) demonstrating that there are robust levels of transcription in the complete absence of JIL-1 and that JIL-1 is not present at developmental or heat shock-induced polytene chromosome puffs, we reexamined JIL-1's possible role in H3S28 phosphorylation and 14-3-3 recruitment. Using two different H3S28ph antibodies we show by immunocytochemistry and immunoblotting that in *Drosophila* the H3S28ph mark is not present at detectable levels above background on polytene chromosomes at interphase but only on chromosomes at pro-, meta-, and anaphase during cell division in S2 cells and third instar larval neuroblasts. Moreover, this mitotic H3S28ph signal is also present in the *JIL-1* null mutant at undiminished levels suggesting that JIL-1 is not the mitotic H3S28ph kinase. We also demonstrate that H3S28ph is not enriched at heat shock puffs. Using two different pan-specific 14-3-3 antibodies as well as an enhancer trap 14-3-3-GFP line we show that 14-3-3, while present in salivary gland nuclei, does not localize to chromosomes but only to the nuclear matrix surrounding the chromosomes. In our hands 14-3-3 is not recruited to developmental or heat shock puffs. Furthermore, using a LacI-JIL-1 targeting system to ectopic sites on polytene chromosomes we show that only H3S10ph is present and upregulated at such sites, not H3S28ph or 14-3-3. Thus, our results argue strongly against a model where JIL-1 is required for H3S28 phosphorylation and 14-3-3 recruitment at active genes. Supported by NIH grant GM62916.

1525

The CENP-A-specific assembly factor HJURP induces chromatin expansion at non-centromeric loci.

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The centromere-specific histone H3 variant, CENP-A, is targeted to centromeres in a complex with its chaperone HJURP, Holliday junction recognition protein. HJURP binds to the pre-nucleosomal CENP-A protein when it exists as a soluble heterodimer with H4 and escorts it specifically to centromeric chromatin in a Mis18 complex dependent manner. The HJURP/CENP-A/H4 pre-nucleosomal complex localizes to centromeric chromatin during a window in early G1 phase when CENP-A is deposited. The Scm3 domain of HJURP is conserved from yeast to humans and is sufficient to bind CENP-A and execute functional CENP-A nucleosome assembly. However, vertebrate HJURP is significantly larger than yeast Scm3 proteins and contains a central conserved/mid domain. The role of this domain in centromere maintenance and CENP-A deposition is unknown. Previously, our lab demonstrated that targeting HJURP to a non-centromeric site within the genome is sufficient to induce the deposition of new CENP-A nucleosomes at that site. Here, we show that the recruitment of HJURP results in a striking unfolding of the targeted chromatin domain. Moreover, we find that the central conserved/mid domain is required for HJURP to induce the chromatin unfolding. This chromatin unfolding resulted in escalated CENP-A deposition levels.

However, the large degree of unfolding was inhibitory to CCAN recruitment, despite the presence of a large quantity of CENP-A at the expanded region. These results suggest that CCAN proteins may be most efficiently recruited to centromeres with a defined arrangement of CENP-A nucleosomes. The replenishment of CENP-A nucleosomes at the centromere occurs during early G1 into pre-existing centromeric chromatin. This differs from the replenishment of canonical histones that occurs on recently replicated DNA in S phase, whose chromatin has been previously disassembled to accommodate the replication machinery. While we observe this striking unfolding, there is no precedent for drastic expansion of endogenous centromeres when HJURP is recruited during G1. This suggests that cells have a mechanism to counter the HJURP-induced unfolding at endogenous centromeres and that chromatin unfolding may be a transient step in centromeric nucleosome deposition. It is plausible HJURP-induced chromatin unfolding is a crucial intermediate step in CENP-A nucleosome assembly at centromeric DNA.

1526

Localization dynamics and complex interactions of the human Mis18 complex.

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Centromeres are chromosomal domains that couple the pulling forces of microtubules to each chromosome in order to faithfully segregate the duplicated genome during mitosis. In human cells, centromeric location is specified epigenetically rather than by a specific DNA sequence. The epigenetic mark that dictates centromere identity is considered to be the presence of nucleosomes that contain the histone H3 variant, centromere protein A (CENP-A). Following S phase, newly synthesized nucleosomes must be deposited into chromatin in order to compensate for nucleosomal dilution that occurs during DNA replication. However, human cells do not deposit newly synthesized CENP-A nucleosomes until after mitotic exit, throughout G1. While centromere architecture varies widely among species, the deposition of CENP-A is controlled by a group of conserved proteins, which includes the CENP-A chaperone and assembly factor, Holliday junction recognition protein (HJURP) and the Mis18 complex. The human Mis18 complex consists of Mis18 α , Mis18 β and Mis18 binding protein 1 (Mis18BP1^{KNL-2}). Our lab has previously shown that the recruitment of Mis18 α and Mis18BP1^{KNL-2} to centromeres is required for HJURP localization and subsequent CENP-A deposition. Mis18BP1^{KNL-2} contains two annotated domains, an N-terminal SANT-associated (SANTA) domain as well as a C-terminal SANT (Swi3, Ada2, N-CoR, TFIIIB) domain. In order to determine the contribution of these regions to the centromere recruitment of the Mis18 complex, we examined the localization of N- and C-terminal Mis18BP1^{KNL-2} fragments. Only the N-terminal portion of Mis18BP1^{KNL-2}, containing the SANTA domain, is able to support localization to centromeric chromatin. The C-terminal fragment, containing the SANT domain, was not found to localize to centromeres. Recruitment of Mis18BP1^{KNL-2} to centromeres has been linked to an interaction with centromere protein C (CENP-C). Targeting the N-terminal, SANTA containing fragment, to a non-centromeric locus was sufficient to recruit CENP-C. The N-terminus was also sufficient to recruit the entire Mis18 complex, including Mis18 α and Mis18 β . Therefore, the SANT domain is not required for the centromeric localization of Mis18BP1^{KNL-2}, or for its interaction with Mis18 α , Mis18 β or CENP-C. Identifying these required centromere-targeting and complex-formation interactions within the Mis18 complex is a key component in elucidating the CENP-A deposition pathway in human cells.

1527

Rabl Organization of Chromosomes in the Yeast NucleusB. Avsaroglu¹, J. Ham², G. Bronk¹, J. E. Haber², J. Kondev¹;¹Physics, Brandeis University, Waltham, MA, ²Biology, Brandeis University, Waltham, MA

The spatial organization of genomes plays an important role in cell biology as it influences chromosome functions such as recombination and repair of broken DNA. The Rabl model proposed in the late 1800's describes the organization of budding yeast chromosomes during interphase, with the centromeres tethered at the spindle pole body (SPB) and the telomeres tethered to the nuclear periphery. Here we address the question, to what extent does the Rabl organization of chromosomes whose conformations are described by a simple polymer model, quantitatively account for the positioning of genetic loci within the interphase nucleus? To investigate this question we performed a combined experimental and theoretical study of the organization of yeast chromosome III. By imaging two fluorescent markers, one at the SPB and the other proximal to the HML locus that is involved in DNA recombination during mating type switching, we measured the distribution of distances. In addition to wild type cells, *yku70Δ* and *esc1Δ* mutants with disrupted telomere tethering and mutants that have a proximal HML marker tethered to the nuclear periphery were also considered. We compared our experimental results with a random-walk polymer model that takes into account different tethering scenarios and confinement of chromosomes in the nucleus, and found that the model recapitulates the observed spatial organization of chromosome III in yeast in quantitative detail. The Rabl model makes specific predictions for chromosome organization in yeast, and suggests new experiments to test them.

1528

Mitotic map of condensin I in vertebrate cells.

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In order for DNA to be segregated properly during mitosis, chromosomes must be packaged and shaped correctly prior to anaphase onset in a process termed chromosome condensation. Condensin I is a multi-subunit protein complex, consisting of two core subunits SMC2 and 4, and three auxiliary components CAP-H, CAP-D2, CAP-G. Condensin I associates with chromosomes after the nuclear envelope breaks down, and is required for correct chromosome condensation and segregation. While its role in chromosome condensation is well-established, where condensin I binds in the vertebrate genome is a key outstanding question. This presentation will illustrate the first genome-wide map of condensin I in vertebrate cells, which will shed light on the role of condensin I in chromosome condensation and help to understand its increasing link to diseases.

To create the first genome-wide map of condensin I, conditional knockouts of SMC2 and CAP-H in chicken DT40 cells were complemented with an affinity tagged SMC2 and CAP-H, respectively, allowing stringent purification of condensin I-associated DNA. The affinity purified DNA was analyzed using the next-generation sequencing.

Condensin I in vertebrate was found to be enriched on tRNA and rRNA genes, in agreement with previous studies in yeast. Strikingly, a high enrichment was observed consistently at gene

promoter regions, suggesting that condensin I may have an additional role beyond chromosome condensation.

1529

Motion analysis of DNA to deduce the visco-elastic properties of chromatin upon DNA damage.

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Cells must maintain chromosome integrity despite constant exposure to a variety of sources of DNA damage including radiation, carcinogens and DNA replication errors. It is essential that double-strand breaks (DSBs) are repaired accurately prior to chromosome segregation, as errors can lead to chromosomal rearrangements, cancer or cell death. Previous studies have shown that chromatin exhibits energy-dependent increased mobility and expansion upon damage. Here we examine the mechanisms underlying the damage-induced decompaction and increased motion using polymer theories to deduce the contributions of the elastic and viscous moduli. We find that the visco-elastic nature of the chromatin is altered upon DSB induction – shifting from being primarily viscous at intermediate time scales (10-50 sec) to predominantly elastic. This mechanical change provides insights into the underlying mechanisms by which chromatin modifications promote DNA repair.

1530

Transcription Factor Binding to a DNA Zip Code Controls Interchromosomal Clustering at the Nuclear Periphery.

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Active genes in yeast can be targeted to the nuclear periphery through interaction of cis-acting "DNA zip codes" with the nuclear pore complex. We find that genes with identical zip codes cluster together. This clustering was specific; pairs of genes that were targeted to the nuclear periphery by different zip codes did not cluster together. Insertion of different zip codes at an ectopic site induced clustering with endogenous genes having that zip code. Targeting to the nuclear periphery and interaction with the nuclear pore is a pre-requisite for gene clustering, but clustering can be maintained in the nucleoplasm. Finally, we find that specific transcription factors and other DNA binding proteins recognize the GRS zip codes to mediate both targeting to the NPC and interchromosomal clustering. These results suggest that zip code-mediated clustering of genes at the nuclear periphery influences the three-dimensional arrangement of the yeast genome.

1531

Dimerization of the CENP-A assembly factor HJURP is required for centromeric nucleosome deposition.

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Histone variants use unique assembly factors to determine the temporal and spatial pattern of variant-containing nucleosome deposition. CENP-A is the histone H3 variant that acts as the

epigenetic mark of centromere position. The deposition of new centromeric nucleosomes requires the CENP-A specific chromatin assembly factor HJURP. CENP-A and HJURP interact as a soluble prenucleosomal complex and are recruited to centromeres during G1 when new CENP-A deposition occurs. Crystallographic and biochemical data demonstrate that HJURP binds a single CENP-A-histone H4 heterodimer. However, the formation of stable CENP-A nucleosomes requires a conserved dimerization interface, suggesting that HJURP forms octameric CENP-A nucleosomes. How an octameric CENP-A nucleosome forms from individual CENP-A-histone H4 heterodimers is unknown. Here we show the CENP-A assembly factor HJURP forms a homodimer through its second HJURP_C domain. HJURP exists as a dimer as a soluble pre-assembly complex and at chromatin during G1 when new CENP-A is deposited by HJURP. Dimerization of HJURP is essential for the deposition of new CENP-A nucleosomes. The recruitment of HJURP to centromeres occurs independent of dimerization and CENP-A binding. These data provide a mechanism whereby the CENP-A prenucleosomal complex achieves assembly of the octameric CENP-A nucleosome by formation of a prenucleosomal complex that contains CENP-A molecules through the dimerization of the CENP-A chaperone HJURP.

1532

Probing Genome-Nuclear Lamina Interactions in Mouse Myoblasts.*F. Wu¹, J. Yao¹; ¹Cell Biology, Yale University, New Haven, CT*

Cytological studies have revealed that a number of genes are preferentially positioned to the nuclear periphery, and changes in their radial positioning are often correlated with changes in gene activities. We are using the myogenic differentiation system to study how subnuclear gene positioning confers regulatory functions on gene transcription. By high resolution cell imaging, we have revealed that the histone mark H3K4me3 and its associated factor TAF3 are localized away from the nuclear periphery (Yao et al, *Genes & Development*, 25: 569-580), which suggests that differential compartmentalizations of histone marks may characterize the chromatin states at the nuclear periphery and influence gene transcription.

We aim to further examine this observed subnuclear compartmentalization at the molecular level. DNA adenine methyltransferase identification (DamID) assay has been used to map genome-nuclear lamina interactions in *Drosophila* and mammalian cells, providing an approach to identify DNA sequences at the nuclear periphery genome-wide. Here, we have used the DamID assay coupled with next generation sequencing and bioinformatic analyses to identify genomic regions that have significantly higher frequencies interacting with the nuclear lamina in mouse 3T3 fibroblasts and C2C12 myoblasts. In 3T3 cells, these regions overlap with 98% of the Lamin Associated Domains (LAD) previously identified (Peric-Hupkes et al, *Molecular Cell*, 38: 603-13). Proximities of these regions to the nuclear periphery were confirmed with fluorescence in situ hybridization. We are analyzing these genomic regions relative to reported genomic and epigenomic features in C2C12 myoblasts, such as transcription start sites, histone modifications and gene expression. We are also examining the localizations of additional histone modifications relative to the nuclear periphery by cell imaging. These findings will be discussed.

1533

Micromechanical properties of mammalian meiotic chromosomes: a high frequency model of genomic instability.

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Prior to cell division, eukaryotic cells undergo a dramatic reorganization whereby their chromosomes are bundled and condensed into compact mitotic or meiotic forms. Proper chromosome condensation is essential for accurate segregation of homologous chromosomes or sister chromatids. The folding of the flexible chromatin fiber into its compacted form is due, in part, to the multi-subunit protein, condensin, and cells deficient in condensin fail to fold chromatin properly, resulting in anaphase bridges. Meiosis is a specialized process with two sequential cell divisions that occur in the absence of an intervening interphase to produce haploid gametes. Compared to mitosis, meiosis in the mammalian egg is remarkably error-prone, and the error frequency increases in female gametes of advanced reproductive age. These errors contribute to aneuploidy and result in pregnancy loss or birth defects, however, their origins and age dependency is not understood. Thus, the mammalian egg represents a robust and unique model system to study molecular mechanisms of chromosome segregation as well as the errors that result in aneuploidy. However, the role of the micromechanical properties of mammalian chromosomes during meiosis is unknown. Measurement of the micromechanical properties of isolated individual chromosomes has provided a powerful tool for understanding the organization of chromosomes in mitotic cells of several species. Here, we demonstrate that we can efficiently isolate individual chromosomes from mammalian (mouse) eggs arrested at metaphase of meiosis II and that these isolated meiotic chromosomes have reversible elasticity and a force constant on the subnanonewton scale. Moreover, we can identify condensin localization on native chromosomes for the first time. We have expanded these studies to investigate the changes in the structure between chromosomes isolated from eggs from young and old mice. Such analysis in a well-established model of increased genomic instability not only will reveal the relationship between changes in the micromechanical properties of meiotic chromosomes and aneuploidy, but will also have broad implications for reproductive health and associated interventions.

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1534

Scaling Chromosome Condensation to Cell Size.

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During embryonic development in metazoans (like the common model organisms *Caenorhabditis elegans* or *Xenopus laevis*), cells decrease in volume by up to two orders of magnitude (from 1,2 mm to 12 μ m in *X. laevis*), a consequence of multiple rounds of cell divisions without growth of the embryo. Using *X. laevis* or *C. elegans* embryos as model organisms, it has been shown that mitotic structures including mitotic spindle length, centrosome size and nucleus size all scale with cell size. However, the exact molecular mechanisms regulating how organelles scale their size to the continuously changing size of the cells are poorly understood. Due to technical limitations, scaling of mitotic chromosome condensation has received less attention. Despite dramatic changes in cell size, the genome size is constant in all diploid cells of an organism. Therefore, it is expected that during

anaphase, condensed mitotic chromosomes must be half the length of the mitotic spindle to be properly segregated to each daughter cells. Using the determined development of *C. elegans* embryos we show that prometaphase condensed chromosomes are smaller in length as cell reduces in size. To assess the hypothesis of a cell autonomous regulated mechanism, we RNAi depleted an importin α protein (*ima-3*), resulting in reduced embryo size and therefore the size of the cells. Surprisingly, we found that experimental reduction of cell size resulted in a corresponding reduction of chromosome size. This chromosome-cell size scaling fit with is seen in WT embryos at a later developmental stage. Currently, we are examining two models that could explain this chromosome-cell size scaling. First, the limiting component model proposes that there is a fixed amount of 'X' factor in the embryo which is redistributed and diluted to each daughter cells after each mitotic divisions. Second, a DNA to cytoplasm ratio could dictate chromosome size. The total amount of 'X' factor would be the same in all cells but as cell reduces in size, the proteins are more concentrated and perhaps more active. To elucidate this mechanism, we used a worm strain with one longer chromosome than the chromosomes present in a wild-type strain resulting from a X/autosomeV end-to-end fusion. We reasoned this longer chromosome would sensitize a worm to any defect in chromosome condensation or segregation in smaller cells of the developing embryo. In fact, a RNAi depletion of one of the condensin I subunits increases the lethality by 3 fold compare to a control RNAi, although it is near 100% viable in a WT strain. Thus we are using RNAi and high resolution imaging to determine the mechanisms of mitotic chromosome size regulation.

1535

The nuclear architectural protein NuMA targets the ISWI ATPase SNF2h to DNA breaks.

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Chromatin remodeling plays an active role in the DNA damage response (DDR). The current view is that chromatin remodelers, including members from the ISWI family, shape the chromatin landscape to facilitate the repair process and to restore damage sites after repair. The ISWI ATPase SNF2h has been proposed to continuously sample chromatin by transient, genome-wide, associations with nucleosomes. SNF2h rapidly accumulates at sites of DNA damage but the mechanism leading its stabilization at damaged sites is unknown. The nuclear architectural protein NuMA has been linked to chromatin organization during breast epithelial differentiation and to H2AX phosphorylation (γ H2AX), an early chromatin response to DNA damage. These observations led us to propose that NuMA coordinates large scale chromatin rearrangement during the DDR.

Here, we identify NuMA as a binding partner of SNF2h in immunoprecipitation experiments. NuMA silencing impairs SNF2h recruitment at DNA breaks in laser-microirradiated cells and fluorescence correlation spectroscopy measurements indicate that NuMA regulates SNF2h diffusion in the nucleoplasm. Although NuMA distributes throughout the nucleus during the interphase, it significantly accumulates at microirradiated sites. This phenomenon is exacerbated upon ATM inhibition. Moreover, NuMA knock-down impairs homologous recombination repair (HRR) and leads to a rapid loss of γ H2AX in cells exposed to ionizing radiations. This effect is not mediated by cell cycle alteration. Both HRR and γ H2AX maintenance have previously been linked to the function of ISWI complexes.

NuMA might provide a dynamic lattice reinforced at DNA damage sites and essential for the stabilization of chromatin remodeling complexes. Our previous work has established a mutual influence between NuMA and the extracellular matrix during mammary epithelial cell differentiation. NuMA may therefore act as a transducer of microenvironmental cues on nuclear organization, thereby regulating genome functions and maintenance.

1536

Investigating regulation of linker histone H1 on mitotic chromosomes in *Xenopus*.*K. Miller¹, R. Heald¹; ¹Molecular and Cell Biology, UC Berkeley, Berkeley, CA*

Histone chaperones are a group of cytoplasmic proteins that aid in dynamic chromatin organization during different cellular processes. Linker histone H1 is a highly dynamic and fundamental chromatin-bound protein required for mitotic chromosome condensation. Here, we report that the histone chaperone Nucleosome Assembly Protein 1 (Nap1), a chaperone that associates with linker histone H1, is required for mitotic chromosome condensation and mitotic spindle assembly. Depletion of Nap1 in *Xenopus* egg extracts causes defects in both remodeling of sperm chromatin and a decrease in microtubule polymerization around these structures. Examination of depleted extracts reveals an increase in H1 association with sperm chromatin, without an increase in core histone localization. Add-back of a truncated mutant of Nap1 does not rescue this effect. Experiments are currently under way to examine how depletion of Nap1 affects the dynamic behavior of H1, how specific the actions of Nap1 are to H1, and how different domains of Nap1 may be modified in a cell-cycle specific manner to regulate H1 function. Our findings illustrate the importance of proper linker histone regulation on mitotic chromosome structure and highlights histone chaperone Nap1 as a novel spindle assembly factor.

1537

On the Role of Active Fluctuations in Genetic Regulation.*K. Raghunathan¹, J. Milstein², J-C. D. Meiners¹; ¹Departments of Biophysics and Physics, University of Michigan, Ann Arbor, MI, ²Physics, University of Toronto, Toronto, ON, Canada*

Cells are not just small test tubes, but present a mechanically complex, ever fluctuating environment in which essential biological processes take place. We investigate how the complex nanomechanical environment of the cell affects the dynamic motion of DNA, and how this in turn affects the formation of regulatory DNA-protein complexes. Using single-molecule particle tracking on doubly-labeled DNA constructs in living cells we show that the fluctuations of the DNA in living cells are two orders of magnitude larger than in dead, yet structurally intact cells. We attribute this enhanced motion to the activity of molecular motors in the cellular environment, which exert random forces on the DNA that are much stronger than thermal forces. This dovetails with our observations in FCS studies of genomic DNA in live prokaryotes, which show a dramatically enhanced persistence of spatiotemporal correlations on the millisecond to second time scale. We will discuss whether observations like this and underlying mechanisms like active motor-induced fluctuations and mechanical constraints on the DNA in vivo can explain the perhaps unexpected high efficiency of regulatory mechanisms. Protein-mediated repressor loops like the lac-repressor, for instance, can repress gene expression several hundredfold, whereas these loops have been show to be open much of the time in single-molecule in-vitro studies, rendering them apparently rather inefficient. To investigate the effects of an actively fluctuating mechanical environment of the cell quantitatively, we simulated such an environment in vitro by introducing fluctuating tensile forces into a single-molecule model system using axial optical tweezers. Increasing the fluctuations in the molecule over its thermal conformational fluctuations by just 5% enhanced loop formation rates twofold. Based on such observations we hold the view that the crowded, constrained, and actively fluctuating environment of the living cell is most likely responsible for much enhanced DNA-protein complex formation in vivo, a mechanism that may well be quite general when it comes to explaining the amazing efficiency of macromolecular assembly in living cells.

1538

Mitotic Stability Functions of the INO80 Chromatin-Remodeling Complex.A. J. Morrison¹; ¹Department of Biology, Stanford University, Stanford, CA

ATP-dependent chromatin-remodeling complexes influence diverse nuclear processes, such as transcription, replication, and DNA damage responses, by specifying nucleosome positioning and composition. The *S. cerevisiae* INO80 complex is composed of 15 subunits, several of which contribute to different functions of the chromatin-remodeling complex. For example, deletion of certain INO80 subunits specifically disrupts double-strand break repair, while another subunit is phosphorylated by the ATM/ATR homologues in yeast, Mec1/Tel1, to influence cell cycle checkpoint pathways. Less defined components of the INO80 complex include actin-related proteins (Arps), which have sequence similarity to actin and are evolutionarily-conserved components of chromatin-remodeling complexes. There are six nuclear Arps in *S. cerevisiae*, all of which are found within the complexes of the SWI/SNF and INO80 chromatin-remodeling subfamilies. The INO80 complex contains Actin and Arps 4, 5 and 8, of which Arp5 and 8 are unique to the INO80 chromatin-remodeling complex. Previous research demonstrates that Arp5 and 8 contribute to the *in vitro* chromatin-remodeling activity of the INO80 complex, while the precise mechanisms by which they function *in vivo* are unclear.

Here we demonstrate that subunits of the *S. cerevisiae* INO80 complex have heterogeneous functions in mitotic stability pathways. Specifically, deletion of *ARP5* and *IES6* results in significant fitness defects when cells are treated with microtubule destabilizing agents. Biochemical characterizations reveal that Arp5 and Ies6 form a functional module within and outside of the INO80 complex. Functional assays demonstrate that the Arp5-Ies6 subcomplex is involved in mitotic stability pathways, as deletion causes ploidy alterations. Deletion of *ARP5* and *IES6* results in mitotic defects, specifically chromosome segregation is delayed or absent in mutant cells. Genetic data demonstrates that these INO80 subunits are involved in the spindle positioning pathway, specifically microtubule regulation. These results demonstrate that the Arp5 and Ies6 subunits have critical genome maintenance functions during mitosis.

1539

Paired Sense and Antisense RNA Transcription during Chromosomal Kissing.J. H. Frenster¹, J. A. Hovsepian²; ¹Medical Oncology, Stanford University School of Medicine, Atherton, CA, ²Diagnostic Imaging, Stanford University School of Medicine, Atherton, CA

Mammalian cells often display chromosomal kissing of one or more distinct chromosomes, resulting in paired sense and antisense RNA synthesis at that kissing gene locus.

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We have used high-resolution electron microscopy to determine the ultrastructure of active euchromatin 10 nm microfibrils within interphase calf thymus lymphocyte nuclei. Interphase euchromatin microfibrils display localized areas of chromatin looping up to 1,000 nm in length. A minority of loops reach out to loops on adjacent chromosomes, forming short linear side-by-side duplexes 20 nm in diameter, and up to 20 nm in length. Such paired-chromatin structures may provide a paired template for paired sense-antisense gene transcription, resembling DNA-DNA microstructures described previously for oncogenic viral integration.

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These paired DNA-DNA tetraplexes can be formed by paranemic (sliding) DNA bonds, rather than by the more usual plectonemic (intertwined) DNA bonds, as described earlier by Lewin B, "Genes", Ed. 7, 428 (2000, Oxford Press).

1540

Comparative Analysis of Genomic Alternations Between Chinese Hamster and CHO Cell Line.

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Chinese hamster ovary (CHO) cell lines, established over five decades ago, are among the most important cell lines used in biomedical research for decades. The low diploid number of chromosomes ($2n = 22$) in the Chinese hamster and the relative ease of deriving mutant lines made these cell lines a valuable cytogenetic tool. In the past two decades, CHO has also become the most prominent host cell for producing therapeutic proteins, acquiring much genomic reorganization in the process. Recently, the genome of a CHO-K1 cell line has been sequenced. Given its importance in biomedical research and in the biopharmaceutical industry, it is of great interest to understand better the genomic foundation that gives rise to the many features that have given CHO cells such prominence.

We have independently sequenced and assembled the genome of a female Chinese hamster by Illumina deep sequencing; and have performed a comparative analysis with the sequenced CHO-K1 genome. The depth of the sequencing attained enabled us to analyze heterozygosity and possible chromosomal reorganization.

At a first glance, a high level of sequence variation was observed between the two genomes. A closer examination of the deeply sequenced regions and further superimposition of RNA seq results uncovered a high level of false positive calls of sequence variants. Using a stringent criterion for base variant calls, the mutation rate of the CHO-K1 genome was deciphered to be substantially lower than the first estimate.

The comparison of the CHO-K1 and Chinese hamster genomes was also performed at a scaffold level. Scaffolds longer than 1 Mbp in size from both assemblies were aligned to each other and scanned for potential rearrangements. Although a majority of scaffolds showed identical order of alignments, reordering was observed in several other scaffolds. The alternation in sequence alignment could either be an assembly error or genuine chromosomal reorganization. Using a long 10 Kbp insert mate pair sequence library, that associate two reads spanning over an estimated 10 Kbp region, we verified the authenticity of rearrangements for a few representative scaffolds.

The availability and application of these genomic resources will facilitate physiological comprehension of cellular behavior, providing further opportunities to enhance CHO cells in their role as recombinant protein producers.

Stem Cells and Pluripotency I

1541

Application of collagen scaffolds for differentiation of mouse induced pluripotent stem cells into cardiomyocytes.

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The surface on which cells are grown plays a key role in determining cell behavior in vitro and in optimizing culture conditions for a particular cell type. The elasticity of the extracellular matrix microenvironment has been identified as an important factor to direct developmental pathways of stem cells. In this study, mouse induced pluripotent stem cells (iPSCs) were cultured on gelatin-coated type I collagen scaffolds which were freeze-dried and then cross-linked with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysulfosuccinimide (NHS). While native collagen scaffolds without cross-linking showed a fiber-like structure, EDC/NHS cross-linked scaffolds demonstrated better interconnectivity and formed a sheet-like sponge structure. Compared with mouse iPSCs cultured on native collagen scaffolds, the cardiomyocyte differentiation of mouse iPSCs on cross-linked scaffolds showed a significant increase in the differentiation rate, especially when higher concentration of EDC/NHS cross-linkers were used to cross-link the scaffolds. In addition, beating cardiomyocytes differentiated from mouse iPSCs were not observed on native scaffolds but on cross-linked scaffolds due to increase in stiffness. The overall results suggest that three dimensional type I collagen scaffolds with suitable cross-linking can recapitulate the mechanical stimuli required for directing the differentiation of iPSCs into cardiomyocytes and these stimuli can play an important role in determining iPSC fate.

1542

Image cytometry provides an optimal approach for automated characterization of stem cell pluripotency and differentiation.

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There is no doubt that stem cell research holds incredible promise in many areas of discovery research, as well as potential human therapeutic applications. Understanding the complex signaling processes, differentiation mechanisms and general physiology of stem cells is critical to their utilization, and often involves painstaking, manual experiments that provide only subjective results. Image cytometry offers a rapid, automated and quantitative approach to investigating stem cell biology at the cellular level, enabling increased productivity and the promise to accelerate this critical area of biological research. Human foreskin iPS cells or HSF-6 human embryonic stem cells were either maintained in a pluripotent state or differentiated into mesoderm, endoderm or ectoderm using standard directed protocols. Indirect immunofluorescence was utilized to stain cells with standard pluripotency and differentiation markers (Oct4, SMA, FOXO2, and/or β -III tubulin). In addition, mouse cortical stem cells were directed to differentiate into neurons, astrocytes and oligodendrocytes and stained for β -III

tubulin, GFAP or O4, respectively. Image cytometry was used to quantitatively characterize the resulting cellular populations. This work demonstrates the advantages of image cytometry, current applications of the technology in stem cell research and demonstrates how image cytometry can provide a reliable technique for determining the pluripotency of stem cells, as well as allow rapid characterization of differentiated cells.

1543

Heterogeneous expression of pluripotency-associated gene in mouse embryonic stem cells visualized by bioluminescence microscopy.

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The embryonic stem (ES) cells consist of various cell subsets which express different levels of pluripotency associated genes such as Nanog, Oct4, and Sox2. The heterogeneous expression of the transcription factors associated with pluripotency within ES cells has been shown by the use of RT-PCR, luciferase reporter assay, flow cytometry, and immunohistochemistry. Although these conventional methods provide the end-point data of gene expression, they cannot monitor the temporal changes within individual cells. Therefore, the temporal and spatial analysis of gene expression at the single cellular level is required for studies on self-renewal and differentiation processes of ES cells. To understand the exact profile of gene expression in the process of ES cell differentiation, gene expression of Nanog in mouse ES cells were monitored by using the single cellular bioluminescence imaging system (LV200, Olympus). The time course imaging analysis using luciferase as a reporter revealed that the promoter activity of Nanog gene mostly fluctuated and the pattern of fluctuation varied among the colonies of ES cells. The promoter activity of Nanog gene decreased in the most of ES cells after FGF-induced differentiation, whereas higher Nanog expression was sustained in small subpopulation of ES cells. We also found that Nanog promoter activity revealed the heterogeneous response in each cell by dual inhibition of mitogen-activated protein kinase signaling pathway using PD184352 and SU5402. Several studies reported that transcription factors associated with pluripotency are expressed in a heterogeneous fashion in ES cell cultures, however, this is the first study to show the temporal and spatial dynamics in Nanog expression of the identical cells at the single cellular level by using real-time imaging method. Our luminescence imaging system has the potential to provide some insight into possible diversity between ES cells in pluripotency and cell fate decision research.

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Patient-derived Neural Progenitors: Setting the Stage for Neurodegeneration.

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Parkinson disease (PD) is a multi-factorial progressive neurodegenerative disorder resulting in loss of dopaminergic neurons in the substantia nigra and characteristic intracellular inclusions called Lewy bodies. Genetic predisposition, such as mutations of the alpha-synuclein (SNCA, PARK1/4) gene e.g. point mutation and copy number variants of the SNCA gene locus (SNCA-Tri), can accelerate disease progression and can result in autosomal-dominant PD. α -synuclein has been suggested to affect the function of membrane associated processes, specifically impairing mitochondrial morphology, dynamics and function in the highly energy-dependent dopaminergic neurons. The impact of PD mutations on the stability and developmental potential of neuronal stem cells may also have wide ranging implications for the development, maintenance, and quality of neuronal networks.

Here, we investigated if changes and impairment in mitochondrial function are already present in neuronal precursor cells (NPCs) derived from induced pluripotent stem cells of a patient with an SNCA triplication. Using medium to high throughput biochemical and imaging assays, we assessed cellular morphology and mitochondrial function in SNCA-Tri NPCs under normal and glucose-deprived growth conditions and under environmental stress. SNCA-Tri NPCs displayed overall normal cellular and mitochondrial morphology, but have a 2-fold increase of alpha-synuclein protein levels. SNCA-Tri NPCs showed changes in growth, mitochondrial protein import, cellular energy metabolism and specifically mitochondrial function especially when exposed to environmental or toxin stress. SNCA-Tri NPCs exhibited higher basal levels of reactive oxygen species and a reduced capacity to mitigate the impact of oxidative stress. These cells were also more sensitive to mitochondrial permeability transition pore opening and to apoptotic cell death.

Given the role of mitochondria not only in cellular energy metabolism, but also their crucial role in development, genetic alterations such as the SNCA gene triplication may set the stage for decreased developmental fitness, accelerated aging and increased neuronal cell loss in patients with genetic forms of PD.

These observations of “stem cell pathology” could have wide ranging implications on both quality and quantity of the neuronal networks in PD and may be applicable to a broader range of neurodegenerative disorders.

1545

The RB tumor suppressor restricts reprogramming by directly silencing pluripotency genes.

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The reprogramming of differentiated cells to an embryonic stem cell-like state provides a powerful system to explore fundamental mechanisms of development, including how mammalian cells establish and maintain pluripotency and long-term self-renewal capability. Based on the similarities between embryonic stem cells and cancer cells, we investigated the potential role of the retinoblastoma tumor suppressor and cell cycle regulator RB in the reprogramming of fibroblasts into induced pluripotent stem cells (iPS cells). Here we show that loss of RB function leads to both an acceleration of the reprogramming process and the generation of more iPS clones from fibroblasts. This effect is largely due to a restrictive role for RB at the early stages of reprogramming. Surprisingly, however, RB inactivation does not enhance the formation of iPS clones by accelerating the proliferation of cells undergoing reprogramming. Rather, a genome-wide investigation of RB targets indicates that RB binds to regulatory regions of pluripotency genes such as *Sox2* and *Oct4* and contributes to their full repression in differentiated cells. This effect correlates with the maintenance of a repressive chromatin structure at these loci. Accordingly, *Rb*-deficient fibroblasts can be reprogrammed into iPS cells even in the absence of exogenous *Sox2*, which is normally required to initiate reprogramming from fibroblasts. These experiments identify a novel barrier in the reprogramming process, mainly the repression of some pluripotency genes such as *Sox2* by RB, which provides a new link between tumor suppressor mechanisms and cellular reprogramming.

1546

Enhanced in vivo regenerative potential of fetal-derived muscle stem cells*M. Tierney¹, A. Sacco¹; ¹Muscle Development & Regeneration, Sanford-Burnham Medical Research Institute, La Jolla, CA*

Adult muscle stem cells (MuSC) are primarily responsible for the homeostatic maintenance and repair of skeletal muscle, residing in a quiescent state until stimulated by external cues. Their developmental precursors, fetal MuSC, are an actively cycling population of Pax7-expressing cells required for embryonic myogenesis and represent a related but intrinsically different cell population. In an effort to decipher their intrinsic properties, we compared their self-renewal and regenerative potential with adult MuSC. Both populations were successfully isolated via flow cytometry using the cell surface markers alpha-7 integrin and CD34 and uniformly expressed Pax7. Fetal MuSC were readily able to form myotubes in vitro, whereas single cell analyses demonstrated comparable clonogenicity. Interestingly, a subset of cycling fetal MuSC did not express MyoD when expanded in culture, suggesting that they are resistant to myogenic commitment. When transplanted in vivo, fetal MuSC displayed a robust proliferative potential and successfully fuse with a substantial number of resident myofibers. Engrafted fetal MuSC were also able to respond to serial injury and give rise to an expanded pool of progenitors, strongly indicating stem cell function. Lastly, fetal single myofibers can be successfully isolated with their associated MuSC in situ, partitioned beneath the developing basal lamina. Ongoing work has revealed differential behavior and self-renewal potential of fetal and adult MuSC in suspension cultures. Future lineage tracing experiments are planned to establish the developmental relationship of adult and fetal MuSC, as well as the timing and extent of fetal MuSC contribution to the quiescent adult satellite cell pool.

1547

BAF53A enforces the epidermal progenitor state by re-targeting the SWI/SNF/BAF chromatin remodeling complex away from differentiation gene promoters.*X. Bao¹, J. Tang², V. Lopez-Pajares¹, S. Tao¹, K. Qu¹, G. R. Crabtree², P. A. Khavari¹; ¹Dermatology, Stanford University, Stanford, CA, ²Howard Hughes Medical Institute, Stanford University, Stanford, CA*

Epidermal progenitors suppress expression of terminal differentiation genes. Recently, epigenetic mediators of DNA and histone modification have been identified as dynamically regulating such repression, including DNMT1, JMJD3, Polycomb, and HDAC1/2. These data indicate a role for multiple classes of epigenetic regulators in this process, however, the impact of epigenetic chromatin remodelers, such as the ATPase driven multi-subunit BAF (SWI/SNF) complex, is not fully characterized. To address this, we performed loss of function studies of multiple complex subunits in epidermal tissue. Conditional BAF53A (ACTL6A) gene deletion in mice was found to abolish epidermal progenitor maintenance and to induce differentiation, leading to a failure of epidermal self-renewal. Similar findings in organotypic human epidermis were observed with BAF53A depletion via RNAi, where ectopically expressed BAF53A also suppressed differentiation, indicating that BAF53A enforces the undifferentiated cell state. Depletion of key components of multiple histone acetyltransferase and chromatin remodeling complexes with which BAF53A can physically associate identified a phenocopy only with the BAF250A (ARID1A) BAF complex subunit. Paradoxically but consistent with prior knockout mouse studies, depletion BRG1/BRM ATPase subunits impaired differentiation gene induction, suggesting that BAF53A functions as an anti-differentiation component of the BAF complex. Consistent with this, BAF53A is down-regulated during normal differentiation. Mechanistically, chromatin immunoprecipitation (ChIP) experiments demonstrated that BAF53A impairs binding

of the BRG1/BRM-containing BAF complex to differentiation gene promoters, including key regulators such as of KLF4, blocking their induction. These data indicated that BAF53A maintains the undifferentiated progenitor state by opposing BAF complex-enabled differentiation via genomic re-targeting of this chromatin remodeling complex away from differentiation gene promoters.

1548

Dissecting the role of histone variant macroH2A in iPS reprogramming and cancer progression.

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MacroH2A is a unique histone variant, consisting of an N-terminal region similar to canonical histone H2A and a non-histone macro domain at its carboxyl terminus, making it the largest known histone variant. The role of macroH2A as a transcriptional repressor has yet to be clarified, and its biological role development and cancer progression are now being unraveled.

Here we show that during development and differentiation, macroH2A1 and macroH2A2 isoforms become enriched in the chromatin fraction, and we hypothesized that macroH2A could create an epigenetic barrier to somatic cell reprogramming towards pluripotency. We show that dermal fibroblasts derived from macroH2A1 and macroH2A2 double knockout (dKO) mice are more efficiently reprogrammed than their wild type counterparts. Using macroH2A single knockout cells and rescue studies, we demonstrate that macroH2A isoforms act synergistically in this process. Furthermore, macroH2A is deposited to regulatory regions of pluripotency factors upon differentiation. Finally, macroH2A dKO iPS cells maintain their ability to differentiate in vitro and in teratomas. Therefore, we propose that macroH2A isoforms are dispensable for differentiation, but provide a redundant silencing layer or terminal differentiation 'lock' that, in turn, presents as an epigenetic barrier when differentiated cells are challenged to reprogram.

Our lab has demonstrated that macroH2A isoforms are lost during melanoma progression. Coupled to our reprogramming studies, we hypothesize that macroH2A may act as global repressor that is evicted from chromatin as a cancer cell becomes more aggressive and undifferentiated. We have observed low levels of macroH2A in triple negative breast cancer cell lines and our data suggests that over-expressing macroH2A in these cells may decrease their tumorigenicity. We are also interested in understanding whether progenitor and tumor initiating cell populations rely on macroH2A downregulation for their maintenance.

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microRNA dynamics in the maintenance and reprogramming of mouse pluripotent stem cells.

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Several microRNA families, such as the 290-cluster and 302-cluster families have been classified as embryonic stem cell specific and have been demonstrated to serve as cell cycle regulators vital to the maintenance of self-renewal. In order to gain a comprehensive understanding of the function of microRNAs within pluripotent cells we examine them within mouse embryonic stem cells over a time course of differentiation induced by loss of key transcription factors, Nanog or Pou5f1(OCT3/4).

We find using deep sequencing that, as expected, the milieu of microRNAs varies with loss of these key pluripotency genes and further show that they are differentially incorporated into the RISC complex via HITS-CLIP studies. In particular, the microRNAs shown from previous

ChIPseq studies to have Nanog and Pou5f1 at their transcript start sites (TSS), e.g. the 290-cluster, are coordinately lost upon transcription factor perturbation and that the respective microRNA target mRNA are released from repression. This demonstrates a complex and multilayered regulatory mechanism mediated by core pluripotency genes beyond transcriptional regulation to non-coding RNA-based epigenetic regulation. Within the same time course, we also measure by mRNAseq the transcriptome to provide additional knowledge of potential microRNA mediated target regulation.

To augment our understanding of this transcription factor-microRNA coordinated regulation in the context of pluripotency, we also examine the expression of microRNAs during reprogramming of mouse embryonic fibroblasts to induced pluripotent stem cells using the four canonical transcription factors, Pou5f1, Sox2, Klf4 and c-Myc using a Tet-inducible cassette. Here we also see a direct upregulation of pluripotency-related microRNAs over the course of reprogramming.

We observe both transcriptional activation and repression of microRNAs with Pou5f1 located near their transcript start sites (TSS) during loss of Pou5f1 and overexpression of Pou5f1 during reprogramming. We seek to integrate our knowledge of microRNA function during the loss and gain of pluripotency to provide a model of microRNA function and participation in the maintenance of self-renewal and pluripotency associated with embryonic stem cells.

1550

Clonal Level Lineage Commitment of Mouse Hematopoietic Stem Cells In Vivo.

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Hematopoietic stem cells (HSCs) sustain the blood and immune systems through a complex lineage commitment process. While this process has been extensively studied at the population level, little is known about the lineage commitment of individual HSC clones. Here, we provide a comprehensive map of *in vivo* HSC clonal development that reveals new HSC regulatory mechanisms undetectable at the population level.

The clonal map was derived from the simultaneous tracking of hundreds of individual mouse HSCs *in vivo* using genetic barcodes. These unique barcodes were delivered into HSCs using a lentiviral vector to obtain a one-to-one mapping between barcodes and HSCs. Barcoded HSCs were then transplanted into recipient mice using standard procedures. Recipients were either conditioned with irradiation (myeloablative) or with an anti-ckit antibody (non-myeloablative) prior to transplantation, or were not conditioned with any regimen. Genetic barcodes from donor derived HSCs and their progeny were examined twenty-two weeks after transplantation using high-throughput sequencing.

While irradiation-mediated transplantation has been widely used as a gold standard to study HSCs, our data suggests that irradiation conditioning alters HSC lineage commitment at the clonal level. After syngeneic transplantation into mice absent of any conditioning regimen, all donor-derived HSC clones homogeneously regenerate blood without exhibiting any measurable dominance or lineage bias. In contrast, when recipient mice are pre-conditioned with irradiation, only a small fraction of donor-derived HSC clones differentiates, while dominantly expanding and exhibiting lineage bias. Interestingly, when recipient mice are pre-treated with an anti-ckit antibody, the lineage commitment of some HSC clones resembles those transplanted into unconditioned mice, exhibiting no clonal dominance or lineage bias. Other clones in the same

mice resemble those transplanted into irradiated mice, exhibiting both clonal dominance and lineage bias.

We have identified the cellular origins of clonal dominance and lineage bias, and have uncovered the lineage commitment pathways that lead HSC clones to differential blood production. This study reveals surprising alterations in HSC regulation by irradiation, and identifies the key hematopoiesis stages that may be manipulated to control blood production and balance.

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Regulation of stem cell growth and differentiation by polypeptide multilayer films.

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Cell growth and differentiation is critical for many physiological events such as embryonic development and wound healing. Polypeptide multilayer films are promising for the development of artificial extracellular matrix for the regulation of stem cell differentiation since they are entirely synthetic and can mimic crucial features of ECM proteins. In addition, this material allows for modulation of thickness, stiffness, and adhesiveness. In this study, mouse induced pluripotent stem cells (iPSCs) were cultured on EDC/NHS cross-linked and gelatin-coated polypeptide multilayer films built up by the alternate adsorption of poly-L-lysine (PLL) and poly-L-glutamic acid (PLGA), and the growth and differentiation of these cells were evaluated. The results showed that though mouse iPSC colonies maintained similar initial adhesion and attachment regardless of layer number of films, they demonstrated better cell spreading and growth on even number layer of films, where the top layers are PLGA films. In addition, compared with cells cultured on 2-layer film, the cardiomyocyte differentiation of mouse iPSCs on 4- and 6-layer films showed a significant increase in the differentiation rate, especially when EDC/NHS cross-linkers with higher concentration were used to form the films. This cellular response on 4- and 6-layer films was coupled with increase in the growth rate. These observations indicate that mouse iPSC growth and differentiation on polypeptide multilayer films can be regulated by the type of the outermost polypeptide layer, the concentration of cross-linkers, and the number of layers in the film.

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Understanding pathogenesis of lissencephaly-a severe neurodevelopmental disorder-with patient-derived induced pluripotent stem cells.

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Miller-Dieker Syndrome (MDS) is the most severe form of lissencephaly or “smooth” brain-a neuronal migration disorder that arises during early development, leading to disorganized cortical layering and devastating neurological consequences such as mental retardation and intractable epilepsy. MDS is always caused by heterozygous deletions of the 17p13.3 locus harboring about twenty genes, with the critical missing region spanning from LIS1 to YWHAE. Attempts to generate mice with such a large deletion have thus far been unsuccessful. While it is commonly accepted that defective neuronal migration is the primary cause of lissencephaly, it is unknown whether brain cells of human patients develop, migrate, mature or function properly. To facilitate our understanding of MDS pathogenesis and to begin elucidating the contributions

of genes within the deleted locus, we generated induced pluripotent stem cells (iPSCs) from fibroblasts of two MDS patients. We found that MDS fibroblasts can be efficiently reprogrammed into functional iPSCs that exhibit wild type characteristics in self-renewal and differentiation capacity in vitro and in vivo. We then optimized a neural differentiation strategy to produce cortical neural progenitors (NPCs) and neurons. We found that while MDS-iPSCs can be specified into cortical NPCs with high efficiency, upon specification they exhibit severely impaired viability and cannot be expanded through suspension or monolayer culture, likely due to increased apoptosis. Since expansion of proliferative neural stem and transit-amplifying cells is a key feature of human brain development that contributes to the structural complexity and foldedness of the neocortex, our results suggest that impaired proliferation of neural stem cells may be a more significant factor in lissencephaly pathogenesis than previously appreciated.

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Genetic and Pharmacological Properties of Human Cardiac Stem Cell-Derived Cardiomyocytes.

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Human Adult Cardiac stem cells (hACSCs) can be directed to differentiate into cardiomyocyte-like cells. These cells express cardiac-specific markers and display functional similarities to their adult counterparts. Based on these properties, hACSCs -derived cardiomyocytes have the potential to be extremely useful in various in vitro applications and to provide the opportunity for cardiac cell replacement therapies. However, before this can become a reality, the molecular and functional characteristics of these cells need to be investigated in more detail. In the present study we differentiate hACSCs into cardiomyocyte-like cells. The fraction of spontaneously beating clusters obtained from the Cardiac Stem Cells averaged approximately 25% of the total number of Cardiac Clusters used. These cell clusters were isolated, dissociated into single-cell suspensions, and frozen for long-term storage. The cryopreserved cells could be successfully thawed and subcultured. Using Florence microscopy, we observed Z discs and tight junctions in the hACSC-derived cardiomyocytes, and by immunohistochemical analysis we detected expression of cardiac-specific markers (cTnI and cMHC). Also, utilizing BrdU labeling we could demonstrate that some of the hACSC-derived cardiomyocytes retain a proliferative capacity. The pharmacological stimulation of the cells resulted in responses indicative of functional adrenergic and muscarinic receptor coupling systems. Taken together, these results lend support to the notion that hACSCs can be used as a source for the procurement of cardiomyocytes for in vitro and in vivo applications.

Germ Cells, Gametogenesis and Fertilization

1554

Expression Dynamics and Evolution of Sperm PIWIL1, PIWIL2, and AGO2.

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Early mammalian development is initiated by fertilization of the egg by the spermatozoon, and followed by dramatic reprogramming of gene expression during embryogenesis and beyond. Sperm provides half of the genome and other macromolecules, including coding and non-coding transcripts, into the oocyte to enable the formation of new organism, zygote. Research in our

laboratory and elsewhere showed that mammalian spermatozoa contain small non-coding RNAs, including piwi-interacting RNAs (piRNAs) and microRNAs (miRNAs). It is also known that spermatozoa lack ribosomal RNAs and that transcription and translation are largely absent in these cells. Although these short transcripts are known to play a vital role in genome protection and regulation of gene expression by RNA interference (RNAi), their functions in the sperm are not known. The objectives of this study were to: 1) analyze the expression profiles of specific binding proteins associated with piRNAs and miRNAs: PIWIL1, PIWIL2, and AGO2, in sperm from a high vs. a low fertility bull, and 2) determine conservation of the protein sequences among mouse, human, cow, dog, and opossum. To accomplish these objectives, we performed immunocytochemistry using bull sperm and clustal alignments to detect the piRNA and miRNA binding proteins and evolutionary conservation, respectively. Our results showed that PIWIL1, PIWIL2, and AGO2 were detectable in the sperm with varying cellular locations including sperm head and tail. In addition, all the three proteins are well conserved across the mammals examined. The degree of conservation was over 88% and 99% for PIWIL1 and 2, and AGO2 proteins, respectively. The results suggest that since the expression of these sncRNA binding proteins are present within the mature sperm cell; this can further implicate that the machinery for the transcriptional control of gene expression is present in the male gamete and that this may play roles in regulating gene expression at the onset of development. Furthermore, regulation of the gene expression by miRNAs, and protection of the genome by piRNAs appear to be a well- conserved mechanism across the mammals.

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Dissecting Auxin and *indeterminate gametophyte1 (ig1)* Signaling in Maize Embryo Sac Development.

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In plants, the female gametophyte produces two gametes, the egg and central cell, which are fertilized by the two male gametes in the process called double fertilization resulting in the embryo and endosperm of the seed. A detailed understanding of embryo sac development is central to better seed production. The female gametophytes of angiosperms have four cell types: the egg cell, synergids, central cell and antipodal cells arranged in the micropylar-chalazal axis. Cereal grasses possess proliferative antipodal cells which persist after fertilization, in contrast to *Arabidopsis* antipodal cells, which degenerate during embryo sac development. A number of maize mutants with altered antipodal cell morphology are being characterized. One of these, *indeterminate gametophyte1 (ig1)*, encodes a Lateral Organ Boundaries(LOB) domain protein with high similarity to Asymmetric Leaves2(AS2) of *Arabidopsis thaliana*. To decipher the mechanism of *ig1* regulation of embryo sac development, interactors of the LOB domain were identified in a yeast two- hybrid screen. Preliminary results identified DNA/chromatin modifiers and proteins involved in cell metabolism as potential interactors. RNA-Seq analysis of *ig1* mutant embryo sacs identified several differentially expressed genes, including several transcription factors. Detailed analysis of the two hybrid interactors and RNA-seq data, including future mutant analyses, could provide critical insights into female gametophyte development in plants.

To explore the role of phytohormone, auxin in maize embryo sacs, expression patterns of two fluorescent reporters in maize (a reporter for auxin levels (DR5::RFP) and auxin efflux carrier (PIN1::PIN1-YFP) were examined. In Maize, unlike in *Arabidopsis* DR5 expression is not present in the embryo sac before cellularization. However, in mature maize embryo sacs both DR5 and PIN1 expression are expressed strongly and specifically in the antipodals. Embryo sac enriched genes from RNA-seq data were consequently searched for cis Auxin Response

Elements (AuxRE) suggestive of these genes potentially regulated by auxin and compared to genes misregulated in *ig1* mutant embryo sacs to identify genes potentially regulated by both. Preliminary in-situ's with Maize Auxin Response Factors (ZmARF's) support presence of auxin in Maize antipodal cells. This raises the possibility that differences in auxin levels and patterns may be partially responsible for antipodal cell biology. Experiments that alter cell type specific auxin levels in the embryo sac are in progress to address this possibility.

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Expression and function of LYAR in male reproduction.

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Mammalian spermatogenesis is a complex process that involves an intrinsic genetic program consisting of germ cell-specific and -predominant genes. In the present study, we analyzed *Ly-1 reactive clone (Lyar)* gene in the mouse. *Lyar* is known to be expressed abundantly in the testis and encodes a nucleolar protein which contains a LYAR-type C2HC zinc finger motif and three copies of nuclear localization signals. We showed that *Lyar* is expressed predominantly in the testis and this expression is specific to germ cells in the testis. Protein analyses with an anti-LYAR antibody demonstrated that the LYAR protein is present in spermatocytes and spermatids, but not in sperm. To assess the functional role of LYAR *in vivo*, we have established a mouse model with the aid of gene trap mutagenesis approach. *Lyar* mutant mice were born live and developed normally. Male mutant mice lacking LYAR were fully fertile and spermatogenesis in the mutant testis was intact. Taken together, our study demonstrates that LYAR is strongly preferred in male germ cells, but has a dispensable role in spermatogenesis and fertility.

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Species Specificity of Energy Metabolisms and Mitochondrial Morphology in Fish Spermatozoa.

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Since fish spermatozoa are spawned and diluted in seawater, they have to swim through utilizing intracellular energy substrates to reach egg. To clarify any relation in sperm motility, energy sources and mitochondrial function and morphology, we compared these factors in spermatozoa freshly prepared from flounders such as Black flounder (*Liopsetta obscura*) and Barfin flounder (*Verasper moseri*), Pacific herring (*Clupea pallasii*). Spermatozoa of both flounders moved linearly, and turned their directions at the egg micropyle where they entered, while herring spermatozoa crawled into the micropyle in rotation movement. When diluted in seawater, spermatozoa of *L. obscura* swam vigorously followed by stopping the movement within one minute. *V. moseri* spermatozoa kept swimming for more than ten minutes. In *C. pallasii* spermatozoa, a very few sperms moved rarely in seawater, although they started vigorous swimming near the egg micropyle. Mitochondria produce the energy needed for biological functions mainly via the electron transport chain, concomitantly generating reactive oxygen species (ROS). Because ROS have the potential to cause cellular damage, cells defend against ROS through either enzymatic and/or non-enzymatic ROS eliminating systems. We also examined ROS eliminating mechanisms in these fish spermatozoa. Spermatozoa of *V. moseri* and *C. pallasii* showed high activities to degrade hydrogen peroxide (H₂O₂) added exogenously,

while the spermatozoa of a flounder, *L. obscura*, exceptionally showed low activity. Morphological observations revealed that *L. obscura* spermatozoa possesses small mitochondria with undeveloped cristae while *V. moseri* and *C. pallasii* possesses large mitochondria with developed cristae. These results, taken together, indicate that the sperm motility and energy metabolism in fish is coupled to its mitochondria morphology, varying from one species to another.

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Effect of streptozotocin-induced diabetes on membrane integrity and acrosome reaction in mice spermatozoa.

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Diabetes mellitus is a well-recognized cause of male sexual dysfunction and impairments of male fertility. This alteration includes endocrine disorders, neuropathy, deleterious effects on male reproductive function and increased oxidative stress. The aim of this study was to investigate the spermatozoa quality, the membrane integrity, acrosome reaction and testicular histology on mice diabetics during 60 days. Male CD1+ mice were divided into two groups of eight animals each: diabetic and control group. Animals were treated with a single injection of streptozotocin (150 mg/kg; ip), or buffer citrate. Blood glucose was estimated after one week and the animals with glucose levels ≥ 250 mg/dl were included in this study. Animals were kept with diabetes during two months before the experiments. We evaluated several parameters in spermatozoa from diabetic and not diabetic mice: i) standard quality analysis according to World Health Organization, ii) integrity of sperm membrane by hypoosmotic swelling test (HOS-T), iii) acrosome reaction by staining method using Coomassie Blue G-250 and iv) histology testicular using technical paraffin-embedded and stained with hematoxylin and eosin. Spermatozoa from diabetic mice exhibited a significant decrease in motility ($p \leq 0.001$), concentration ($p \leq 0.0042$), viability ($p \leq 0.001$), a significant increase on alteration in membrane integrity ($p \leq 0.001$), and a significant increase in the loss of acrosome ($p \leq 0.001$), relative to spermatozoa from control group. Finally, the histoarchitecture in testis was visibly altered diabetic mice compared with control group. These observations suggest that subchronic diabetes causes an important alteration in the integrity of sperm membrane.

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PI(4,5)P2 is required for chromatin reorganization during spermiogenesis.

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Phosphoinositides (PIPs) regulate several aspects of *Drosophila* spermiogenesis, such as axoneme biogenesis, cell polarity and membrane trafficking. Here we show that PIPs are also required for nuclear morphogenesis, a process essential for male fertility and which requires interactions between nuclear membrane, chromatin and cytoskeleton. Nuclear morphogenesis includes dramatic changes in the shape and size of the sperm head and also in the state of chromatin condensation. These changes are associated with microtubule-dependent nuclear elongation, when the perinuclear microtubule cytoskeleton is reorganized to provide additional support to the elongating nucleus. Chromatin is reorganized by switching from a histone-based chromatin configuration, present in early round spermatid nuclei, to a protamine-based configuration, present in mature sperm nuclei. Spermatids in which levels of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) have been reduced show profound defects in chromatin organization, lack of nuclear maturation, and male sterility. Posttranslational modification of histones is impaired and their removal is delayed. Protamines get incorporated into nuclei

despite histones not being completely removed. Transition proteins are not present in these spermatids. Localization of inner nuclear membrane proteins is defective, sumoylation is impaired, and repair of double-stranded DNA breaks is incomplete. Our present data suggest that normal levels of PI(4,5)P2 are required to coordinate interactions between the nuclear membrane and chromatin.

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Spindle assembly checkpoint plays a role in DNA-damage-induced cell cycle arrest in *C. elegans* male germ line.

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Persistent DNA damage in germline stem cells leads to embryonic lethality, progeny inviability or germline tumors. Consequently, cells closely monitor genomic integrity and can delay their progress through the cell cycle so that repair precedes division. In *C. elegans*, genotoxic perturbations to proliferative cells in the distal tip of the gonad activate the DNA damage response (DDR) pathway, resulting in cell cycle arrest. When this arrest is in response to stalled replication forks induced by hydroxyurea (HU), it is characterized by enlarged nuclei and can be visualized cytologically.

We have found that while RNAi knockdown of several genes in the DDR pathway disrupts checkpoint output in hermaphrodites, the same treatment does not prevent HU-induced arrest in males, suggesting that not all components of the DDR are essential for male cell-cycle arrest in response to stalled replication forks.

We next investigated functional redundancy between the DDR and the spindle assembly checkpoint (SAC), which is most often associated with regulating kinetochore attachment to spindles during prometaphase/metaphase of mitosis and meiosis. RNAi knockdown of several SAC components alone did not affect HU-induced cell-cycle arrest in males; however, knockdown of both the DDR and SAC resulted in a failure to arrest in the presence of HU. This result suggests that males use two independent pathways to elicit arrest in response to stalled forks. Preliminary data from experiments utilizing cell cycle markers suggests that the SAC, like the DDR, mediates an S phase arrest not predictive of its expected role as an inhibitor of *cdc20* at metaphase. Future work aims to understand this novel role for SAC components and investigate the mechanisms used by the SAC to induce an S phase arrest in males.

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Excess Consumption of Multiple Sugars Reduces Fertility in *Caenorhabditis elegans*: Roles for Hexosamine Signaling and Sex-Specificity.

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High sugar diets have been linked to several diseases: increased glucose intake correlates with obesity, diabetes, and cardiovascular disease, and increased galactose intake can lead to liver failure and neurotoxicity. The nematode worm *Caenorhabditis elegans* has a conserved insulin-signaling pathway and is thus a good model for the response to high sugar diets. Previous studies have shown that exposing hermaphrodite worms to high glucose conditions decreases fertility and lifespan (Lee *et al.* 2009, Mondoux *et al.* 2011). Among its many functions in the cell, glucose serves as the precursor for O-linked N-acetyl-glucosamine (O-GlcNAc), a post-translational protein modification. Mutants that lack the O-GlcNAc transferase, *ogt-1*, have decreased fertility and lifespan even at low glucose concentrations. We wanted to test whether the fertility response to sugar stress was sex-specific and/or glucose-specific.

We tested sex-specificity by mating hermaphrodites and males in the presence of glucose stress and found that the number of progeny and proportion of male progeny were reduced by ~20% compared to mating on control plates, indicating decreased male fertilization success. In order to determine the mechanism of this response, we performed a courtship ability assay and scored the location of males and their interactions with hermaphrodites. Interestingly, males on high glucose had no loss of courtship ability, indicating that their ability to find mates was not limited by glucose exposure. These results also showed a uniquely male sensitivity to glucose stress, as male fertilization success was compromised at concentrations that had no effect on hermaphrodite fertility.

We tested glucose specificity by assaying fertility in response to a high galactose diet. Glucose and galactose have the same molecular formula, and only differ in their spatial orientation, leading us to hypothesize that fertility levels would be similar for both sugars at the same concentration. However, we found that wild type *C. elegans* had no fertility defect at 333mM galactose, even though fertility is decreased 30% at that concentration of glucose. *C. elegans* was sensitive to galactose stress, as 500 mM galactose reduced fertility by 50% and caused a delay in reproductive timing. *ogt-1* mutant worms, in contrast, had extremely low fertility on 333mM galactose (< 10% fertility compared to wild-type); even lower than the published fertility levels on 333mM glucose.

Together, our data suggest that *C. elegans* is sensitive to multiple types of sugar stress but that different genetic backgrounds and sexes have different sensitivities to different sugars. Future experiments will test the role of germline apoptosis in both males and hermaphrodites and elucidate the role O-GlcNAc might play in this process.

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Sperm Parameters and Leydig cells function in adult rats are impaired by maternal obesity and overnutrition postnatal.

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Sperm production and steroidogenic activity of Leydig cells are impaired by obesity in adult life. This investigation evaluates the effects of obesogenic environment (OE) during different periods of development on sperm parameters and Leydig cell function of adult rats. Wistar rats were subjected to a balanced feeding from gestation to adulthood (control group, 4% lipid in diet) or to OE during gestation (O1 group), from gestation to weaning (O2 group), from weaning to adulthood (O3 group), from breast-feeding to adulthood (O4 group) or from gestation to adulthood (O5 group). Obesity was induced to female rats before the pregnancy (group O1, O2 and O5) and also to offspring of groups O3, O4 and O5 by high-fat diet (20% saturated lipid) for 15 weeks. Animals were killed with 18-week-old and body weight, adiposity index and plasma leptin and testosterone were determined. It was performed the sperm counts in the testis and epididymal regions, the daily sperm production (DSP) and the transit time of spermatozoa in the epididymis. In addition, testes were subjected to immunohistochemistry and western blotting for androgen receptor (AR) and 17 β -hydroxysteroid dehydrogenase (17 β -HSD). All groups exposed to OE were obese at adulthood, as indicated by increase in body weight, adiposity index and plasma leptin. Obese groups presented a decrease in circulating testosterone. Furthermore, the number of spermatids in the testis and the DSP decreased 16% for group O1, 20% for groups O2 and O4, and 26% for groups O3 and O5. The number of sperm in the region caput/corpus epididymis decreased about 17% for groups O1, O2, O3 and O4, and 26% for

group O5. This reduction was 9% for group O1, 20% for groups O2, O3 and O4, and 27% for group O5 in the cauda; however, the transit time of spermatozoa through the caput/corpus and cauda of the epididymis was not affected. The number of Leydig cells AR- and 17 β -HSD-positive was unaffected in obese rats but the content of these proteins in crude testis samples varied among obese groups. A low expression of 17 β -HSD was detected to all obese groups. The OE during gestation/breast-feeding led to overexpression of AR whereas the others obese groups showed a reduction the AR content of testis in relation to control. In conclusion, the results observed in this study indicate that maternal obesity and overnutrition postnatal reduces the production and reserve of spermatozoa, as well as commits the function of Leydig cell in adult. This was probably due to decrease in circulating testosterone and reduction in AR and 17 β -HSD expression. Financial Support: FAPESP.

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A role for a sperm protease at fertilization.

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During fertilization, the sperm and egg membranes fuse and the zygote metabolism is activated. While many of the signaling molecules involved in fertilization have been discovered, little is known about the cell surface molecules that allow for sperm-egg fusion and egg activation. A number of proteases have been identified on the sperm acrosome and could potentially play a role in the initiation of fertilization. Among these are trypsin-like proteases (other than acrosin) whose function(s) have not been well established. A sperm acrosomal protease could participate in fertilization at several levels, functioning in: 1) the acrosome reaction that remodels the sperm surface to prepare it for fertilization; 2) penetration of the large jelly layer and/or the vitelline coat that surround the egg; 3) cleaving a receptor on the egg membrane, changing its conformation and initiating signaling pathways; or 4) remodeling an egg cell surface protein to generate a fusion protein to facilitate sperm-egg membrane fusion. The starfish represents a model system to test these ideas because of its amenability to in vitro fertilization following removal of either the egg jelly or the vitelline envelope, and it is possible to stimulate the acrosome-reaction in sperm separately from the eggs. To identify membrane proteins, the starfish eggs were biotinylated. Following fertilization, biotinylated fragments from cleaved cell surface proteins were recovered from sea water indicating that a protease does have a function. Next, to test possible protease functions at fertilization in the starfish, different egg preparations were fertilized in seawater with or without the presence of soybean trypsin inhibitor (SBTI) and scored for egg activation by vitelline envelope elevation. SBTI (1mg/ml, 2mg/ml, and 3mg/ml) inhibits fertilization envelope elevation in jelly-, and vitelline envelope-intact eggs. This suggests that the sperm protease is necessary for either the acrosome reaction and/or penetration of the egg jelly or vitelline envelope. Fertilization envelope elevation was also blocked when jelly-free eggs were treated with SBTI and fertilized with acrosome-reacted sperm, which strongly suggested that penetration of the vitelline envelope is dependent upon the sperm trypsin-like protease activity. When performing the SBTI experiment on jelly-free and vitelline-free (naked) eggs, they were observed after fertilization for cell division (as they do not have a vitelline envelope to elevate). At 2 hours after sperm were added, division was observed in naked eggs, suggesting the protease is needed for vitelline envelope penetration.

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Zap70 and its downstream gene RanBP2 regulate meiotic cell cycle speed in oocytes.

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Previously, we demonstrated that Zap70 (Zeta-chain-associated protein kinase) expressed in the mouse oocyte and played significant role in completion of meiosis. Aim of the present study was to determine the mechanisms of action of Zap70 during oocyte maturation by evaluating downstream signal networking after Zap70-RNAi.

To elucidate RNAi effects, Zap70 and RanBP2 dsRNA were microinjected into the GV (germinal vesicle) oocyte cytoplasm, and RNAi-treated oocytes were incubated in M16 medium containing IBMX (3-isobutyl-methyl-xanthine) for 8 hours followed by culture in the plain M16 for 8 hours. We examined maturational changes and the gene expression profiles by microarray analysis. The probe hybridization and data analysis were used by Affymetrix Gene Chip Mouse Genome 430 2.0 array and GenPlex 3.0 (ISTECH, Korea) software. Expression of Zap70 and RanBP2 during meiotic maturation was observed by immunofluorescence staining.

Immunofluorescence analysis showed that the Zap70 was localized in the GV cytoplasm and moved on to the chromosomes during *in vitro* oocyte maturation. Also, we found that GVBD in the Zap70-RNAi oocytes occurs 30 min faster than in control group. Zap70-RNAi resulted in arrest at MI stage with elongated spindles with prematurely divided chromosomes at 6~8 hr stages, that usually found in anaphase (12~16 hr). Among the genes regulated by Zap70-RNAi, we selected 10.7 folds down-regulated RanBP2 (Ran binding protein 2), since RanBP2 has been known to be involved in NEBD (Nuclear envelope breakdown). RanBP2 was localized on the nuclear envelope and its depletion by RNAi caused exactly similar results as found in the Zap70-RNAi oocytes.

We concluded that 1) the Zap70 is a crucial factor during oocyte maturation; especially for regulating the exact timing of meiosis, and 2) Zap70 function involves the role of RanBP2. These results are the first report regarding the function of Zap70 and RanBP2 in the oocyte maturation and regulation of meiotic cell cycle.

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Identification of germ cell genes expressed in F9 cell line.

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Mammalian spermatogenesis is a complex process involving the differentiation of spermatogonial stem cells into spermatozoa. During this process, spermatogonia-, spermatocytes-, and spermatid-specific genes are expressed in germ cells, following a precise spatial and temporal order. Because of the absence of permanent germ cell lines, the transcriptional and functional mechanisms have not been studied in depth for germ cell-specific genes. F9 cell line is a teratocarcinoma cell line, induced by ectopically implanting embryos into the testis. Several laboratories have used this cell line in promoter-report assays for germ cell-

specific genes. It is possible that F9 cell line can be used as a cell model for research of germ cell-specific gene expression. To identify germ cell genes expressed in F9 cell line, we used a microarray-based approach. We found that 1088 genes were co-expressed in male germ cells and F9 cells. The numbers of genes expressed in spermatogonia, spermatocytes and spermatid are 532, 353 and 203, respectively. To identify genes with testis-specific expression, we performed *in silico* analysis with the aid of UniGene database. Among 1088 genes, nine genes were predicted as testis-specific genes. We are performing further studies to validate testis-specific expression and stage-specific expression of those genes. We will investigate their promoter regions responsible for the testis- and germ cell-specific expression using F9 cell line.

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Identification of a testis-specific KRAB gene in mice.

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C2H2 type-zinc fingers represent one of the largest families of transcription factor in mammals. One-third of them encode *Kruppel*-associated box (KRAB) domain which has a strong repressive activity in their N-terminal region. The KRAB domain is a conserved domain spanning about 40~75 amino acids. Structurally, it is composed of two subdomains, A and B boxes. KRAB containing zinc finger (KZNF) genes reside in large familial clusters, and many of them contain substantial numbers of lineage-specific genes. Most KZNF genes remain completely uncharacterized. To identify and characterize KZNF genes in the mouse genome, we performed *in silico* analysis and identified 191 KZNF genes. Based on tissue specificity, we focused to Mm.159188 gene which showed high specificity in testis. The Mm.159188 gene, also named as zinc finger protein 819 (Zfp819), consists of the KRAB domain and 14 zinc fingers. Tissue distribution analysis showed that the Mm.159188 gene is specifically transcribed in testis. Subcellular localization analysis revealed that GFP tagged-Mm.159188 protein is located in nucleus in NIH3T3 cells. Luciferase assay showed that the Mm.159188 has a repressive activity in transcription. Microarray analysis of cells overexpressing Mm.159188 identified 95 genes modulated more than 1.3-fold in expression level. Among them, 49 genes were down-regulated. Future analysis of these genes will elucidate the functional importance of the Mm.159188 gene in male germ cell development.

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Molecular cell biology of spermatid coiling and release in *Drosophila melanogaster*.

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Spermatogenesis in *Drosophila* testis takes place within an enclosure formed by two somatic-origin cyst cells. During the final stages, mature spermatids coil up inside the cyst enclosure at the base of testis, and subsequently, they are released into the testis lumen. This process is known as spermiation. Till date, very few attempts were made to understand how the seemingly mature sperm, trapped in the cyst cell enclosure, come out. Earlier studies hypothesized that the spermatids would pass through head cyst cell and terminal epithelium interface during the release process. We observed that mature spermatids are, however, pulled from the head cyst cells before passaging through the sperm roller into the seminal vesicle, in live testis preparations. The process shares similarity with spermatid-release from Sertoli cells in mammalian testis. In addition, a directed screen using protein-trap lines, for their localization during late stages of spermatogenesis, helped to identify dynamic, Disc large (*Dlg1*)-enriched structures, containing other septate junction markers, around the elongated and coiled spermatids. Disruption of these junctions, by *Dlg1* knockdown in the cyst cells, caused

premature spermatid release, suggesting an essential role of septate junctions in regulating spermiation. These results will help to elucidate the molecular cell biology of sperm maturation and release in *Drosophila*, and identify its parallels in mammalian testes.

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The protein prenylation alteration in Sertoli cells is associated with adult infertility resulted from childhood Mumps infection.

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Abstract; Mumps commonly affects children aged 5-9 years, often leading to mumps orchitis and resulting in permanent adult sterility in rare cases. However, the etiology of this long-term effect remains unclear. Mumps orchitis results in progressive degeneration of seminiferous epithelium and sometimes leads to Sertoli cell-only syndrome. Thus the left Sertoli cells may be critical to spermatogenesis recovery after orchitis healing. We found that the protein prenylation alteration in Sertoli cells during childhood might determine adulthood sterility. Mumps infection during childhood decreases the expression of geranylgeranyl diphosphate synthase 1 (GGPPS) in human testes. When we altered farnesylation/geranylgeranylation balance by specifically deleting GGPPS gene in Sertoli cell, Sertoli cell itself remained intact, while adjacent spermatogonium significantly decreased after the 5th postnatal day. The proinflammatory signaling pathways MAPK and NF- κ B were constitutively activated because the farnesylation of H-Ras was enhanced. GGPPS^{-/-} Sertoli cells synthesize an array of cytokines to stimulate spermatogonia apoptosis and chemokines to induce macrophage invasion into seminiferous tubules because the neonatal testes of mice had no BTB. Invaded macrophages further blocked spermatogonia development, thus resulting in a long-term effect on adult infertility. Our results suggest a novel mechanism by which mumps viral infection during human childhood results in adult infertility.

Conclusions: In summary, inactivating GGPPS in testicular Sertoli cell resulted in sustained activation of H-RAS proteins and spontaneous hyper-inflammation response in the early stage of testis development when there was no BTB formation between Sertoli cells of mice. Our results indicated that the protein prenylation in Sertoli cell was critical to regulate orchitis before puberty. This phenotype can mimic the assault against Sertoli cell during human childhood like mumps infection that would result in adulthood infertility.

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Relationship of testicular androgen receptor protein expression with in vitro fertilizability of epididymal sperm in mice.

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Strain differences in in vitro fertilizability still constitute a serious problem in mouse reproduction. We previously reported (Kawai et al., J Reprod Dev 52:561-568) that the in vitro fertilizability of sperm in five strains (mean \pm SEM, n=5) was 31.3 \pm 4.9%, 29.5 \pm 3.2%, 47.8 \pm 3.3%, 68.9 \pm 3.8%, and 97.4 \pm 4.3% in 129X1, C57BL/6, C3H, BALB/c (n=10), and DBA/2, respectively. To elucidate the molecular mechanism of strain difference in the in vitro fertilizability of mouse sperm, we examined the androgen receptor (AR) protein expression in testes of the five mouse strains.

Testes of five males per strain at 12 weeks of age were collected. Proteins from the testes were extracted using ReadyPrep protein extraction kit for Total Proteins (Bio-Rad). Quantitative Western blotting (QWB) using testicular protein extracts was conducted with anti-AR antibodies (Millipore, rabbit) and antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Millipore, mouse) as an internal control. Antibody-reacted bands were visualized with corresponding peroxidase-conjugated secondary antibodies (anti-Rabbit and anti-mouse IgG; Jackson ImmunoResearch) and chemiluminescent reagent (Pierce). Band intensities were quantified after image captures by a CCD-camera (LAS-3000, Fuji Film). AR protein expressions were obtained as AR/GAPDH ratios and statistically compared among strains by Kruskal-Wallis one way analysis of variance (ANOVA) on ranks (SigmaPlot 12, Systat software) because normality test (Shapiro-Wilk) was OK but equal equivalence test (Levene) failed. QWB showed significant strain difference in the AR protein expression ($n=5$ per strain; $p<0.05$). The expressions were 0.498 ± 0.081 , 0.526 ± 0.042 , 0.834 ± 0.078 , 0.950 ± 0.164 , 1.356 ± 0.160 in 129X1, C57BL/6, C3H, BALB/c, and DBA/2, respectively (Arbitrary units; mean \pm SEM). Pairwise comparisons by Tukey test indicated significant differences ($p<0.05$) between 129X1 and DBA/2, and between C57BL/6 and DBA/2. Linear regression analysis by ANOVA with average values between AR expression and in vitro fertilizability (SigmaPlot) showed significant association ($p=0.002$) with $R^2=0.974$, suggesting that the differential AR expression in testes might cause strain difference in the in vitro fertilizability.

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Oocyte Growth Depends on Phosphorylation of Specific Serine Residues in the C-terminal Cytoplasmic Tail of Connexin43.

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Connexin43 (CX43) forms gap junction channels that metabolically couple the granulosa cells of developing ovarian follicles. Research with knockout mice has demonstrated a requirement for this connexin in supporting follicle and oocyte growth: CX43 null mutant follicles fail to develop beyond the primary stage on the C57BL/6 background due to failure of the granulosa cells to proliferate. We have used the CX43 null mutant mouse line and the recombinant-reaggregated ovary technique to examine the importance of specific CX43 phosphorylation sites in supporting oocyte growth. Serines at 255, 262, 279 and 282 are MAPK family substrates that become phosphorylated in response to EGF and other growth factors, causing a decrease in coupling levels. Mutant forms of CX43 were constructed with these serines replaced with amino acids that cannot be phosphorylated. These mutants, singly and in combination, were retrovirally transduced into CX43 null granulosa cells and combined with wildtype oocytes to make recombinant ovaries. The ovaries were grafted under the kidney capsules of immunocompromised female mice permitting follicle growth in vivo. Oocyte growth was retarded when the empty vector was introduced to CX43 null granulosa cells compared to granulosa cells infected with the CX43 wildtype (WT) control (35.47 ± 1.12 μ m and 61.34 ± 1.47 μ m diameter respectively). CX43 with a single serine to alanine mutation at residue 255, or with a single serine to aspartic acid mutation at either residue 255 or 262, were able to rescue the null phenotype, restoring complete oocyte growth (60.43 ± 1.69 μ m, 60.96 ± 1.22 μ m, and 62.11 ± 1.18 μ m respectively). In contrast, CX43 with serine to alanine mutations at either residue 279 or 282 failed to permit a complete rescue, with follicle development largely limited to the early secondary stage and oocytes remaining significantly smaller than those in WT CX43 controls (34.84 ± 1.20 μ m and 43.49 ± 1.74 μ m respectively). Mutating all four residues from serine to alanine caused complete arrest of folliculogenesis and retardation of oocyte growth in the context of our system (47.05 ± 1.63 μ m). Immunofluorescence analysis revealed that the mutant

molecules failing to rescue folliculogenesis were largely confined to intracellular sites, with few gap junctions visible. Using an *in vitro* EdU proliferation assay, we observed a decrease in proliferation in granulosa cells containing the mutated 279 and 282 construct compared to those receiving a non-mutated CX43 construct. These results indicate that CX43 phosphorylation is regulated by MAPK in granulosa cells and that this regulation is essential for supporting oocyte growth. The authors acknowledge Dan Li and Kevin J. Barr for technical assistance and the Canadian Institutes of Health Research and U.S. National Institutes of Health for funding.

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Interactions of 14-3-3 (YWHA) protein isoforms with CDC25B phosphatase in mouse oocytes.

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Immature mammalian oocytes are arrested at prophase I of meiosis by an inhibitory phosphorylation on Cyclin-Dependent Kinase I (CDK1). Release from this meiotic arrest is dependent on dephosphorylation of CDK1 by M-phase inducer phosphatase 2 (CDC25B). Evidence suggests that phosphorylated CDC25B is bound to 14-3-3 (YWHA) proteins in the cytoplasm and rendered inactive. We previously reported expression of all seven mammalian isoforms of 14-3-3 in mouse oocytes and eggs. To examine the interactions of 14-3-3 isoforms with CDC25B in oocytes and eggs, we performed an *in situ* Proximity Ligation Assay (PLA; Duolink in cell Co-IP, Olink Bioscience) that can detect protein-protein interactions at the single molecule level and allows visualization of the actual intracellular sites of the interactions. We observed prominent interaction of all seven 14-3-3 isoforms with CDC25B throughout cytoplasm and nuclei of mouse oocytes, along with reduced interactions for each individual isoform in eggs compared to oocytes. Co-immunoprecipitation studies with extracts of mouse oocytes also demonstrated interaction of CDC25B with six 14-3-3 isoforms. These results suggest that any of the 14-3-3 isoforms may have the potential to hold CDC25B inactive in oocytes to maintain the meiotic arrest. In preliminary experiments to explore if interactions of 14-3-3 with other proteins are important for maintaining meiosis I arrest, we microinjected oocytes with 0.5µg/µL R18, a synthetic non-isoform specific 14-3-3-blocking peptide. Injected oocytes were incubated overnight in media containing a threshold concentration of dibutyryl cAMP (0.05mg/mL) which normally holds oocytes arrested through activation of Protein Kinase A (PKA) and phosphorylation of CDC25B and CDK1. We observed a marked increase in germinal vesicle breakdown (GVBD), compared to control oocytes. To investigate which specific isoform(s) of 14-3-3 is/are responsible for maintaining the meiotic arrest, we reduced the synthesis of each 14-3-3 isoform in mouse oocytes by intracytoplasmic microinjection of 0.1mM translation-blocking morpholino oligonucleotide against the corresponding isoform mRNA. Injected oocytes were held arrested at prophase I for 24 hours, and then incubated overnight in media containing the threshold concentration of dbcAMP. GVBD was observed in 70% of oocytes microinjected with morpholino against 14-3-3 eta, despite the presence of dbcAMP. Injection of morpholinos targeting other 14-3-3 isoforms caused little or no GVBD. Thus, reduction in 14-3-3 eta/CDC25B interaction releases the mouse oocyte from meiotic arrest. These results suggest that, while all 14-3-3 isoforms interact with CDC25B in mouse oocytes, 14-3-3 eta is essential for maintaining the prophase I meiotic arrest.

Embryogenesis

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eIF3m stabilizes the eIF3 complex and is essential for embryonic development and tissue homeostasis.

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eIF3, the largest eukaryotic initiation factor composed of approximately 13 subunits, plays a key role in protein biosynthesis and cell proliferation. How the different subunits contribute to its structural integrity and functions, however, is not fully understood. Here we show that eIF3m, a non-core subunit of eIF3 also known as GA17, is essential for mouse embryonic development and tissue functions. eIF3m null embryos were lethal at peri-implantation stage. The heterozygotes were normal, except for their diminished body weight. Compound heterozygotes or embryos with eIF3m deficiency in adipose tissue and the central nervous system were lethal at early embryonic stages as well. Acute ablation of eIF3m in the liver of adult mice led to rapidly decreased body weight and death in a week, correlated with severe decline of protein biogenesis. Its acute ablation in K-ras-expressing lung cells markedly repressed the K-ras-induced cancer formation in the mouse lung. Molecular analysis revealed that the eIF3m deficiency significantly impaired integrity of the eIF3 complex. Two of the subunits, to which eIF3m directly bound, were destabilized. Therefore, eIF3m is required for the structural integrity and the translation initiation functions of eIF3 fatal to the maintenance of cell viability.

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The transcriptional co-factor Jab1 is essential for early mouse limb development *in vivo*.

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The transcriptional cofactor Jab1 controls cell proliferation, apoptosis, and differentiation in many developmental processes by regulating the activity of numerous transcription factors. However, the physiological role of Jab1 in skeletal development is still poorly understood. We previously reported that the specific deletion of Jab1 in differentiating chondrocytes in mice leads to neonatal lethal chondrodysplasia with severe dwarfism. Jab1 mutant chondrocytes exhibited increased apoptosis and accelerated chondrocyte hypertrophy. Notably, there was a heightened expression of BMP signaling components and targets in Jab1 mutant chondrocytes, suggesting that Jab1 is a novel inhibitor of BMP signaling in differentiating chondrocytes *in vivo*. In this study, to determine the function of Jab1 in even earlier osteochondro progenitor cells (OPCs), we bred *Jab1^{fllox/fllox}* mice with *Prx1-Cre* transgenes to delete *Jab1* specifically in limb buds. The *Jab1^{fllox/fllox}; Prx1-Cre (cKO)* mutant mice displayed drastically shortened limbs starting at E13.5, strikingly mirroring the human skeletal disorder phocomelia. Phocomelia, a birth defect of severe limb truncation, is predominantly caused by the use of thalidomide, a potent teratogen, during early pregnancy. Children with such conditions are still being born today worldwide, especially in developing countries. Furthermore, thalidomide is being used to treat multiple myeloma, Crohn's disease, AIDS, and some cancers in the United States these days. Thus, even under strict guidelines, the thalidomide-induced phocomelia is still a real threat today. Recent studies in chicken models suggest that the etiology of phocomelia may lie in a defect in the survival and differentiation of OPCs. The molecular basis of phocomelia, however, is still poorly understood and the mouse model for this disease is also very much lacking. Thus,

the *Jab1 cKO* mice can serve as a novel model for phocomelia study. Indeed, the *Jab1 cKO* mutant limbs exhibited drastically reduced numbers of hypertrophic chondrocytes, disorganized chondrocyte columns, delayed primary ossification center formation and complete absence of secondary ossification center, and increased apoptosis during embryonic development. To determine the underlying mechanism for the severe short-limb defect in the *Jab1 cKO* mutants, we used a micromass culture of E11.5 *Jab1* mutant and wild-type limb mesenchyme cells to study chondrogenesis *ex vivo*. Alcian blue staining showed significantly decreased chondrogenesis in *Jab1* mutant cultures. Real time RT-PCR confirmed that the expression of chondrocyte differentiation markers, including *Sox9* and *Col2a1*, were significantly decreased in *Jab1* mutants. Western blot analysis indicated that FGF and BMP signalings were altered in *Jab1 cKO* mutants, suggesting that *Jab1* affects complex developmental cues during early limb development. Additionally, the deletion of *Jab1* in primary mouse osteoblastic precursors with *Ade-Cre* abolished overt osteoblast differentiation *ex vivo*. In conclusion, our study demonstrates that *Jab1* is a novel, essential regulator of early mouse limb development *in vivo* and the *Jab1 cKO* mutant mice in our study can serve as a powerful model for the development of novel therapeutics for phocomelia and other severe limb reduction defects in the future.

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Pericentrin null mice exhibit features of primordial dwarfism, defects in centrosome organization and aberrant stem cell division.

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Majewski Osteodysplastic Primordial Dwarfism type II (MOPDII) is one of the primordial dwarfisms that are characterized by extreme intrauterine growth retardation that continues throughout adulthood. The etiology of MOPDII was recently linked to the centrosome gene *pericentrin* (*PCNT*) but the underlying mechanisms at cellular and molecular levels remain obscure. To address this, we developed *PCNT*-null mice that fully recapitulate major features of MOPDII, including small stature, microcephaly and cardiovascular defects. Cardiovascular defects include atrial and ventricular septal defects, mitral valve dysfunction, increased vascularization and hemorrhaging of brain vessels. At the cellular level, prominent phenotypes include supernumerary centrioles or centrosomes, and misoriented cell division plane in both stem and committed cells. The former could delay mitotic progression or cause mitotic catastrophe, and the later could account for premature differentiation divisions and/or mitotic exit *in vivo*. Both may contribute to the decrease in overall cell number and the smaller body size, as observed in humans. At the molecular level, *PCNT* deletion delocalizes a subset of centriole and centrosome proteins that is consistent with the cellular defects. We conclude that the disease phenotypes in both mice and humans harboring *PCNT* mutations can be attributed to compromised centrosomes. We propose that disrupted centrosomes, in turn, causes spindle disorganization and misoriented cell division, which contributes to primordial dwarfism.

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The ciliary G protein-coupled receptor Gpr161 negatively regulates the sonic hedgehog pathway via cAMP signaling.

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Graded signals, referred to as morphogens, provide positional information for organizing developing tissues; particularly, a gradient of Sonic Hedgehog (Shh) controls ventral patterning in the neural tube in vertebrates. The primary cilium is required for vertebrate Shh signaling. The intraflagellar transport (IFT) machinery is fundamental for assembly and maintenance of primary cilia. Thus, mutants encoding components of the IFT-B complex (implicated in anterograde IFT), as well as the IFT motors exhibit strongly decreased Shh signaling in the neural tube. Paradoxically, mutations in the ciliary IFT-A complex, which is implicated in retrograde IFT, cause increased Shh signaling. The opposing neural tube phenotypes of IFT-A from the other IFT mutants suggest that the IFT-A complex has functions in addition to its postulated role in retrograde IFT. Similar to the IFT-A mutants, mutations in the tubby-like protein *Tulp3*, also exhibit increased Shh signaling in the neural tube. We previously showed that the IFT-A complex binds to *Tulp3*, and recruits it to cilia. *Tulp3* in turn promotes trafficking of specific rhodopsin family G protein-coupled receptors (GPCRs) to cilia (Mukhopadhyay et al, *Genes Dev* 24, 2180-2193). The function of IFT-A/*Tulp3* in mediating GPCR trafficking to the cilia suggests the role of a novel GPCR in the Shh pathway. In order to identify GPCRs important in vertebrate Shh signaling, we performed a screen to identify candidate GPCRs that are ciliary and are expressed early during development. This led us to the discovery of an "orphan" GPCR, Gpr161 that is localized to the cilia. This receptor is expressed ubiquitously as early as E8.5, and is mostly concentrated in the developing nervous system in later stages. Furthermore, depletion of *Tulp3* and IFT-A prevents its ciliary localization. Most importantly, a null knockout mice model for this receptor results in embryonic lethality by E10.5 and phenocopies the *Tulp3*/IFT-A mutants by showing increased Shh signaling in the developing neural tube. These data suggest that Gpr161 functions as a negative regulator of the Shh pathway. The cAMP-activated protein kinase A (PKA) is pivotal in regulating the basal repression mechanism in Shh pathway by mediating the processing of Gli transcription factors into Gli repressors; however, the cAMP regulating pathways that mediate activation of PKA remain unknown. Interestingly, constitutive Gpr161 activity increases cAMP levels and represses Shh signaling by determining the processing of the Gli transcription factor Gli3 to its repressor form. Simultaneously, Shh signaling directs Gpr161 to be internalized from cilia preventing its activity. Thus, Gpr161 defines a new pathway coupling PKA activation to Shh signaling during neural tube morphogenesis.

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Novel *Drosophila* long non-coding RNAs regulate transcription of the Hox gene *Sex combs reduced in cis and trans*.

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Hox genes encode a conserved family of clustered transcription factors specifying segmental identity in all animals. The Hox intergenic regions exemplify a complex landscape containing many long range interactions between cis-regulatory domains and promoter elements that are responsible for establishing and maintaining proper Hox gene expression. Recently it has become clear that Hox cluster intergenic sequences exhibit extensive non-coding transcription, and that many of the classically defined 'regulatory elements' are themselves transcribed. Here,

we have identified and functionally characterized two new conserved ncRNAs in the fruit fly *Drosophila* upstream of the Hox gene *Sex combs reduced (Scr)*. Both ncRNAs are transcribed from genomic regions previously defined as 'Scr regulatory elements'. We use transgenic overexpression and knockdown to confirm that both ncRNA transcripts are required for proper activation of *Scr* expression and repression, via a 'tethered RNA' mechanism. Analysis of a naturally occurring GOF mutation establishes that ectopic transcription of the endogenous ncRNA is sufficient to activate *Scr* expression, and surprisingly that this ncRNA-mediated activation of *Scr* occurs in *trans*, between homologs. This work contributes to our mechanistic understanding of the role non-coding RNAs play in long range enhancer promoter interaction and transvection both within and between chromosomes.

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Lipid droplets control the maternal histone supply of *Drosophila* embryos.

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Histones are essential for chromatin packing, yet free histones not incorporated into chromatin are toxic. In most cells, multiple regulatory mechanisms prevent accumulation of excess histones; early *Drosophila* embryos, in contrast, contain a thousand-fold excess of histones over DNA. The function of these maternal histone deposits is unclear, in particular because these embryos also contain maternally provided histone messages. Excess histones H2A, H2B, and H2Av are bound to lipid droplets, ubiquitous fat storage organelles that are especially abundant in embryos. We had previously proposed that sequestration on lipid droplets allows safe transient storage of excess histones. The studies described here critically test this sequestration hypothesis. We find that histones are anchored to lipid droplets via the previously uncharacterized protein Jabba: Jabba localizes to droplets, co-immunoprecipitates with histones, and is necessary to recruit histones to droplets. *Jabba* mutants lack the maternal H2A, H2B, and H2Av deposits altogether; presumably, these excess histones are degraded unless sequestered on droplets. *Jabba* mutant embryos compensate for this histone deficit by translating maternal histone mRNAs. Mild disruption of histone expression has no discernable effect in a wild-type genetic background, but in the absence of Jabba results in defective mitoses and embryonic death. Our findings are of broad general importance because a growing number of proteins from other cellular compartments have been found to transiently associate with lipid droplets. Our studies provide the first insight into mechanism and functional relevance of such sequestration. We conclude that sequestration on lipid droplets allows embryos to build up extra-nuclear histones stores and provides histones for chromatin assembly during times of high demand. This work demonstrates a novel function of lipid droplets for development, as storage sites for unstable or detrimental proteins.

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Plasma membrane partitioning of syncytial blastoderm into individual cells is critical for tissue invagination during *Drosophila* ventral furrow formation.

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Epithelial morphogenesis is a fundamental mechanical process that generates changes in shape and motility utilizing the response of biomaterials to cellular forces. During *Drosophila* gastrulation, an apical actomyosin contractile network powers the internalization of mesoderm precursor cells to form a ventral furrow (VF) in the epithelial monolayer. In this process, the ventral cells first constrict their apex and elongate along the apical-basal axis, then are rapidly internalized into the embryo as they return back to their original length. It remains unclear how apically generated forces are transmitted across the entire apical-basal axis and how cells transition from lengthening to shortening to internalize VF. Here we apply particle tracking microrheology with injected fluorescent micro-beads to measure the movement of cytoplasm and plasma membrane within individual cells undergoing VF formation. We find that during the lengthening phase the apical cytoplasm responds as a viscous fluid to apical constriction, gradually elongating along the apical-basal direction, and thus accounting for much of the lengthening observed in the cell. Within a cell, the nucleus and the basal cytoplasm are displaced basally with no apparent mixing between compartments. Surprisingly, beads bound to the lateral plasma membrane showed a pattern of basal movement very similar to that of beads in the cytoplasm, as if the lateral membrane is flexible to apical-basal stretching and passively follows cytoplasm during lengthening. To address the role of the lateral membrane in VF formation, we generated mutant embryos that do not form cells prior to gastrulation, while the dorsal-ventral patterning, the developmental timing of gastrulation, and the apical myosin activity remain intact. In such “acellular” embryos, the movements of the cytoplasm and nuclei during the “lengthening phase” resemble those in wild type; however, the “shortening phase” is absent and the ventral tissue fails to internalize. We propose that the lengthening and shortening phases of VF formation have distinct requirements for the basolateral plasma membrane. While the viscous cytoplasm is sufficient to transmit force across a distance to drive cell elongation without the basolateral membrane, the membrane partition of common cytoplasm into individual cells is critical for shortening and tissue invagination.

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The role of miR-206 in *Xenopus laevis* somitogenesis.

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MicroRNAs (miRNAs) are highly conserved small non-coding RNA molecules that regulate gene expression post-transcriptionally in multicellular organisms. Each miRNA has a distinct and specific expression pattern and can target multiple messenger RNAs. miR-206 is part of the muscle-specific family of miRNAs (myomiRs) but it is the only miRNA expressed in skeletal muscle. Unlike other myomiRs, miR-206 is found exclusively in vertebrates and recent work has linked miR-206 to several diseases including muscle dystrophy. Here, we have characterized the importance of miR-206 in muscle formation using *Xenopus laevis* embryos. Similar to other studies, our *in situ* hybridization analysis shows that miR206 is found exclusively in muscle. Expression analysis using qRT-PCR shows that a morpholino directed against the mature form of miR-206 downregulates its expression without significantly affecting the expression of another myomiR, miR-133b. Embryos lacking miR-206 show abnormal muscle formation and an abnormal gap between the somites and notochord. Scanning electron microscopy results show

that this abnormal gap is comprised of extracellular matrix (ECM). Expression analysis of several ECM components surrounding the notochord and somites reveal their abnormal distribution during somite formation in miR-206 morphants. We hypothesize that the main role of miR-206 in *Xenopus laevis* is to regulate the deposition of matrix molecules during somite boundary formation. The abnormal formation of these intersegmental boundaries then interferes with the ability of muscle fibers to adapt the normal parallel alignment observed in wild-type embryos.

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Centrosome positioning via dynein-powered intracellular cargo transport.

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Correct positioning of the centrosome is critical for the survival of the cell. For small and medium-sized cells, the force required to move the centrosome can arise from either microtubule pushing on the cortex, or cortically-attached dynein pulling on microtubules. However, in large cells, such as the fertilized *Xenopus laevis* embryo, where microtubules are too long to support pushing forces or they do not reach all boundaries before centrosome centering begins, a different force-generating mechanism must exist. Here, we present a centrosome positioning model in which the cytosolic drag experienced by cargos hauled by cytoplasmic dynein on the sperm aster microtubules can move the centrosome towards the cell's center. We find that small, fast cargos (diameter ~ 100nm, cargo velocity ~ 2µm/s) are sufficient to move the centrosome in the geometry of the *Xenopus laevis* embryo within the experimentally observed length and time scales.

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Regulation of TRPM7 By 80K-H and Wnt Signaling During Early Embryonic Development.

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TRPM7 is a bifunctional protein that contains both ion channel and kinase domains. TRPM7's channel is permeable to Mg²⁺, Ca²⁺, and other divalent cations such as Zn²⁺, while the function of the kinase has remained relatively elusive. Our studies have identified TRPM7 as an essential component of the non-canonical Wnt pathway regulating gastrulation, a pivotal process during early embryonic life that executes the emergence of the body plan and closure of the neural tube. The mechanism by which TRPM7 is regulated during early development is unknown. To gain insight into how the channel-kinase is controlled we conducted a yeast two hybrid screen using the COOH-terminus of TRPM7, which revealed 80K-H as a potential binding partner. Pulldown purification assays and immunoprecipitation experiments confirmed 80K-H as a bona fide TRPM7-interacting protein. Surprisingly, the strength of the interaction between 80K-H and TRPM7 was dependent upon growth factor stimulation. 80K-H has previously been shown to be involved in regulating TRPP2 protein expression. Our studies revealed that knockdown of 80K-H decreased TRPM7 protein expression, which can be prevented by application of MG132, a proteasome inhibitor. Depletion of 80K-H from *Xenopus laevis* embryos produced a gastrulation defect, which can be made more severe by the simultaneous depletion of 80K-H and TRPM7. These results uncover a novel role for 80K-H in early development and suggest a model in which 80K-H regulates TRPM7 during early embryonic development by regulating its expression level.

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Two isoforms of protein phosphatase 1 beta assemble the zebrafish myosin phosphatase.

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The myosin phosphatase is a highly conserved regulator of actomyosin contractility. Zebrafish has emerged as an ideal model system to study the *in vivo* role of myosin phosphatase in controlling cell contractility, cell movement and epithelial biology. Most work in zebrafish has focused on the regulatory subunit of the myosin phosphatase called Mypt1. In this work, we examined the critical role of Protein Phosphatase 1, PP1, the catalytic subunit of the myosin phosphatase. We observed that two isoforms of PP1 β , called PP1 Ba and PP1 Bb, are both expressed ubiquitously during early zebrafish development. Furthermore, we found that both isoforms can interact with Mypt1 and assemble an active myosin phosphatase complex *in vitro*. Expression of this complex results in dephosphorylation of the myosin regulatory light chain and large scale rearrangements of the actin cytoskeleton in cell culture. Morpholino antisense knockdown of either isoform results in a dose-dependent defect in morphogenetic cell movements during zebrafish gastrulation. Furthermore, co-injection of mRNA of either isoform can rescue the gastrulation phenotype indicating functional redundancy. Taken together, this work provides evidence that zebrafish express two isoforms of PP1 β that both assemble the myosin phosphatase and are required in a dose-dependent manner to regulate morphogenetic cell movements during gastrulation. This work will provide valuable insight and tools for the study of the role of actomyosin contractility in epithelial biology, neural development and morphogenetic cell movement.

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Characterization of the mitochondrial transport complex in developing zebrafish.

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Inside a cell, mitochondria exhibit dynamic locomotion and spatial rearrangement. This movement is necessary for a cell to maintain basic metabolic functions, and disruption of this motility often results in cell death. Miro is a mitochondrial outer membrane Rho GTPase essential for mitochondrial movement in diverse systems. In fruit flies and mammals, Miro interacts with a protein called Milton/Trak to link mitochondria to kinesin (KIF5) for trafficking along microtubules. We sought to characterize the Miro and Trak protein families in zebrafish. We confirmed that, like human Miro, the zebrafish Miro proteins (Rhot1a, Rhot1b, and Rhot2) localized to mitochondria in mammalian tissue culture cells by both biochemical fractionation and immunofluorescent colocalization. In addition, using whole mount *in situ* hybridization, we observed ubiquitous expression of all three mRNAs throughout development. By microinjecting three antisense morpholino oligonucleotides targeted to each of the mRNAs, we knocked down all three proteins in developing zebrafish embryos. The triple morphants demonstrated a dose-dependent defect in posterior body-axis elongation, while a single knockdown of each protein at the same dose produced no effect. At higher doses of a triple knockdown, embryos failed in gastrulation and died during epiboly. We are currently working to rescue the morphants with human Miro mRNA. Furthermore, in contrast to the two human Trak proteins, zebrafish Trak2, but not Trak1, localized to mitochondria when overexpressed in COS-7 cells. Given the low amino acid conservation between these two proteins, it is not surprising that these two zebrafish

proteins might have different cellular functions. Taken altogether, this research will lay the foundation for studies in the role of mitochondrial motility during vertebrate development.

1584

Optimizing Conditions for Preserving Sea Urchin Eggs.

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This study is to optimize conditions for maintaining viable sea urchins eggs in the laboratory for extended periods of time so that they are more readily useful for research and for classroom experiments. After spawning, sea urchin eggs have an average shelf life of 24 to 36 hours. Previously, we have shown that 0.3 mM of empty liposomes and liposomal glutathione (L-GSH) at a concentration of 1.08 mg/mL glutathione can preserve the integrity of spawned sea urchin eggs for up to seven days. In addition, eggs incubated in these agents could be fertilized by fresh sperm for up to 24 hours. Although both L GSH and empty liposomes were shown to maintain the integrity of the eggs, the liposome concentration and the type of liposomes in each case was not directly comparable. The aim of the experiments outlined here is to determine optimal conditions using L GSH and empty liposomes at uniform concentrations. We will use (1) custom prepared empty HSPC-chol liposomes (98-103 nm diameter) at 0.3 mM and (2) custom prepared liposomal glutathione (L GSH) with HSPC-chol liposomes (98-103 nm diameter) at 0.3 mM of liposomes with a concentration of 1.08 mg/mL of GSH. Using these agents for incubation, eggs will be observed and counted each day for the duration that they remain intact. Viability will be confirmed by observing the elevation of a fertilization membrane following exposure to live sperm. In a separate experiment, we will also determine optimal incubation temperature for extended preservation.

1585

Examining Protist Infestation of Harvested Sea Urchin Eggs.

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Without treatment, harvested sea urchin eggs have a poor shelf life of less than 36 hours during which they become heavily infested with protists. Previously, we have shown that sea urchin eggs incubated in the presence of liposomal glutathione (L-GSH) and empty liposomes remained intact for up to seven days and could be fertilized for up to 24 hours. In addition, these agents protected against infestation of the eggs by protists. Preliminary results indicated that there was an inverse relationship between the concentration of L GSH or liposomes and the number of protists observed. We are continuing to investigate the role of these agents in controlling protist infestation of harvested sea urchin eggs. We will monitor and quantitate protist appearance and survival rates in the presence of various concentrations of both L GSH and empty liposomes. In addition, we will examine whether the observed protists are endogenous to sea urchin stocks or appear later as contaminants. Finally, we will identify the type or types of salt water protists that are isolated from our sea urchin egg cultures.

Chaperones, Protein Folding, and Quality Control II

1586

Construction of a ubiquitin-2 interaction network affected in amyotrophic lateral sclerosis.

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Amyotrophic lateral sclerosis (ALS) is a rapidly progressive debilitating disease that affects one in every 50,000 people. Like other neurodegenerative diseases, the mechanisms that cause ALS remain unresolved. However, recent evidence suggests a possible common link between mutations in different genes and the neuropathology seen in the disease. One such connection is the finding that mutations in ubiquitin-2 (ubq1n-2) and in other ALS susceptibility genes are linked with ubq1n neuropathology. Yet the underlying mechanisms of how mutations in ubq1n-2 or ubq1n pathology contribute to ALS pathogenesis are unclear. To understand how ubq1n proteins are involved in ALS we are constructing a map of ubq1n-2-interacting proteins. Ubq1n-2 is one of four ubq1n proteins expressed in humans. The proteins are ~600 amino acids long and contain conserved UBL and UBA domains that flank a more variable central domain. Each ubq1n protein is distinguished by containing unique short inserts. Interestingly, the missense mutations in ubq1n-2 that cause ALS map to one of these inserts that is rich in PXX repeats. We used almost the entire central domain of ubq1n-2 and the segment containing the PXX repeats as baits to screen a human brain cDNA library using the yeast two-hybrid system. Through this screen we recovered several candidates that interacted strongly with each of the baits. Among the interactors were factors that function in RNA splicing, protein folding, protein translation and cell stress. Several of these factors function in pathways that have been implicated in ALS. The clones are being screened to determine whether they interact differently with wild type and ALS mutant (P497H, P497S, P506T, P509S, and P525S) ubq1n-2 baits. Clones that display altered binding to the ALS mutants are excellent candidates for genes that might be disrupted in ALS pathogenesis. We will report on these findings and on the further characterization of the clones.

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Validating ubiquitin-1 overexpression as a therapy for Huntington's disease.

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Huntington's disease (HD) is a debilitating neurodegenerative disorder caused by a polyglutamine expansion in huntingtin (htt) protein. The mechanism(s) by which mutant htt proteins cause disease remains unresolved. However, recent therapeutic strategies have focused on ways to either silence or clear mutant huntingtin. Ubiquitin-1, one of four ubiquitin isoforms in humans, contains conserved N- and C-terminal UBL and UBA domains, which flank a longer central domain that differs among the ubiquitin isoforms. This structure facilitates the function of the protein as a shuttle factor, binding polyubiquitinated proteins and delivering them to the proteasome for degradation. Moreover, ubiquitin-1 has been implicated in both endoplasmic reticulum-associated degradation and autophagy. We previously demonstrated that overexpression of ubiquitin-1 in cell and *C. elegans* models of HD reduces huntingtin aggregates and toxicity. Importantly, we found that ubiquitin-1 overexpression selectively enhances the clearance of mutant htt protein without affecting normal htt protein. Two key questions need to be addressed in order to further validate ubiquitin-1 as a therapeutic target for HD. First, does overexpression of ubiquitin-1 prevent or delay neurodegeneration in mouse models of HD? Second, can factors be found to modulate ubiquitin-1 expression? Toward the first goal we have generated transgenic mice that overexpress human ubiquitin-1 driven by the

neuron specific Thy1.2 promoter. Transgene integration was verified via PCR and Southern blotting, and ubiquilin-1 protein overexpression through immunoblotting. Immunoblotting revealed that one of these lines overexpresses ubiquilin protein as much as 1.8 fold in brain lysates. Interestingly, we also observed significantly reduced ubiquilin-1 levels in brain lysates of R6/2 HD model mice, as well as colocalization of ubiquilin-1 with huntingtin aggregates. We therefore crossed our ubiquilin-1 transgenic line with R6/2 mice in order to assess whether we could restore ubiquilin-1 levels and potentially delay pathology in these mice. We will report of the assessment of the motor phenotype, neuropathology, and survival of ubiquilin-1-R6/2 double transgenic mice. In further attempts to manipulate ubiquilin expression, we are studying factors that regulate ubiquilin-1 expression using luciferase reporter constructs and real-time PCR. Initial findings indicate that ER-stressors potently upregulate ubiquilin-1. The regulation of ubiquilin-2 in which mutations were found to cause amyotrophic lateral sclerosis is also being examined.

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Cross-Talk Between Proteasomes and Lysosomes Determines the Fate of Amyloid Peptides in Pancreatic Cells.

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Self-assembly of the human pancreatic hormone amylin into toxic oligomers and aggregates is linked to the dysfunction of islet beta-cells and pathogenesis of type-2 Diabetes Mellitus (T2DM). The other aggregation-prone peptide β -amyloid, previously linked to pathology of Alzheimer's disease, was recently found, together with amylin aggregates, in the extracellular plaques of human islets, suggesting a common etiology of the two amyloid diseases. However, the trafficking mechanism and identity of degradation compartments involved in the clearance of these two misfolded amyloid peptides, and their causal roles in islet amyloid formation and T2DM remain poorly understood. Hence, in the current study, we investigated amylin and β -amyloid trafficking and degradation pathways in pancreatic rat insulinoma (RIN-m5F) and human islet cells, and explored the causal connection between amyloid peptide turnover and toxicity in these cells. Confocal microscopy analysis of fluorescently-labeled amyloid peptides and organelle/ trafficking markers revealed a common trafficking itinerary for amylin and β -amyloid in pancreatic cells. Dominant negative (DN) clathrin (AP180c) and dynamin (Dyn1/2K44A) constructs or low temperature (+4° C) markedly inhibited ($\geq 90\%$) internalization of both peptides and completely inhibited entry of other proteins such as cholera toxin (CTX) and transferrin (Trf), indicating endocytosis as major mechanism in amylin and β -amyloid uptake by cells. Amylin and β -amyloid co-trafficked with CTX but not with Trf to juxtannuclear compartments indicating possible association with Golgi and/or lysosomes. Supporting this finding, high colocalization between amyloid peptides and lysosomes and their partial colocalization with autophagosomes in RIN-m5F and human islet cells were detected. Although amyloid peptides and Trf uptakes require clathrin, amylin and β -amyloid were absent from Trf-labeled endosomes, suggesting little association of amyloid peptides with recycling compartment under control conditions. Thus, degrading compartments appear to be a final common destination for both peptides. Interestingly, significant colocalization between amyloid peptides and 20S proteasome subunit was demonstrated in our studies. Inhibition of proteasome function using specific proteasome inhibitor lactacystin increased co-localization between amylin and lysosomes. Similarly, inhibition of lysosome proteolytic function with pepstatin and ammonium-chloride re-directed amylin to the proteasome. Increased accumulation of amyloid peptides in culture and apoptosis was observed by western blot and ELISA following inhibition of amylin degradation in lysosomes or proteasomes. Thus, our

studies indicate that the evolutionarily conserved endocytotic pathway regulates turnover and toxicity of amyloid peptides in pancreatic cells via two distinct but closely interacting proteolytic compartments, proteasomes and autolysosomes.

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Alternative nucleotides to rescue the function of disease-causing mutant septins.

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Septins are a family of GTP-binding proteins that form cortical filamentous arrays in a wide variety of eukaryotic cell types and play diverse roles in processes such as cytokinesis and the establishment of cell polarity/morphogenesis. Mutation or misregulation of septin-encoding genes has been linked to a panoply of human diseases, including cancer, neurodegeneration, and male infertility. Septin proteins typically co-assemble into rod-shaped hetero-oligomeric complexes composed of two molecules of each of three or four distinct polypeptides. Septin rods polymerize into filaments and associate with the plasma membrane and/or other elements of the cytoskeleton to regulate vesicle trafficking and membrane dynamics by functioning as scaffolds and/or membrane diffusion barriers. Despite significant progress in the last few years, how septin ultrastructure relates to septin function remains largely unknown, a problem whose importance has been highlighted by the identification of disease-associated septin mutations that appear to target higher-order assembly properties.

For example, whereas male infertility has been experimentally induced in transgenic mice using deletion alleles of specific septin genes, all of the septin mutations identified in infertile men cause substitutions at specific residues predicted to make important contacts either with GTP bound in the nucleotide-binding pocket, or between septin subunits in the context of septin filaments. Thus, surprisingly, naturally-occurring disease-causing mutations are not null alleles, and instead resemble the original septin mutants identified in Lee Hartwell's pioneering cell division cycle screen of temperature-sensitive mutants in *Saccharomyces cerevisiae*, several of which have been sequenced and determined to carry single nucleotide-binding-pocket mutations. Indeed, we discovered that one of the infertility-causing mutations (SEPT12 Asp197Asn) is identical to a temperature-sensitivity-inducing mutation isolated in the Hartwell screen (Cdc10 Asp182Asn). In septins and many other GTP-binding proteins, the Asp in the position corresponding to SEPT12 Asp197 or Cdc10 Asp182 contacts the base of the bound nucleotide. Remarkably, for many such proteins (most famously EF-Tu) substitution of this residue to Asn switches the nucleotide-binding specificity from GTP to xanthosine triphosphate (XTP), a nucleotide that is not typically produced *in vivo*. Experimental manipulations providing an intracellular source of XTP were able to rescue function of one such mutant protein, the *Drosophila* P element transposase (PMID: 9250688). Nucleotide binding by septins plays important roles in the folding of septin proteins and their assembly into complexes and filaments, suggesting that the functional defects of GTP-binding-defective septins might be overcome if another nucleotide could occupy the mutant septin's pocket. Our studies indicate that additional mutant septins bind other alternative nucleotides, and that generating these nucleotides *in vivo* rescues function. Thus, we propose a novel approach to remedy the dysfunction of nucleotide-binding-pocket septin mutants and thereby treat disorders arising from such mutations.

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FKBP14 is an essential gene that regulates Presenilin protein and Notch signaling in *Drosophila* development.

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Presenilins were identified as causative factors in Familial Alzheimer's Disease and also play an essential role in Notch signalling during development. We previously identified FKBP14, a member of the family of FK506-binding proteins (FKBPs), as a modifier of *presenilin* in *Drosophila*. FKBPs are highly conserved peptidyl-prolyl cis-trans isomerases that play integral roles in protein folding, assembly and trafficking. Although FKBPs have been implicated in a broad range of biological processes, they are non-essential in yeast and their role in the development of multicellular organisms remains unclear. We show that FKBP14 is an essential gene in *Drosophila* and that loss of FKBP14 gives rise to specific defects in eye, bristle and wing development. FKBP14 mutants genetically interact with components of the Notch pathway, indicating that these phenotypes are associated, at least in part, with dysregulation of Notch signaling. We show that while Notch trafficking to the membrane is unaffected in FKBP14 mutants, levels of Notch target genes are reduced, suggesting that FKBP14 acts downstream of Notch activation at the membrane. Consistent with this model, we find that Presenilin protein levels are significantly reduced in FKBP14 null mutants. We also find evidence of critical, apparently Notch-independent roles for FKBP14, including a requirement in cell viability. Altogether, our data demonstrate that FKBP14 plays an essential role in development, one aspect of which includes regulating members of the Notch signaling pathway.

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Influence of Bag2 cochaperone overexpression on the hyperphosphorylation of Tau.

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Introduction: Tau inclusions are classic hallmarks of neurodegenerative diseases, including Alzheimer's diseases. Impairment of degradation pathway, besides protein misfolding altogether contribute to deficit in cellular homeostasis, such as: molecules traffic disturbance and synaptic deficit. The Bag2 cochaperone participates of protein triage and clearance through ubiquitin-independent degradation pathways. The goal of this study was to analyze the effect of Bag-2 overexpression on the hyperphosphorylated Tau (p-Tau). Methods: All the procedures were performed in accordance with the Institutional Guidelines for Animal Experimentation (CEA/IB-USP protocol 136 /2011). Hippocampal cell cultures were made using one-day old Lewis rat. Cultures were exposed to either 0.3; 0.5 or 0.7nM of rotenone for 48h, followed by Bag-2 transfection. Western blotting technique was employed to determine the total Tau and p-Tau levels. Films were analyzed by ImageJ software. Results are shown as mean \pm standard error. Two-way ANOVA was used for statistical analysis. N=3. Results: Levels of p-Tau decreased in the presence of Bag-2 overexpression as compared with Bag-2 absence in control and rotenone groups; DMSO (75.17 \pm 1.167 vs 99.00 \pm 0.3333), 0.3nM (50.33 \pm 0.6667 vs 92.83 \pm 0.5000), 0.5nM (63.67 \pm 0.3333 vs 111.5 \pm 0.5000) and 0.7nM (55.17 \pm 1.167 vs 121.5 \pm 0.1667). Bag-2 overexpression also increased the levels of total Tau when compared to cells in the absence of Bag2 at control and rotenone group; DMSO (5026 \pm 28.50 vs 8778 \pm 43.00) and 0.5nM (5612 \pm 80.00 vs 8660 \pm 207.5). Total Tau did not change in cultures exposed to 0.3 and 0.7nM of rotenone after Bag-2 overexpression. Discussion: Our findings revealed that Bag-2 overexpression was able to decreased p-Tau levels. In addition, the cochaperone may

increase the total Tau levels. The results presented herein reveal new venues of investigation regarding Bag-2 role in the neurodegenerative diseases involving the formation of neurofibrillary tangles. Financial Support: CAPES, FAPESP and CNPq.

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Neuroprotection Against Beta-Amyloid Mediated by a Targeted Antioxidant.

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Alzheimer's disease is a progressive neurodegenerative disorder characterized, at least in part, by accumulation of amyloid β -protein ($A\beta$) in the brain. $A\beta$, produced by proteolytic cleavage from the β -amyloid precursor protein, either assembles extracellularly into fibrils or remains as soluble globular oligomers, both of which appear capable of initiating the cellular and molecular reactions associated with the disease. How, mechanistically, fibrillar or soluble oligomeric amyloid induces neuronal toxicity remains an area of intense investigation. Affected neurons certainly experience oxidative stress, generate a number of pro-inflammatory cytokines, and appear to activate the endoplasmic reticulum-mediated unfolded protein response. Synaptic circuitry is compromised and patients eventually suffer neurological impairment.

The goal of this work was to document the neuroprotective effects of a cell-penetrating enzyme with powerful antioxidant and anti-inflammatory properties. To accomplish this, β -amyloid fibrils or soluble monomers were generated in vitro and used to treat primary rat hippocampal/cortical neuron co-cultures. We show that $A\beta$ induces production of reactive oxygen species (ROS) in these cells, and viability is compromised. We also demonstrate that our targeted antioxidant, catalase-SKL (CAT-SKL) dramatically protects cells, reducing ROS levels and enhancing their survival. We are currently examining the production of a number of inflammatory cytokines (e.g. IL-6, IL-8, and TNF- α) in response to $A\beta$ and will similarly examine the effects of CAT-SKL.

The results obtained to date constitute the preliminary observations/proof-of-concept validation necessary for follow-up studies with appropriate Alzheimer's disease animal models in a preclinical setting.

1593

The Role of the Interaction Between the ER and the Mitochondria in the Development of the Dementia of Alzheimer's Disease.

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Studies from my laboratory have suggested that there is a decline with age in the capacity of the ER to catalyze the posttranslational processing of nascent proteins. This loss is associated with a decline with age in the content of the ER chaperones. Data from other laboratories indicate that declines in N-glycosylation may also play a role. Together these findings suggest that the dementia of Alzheimer's disease may be due to a decrease in the capacity of neurons to synthesize the synaptic, membrane proteins that are necessary for a functioning memory. It has also been thought that the decline in cognition may be secondary to a loss of mitochondrial function with age. Recent studies have indicated possible biochemical mechanisms whereby this decline may be related to our observed loss of ER function, since the ER plays a critical role in maintaining normal mitochondrial activity. The ER and mitochondria are connected through the Mitochondria Associated Membrane (MAM). This structure serves a number of critical functions including transporting phospholipids from the ER, where they are synthesized, into the mitochondria. It also regulates the fusion and fission of mitochondria. These processes are important because as the organism ages the mitochondrial proteins undergo oxidative damage.

During fusion and fission the damaged proteins are isolated to one of the daughter mitochondria that then undergo autophagy. The proteins which form the MAM and the autophagic vesicles are processed in the ER. Hence, declines in protein processing in the ER would compromise both of these systems. Furthermore, these findings provide a biochemical basis for the well recognized role of polymorphisms in apolipoprotein E (apoE) in the early development of Alzheimer's disease. The gene for apoE is 30 kb down stream from the gene for TOM40. TOM40 is a pore protein necessary for the transport of nascent proteins from the cytosol into the mitochondria. Furthermore, the apoE4 gene is tightly linked to a poly T polymorphism in intron 6 of TOM40. Individuals with this polymorphism have low mitochondrial function at all ages. I would postulate that these compromised mitochondria will be more susceptible to the age related decline in ER processing than the more robust mitochondria found in individuals with short poly T polymorphisms in intron 6.

1594

A prion-containing yeast cell can switch into agar-invasive growth while an isogenic non-prion strain cannot.

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Yeast prions consist of infectious, self-propagating protein particles which act as epigenetic elements of inheritance. Yeast prions may provide an epigenetic mechanism for allowing inheritable diversity that promotes survival in fluctuating stressful environments by switching into pseudohyphal filamentous growth in order to stimulate migration to find new food sources.

Yeast cells enter different differentiation pathways according to nutrient availability and environmental conditions. Our hypothesis is that [PSI⁺], a prion-containing strain can switch to filamentous pseudohyphal growth under stressful conditions while a prion-negative [psi⁻] strain cannot.

Under nitrogen-limiting media conditions, [PSI⁺], a Sup35 prion-containing cell, enters filamentous growth and invades agar. [psi⁻], which contains Sup35 in its native functional form, is not invasive even when grown under the same nitrogen-limiting conditions. In order to check whether these strains are invasive/non-invasive under different stressful conditions, we tested the strains in different growth media using three different growth and invasion assays, two of which are novel and developed in our laboratory.

[PSI⁺] and [psi⁻] strains were tested for invasive growth using 1) an agar washing assay, 2) a test tube invasion assay, or 3) a confocal invasion assay. In all three assays carried out under stressful conditions, the [PSI⁺] prion-containing strain invaded the agar while the [psi⁻] strain did not. [PSI⁺] prion-containing strains are able to read through stop codons and this ability of [PSI⁺] may explain our results. The ability of [PSI⁺] to invade agar may allow it to survive in stressful conditions and is evidence that prion formation may sometimes result in the acquisition of traits that can be advantageous to the organism.

1595

Exposure to Sodium 4-phenylbutyrate Does Not Increase Grp78 Levels in *C. elegans* following E.R. Stress Treatment.

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Unfolded protein response (UPR) is the basic cellular process that underlies a wide range of diseases such as Parkinson's disease, prion disease, and other chronic metabolic diseases such as obesity and type 2 diabetes. In this research, *C. elegans* were exposed to cadmium chloride at concentrations of 3 mM and 7 mM and tunicamycin at concentrations of 3 µg/mL and

7 µg/mL. The worms were either grown on nutrient agar medium plates supplemented with sodium 4-phenylbutyrate (PBA) at a concentration of 3mM or on nutrient agar medium plates alone. After the experiment, all worms were lysed and the protein concentrations in each sample were determined using the Bradford assay. The same amount of protein was used for Western Blot analysis to determine the amount of Grp78 present in each sample. The initial findings suggested that PBA treatment did not increase Grp78 level.

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Allosteric control of the IRE1 α endoribonuclease using kinase inhibitors.

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The unfolded protein response (UPR) is an evolutionarily conserved intracellular signaling pathway triggered when unfolded proteins accumulate in the endoplasmic reticulum (ER). Aberrant UPR contributes to cell degenerative disorders and the inappropriate survival of secretory cell tumors. To control the UPR's cell fate outcomes may provide new therapeutic options for these diseases. IRE1 α is an ER-transmembrane kinase/endoribonuclease (RNase) that senses unfolded proteins, oligomerizes, autophosphorylates, and initiates splicing of XBP1 mRNA, thus triggering the UPR. Although activation of IRE1 α 's RNase is normally dependent on kinase autophosphorylation, using chemical-genetic systems we had previously shown that a conformational change in the kinase domain triggered by ATP-competitive inhibitors bypasses autophosphorylation requirement in genetically modified versions of IRE1 α to allosterically activate IRE1 α 's RNase domain. Here we show that novel kinase inhibitors occupy the ATP-binding site of endogenous IRE1 α , to affect RNase activity from a distance. As dysregulation of the UPR has been implicated in a variety of cell degenerative and neoplastic disorders, small molecule control over endogenous IRE1 α should advance efforts to understand the UPR's role in pathophysiology and to develop drugs for ER stress-related diseases.

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Calreticulin and Calnexin, Ca²⁺ Binding Chaperones in ER, Regulate Olfactory Sensory Animal Behavior.

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Calreticulin and calnexin, molecular chaperone proteins, are Ca²⁺ binding proteins residing in endoplasmic reticulum (ER). These proteins control Ca²⁺ homeostasis and facilitate the proper folding of newly synthesized proteins in the lumen of ER. In order to study the function of calreticulin and calnexin in chemosensory behaviors, we investigated the behavioral phenotype of calreticulin (crt-1) and calnexin (cnx-1) mutants of *Caenorhabditis elegans*. Both crt-1 and cnx-1 mutants exhibited normal chemotaxis responding to AWC sensed odorants such as isoamyl alcohol, benzaldehyde and butanone. The crt-1 mutants exhibited hyperadaptation to AWC sensed odors whereas cnx-1 mutants failed to adapt, when compared with wildtype. These results imply that CNX-1 and CRT-1 may be involved in adaptation of AWC-mediated chemosensation. The expression of str-2::GFP, the neural developmental asymmetry indicator, in crt-1 or cnx-1 mutant background showed defects in the asymmetry of AWC neurons. We are further investigating how these ER resident proteins modulate to the chemosensory behavior and the underlying mechanism.

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Single Molecule Studies on Protein Unfolding and Polypeptide Translocation by an ATP-dependent Protease.

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The process of controlled protein degradation is critical for the maintenance of cellular homeostasis in all organisms. This task is strictly regulated and mainly performed by ATP-dependent proteases. These energy-dependent proteases have two components: a barrel-shaped peptidase with active sites sequestered deep in its cavity, and a hexameric, ring-shaped unfoldase that uses the energy of ATP hydrolysis to unfold protein substrates and translocate them into the associated peptidase. In this study, we developed a single-molecule optical tweezers assay to monitor in real time protein unfolding and polypeptide translocation by ClpXP, a model ATP-dependent protease from *Escherichia coli*. We conducted these experiments with the ClpX unfoldase alone, and in complex with the ClpP peptidase. Our results showed that upon ATP hydrolysis, ClpX generates mechanical force to unfold and translocate polypeptides through its central pore. Polypeptide translocation is interrupted by pauses that are found to be off the main translocation pathway. The translocation velocity of ClpX is force dependent, reaching a maximum of 8 nm/s (~80 aa/s) at near zero force and vanishing at around 20 pN. During translocation, ClpX takes 1, 2, or 3 nm steps, suggesting a fundamental step size of 1 nm and a certain degree of intersubunit coordination. In front of a folded substrate like GFP, ClpX either overcomes this mechanical barrier by unfolding it, or slips on the polypeptide before making another unfolding attempt. Binding of ClpP decreases the slip probability and enhances the unfolding efficiency of ClpX. The mechanical unfolding of GFP by ClpXP is cooperative and displays well-defined intermediate states. Because ClpXP shares its basic structural design with other ATP-dependent proteases, these studies provide important insight into the fundamental operating principles used by this group of enzymes.

Autophagy

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Plasma membrane to vacuole traffic mediates cell survival in glucose starvation and inhibits canonical macroautophagy.

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Starvation is a common event in the life of a cell. Facing starvation, the cell deploys highly conserved responses that modify cell behavior to survive. These responses frequently differ depending on the identity of the scarce nutrient or nutrients. It is thought that macroautophagy, a process by which the cell recycles its own cytoplasmic components, is an integral response to many types of starvation. Despite its critical role in many types of starvation, numerous observations suggest that there are additional alternative mechanisms to survive starvation. Here we report a new form of cell catabolism that occurs in response to glucose starvation in the yeast *Saccharomyces cerevisiae*. This process involves massive plasma membrane to

vacuole traffic. This process also actively inhibits the onset of macroautophagy via a TORC1 dependent pathway. We find that plasma membrane to vacuole traffic is necessary for cell survival, while macroautophagy is, in fact, dispensable. These results identify the plasma membrane to vacuole pathway as the mechanism for cell catabolism that directs cell survival in glucose starvation in yeast and thus expands the repertoire of response pathways available to a starving cell.

1600

LC3 constitutively associates with a high molecular weight complex in both the cytoplasm and nucleus.

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LC3 is a key component of the autophagy pathway, where it functions in autophagosome formation and cargo recruitment. Recent proteomics analysis indicates LC3 and other members of the ATG8 family interact with 67 different proteins, many of which appear to bind to common sites on LC3. However, it is currently unknown how many proteins are bound to LC3 at a given time. In addition, it is unclear if cytoplasmic and nuclear forms of LC3 interact with similar or different proteins. Here, we report several independent lines of evidence that suggest LC3 is constitutively associated with a high molecular weight complex in both the cytoplasm and nucleus. First, confocal FRAP measurements indicate that Venus-LC3 diffuses significantly more slowly than predicted based on its molecular weight in both the cytoplasm and nucleus of living cells. Second, fluorescence correlation spectroscopy measurements of freshly prepared cell homogenates report the correlation time of Venus-LC3 is longer than that of Venus alone, again suggesting it is contained within a large complex. Third, both cytoplasmic and nuclear forms of endogenous LC3 and Venus-LC3 migrate as part of a high molecular weight complex in blue native gel electrophoresis. To test whether multiple copies of LC3 are contained within the same complex, we performed FRET analysis between Venus- and Cerulean-tagged LC3, homoFRET analysis of Venus-LC3, and brightness analysis of Venus-LC3. No FRET was detected between LC3 molecules in the cytoplasm or in cell homogenates, and the brightness of LC3 was consistent with approximately 1 Venus-LC3 per complex. However, a small level of FRET was measured between LC3 molecules in the nucleus, suggesting multiple copies of the protein may be present within the same complex. Experiments are currently underway to determine how the formation of these complexes is regulated as well as to identify other complex components. We suggest that the incorporation of LC3 into multi-protein complexes may represent a currently unrecognized mechanism for regulating autophagy.

1601

Autophagy Inhibition by irreversible deconjugation of Atg8 proteins from membranes.

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Eukaryotic cells use the autophagy pathway to defend against microbes that gain access to the cytosol or reside in pathogen-modified vacuoles. It remains unclear if pathogens have evolved specific mechanisms to manipulate autophagy. Here we demonstrate that the intracellular

pathogen *Legionella pneumophila* interferes with autophagy using the bacterial effector protein RavZ, which is an enzyme that directly uncouples Atg8 proteins attached to phosphatidylethanolamine on autophagosome membranes. RavZ hydrolyzes the amide bond between the carboxyl-terminal glycine residue and an adjacent aromatic residue in Atg8 proteins. The result is an Atg8 protein that is no longer a substrate for re-conjugation by Atg7 and Atg3. Thus, one mechanism by which intracellular pathogens inhibit autophagy is to irreversibly inactivate Atg8 proteins on the early phagophore membrane.

1602

Phosphorylation and proteolysis-dependent regulation of Acinus stability modulates autophagy and resistance to starvation stress.

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Acinus is a conserved nuclear protein that has been implicated in the regulation of cell death and autophagy. We have previously shown that loss-of-function mutants of *Drosophila* Acinus (*dAcn*) interfere with autophagosome maturation in the larval fatbody, whereas elevated levels of *dAcn* induce autophagy downstream of mTOR (1). Therefore we were interested in defining the mechanisms that regulate the dynamic, cell-type-specific changes in *dAcn* protein levels. In a targeted RNAi screen, we identified the Caspase-3 homolog Dcp-1 as a protease regulating *dAcn* stability. Loss of Dcp-1 stabilized *dAcn* and increased *dAcn*-dependent autophagy. Furthermore, a mutation in a conserved Caspase-3 cleavage site (*dAcn*^{D527A}) stabilized *dAcn* and enhanced autophagy. In fed and starved larvae, fat bodies expressing the *dAcn*^{D527A} mutant - instead of wild-type *dAcn* - showed robustly enhanced autophagy as detected by a 3MA-dependent accumulation of lysotracker and an increase in Atg8 staining. The Dcp-1-mediated cleavage of *dAcn* appears to be regulated by phosphorylation. We identified two conserved potential phosphorylation sites in the C-terminus of *dAcn*. To explore their role we generated flies in which - instead of wild-type *dAcn* - either a phospho-mimetic (*dAcn*^{S641,731D}) or a phospho-inert (*dAcn*^{S641,731A}) mutant was expressed under control of the endogenous *dAcn* promoter. Proteolysis of the phospho-mimetic *dAcn*^{S641,731D} protein was reduced and these flies exhibited enhanced autophagy similar to flies expressing the Caspase-3 cleavage-resistant *dAcn*^{D527A} mutant. By contrast, the *dAcn*^{S641,731A} mutant was destabilized and autophagy was reduced even after starvation. This regulation of *dAcn* stability is important on the organismal level, as increased induction of autophagy paralleled an increased resistance to starvation stress: flies expressing the stabilized *dAcn*^{S641,731D} or *dAcn*^{D527A} mutants lived significantly longer compared to a wild-type *dAcn* control, whereas flies expressing the destabilized *dAcn*^{S641,731A} protein died faster. These results are consistent with a model in which phosphorylation of serines 641 and 731 counteract DCP-1-mediated cleavage of Acinus. In turn, elevated levels of *dAcn* protein play a critical role in regulating developmental decisions and promoting survival of animals exposed to starvation stress.

(1) Haberman et al. (2010) *Development* 137: 2157-2166.

1603

Dynamic Live Cell-based Assays for Autophagy.

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Autophagy is a complex physiologic cellular process essential for cell survival under stressed conditions. Probing into the pathway has offered novel targets for new drug development for cancer and neuronal diseases. By performing live-cell imaging on cells transduced with the autophagosome marker LC3 genetically fused to green fluorescent protein (GFP), the

autophagy process can be visualized on a fluorescent microscope. However, the dynamics of the lysosomal degradation phase of the autophagy process are less easily assessed than the preceding autophagosome formation phase. To better profile the effects of molecules acting on the whole autophagy pathway, a dynamic live cell-based assay has been developed to monitor both the rate of autophagosome formation and the decay for different autophagy-related perturbations. In this study, we interrogated GFP-LC3 CHO reporter cells with multiple reagents at the designated time points and visualized the autophagy process by fluorescence microscopy. In order to observe the dynamics of the autophagy process, we also exploited a commercially available microfluidic live cell imaging platform that has temperature and gaseous microenvironment control along with a solution switching capability.

The dynamic assay was composed of four continuous phases: (1) a standard culture phase where the native background fluorescent signal was recorded; (2) a stressed phase where a stressor was applied to induce autophagy; (3) a lysosome inhibition (with stressor) phase where the rate of autophagosome formation was recorded by enumeration with the Cell Profiler software; and finally (4) a recovery phase where culture medium was perfused continuously to return the cells from autophagy while monitoring the degradation rate of autophagosomes. To validate the dynamic cell-based assay, we applied known stressors such as starvation with EBSS, as well as a model inducer Rapamycin at various concentrations. The results have successfully shown that the entire dynamic profiling of these stressors for autophagy is feasible through the established live-cell imaging system on a fluorescent microscope. Through software analysis, we also observed that the complete lysosome degradation in the GFP-LC3 CHO reporter cells occurred linearly within 180 minutes after the recovery growth medium was added. Here we describe an autophagy assay that provides the quantitative information of the study of both autophagosome formation and the lysosome degradation machinery, which may facilitate the characterization of existing targets and discovery of new targets for drug development for multiple autophagy-related diseases.

1604

The clearance of protein aggregates: Elucidating a role for small heat shock proteins versus selective macroautophagy.

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Macroautophagy is the cellular pathway by which the lysosome eliminates cytosolic proteins through fusion with a de novo synthesized organelle known as the autophagosome. The ability of cargo to be targeted for degradation can rely on autophagic receptors and selectivity adaptors, which may be required for the degradation of some cargo, but not for macroautophagic degradation as a whole. For example, the selective degradation of aggregated proteins relies on the selectivity adaptor, Alfy (autophagy linked FYVE protein), which acts as a molecular scaffold between its cargo and the key macroautophagic protein Atg5. Overexpression of Alfy decreases the presence of cytoplasmic protein aggregates and can be cytoprotective against toxic aggregates in vivo. One of the key questions about Alfy, however, is how it recognizes aggregated proteins; Alfy is a nuclear protein that shuttles to the cytosol in the presence of aggregation. Similarly, it has recently been shown that the small heat shock protein (sHSP), HSPB7 also modulates protein aggregation in a manner similar to Alfy: its overexpression diminishes aggregation and is cytoprotective in vivo. Moreover, it has been suggested that unlike classic sHSPs, HSPB7 relies on the macroautophagic machinery to exert its functions, although how remains unclear. In this study, we test the hypothesis that HSPB7 contributes towards Alfy-mediated selective macroautophagy to promote the degradation of toxic protein aggregates. Using fragments of the huntingtin protein with an expanded

polyglutamine (polyQ) repeat as a model aggregation-protein protein, we use a combination of biochemical (segregation of detergent soluble and insoluble proteins) and cell biological methods to examine the role of Alfy and HSPB7 on polyQ protein. First, we confirmed previous published data showing that overexpression of Alfy in a stable polyQ cell line and co-transfection of HSPB7 with polyQ constructs leads to diminished protein aggregation. Next, when investigating the role of stable expression versus transient transfection of polyQ proteins, we found that unlike Alfy, HSPB7 failed to reduce the amount of detergent insoluble proteins under conditions of stable expression, suggesting that HSPB7 cannot eliminate preformed inclusions, and works independently of Alfy. This was further confirmed when the anti-aggregation activity of HSPB7 was maintained in Alfy knockout MEFs. These findings indicate that distinct pathways may be used by the cell to reduce protein aggregation and promote cytoprotection.

1605

Recycle or Destroy? The Duplicitous Roles of Autophagy in Cadmium-induced Apoptosis in *Saccharomyces cerevisiae*

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Autophagy, or self-eating, is a conserved cellular stress response that gives rise to the controlled breakdown of unwanted cytosolic material, enhancing the potential for cellular survival. As such, it can be considered to be a protective mechanism. The process begins with the formation of membrane vesicles, or preautophagosome structures (PAS), which engulf the cytosolic contents to be digested. The PAS then mature in to so-called autophagosomes, which are double-membrane structures that ultimately fuse with the lysosome to facilitate the further degradation and recycling of their contents. Autophagy can be induced by variety of cellular stressors, such as nutrient deprivation, accumulation of dysfunctional proteins or exposure to heavy metals. The overly simplified view of autophagy as being a mechanism to conserve cellular function has been challenged, of late, as autophagic activity has been correlated, in a number of different cells, with the augmentation of programmed cellular demise, or apoptosis. Herein, we confirm that the autophagic process is indeed necessary for the induction of the caspase-dependent apoptotic response in *S. cerevisiae* to the presence of the heavy metal, cadmium. We demonstrate further that the initial phase of autophagy is crucial in determining whether autophagy or apoptosis predominate. We also show that the timing of the autophagic induction is a key factor in deciding which role it plays in cell survival: in that induction of autophagy prior to exposure of the cell to cadmium serves to negate the apoptotic response, while the independent induction of autophagy after caspase induction serves only to facilitate the apoptotic potential of the cell.

1606

The Role of the NAADP/Two Pore Channel 2 (TPC2)/Ca²⁺ Signaling in the Maturation of Autophagosome.

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Autophagy, an evolutionarily conserved lysosomal degradation pathway, has been implicated in a wide variety of cellular processes, and the underlying mechanisms have been extensively studied. Yet, the fusion between autophagosome and lysosome is poorly understood. Nicotinic acid adenine dinucleotide phosphate (NAADP), an endogenous Ca²⁺ mobilizing nucleotide, mobilizes Ca²⁺ from acidic organelles, e.g. lysosome, through two pore channel 2 (TPC2). Here

we reported that overexpression of TPC2 in HeLa cells, which lack the expression of endogenous TPC2, inhibited the fusion between autophagosome and lysosome by raising lysosomal pH, whereas overexpression of a TPC2 inactive mutant failed to inhibit the fusion. Transient treatment of the cells expressing the wildtype, not mutant, TPC2 with a cell permeant NAADP analogue further inhibited the fusion. Interestingly, autophagy occurs during the in vitro neural differentiation of mouse embryonic stem (ES) cells initiated by the monolayer culture, and the expression of endogenous TPC2 was markedly decreased during the initial ES cell entry into neural progenitors. Consistently, TPC2 knockdown or overexpression in mouse ES cells promoted or inhibited the fusion between autophagosome and lysosome during the in vitro neural differentiation, respectively. Correspondingly, TPC2 knockdown or overexpression in ES cells accelerated or delayed mouse ES cells entry into neural lineages, respectively. Taken together, our results indicated that the NAADP/TPC2/Ca²⁺ signaling inhibited the maturation of autophagosome to antagonize the neural differentiation of mouse ES cells. The development of novel agonists or antagonists of TPC2 based on NAADP might be a good approach to control the maturation processes of autophagosome.

1607

The autophagy lipidation enzyme, Atg3, is a membrane curvature sensor.

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Although a wide variety of autophagy-related proteins can target the cup-like autophagosomal isolation membrane, once the autophagosome closes only lipidated Atg8 proteins remain associated with the outer surface. It is not yet known what features of the isolation membrane distinguish it from the mature autophagosome, but one possibility is that the highly curved rim of the cup is an important marker for transiently-associated components of the autophagic machinery. Here we use a fully reconstituted in vitro assay of protein lipidation to establish several first principles in the ubiquitin-like lipidation of Atg8. We show that the E2-like enzyme, Atg3, is a membrane hydrophobicity sensor and requires extensive exposure of the membrane hydrophobic core to facilitate Atg3 function. This detection depends upon an amino-terminal ALPS-like amphipathic helix similar to motifs found on other proteins targeting highly curved intracellular membranes. Indeed, in vitro Atg3 function is dramatically increased on membranes exhibiting strident curvature. By tuning the amphipathicity and hydrophobicity of the ALPS motif, we can promote or inhibit LC3 lipidation both in vitro and in rescue experiments in Atg3 knockout MEFs, implying a physiologic role for this sensing. The need for extensive hydrophobic membrane exposure suggests that Atg3 is designed to work at highly-curved membranes perhaps including the limiting edge of the growing phagophore.

1608

Parkin regulates the clearance of misfolded proteins by the aggresome-autophagy pathway.

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Parkinson disease (PD) and many other human diseases (e.g., cystic fibrosis and Alzheimer disease) are often referred to as “protein misfolding diseases” because their pathogenesis involves protein misfolding and aggregation. The accumulation of misfolded proteins in these diseases is likely due to a chronic imbalance in the generation and clearance of misfolded proteins, and it suggests that failure of cells to cope with excess misfolded proteins may be a common pathological mechanism linking these clinically distinct diseases. Recent evidence

indicates that the aggresome-autophagy pathway plays a cytoprotective role by mediating selective clearance of misfolded and aggregated proteins under conditions of proteotoxic stress. The molecular mechanisms that regulate the aggresome-autophagy pathway remain poorly understood. We have shown that the PD-linked E3 ubiquitin-protein ligase parkin selectively binds and catalyzes K63-linked polyubiquitination of misfolded DJ-1 but not wild-type DJ-1. Furthermore, our study reveals that the K63-linked polyubiquitination serves as a signal for targeting misfolded DJ-1 to the aggresome-autophagy pathway. We have identified another misfolded protein (MFP) that is specifically recognized and ubiquitinated by parkin. We find that parkin overexpression promotes clearance of MFP by the aggresome-autophagy pathway and protects cells against MFP-induced toxicity. Our findings support a general role of parkin-mediated K63-linked polyubiquitination in regulating misfolded protein clearance by the aggresome-autophagy pathway.

1609

Microbial detection controls defective ribosomal proteins degradation by autophagy and subsequent endogenous MHC II-restricted presentation in dendritic cells.

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Dendritic cells (DCs) are antigen presenting cells with the unique capacity to initiate primary immune responses. DCs have a remarkable pattern of differentiation (maturation) that exhibits highly specific mechanisms to control antigen presentation in response to microbial stimuli. In particular, expression, transport and loading of MHC class I and II molecules are strongly activated during DC maturation. MHC class I molecules present to CD8+ cytotoxic T cells peptides mostly derived from defective cytosolic proteins (DRiPS), which are ubiquitinated and degraded by the proteasome. Here, we show that newly synthesized defective proteins are also targeted to autophagosomes and degraded. Upon DC maturation, autophagy is inhibited leading to the accumulation of these DRiPs in large cytosolic structures. Interestingly, these Dendritic cell Aggresome Like Induced Structures (DALIS) are transient, and require both translation inhibition and proteasome activity for their disappearance. Thus, autophagy affects the efficiency of the classical proteasome-mediated antigen processing by competing for a fraction of the same DRiPs substrates. Autophagy was estimated to reduce the level MHC I presentation by 20%. We also demonstrate, that this pathway is used by DCs to present endogenous proteins on the MHC II molecules and that immature cells have the capacity of presenting peptides derived from the same endogenous antigens both on the MHC I and MHC II. Upon microbial activation MHC II presentation of the endogenous antigens is lost, while the MHC I antigens source changes from newly synthesized proteins to other undefined sources. Moreover, we demonstrate that several cytokines can revert this process and induce alternative pathways for the autophagic processing of antigens as well as different immunological functions. This phenomenon is likely to be important for regulating MHC class I and II presentation and the establishment of tolerogenic or immunostimulatory conditions during the necessary functional switch of DC maturation and CD4+ T cells licensing

1610

The Functional Analysis of Autophagy Using a New Class of Lysosome-Specific Fluorescent Indicators.

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Lysosomes are cellular organelles which contain acid hydrolase enzymes to break up waste materials and cellular debris. Lysosomes digest excess or worn-out organelles, food particles, and engulfed viruses or bacteria from phagocytosis, endocytosis, and autophagy. Autophagy is

a process that degrades damaged organelles, cell membranes and proteins. The failure of autophagy is thought to be one of the main reasons for the accumulation of cell damage and aging.

We report the functional analysis of autophagy using a new class of lysosome-selective fluorescent indicators (called LysoBrite). These newly developed fluorescent lysosome indicators are well retained in cells with minimum cell cytotoxicity. These hydrophobic compounds easily permeate into intact cells, and become trapped in lysosomes after passing through the cell membranes. Their fluorescence is significantly enhanced upon entering lysosomes.

We demonstrated these new lysosome dyes are a set of valuable tools for monitoring autophagy in live cells. The signal intensity, photostability, cellular retention, as well as cell cytotoxicity of these new lysosome indicators were evaluated in HeLa and Jurkat cells with fluorescence microscopy and flow cytometry. The LysoBrite dyes has significantly improved signal intensity that can be used for tracking more than 6 passages of HeLa cells, and the indicators stay in lysosomes for more than 20 days in Jurkat cells. Furthermore, there is no fading observed even after a 2 minute exposure time. The cells show no cytotoxicity effect after exposure to the dyes over 24 hours.

In summary, LysoBrite dyes have extremely high photostability as well as excellent cellular retention, making them useful for a variety of applications, including cell adhesion, chemotaxis, multidrug resistance, cell viability, apoptosis and cytotoxicity. The dye is suitable for proliferating and non-proliferating cells, and can be used for both suspension and adherent cells.

Oncogenes and Tumor Suppressors II

1611

Development of mouse brain tumor models derived from neural stem cells expressing activated ALK.

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Neural Stem Cells (NSCs), having self-renewal and multipotent ability, are considered one of the cells-of-origin of glioblastoma multiforme (GBM). Although NSCs can be enriched in neurosphere floating culture with serum-free media containing EGF/FGF as a classical method, neurosphere is composed of not only NSCs but also differentiated and apoptotic cells. Therefore, we utilize the method for efficient derivation of NSCs with long-term self-renewal and differentiate capacity in adherent culture. Using this method, we cultured the genetically modified NSCs. Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase first identified in a chromosomal translocation associated with anaplastic large cell lymphomas. Subsequently, new ALK translocations were found in a fraction of non-small-cell lung cancers and in other solid tumors. The function of full-length ALK is involved in neuronal cell differentiation, regeneration and synapse formation. Recently, gene amplification and mutations of full-length ALK were identified in neuroblastoma. Furthermore, many of the GBM samples and cell lines were observed to have overexpression of ALK. Although constitutive activation of ALK signaling results in cell transformation, little is known about the tumorigenic mechanisms induced by activated ALK. We have established a stable mouse model of brain tumor transplanting the genetically modified NSCs. The NSCs derived from *Ink4a/Arf* KO mice transduced with

constitutively active mutants of ALK (F1174L, R1275Q), identified in neuroblastoma, rapidly formed highly proliferative and invasive brain tumors. Histological characteristics of these tumors resembled human GBM phenotype demonstrating necrosis, perivascular cuffing and giant cell formation. Immunostaining analysis revealed that the tumors have astrocytic differentiation and highly proliferative characteristics. In addition, *WT* mice NSCs transduced with ALK R1275Q, but not F1174L, formed brain tumors same as *Ink4a/Arf* KO mice NSCs. On the basis these findings, we propose a specific regulation of tumorigenic signaling induced by activated ALK.

1612

The Role of MXD3 in Human Precursor B cell Acute Lymphoblastic Leukemia.

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MXD3 is a transcription factor previously shown in our lab to be a novel member of the Hh pathway (Yun, Rust, Ishimaru, Diaz, Mol Bio Cell, 2007). We have previously shown that MXD3 is expressed in medulloblastoma and that its knockdown reduces proliferation of human medulloblastoma cell lines (Barisone et al., PLoS, 2012). In the current study, we investigated a possible role for MXD3 in precursor B (preB) acute lymphoblastic leukemia (ALL) cell proliferation. Using qRT-PCR we observed 13 to 35 times higher levels of MXD3 mRNA expression in 8 primary preB ALL samples, as well as in the preB ALL cell lines Reh and JM1, than in mobilized peripheral blood mononuclear cells from healthy donors, mouse bone marrow and spleen. Immunoblot analysis with anti-MXD3 monoclonal antibodies confirmed that the protein was present in the ALL samples but not in normal cells. We investigated the role of MXD3 in cell proliferation and survival by silencing MXD3 in the Reh cell line. We used lentiviral delivery to knockdown MXD3 using an RNA interference approach. Upon transduction by the viral vector, MXD3 knockdown was confirmed at both the RNA and protein level. Within 48 hours, MXD3 protein levels were reduced >90% in cells infected with the shMXD3 virus but not with the control virus. MXD3 knockdown resulted in decreased proliferation in Reh cells, supporting our hypothesis that it may be involved in the maintenance of ALL. To understand the role of MXD3 in leukemia cells, we analyzed cell cycle progression and apoptosis levels after knockdown using flow cytometry. We observed no significant differences in the G0/G1, S or G2/M populations between experimental and control samples. However, samples where MXD3 had been knocked down showed higher levels of apoptosis when compared to controls. Our results suggest that MXD3 is important for preB ALL cell proliferation, possibly by acting as an anti-apoptotic factor. Therefore, MXD3 is a potential candidate for targeted therapies against preB ALL.

1613

The protooncprotein TCL1 activates the endoplasmic reticulum stress response to promote leukemic progression in mice.

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T cell leukemia/lymphoma-1 (TCL1) protein is expressed in approximately 90% of human B-cell chronic lymphocytic leukemia (B-CLL) samples. Transgenic expression of TCL1 in murine B

cells (E α -TCL1) results in mouse CLL. Much still needs to be explored to understand how the expression of this 14-kDa TCL1 protein leads to leukemia. Here we show that TCL1 can associate with X-box binding protein 1 (XBP-1), and transcriptionally upregulate the expression of several endoplasmic reticulum (ER) chaperones, which include glucose-regulated protein 78 (GRP78/BiP), GRP94 and protein disulfide isomerase. Highly elevated expression of XBP-1 in TCL1-overexpressing mouse CLL cells causes dysregulated expression of paired box protein 5 (Pax5), interferon regulatory factor 4 (IRF4), B lymphocyte-induced maturation protein 1 (Blimp-1), and activation-induced cytidine deaminase (AID). To establish that XBP-1 supports the growth of TCL1-overexpressing CLL cells in mice, we developed an inhibitor that specifically targets the ribonuclease activity of the ER-resident transmembrane kinase-endoribonuclease inositol-requiring enzyme 1 (IRE-1), which is responsible for the splicing of XBP-1 mRNA and the expression of the functional XBP-1 protein. Inhibitor-treated B cells mimic the phenotypes of B cells deleted with the XBP-1 gene, as evidenced by the upregulated expression of IRE-1 and a specific inhibitory effect on the expression of secretory IgM but not membrane-bound IgM in these B cells. This inhibitor also does not affect general protein synthesis and trafficking in B cells. When we blocked the IRE-1/XBP-1 pathway using the inhibitor, we observed apoptosis and significantly stalled growth of CLL cells in vitro and in mice. This inhibitor does not affect naïve B cells because these cells do not express XBP-1. Our studies reveal an important role of TCL1 in activating the ER stress response in support for malignant progression of CLL.

1614

The T-cell acute lymphoblastic leukemia-associated rpl10-R98S mutation prevents the release of the nuclear export adapter Nmd3 in yeast.

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T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive neoplastic disorder that is caused by accumulation of multiple oncogenic lesions in developing T-cells. Major driver mutations in T-ALL include hyperactivity of the NOTCH1 pathway, activation of JAK/STAT signaling, overexpression of a set of transcription factors, and loss of cell cycle control. Whole exome sequencing of 67 T-ALL samples identified mutations in large ribosomal subunit proteins in 13.3% of pediatric samples with mutation of arginine 98 to serine in RPL10 occurring in 9.5% of patients. These results identify mutation of the RPL10 as a new driver of T-ALL. R98 is highly conserved in eukaryotic Rpl10 and is found at the base of a β -hairpin that supports the P-site loop of Rpl10. We have recently shown that mutations in the P-site loop of Rpl10 (eg rpl10-S104D) cause a failure in the cytoplasmic maturation of the 60S subunits by blocking the release of the anti-association factor Tif6 from the subunit. As a consequence, the export adapter Nmd3 also fails to be released. The phenotypes of rpl10-S104D are similar to those of loss of Sdo1, the protein associated with Shwachman-Diamond syndrome. The T-ALL-associated rpl10-R98S mutation similarly blocks the release of both Tif6 and Nmd3. However, rpl10-S104D and sdo1^{#8710}; can be suppressed by mutations in TIF6, but not in NMD3. In contrast, rpl10-R98S is strongly suppressed by mutations in NMD3 but not in TIF6, identifying release of Nmd3 as the step that is specifically impaired in the rpl10-R98S mutant. These results suggest related underlying defects in ribosome maturation in both Shwachman-Diamond syndrome and cases of T-ALL in which RPL10 is mutated.

1615

Tumor cells derived from different cells of origin show distinct therapeutic sensitivities in Myc-induced lymphoid tumor model.

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Understanding the cell of origin of tumors is important not only for elucidating detailed mechanisms of tumorigenesis but also for characterizing the context in which tumor cells develop, both of which provide valuable information that can inform preventive therapy and therapeutic strategies in the clinical setting. However **the factors determining the cell of origin and difference of therapeutic outcome between tumor cells derived from distinct cells of origin remain unclear.**

In this study, **a mouse model of precursor-B acute lymphoblastic leukemia/lymphoma (pre-B ALL/LBL) was established by retroviral transduction of *N-Myc* oncogenes into mouse bone marrow immature cells.** To identify the cell of origin of this tumor, we fractionated *N-Myc*-transduced hematopoietic stem cells (HSCs), lymphoid progenitors, myeloid progenitors and committed progenitor B cells, and then transplanted into recipient mice in a limiting dilution manner. As a result, HSCs showed the highest susceptibility to pre-B ALL/LBL versus lymphoid progenitors, myeloid progenitors. Although *N-Myc* was unable to induce any tumor directly from committed B cells, *N-Myc* was able to induce tumor directly from those cells in the absence of *Ink4a* and *Arf* (*CDKN2A*) genes whose expression is normally maintained at low levels for endowing of self-renewal capacity to HSCs. Furthermore, since *Arf* was expressed higher in progenitor B cells than *Ink4a* and *N-Myc* could induce pre-B ALL/LBL directly from *Arf* single deficient progenitor B cells, ***Arf* might play a predominant role in determining the cell of origin.**

Next, we compared proliferation and drug sensitivities in tumor cells derived from distinct cells of origin. Tumor cells derived from *Ink4a/Arf*^{-/-} progenitor B cells exhibited faster growth and were more resistant to an anti-cancer drug Ara-C (Cytarabine) treatment than those derived from wild-type (*Ink4a/Arf*^{+/+}) HSCs in vivo and in vitro. Given that *Arf* functions as an inhibitor of Mdm2, the E3 ubiquitin ligase for the tumor suppressor p53, we tried to treat the Mdm2 inhibitor Nutlin-3 to both cells. As a result, Nutlin-3 restored p53 and thereby induced massive apoptosis in tumor cells derived from *Ink4a/Arf*^{-/-} cells but not in those derived from wild-type (*Ink4a/Arf*^{+/+}) HSCs due to p53 mutations. Furthermore, Nutlin-3 significantly induced apoptosis in human B-ALL cell lines with wild-type *p53* and lacking *Ink4a* and *Arf* expression. Therefore, **Mdm2 inhibition might be a promising therapeutic approach to the treatment of *Ink4a/Arf* deficient B-ALL that is frequently found in Ph⁺ ALL and relapsed ALL.**

Collectively these findings suggest that ***Ink4a* and *Arf*, especially *Arf*, are critical determining factors of the cell of origin of pre-B ALL/LBL**, and that **tumor cells derived from distinct cells of origin showed different drug sensitivities to Ara-C and Nutlin-3**, which provides a novel insight into preventive therapy and different therapeutic approaches depending on genetic background of tumor cells.

1616

***Drosophila* Ard1 RNA interference knockdown causes massive cell death.**

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Cancer treatment and prevention is a vital part of biomedical research today considering the millions of those who suffer from a wide variety of cancers. N-acetylation is a major form of protein regulation in humans, where most of this activity is carried out by NatA, an N α -acetyltransferase complex. The catalytic subunit of NatA, Ard1, has been shown to be highly overexpressed in certain forms of cancer, such as liver cancer, colon cancer, and lung cancer. We believe that Ard1 regulates programmed cell death and may contribute to cancer progression. *Drosophila* lines were crossed to allow knockdown of Ard1 via RNA interference in the cells of the larval wing disc. Cell death was observed using fluorescent acridine orange staining. The knockdown of Ard1 in the wing resulted in large scale cell death. Since Ard1 is a highly conserved gene between species, using *Drosophila* as a model organism is an ideal way to unfold the mystery behind the function of Ard1. Considering Ard1 is highly overexpressed in many cancers, understanding how Ard1 functions could be a key element in the prevention and cure to those cancers.

1617

The genetic interaction of *Drosophila* Ard1 with DIAP1 suggests that Ard1 regulates apoptosis.

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Cancer is a widespread problem, and estimated 1.6 million people are diagnosed with cancer every year. Ard1 is the catalytic subunit of the NatA complex, an N α -terminal acetyltransferase. Ard1 is expressed in most tissues, but the Ard1 protein has been found to be overexpressed in a wide range of cancer lines. Our present study investigates how the Ard1 plays a role in cell survival in *Drosophila*. Genetic crosses were used to show that RNAi knockdown of Ard1 in the eye causes a small eye phenotype. Expression of DIAP1, a caspase inhibitor, rescued the Ard1 knockdown phenotype. This genetic interaction suggests that Ard1 is a regulator of apoptosis. Our research into the function of Ard1 and the role it plays in cell survival may help to advance our understanding of cancer genetics.

1618

BCAS2 is essential for *Drosophila* viability and functions in pre-mRNA splicing.

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Here, we demonstrate that dBCAS2 (CG4980, human Breast Carcinoma Amplified Sequence 2 ortholog) is essential for the viability of *Drosophila melanogaster*. We find that whole-body and tissue specifically silencing dBCAS2 leads to larval lethality, wing deformities and impaired splicing. More importantly, overexpression of hBCAS2 rescues these defects. Furthermore, the C-terminal coiled-coil domain of hBCAS2 directly binds to CDC5L and recruits hPrp19/PLRG1 to form a core complex for splicing in mammalian cells; and can restore wing damage induced by silenced dBCAS2 in flies. In summary, *Drosophila* and human BCAS2 share similar function in RNA splicing, which affect cell growth viability

1619

Synthetic Lethality with Raf-Transformation and Dysregulated Intracellular pH.

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Constitutively increased intracellular pH (pHi) is a hallmark of cancer cells, regardless of genetic lesion or tissue of origin. Increased pHi is thought to facilitate adaptive behaviors of cancer cells, suggesting pHi reduction as a therapeutic approach to limit cancer progression. However, this has not been experimentally confirmed *in situ*. Using *Drosophila melanogaster*, we genetically deleted *Dnhe2*, the homolog of the mammalian plasma membrane Na⁺-H⁺ exchanger NHE1 that extrudes protons to increase pHi. *Dnhe2*^{null} mutations are semi-lethal; fewer than 10% of homozygous flies survive to adulthood, although those that survive appear morphologically normal. Expression of oncogenic Raf in the eye (*GMR-Raf*) causes externally rough adult eyes and sections reveal dysplasia with missing or extra photoreceptors and orientation defects. We predicted that removing *Dnhe2* would limit Raf-induced dysplasia. Unexpectedly, loss of *Dnhe2* with *GMR-Raf* expression resulted in necrosis, with a profound loss of retinal organization and cellular structure. The cell death appears to be progressive in nature as there is no evidence of cell death in pupal eyes, suggesting an onset very late in eye development. *GMR-Raf* expression induces cell morphology defects that are evident in late pupal eyes, but cell-cell junctions and cell fate markers are present, even in the absence of *Dnhe2*. To investigate effects of dysregulated pHi on proliferation, we analyzed *Dnhe2*^{null} third instar imaginal eye discs and found reduced proliferation compared with wild type. *GMR-Raf* induced expanded proliferation of retinal cells, and removing *Dnhe2* in *GMR-Raf* flies reduced the over-proliferation, yielding levels similar to wild type. These results suggest that Raf-induced proliferative signals require *Dnhe2* activity and increased pHi. These results led to the prediction that Raf-transformed cells lacking *Dnhe2* abnormally accumulate protons, decreasing pHi. To test this prediction, we generated transgenic flies with a genetically encoded fluorescent biosensor to measure pHi *in situ*. Wild type and *Dnhe2*^{null} *Drosophila* retinal tissue exhibited a pHi of ~7.1 while Raf expression increases pHi to 7.4. Deletion of *Dnhe2* with Raf expression progressively decreased pHi to 6.8 in late pupal eyes, which is consistent with increased cell death. Our data suggest that dysregulated pHi via loss of *Dnhe2* acts as a synthetic lethal in transformed but not normal cells, which suggests inhibition of proton efflux may induce the selective death of transformed cells.

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Loss of PTP-PEST enhances STAT3 activation downstream of integrins and cytokine receptors and promotes tumor growth, invasion, and metastasis in colon cancer.

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The transcription factor STAT3 is hyperactivated by phosphorylation on Y705 in over 75% of metastatic colon cancers and is associated with EMT, cancer stem cells, survival and chemoresistance. The mechanisms of STAT3 inactivation are not well-defined. Here, we identified the protein tyrosine phosphatase, PTP-PEST, as a novel negative regulator of STAT3 in colon carcinoma cells. Using lentiviral shRNA silencing of PTP-PEST in KM12C and HCT116 colon carcinoma cells, we show that PTP-PEST knockdown enhances activation of STAT3. Whereas STAT3 was activated by cell attachment to collagen in control cells expressing non-targeting shRNA, PTP-PEST knockdown resulted in constitutive activation of STAT3 in an anchorage-independent manner. STAT3 also exhibited elevated and sustained activation in

response to IL-6 stimulation in PTP-PEST knockdown cells, which was further enhanced in the presence of collagen. PTP-PEST knockdown cells also showed enhanced chemotaxis to IL-6 and increased activation of Rac1, an upstream activator of STAT3, in response to IL-6 and collagen and expression of STAT3 target genes, MMP2 and mcl-1, was elevated. In vitro assays showed that silencing of PTP-PEST does not affect proliferation, but does enhance anchorage-independent and tumorsphere growth, matrigel invasion, and resistance to 5-fluorouracil in 3-D cultures. Subcutaneous injection of PTP-PEST knockdown cells showed enhanced tumor size while intrasplenic injection showed enhanced metastatic potential as assessed by quantitative PCR for human DNA content in mouse liver. Finally, reduced PTP-PEST expression correlates with metastasis in clinical specimens from colon cancer patients. Taken together, these findings suggest that down-regulation of PTP-PEST in colon cancer may promote metastasis through enhanced activation of STAT3.

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The Effect of StarD13 on Colorectal Cancer Proliferation and Invasion.

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Colon cancer, also considered as colorectal cancer or bowel cancer, is the cancer of the epithelial cells lining the colon. There are many risk factors and many prophylactic measurements against this cancer. 95% of colon cancer is known as adenocarcinoma, mainly divided into different stages according to invasiveness and metastatic ability of the tumor. Many mutations are acquired, leading to this malignancy. Mainly, these occur in entities that greatly alter and affect the cell cycle, cell signaling pathways, cell movement, which all involve the action of Rho GTPases. The protein of our interest is DLC2, also known as StarD13 or START-GAP2, a GAP for Rho and Cdc42. Literature states that this protein is considered a tumor-suppressor in hepatocellular carcinoma. Previous work in our lab proved StarD13 to be a tumor suppressor in brain tumor and in breast cancer. In this work, we studied the role of StarD13 in colon cancer.

When overexpressed, StarD13 led to a decrease in cell proliferation in colon cancer cells. This was measured by WST MTT kits. Knocking down StarD13 using StarD13 siRNA led to an increase in cell proliferation. This showed that, similarly to its role in astrocytoma and breast cancer, StarD13 seems to be a tumor suppressor in colon cancer as well. We were also interested in examining the role of StarD13 in cell motility. StarD13 knock down resulted in an inhibition of cell motility, as seen by time lapse microscopy. This might be due to the inhibition of Rho, thus Rac-dependant focal complexes are not formed nor detached for the cells to move forward. future work is still needed to determine the exact mechanism of inhibition.

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Identification of an E3 Ubiquitin Ligase as a Novel PDZ-containing Target of Oncogenic Human Papillomavirus E6 proteins.

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High-risk human papillomaviruses (HPVs) are the causative agents of several malignancies, including cervical, anal, penile and head-and-neck cancers. The E6 oncoprotein of the high-risk HPV types has a PDZ-binding motif (PBM) at its extreme carboxy terminus, which is not found in the E6 proteins of the low-risk, non-cancer-causing HPV types. Thus the PDZ-binding activities of the high-risk E6 proteins are thought to contribute to the malignant potential of the virus. High-risk E6 proteins have been shown to direct the proteasomal degradation of a number of their cellular target proteins through interaction with cellular ubiquitin-protein ligases, such as E6AP (also known as UBE3A) or EDD. Their cellular targets include the tumour suppressor,

p53, as well as a number of PDZ-containing proteins, including Dlg, hScrib, and the MAGI proteins.

In this report we identify PDZRN3 as a new PDZ domain-containing target of high-risk HPV E6 proteins. PDZRN3 is a PDZ-containing ubiquitin-protein ligase, whose ligase activity occurs through a RING domain. This is in contrast to E6AP and EDD, both of which function through a HECT domain. PDZRN3 is found as two isoforms PDZRN3A and B, both of which are targeted by high-risk HPV E6s. We have found that PDZRN3 levels are strongly reduced in the presence of high-risk E6 protein, and that this reduction is dependent upon the PBM of E6.

Given that PDZRN3 has been shown to inhibit canonical Wnt- β -catenin signaling, it is possible that the E6-induced reduction in PDZRN3 contributes to its oncogenic potential. Studies are in progress to investigate the role of this interaction in HPV-transformed cervical tumour-derived cells.

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Prevalence of human papillomavirus (HPV) infection in oral cancer.

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High-risk human papillomavirus (HPV) is the main source of cervical cancer, accounting for nearly 100% of all cases. Recently, the association of HPV with oropharyngeal cancer has been established, but the relationship between HPV and oral cancer is not yet known. A prognosis of HPV-positive cancer is known to be better than that of HPV-negative cancer. The purpose of this study was to determine the incidence and types of HPV-positive oral cancer in Korea using a HPV DNA chip and immunohistochemical analysis with HPV6, HPV11, HPV16 L1 and HPV18 E6 monoclonal antibodies.

106 patients who were previously diagnosed with invasive cancer of the oral cavity for HPV detection were examined. A DNA chip (MY-HPV chip kit, Mygene Co., Korea) was used for detecting low risk (6, 11, 34, 40, 42, 43, 44) and high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58) HPV types. Specimens used for HPV detection were extracted from the main mass during surgery and were tested for HPV typing using a DNA chip. First, DNA isolation was performed from the specimens using DNA extraction buffer via centrifugation. DNA amplification was performed by polymerase chain reaction (PCR) using primers, B-globin primer for the control group, Taq polymerase, template DNA, four types of deoxynucleotides (dNTP), and buffer solution. The second PCR product was mixed with hybridization buffer for hybridization. After chip scanning, we analyzed the result.

Immunohistochemical examination was performed with formalin-fixed, paraffin-embedded tissue of normal and cancer specimens. HPV 6, HPV 11, HPV 16 L1, and HPV 18 E6 monoclonal antibody was diluted in 1:300, incubated with samples and visualized by incubating with diaminobenzidine. Counter staining was performed with Mayer's hematoxylin, and we analyzed the result.

Of the 106 oral cancer patients in this study, histopathologic results showed squamous cell carcinoma, mucoepidermoid carcinoma, cystadenocarcinoma, and leiomyosarcoma. Among HPV-positive cancer patients, 1 patient was high-risk HPV type 16 positive, 1 patient as both high-risk HPV type 16 and low risk HPV type 6 positive, 3 patients were low risk HPV type 6 positive, and 2 patients were unknown type HPV positive. Different sites in the oral cavity were involved including tongue, palate, buccal mucosa, and mandibular body. Of the 7 patients who were HPV positive, 4 cancers were located on the tongue and 3 were on the lower gingiva.

Our study showed that the incidence of HPV infection in oral cancer was very low, and HPV type 16 in high risk HPV types and HPV type 6 in low risk HPV types were the most frequently detected HPV types in Korean oral cancer patients. "This research was supported by grant no 03-2012-0028 from the SNUHD Research Fund."

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A Cullin3-KLHL20 Ubiquitin Ligase-Dependent Pathway Targets PML to Potentiate HIF-1 Signaling and Prostate Cancer Progression.

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Tumor hypoxia is associated with disease progression and treatment failure, but the hypoxia signaling mechanism is not fully understood. Here, we show that KLHL20, a Cullin3 (Cul3) substrate adaptor induced by HIF-1, coordinates with the actions of CDK1/2 and Pin1 to mediate hypoxia-induced PML proteasomal degradation. Furthermore, this PML destruction pathway participates in a feedback mechanism to maximize HIF-1 α induction, thereby potentiating multiple tumor hypoxia responses, including metabolic reprogramming, epithelial-mesenchymal transition, migration, tumor growth, angiogenesis, and chemoresistance. In human prostate cancer, overexpression of HIF-1 α , KLHL20, and Pin1 correlates with PML down-regulation, and hyperactivation of the PML destruction pathway is associated with disease progression. Our study indicates that the KLHL20-mediated PML degradation and HIF-1 α autoregulation play key roles in tumor progression.

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Profiling differential microRNA expression in African American and Caucasian Prostate Samples.

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Purpose: African American men have almost twice the incidence and death rates related to prostate cancer compared to Caucasian men. Several studies have suggested that some of these differences may be attributed to the elevated expression of different genes. Recently, a new class of genetic regulators has emerged known as microRNAs. Many reports have found microRNAs to be implicated in cell growth, cell differentiation and the onset of many diseases including prostate cancer. To date, many studies have investigated differences of gene expression in Africa Americans and Caucasians, but no comprehensive analysis of microRNA differences between races has been investigated. Thus, we sought to investigate whether microRNAs are differentially expressed in African American and Caucasian prostate samples.

Methods: We analyzed the expression of over 300 microRNAs in 11 African American and Caucasian cell lines utilizing Affymetrix Microarray Chip. Validations of the most significant miRNAs were done by qRT-PCR. Characterization of the miRNA was carried out by performing MTT assay, Flow Cytometry, Western Blot, and Immunofluorescence.

Results: We identified 15 microRNAs that were differentially expressed between the African American and Caucasian cell lines. Further validations led to the identification of miR-152 which is significantly expressed among both groups. MiR-152 is responsible for inhibiting DNA methyltransferase 1. Our results showed that miR-152 reduced cellular proliferation in PC-3, DU-145 and LNCaP prostate cancer cell lines. In addition, miR-152 inhibited DNMT1 expression as well as initiated G2-M cell cycle arrest.

Conclusion: These results suggest that microRNAs may play a role in the advance progression seen in prostate cancer in African Americans. Ongoing research will validate these findings in normal-tumor paired African American and Caucasian tissue samples. Research supported by G12 RR03059-21A1(NIH/RCMI), and CA118623 (NIH/NCI).

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Finasteride alters microRNAs expression in tumoral prostatic cells.

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Prostate cancer (PCa) continues to be one of the most prevalent cancers in men of western countries and studies have shown that many microRNAs are differentially expressed and deregulated in PCa, acting as oncogenes or tumor suppressors. Androgen deprivation is an effective therapeutic approach for advanced PCa and finasteride (an inhibitor of type 2 5- α reductase) had been suggested to act as a PCa chemopreventive agent, but its prescription is still controversial between urologists. Considering that microRNAs play a major role on PCa progression and the current discussion about the safety use of finasteride, we investigated microRNAs 16, 205 (tumor suppressor genes) and 21, 125b, 141, 221 and 222 (oncogenes) expression in normal prostatic epithelial cells (RWPE-1) and in androgen-dependent prostatic cancer cells (LNCaP) treated with finasteride. Cells were cultivated in specific media supplemented with 10 μ M of finasteride dissolved in 0.1% DMSO or with 0.1% DMSO only (control group). After 1, 5 and 10 days of finasteride exposure, total RNA was isolated using TRIzol. The relative microRNA expression levels of target and housekeeping (U6) genes were quantified using real-time PCR analysis with an Applied Biosystems 7300 Real-Time PCR System. The data analysis showed that 125b, 141, 221 and 222 microRNAs are up regulated in LNCaP cells treated with finasteride. The fold change increase was time-dependent to all microRNAs up regulated, being higher at day-10 of treatment. miR-16, miR-205 and miR-21 expression was not altered in LNCaP cells. By contrast, finasteride did not alter microRNAs expression in RWPE-1 cells. The increased expression of the oncomirs 125b, 141 (both involved in the evolution of PCa), and 221 and 222 (both involved in the progression of PCa to androgen independent stage) induced by finasteride treatment in LNCaP cells can contribute to the malignancy and decrease the response of these cells to antiandrogenic therapy. Concomitantly, no change in oncomirs expression of normal cells corroborates with previous studies that point finasteride as a potential chemopreventive against PCa. In summary, our study provides a significant contribution to the molecular mechanisms behind finasteride effects on prostatic cancer cells and encourages its analysis on in vivo models of PCa.

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Characterization of the Roles of Vitamin D and Androgens on CYP24A1 Expression in Prostate Cancer Cells.

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The second leading cause of cancer death in males in the US involves the growth of a malignant tumor in the prostate. CYP24A1 is associated with prostate cancer metastasis. This gene product is a multi-catalytic CYP24 enzyme that metabolizes vitamin D³. The expression of CYP24A1 is stimulated by vitamin D³. Human studies in the Lamb lab indicate that CYP24A1 expression increases in advance staged cancer. Although vitamin D is known to have anti-proliferative effects on tumors, CYP24A1 is a classical vitamin D-stimulated gene. Androgen is thought to have biphasic effects on CYP24A1 expression—at low doses promoting while high doses inhibiting tumor growth. Vitamin D3 and androgen together have a synergistic anti-proliferative effect on androgen-dependent prostate cancer cell growth. The primary objective of this project is to understand the synergistic, growth inhibitory actions of vitamin D and androgen in prostate cancer cells and to study the independent and combined roles of androgen and vitamin D in the regulation of CYP24A1 expression in androgen-dependent and – independent prostate cancer cells. I hypothesize that ligand activated androgen receptor acts as a dominant inhibitor or repressor of CYP24A1 expression in LNCaP prostate cancer cells.

In order to determine the actions of vitamin D and androgen on CYP24A1 expression, LNCaP and C42 cells were subjected to a series of treatments involving vitamin D, R1881, and different combinations of both. Immunoprecipitation with CYP24A1 revealed the presence of a 37 KDa band when immuno-blotting with CYP24A1 antibody. No band was present when immuno-blotting with either AR (androgen receptor) or VDR (vitamin D receptor) antibodies. Immunoprecipitation with AR revealed the presence of a 110 KDa band when immuno-blotting with AR antibody. No band was detected when immuno-blotting with CYP24A1. Interestingly, immuno-blotting with VDR antibody revealed a possible protein-protein interaction with AR. I conclude from these results that AR does not form a complex with CYP24A1. By means of real-time qPCR, our objective was to understand the fold effect of androgen and vitamin D on AR, VDR, and CYP24A1. In C42 cells, vitamin D induced a 23-fold increase in CYP24A1 expression. These results were not observed in LNCaP cells.

To determine mechanisms of transcriptional regulation of CYP24A1 I first interrogated the proximal promoter regions of CYP24 for the presence of cis-acting elements indicative of androgen receptor trans-acting factors. Future studies will focus on transcriptional mechanisms underlying the synergistic interactions of androgen and vitamin D and on biphasic actions of androgen on CYP24A1 expression. These studies aim to identify important pathways that lead to prostate cancer progression, which may be used to improve proper treatment and diagnoses, such as preventing androgen castration resistance in advanced prostate cancer.

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The Y-located TSPY proto-oncogene amplifies its expression in prostate cancer cells through a positive feedback loop.

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The testis-specific protein Y-encoded (TSPY) gene is the proto-oncogene for the gonadoblastoma locus on the Y-chromosome (GBY). It is present in the genome of XY sex-reversed patients and predisposes their dysgenetic gonads to gonadoblastoma development. It is also abundantly expressed in other testicular germ cell tumors and numerous types of somatic cancers, including hepatocellular carcinoma, melanoma and prostate cancer. TSPY accelerates cell cycle progression, particularly the G2/M stage, and contributes to oncogenesis by promoting abnormal cell proliferation. Besides its effects on cell cycle, TSPY could exert oncogenic actions via other mechanisms. In the present study, we demonstrated that an exogenously transfected TSPY transgene could enhance the expression of the endogenous TSPY gene in LNCaP cells. Similar transfection analysis showed that it could significantly enhance the activities of luciferase-reporter harboring first exon sequence of TSPY gene. Consistently, chromatin immunoprecipitation and promoter tiling microarray (ChIP-Chip) analysis showed that TSPY could bind to the first exon sequence of the endogenous TSPY genes. Using a Gal4DBD-TSPY fusion protein expression plasmid and a Gal4UAS-luciferase reporter plasmid in a mammalian one-hybrid system, we demonstrated that Gal4DBD-TSPY strongly activated the Gal4UAS-luciferase reporter, while TSPY alone did not. Hence, TSPY could play an important role as a transactivator, capable of amplifying its expression in a positive feedback manner in prostate cancer cells and accelerate its oncogenic actions in affected cells and tissues.

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Chemical genetics *ex vivo* reveals that Lkb1 regulates organogenesis and oncogenesis along AMPK-dependent and -independent pathways.

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The tumor suppressor Lkb1 is a regulator of cellular energy, proliferation, and polarity, yet the mechanism by which it controls tissue morphogenesis or cancer remains poorly defined. We generated mice harboring a mutant Lkb1 knock-in allele that allows for rapid inhibition of Lkb1 kinase. By culturing embryonic tissues, we show that acute loss of kinase activity perturbs epithelial morphogenesis without a loss in apical-basal polarity. In pancreas, cystic structures developed rapidly following Lkb1 inhibition. In lung, inhibition resulted in epithelial cell-autonomous defects in branching morphogenesis. While the lung phenotype was rescued by bypassing Lkb1 inhibition using an activator of the Lkb1 target AMP kinase, pancreatic cyst development was independent of AMP kinase signaling. The pancreatic phenotype and alterations in gene expression resembled pre-cancerous lesions also induced by expression of mutant K-Ras together with p16/p19 deletion. These observations demonstrate combining the culture of embryonic tissues with chemical inhibition provides a powerful approach to both unraveling developmental programs and evaluating new approaches to cancer therapy.

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A Molecular Signature of Tubulogenesis in Pancreatic Cancer and Oncogenic Role of ASPM.

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Pancreatic ductal adenocarcinoma (PDAC) is a devastating malignancy and only a small subpopulation of the patients with PDAC present with resectable tumors. Unfortunately, the diseases invariably recur following surgery and it is thus imperative to identify patients with risk of early relapse as they may benefit from aggressive neoadjuvant or adjuvant therapy. Disruption of tissue microarchitectures represents an early step of pancreatic tumorigenesis and the degree of which has been used for the histopathological grading of PDAC. We here performed comparative genomic analysis of pancreatic tubules generated in a three-dimensional organotypic culture system which recapitulated the differentiated morphology and exocrine functions of a human pancreatic ductal epithelial tissue. We identified a pancreatic tubulogenesis-specific gene profile and applied it to several independent cohorts of patients with PDAC. We found that the patients with tumors expressing this profile, which we designated “tubule-like” PDAC, fared remarkably well following surgery. Based on this profile we established a 28-gene “tubulogenesis signature” that has strong and robust prognostic value across multiple independent cohorts (total n = 128). We identified from this signature a microcephaly- and stem cell-related gene ASPM as a novel prognostic marker in PDAC. Follow-on functional studies using organotypic cultures and orthotopic tumor xenograft models established the role of ASPM in the malignant progression of PDAC. Thus, by exploiting the genomic program associated with pancreatic tubulogenesis we identified tubule-like PDAC and related molecular markers that significantly enhance prognostic prediction of human PDAC (Supported by grant CA-100-PP-19 from National Health Research Institutes and grant DOH101-TD-C-111-004 from Department of Health of Taiwan for K. Tsai).

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Association between Expression of ERCC1, XPA and 8092C>A of ERCC1, 5'UTR of XPA Polymorphisms and Clinical Response to Cisplatin-Based Chemotherapy and Survival in Patients with Non-Seminomatous Testicular Germ Cell Tumors.

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Purpose: To explore role of expression of ERCC1 and XPA in cancer cell lines and its association to cisplatin-sensitivity (CPS); and to define the association of expression of ERCC1 and XPA, and single nucleotide polymorphisms 8092C>A and 5'UTR, respectively, with CPS and overall survival (OS) in patients with non-seminomatous testicular germ cell tumors (TGCT) treated with bleomycin, etoposide, cisplatin (BEP). **Methods:** ERCC1 and XPA cisplatin-treated expression and DNA damage was tested in cancer cell lines. ERCC1 and XPA expression was analyzed in fresh samples of non-seminomatous TGCT from patients chemo-naïve to BEP. Genomic DNA was extracted from paraffin-embedded samples of patients with non-seminomatous TGCT treated with BEP were included; 8092C>A ERCC1 and 5'UTR XPA polymorphisms were defined; immunohistochemistry for ERCC1 expression was done. **Results:**

The *ERCC1* expression was increased in non-CPS cancer cell lines and increased further after cisplatin treatment. Increased γ H2AX was observed in non-sensitive cancer cell lines after cisplatin treatment compared with testicular germ cell tumor cell line. Increased expression of *ERCC1* in non-CPS patients was found, but not *XPA*. Polymorphisms were not associated to CPS or OS, neither with clinical-pathological variables. Expression of *ERCC1* in TGCT patients was associated to CPS ($p=0.011$) but not to OS ($p=0.231$). **Conclusions:** Diminished expression of *ERCC1* is associated to CPS, as well as the presence of *ERCC1* is associated with non-CPS. This study proposes the use of *ERCC1* as a possible marker of clinical response to cisplatin based chemotherapy in patients with non-seminomatous testicular germ cell tumors. **Acknowledgements:** This work was supported by CONACYT 83959 and PAPIIT IN213311-3.

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Role of Shc1 protein during 8-Cl-cAMP induced growth inhibition of cancer cells.

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8-Cl-cAMP, which induces differentiation, growth inhibition and apoptosis in various cancer cells, has been investigated as a putative anti-cancer drug. The Shc1 protein is an adapter protein that is known to be involved in the signaling of numerous cell surface receptors such as growth factor receptors, antigen receptors, cytokine receptors and hormone receptors. Shc1 protein is expressed as three isoforms (46, 52 and 66 kDa) and the hyper-phosphorylation of Shc1 proteins were shown in many different types of tumor cells. We were able to show that the phosphorylation of Shc1 protein was decreased during 8-Cl-cAMP induced cancer cell growth inhibition. ABT702 (an adenosine kinase inhibitor) pre-treatment could restore the decreased phosphorylation of Shc1 protein by 8-Cl-cAMP treatment. Furthermore, whereas Compound C (an AMPK inhibitor) and SB203580 (a p38 MAPK inhibitor) did not block the 8-Cl-cAMP-induced decrease of Shc1 phosphorylation, AMPK and p38 MAPK were phosphorylated by introducing Shc1-targeted siRNA. These results suggest that reduced level of Shc1 phosphorylation acted upon the phosphorylation of AMPK and p38 MAPK during 8-Cl-cAMP-induced growth inhibition.

Tumor Invasion and Metastasis II

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Palladin expression modulates RhoG activation, migration and invasion of breast cancer cells.

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Metastasis is the leading cause of death related to cancer. Breast cancer patients are particularly susceptible to metastasis, with survival rates decreasing from 98.3% for local tumors to 23.3% for distant tumors. Our previous results showed that palladin levels in breast cancer patients are higher in malignant tissues than in normal breast samples. In addition, highly invasive breast cancer cell lines express higher levels of palladin than non-invasive cell types. Rho GTPases control many aspects of the metastatic process including cell migration, cell adhesion, cell cycle progression, gene expression and cell polarity. Thus, the aim of our study was to evaluate the role of palladin in GTPase activation, and cell migration and invasion. In our experimental model, we used three cell lines that differ in their levels of palladin expression and

invasive potential. We overexpressed palladin in the non-invasive MCF7 cell line and we generated palladin stable knock-down cell lines for invasive MDA-MB-231 and for the highly metastatic MDA-MB-231-BR. Our results showed that palladin expression is correlated with high levels of active RhoG. This increase in active RhoG was also dependent on the levels of palladin overexpression. In addition, palladin knockdown lines showed a decrease in cell migration and invasion when compared with control lines.

These results suggest that palladin plays an important role in RhoG activation, and thus high palladin expression levels may contribute to the invasive motility of metastatic breast cancer cells. Further analyses are necessary to determine the molecular mechanisms implicated in these events.

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The integrin α V β 3 alters cellular biomechanical properties during cancer cell invasion.

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The process of cancer cell invasion through the extracellular matrix (ECM) of connective tissue plays a prominent role in tumor progression and is based fundamentally on biomechanics. Cancer cell invasion usually requires cell adhesion to the ECM through the cell-matrix adhesion receptors integrins. The expression of the α β 3 integrin is increased in several tumor types and is consistently associated with increased metastasis formation in patients. The hypothesis was that the α β 3 integrin expression increases the invasiveness of cancer cells through increased cellular stiffness, and reduced cytoskeletal remodeling dynamics. Here, the invasion of cancer cells with different α β 3 integrin expression levels into loose and dense 3D-ECMs has been studied. Using a cell sorter, two subcell lines expressing either high or low amounts of α β 3 integrins (α β 3^{high} or α β 3^{low} cells, respectively) have been isolated from parental MDA-MB-231 breast cancer cells. α β 3^{high} cells showed a threefold increased cell invasion compared to α β 3^{low} cells. Similar results were obtained for A375 melanoma, 786-O kidney and T24 bladder carcinoma cells, and cells in which the β 3 integrin subunit was knocked down using specific siRNA. To investigate whether contractile forces are essential for α β 3 integrin-mediated increased cellular stiffness and subsequently enhanced cancer cell invasion, invasion assays were performed in the presence of myosin light chain kinase inhibitor ML-7 and Rho kinase inhibitor Y27632. Indeed, cancer cell invasiveness was reduced after addition of ML-7 and Y27632 in α β 3^{high} cells but not in α β 3^{low} cells. Moreover, after addition calyculin A, an increase in pre-stress in α β 3^{low} cells was observed, which enhanced cellular invasiveness. In addition, inhibition of the Src kinase, STAT3 or Rac1 strongly reduced the invasiveness of α β 3^{high} cells, whereas the invasiveness of β 3 knock-down cells and α β 3^{low} cells was not altered. In summary, these results suggest that the α β 3 integrin enhances cancer cell invasion through increased cellular stiffness, which enables the cells to generate and transmit contractile forces to overcome the steric hindrance of 3D-ECMs.

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Tumor necrosis factor- α converting enzyme (TACE) mediates TGF- β dependent shedding of epithin.

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Epithin, a type II transmembrane serine protease, is broadly expressed in epithelial and carcinoma cells. It is known that epithin plays critical roles in cancer metastasis as well as in normal epithelial functions such as epidermal differentiation and barrier formation. Previously, we have reported that epithin undergoes ectodomain shedding in response to phorbol myristate acetate (PMA) stimulation. However, the endogenous ligands that induce epithin shedding and the responsible sheddase have not been characterized well. In this study, it is shown that TGF- β induces epithin shedding strongly in thymic epithelial cell, 427.1.86 and tumor necrosis factor- α -converting enzyme (TACE) mediates this shedding. TGF- β induced epithin shedding was greatly reduced by the treatment of TACE inhibitor, TAPI-0 and also by the inhibition of TACE expression with TACE siRNA. TGF- β treatment also induces translocation of intracellular pool of TACE to the membrane where epithin is located. These findings suggest that TGF- β induces epithin shedding by the translocation of epithin sheddase, TACE, to the membrane.

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The hemopexin domain of matrix metalloproteinase-9 binds $\alpha 4\beta 1$ integrin in B-cell chronic lymphocytic leukemia cells. Biochemical and functional characterization.

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We previously showed that pro-matrix metalloproteinase-9 (prommp-9) binds to b chronic lymphocytic leukemia (b-cll) cells and contributes to b-cll progression by regulating cell migration and survival (Redondo-Muñoz et al, Blood 2008; Cancer Cell 2010). Induction of cell survival involves a non-proteolytic mechanism and the prommp-9 hemopexin domain (pex9). To help design specific inhibitors of prommp-9-cell binding, we have characterized b-cll cell interaction with the isolated pex9. B-cll cells bound soluble and immobilized gst-pex9, but not gst, and binding was mediated by $\alpha 4\beta 1$ integrin. The ability to recognize pex9 was observed in all 20 primary samples studied, irrespectively of their clinical stage or prognostic marker phenotype. By preparing truncated forms of gst-pex9 containing structural blades b1b2 or b3b4, we identified b3b4 as the primary $\alpha 4\beta 1$ integrin-interacting region within pex9. Overlapping synthetic peptides spanning b3b4 were then tested in functional assays. Peptide p3 (fpgvpldthdvhfyrekyfc), a sequence present in b4 or smaller versions of this sequence (peptides p3a/p3b), inhibited b-cll cell adhesion to gst-pex9 or prommp-9, with ic50 values of 138 and 279 μ molar, respectively. Mutating the two aspartate residues to alanine rendered the peptides inactive. An anti-p3 antibody also inhibited adhesion to gst-pex9 and prommp-9. Gst-pex9, gst-b3b4 and p3/p3a/p3b peptides inhibited b-cll cell transendothelial migration, while the mutated peptide did not. B-cll cell incubation with gst-pex9 induced intracellular survival signals, namely lyn phosphorylation and mcl-1 upregulation and this was also prevented by the p3 peptides. The p3 sequence may therefore constitute an excellent target to prevent prommp-9 contribution to b-cll pathogenesis. Functionally, adhesion of b-cll cells to gst-pex9 increased vegf production, suggesting a direct regulation of angiogenesis by pex9. We are currently studying whether binding of pex9 (or mmp-9) provides resistance of b-cll cells to therapeutic drugs. Our results expand the role of non-catalytic effects of mmp-9 in b-cll and support the

contribution of mmp-9 to b-*c*ll pathogenesis. Supported by grants saf2009-07035 and rd06/0020/0011, ministerio de ciencia e innovación, Madrid, Spain.

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EGF receptor activates the ADP-ribosylation factor 1 to promote invasion of breast cancer cells.

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Cell invasion is a process tightly regulated by multiple signalling proteins such as plasma membrane receptors, adaptors and small GTP-binding proteins. During cancer progression, transformed cells migrate and invade surrounding tissues to form metastasis. Expression of small GTPases and their regulatory factors is frequently modulated in human cancers. Recently, the ADP-ribosylation factor (ARF) family of GTP-binding proteins have been associated with cancer progression. We have shown that the GTPase ARF1 is overexpressed in highly invasive cancer cells and that EGF stimulation can activate this ARF isoform to regulate migration. Here, we report that modulation of ARF1 expression levels and activity markedly impaired the ability of invasive breast cancer cells to degrade the extracellular and invade its surrounding. Our results indicate that ARF1 impacts shedding of membrane derived microvesicles and metalloprotease activity. ARF1 is required for basal secretion and activation of MMP-9, but not MMP-2. This study therefore shows that ARF1 is a molecular switch regulating key events important for cellular invasion.

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XRCC3/RAD51C Downregulation Induces Invasiveness in Pre-invasive and Invasive Breast Cancer and Bystander Cells via Cyclophilin A.

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Ectopic overexpression of the homologous recombinational repair protein XRCC3 has been shown to increase breast cell invasiveness. This raises the question of whether XRCC3 is a positive modulator of invasion or an overexpression dominant negative effector which acts by sequestering its partner RAD51C in a non-functional complex when overexpressed. To address this question, and to determine if downregulation of XRCC3 levels has therapeutic implications in reducing invasion and metastasis, we used siRNAs against XRCC3 and RAD51C to transiently downregulate the XRCC3-RAD51C complex in an ER/PR/HER2 negative basal-like human cell line model of pre-invasive to invasive transition in breast cancer. We find that XRCC3 and RAD51C downregulation enhances cell invasiveness. XRCC3 downregulation increases FAK activation on Y397 and signaling via Y861. The FAK effect is abolished when cells are treated with EGF or TGFbeta suggesting that XRCC3 is upstream of these growth factors. Global gene expression analysis shows that XRCC3 downregulation most significantly alters a network of proteins involved in cell movement, including a large number of secreted factors. Consistent with this, conditioned medium collected from XRCC3-downregulated cells has a paracrine enhancement effect on the invasion of naïve pre-invasive and invasive cells. Mass spectrometric analysis reveals that secreted cyclophilin A (CYPA) is upregulated by XRCC3-siRNA. The addition of nanomolar concentrations of recombinant CYPA to XRCC3-wild-type cells enhances invasiveness, suggesting that CYPA is downstream of XRCC3 and is sufficient to induce bystander cell invasiveness. Inhibiting CYPA using cyclosporine A inhibits cell invasiveness at concentrations that do not affect cell proliferation. In addition, using cyclosporine A to block CYPA in the conditioned medium from XRCC3 siRNA treated cells is

sufficient to diminish the ability of this conditioned medium to induce invasiveness in bystander cells. We conclude that XRCC3 is a negative modulator of cell invasiveness working with RAD51C upstream of cell adhesion, growth factor, and paracrine effectors, such as CYPA. These results support the novel idea that DNA repair stress induced by the misregulation of repair proteins could affect the breast tumor microenvironment by altering bystander cell invasion.

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CRN2 (coronin 1C, coronin 3) promotes invasion of tumor cells.

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Spreading of tumor cells from the solid tumor to the surrounding tissue is a critical process in cancer progression. Movement of tumor cells relies on the dynamic remodeling of the actin cytoskeleton regulated by actin binding proteins. One of these actin binding proteins is the WD40-repeat domain protein CRN2, which is involved in cellular processes like protrusion formation, secretion, migration and invasion. Its expression correlates with the malignant phenotype of diffuse gliomas as well as with initiation of pulmonary metastasis of hepatocellular carcinoma cells. Moreover, CRN2 binds to F-actin via its N-terminal β -propeller as well as its C-terminal coiled-coil domain resulting in F-actin bundling and an inhibition of actin polymerization. The CRN2–F-actin interaction is negatively regulated by CK2 dependent phosphorylation at serine 463. This work aimed at a more profound understanding of the CRN2 functions in migration and invasion of tumor cells. We generated stably transduced human U373 glioblastoma cell lines with a knock-down of CRN2 or an over-expression of CRN2 variants. These cells were employed in organotypic brain slice cultures serving as a more physiological model of tumor cell invasion. We could show that CRN2 over-expression increases invasion, whereas CRN2 knock-down decreases invasion of glioblastoma cells into the brain tissue. Additionally invasion is reduced in cells expressing S463D phosphomimetic CRN2 compared to cells expressing the S463A phosphoresistent CRN2. Immunofluorescence studies showed an altered co-localization of CRN2 and F-actin in rod shaped cell extensions in glioblastoma cells expressing the phosphomimetic CRN2 mutant. Our results support the hypothesis that CRN2 plays an important role in actin cytoskeleton dynamics of invasive tumor cells and that phosphorylation at serine 463 modulates this process.

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SGEF is overexpressed in glioblastoma and mediates tumor cell invasion and survival.

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Glioblastoma (GB) is the highest grade and most common form of primary adult brain tumors. GB tumors are characterized by a poorly defined mass due to a highly invasive cell population attributed with having a decreased sensitivity to radiation and chemotherapy with temozolomide (TMZ). Importantly, current therapies for GB do not target invading cells. Thus, the identification and characterization of genes important for cell motility could direct the development of therapies targeting invading cells in an effort to confer an increased sensitivity to radiation and TMZ. We have previously shown that several guanine nucleotide exchange factors (GEFs) for Rho family small GTPases are overexpressed in GB tumors and play important roles in glioma invasion. Here we report a role for SGEF, a RhoG specific GEF, in

mediating glioma invasion and survival. A genome-wide determination of NF- κ B-controlled genes in TMZ-resistant primary GB tumor grafts (GB14-TMZ-R) revealed an increased occupancy of NF- κ B on the SGEF gene promoter region via ChIP-on-chip analysis as compared to non-TMZ-resistant GB tumor grafts (GB14), and SGEF mRNA and protein expression were found to be inducible under the pro-survival and pro-invasive TWEAK-Fn14 cytokine-receptor signaling axis in an NF- κ B dependent manner. Moreover, the resistant line GB14-TMZ-R migrated at an increased rate compared to non-TMZ-resistant GB14. In addition, among human tumor specimens, SGEF mRNA expression is increased in high grade gliomas and levels of SGEF expression in GB tumors inversely correlate with patient survival. Laser capture microdissection of GB tumors showed that SGEF mRNA expression is increased at the invasive tumor rim relative to tumor core, and depletion of SGEF expression by shRNA decreases *in vitro* glioma cell migration and *ex vivo* glioma cell invasion without affecting cell proliferation. In addition, depletion of SGEF sensitizes glioma cells to TMZ-induced apoptosis and impairs colony formation following TMZ insult. Understanding the role of SGEF in promoting cell motility and chemotherapeutic resistance may direct the development of novel targeted therapeutics for invasive GB cells.

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Recruitment and Regulation of Glycolysis in Cancer by Phosphofruktokinase-1.

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Increased aerobic glycolysis and increased cell motility are two hallmarks of metastatic cancer cells. Metastatic cells are highly polarized, suggesting glycolytic enzymes in cancer cells could be spatially regulated. Phosphofruktokinase-1 (PFK-1) is the first rate-limiting step in glycolysis, and we hypothesized that it may be spatially and temporally regulated. We found that PFK-1 is dynamically recruited from the cytosol to the lamellipodia during cell motility. In response to growth factors, endogenous PFK-1 is enriched at the distal margin of lamellipodia of MTLn3 mammary carcinoma cells and CCL39 lung fibroblasts. Other members of the glycolytic metabolon that have increased expression in cancers, including pyruvate kinase isoform PKM2 and lactate dehydrogenase A, show a similar recruitment and co-localize with PFK-1 at lamellipodia. PFK-1 does not co-localize with mitochondria, which are excluded from lamellipodia. We generated an MTLn3 cell line expressing GFP-tagged PFK-1 and used live-cell imaging to show that GFP-PFK-1 is dynamically recruited to the lamellipodia and that enrichment of GFP-PFK-1 in lamellipodia occurs after the extension of the membrane. Polymerization of metabolic enzymes is an area of renewed interest because has been shown to regulate metabolic flux. We asked if PFK-1 can form filaments by electron microscopy and light scattering analysis. In the absence of substrate, recombinant PFK-1 is tetrameric and occasionally forms short filaments. The addition of substrate rapidly induces the formation of higher longer filaments that coincides with increased catalytic activity. A mutant, His199Tyr, which is inactive but retains the tetrameric structure, does not form filaments upon the addition of substrate. Further, ectopically expressed PFK-1 His199Tyr is not enriched in the lamellipodia. To examine the role of PFK-1 in increased glycolytic flux observed in cancer, we generated several cancer-specific mutations of PFK-1. Although these mutations were located in distinct areas of PFK-1, several of these mutations confer a ~2-fold increase in PFK-1 activity. Our data indicate a structural and functional coupling between metabolic adaptation and increased pHi in cancer cells and further suggest molecular mechanisms that may provide novel targets for pharmaceutical intervention. Further, the dynamic recruitment and enrichment of glycolytic enzymes at the distal margin of lamellipodia that lack mitochondria suggest the intriguing possibility of localized energy production for membrane protrusion and cell migration.

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Anaphylatoxin C5a enhances human cancer cell motility and invasiveness via aberrantly expressed C5a-receptor (CD88).

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The anaphylatoxin C5a is a chemoattractant that induces leukocyte migration via C5a receptor (C5aR). There is emerging evidence that C5a is generated in the cancer microenvironment. We analyzed C5aR expression in human cancer specimens, and sought direct influence of C5a-C5aR axis on cancer cells.

Immunohistochemical staining for human cancer tissues revealed that C5aR was aberrantly expressed in various cancer cells but not in noncancerous epithelial cells except renal tubular cells. The C5aR-positive ratio varied in origins of cancer cells. C5aR was expressed on the cell surface in the cancer cell lines examined. In order to analyze the biological effects of C5aR expression in cancer cells under C5a stimuli, C5aR-negative HuCCT1 cells derived from bile duct carcinoma, in which C5aR expression ratio was the highest, were transfected with C5aR cDNA to establish stably C5aR-expressing HuCCT1 cells. C5a triggered cytoskeletal rearrangement in C5aR-expressing HuCCT1 cells, which was visualized by immunofluorescence staining of F-actin. Time lapse analysis showed that C5a enhanced cell motility 3-fold. C5a also enhanced cell invasiveness 13-fold in Matrigel chamber assay. However, such enhancing effect of C5a was not observed in C5aR-negative control cancer cells. Cancer cell invasion was still enhanced in the absence of C5a concentration gradient and even after the removal of C5a stimulation, suggesting that random cell locomotion plays an important role in C5a-triggered cancer cell invasion. C5a increased the release of matrix metalloproteinases (MMPs) from cancer cells by 2 to 11-fold, and inhibition of MMP activity with GM 6001 abolished the C5a enhancing effect on cancer cell invasion. Compared with C5aR-negative control cells, C5aR-expressing HuCCT1 cells spread 1.8-fold more broadly when implanted in nude mouse skin only when stimulated with C5a.

These results illustrate a novel activity of C5a-C5aR axis that promotes cancer cell invasion through motility activation and MMP release. Targeting this signaling pathway may provide a useful therapeutic option for cancer treatment.

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Down regulation of Cell Proliferation and ROCK-1 Protein Expression in Metastatic Breast Cancer Cells in Response to Treatment with Melatonin and Y27632.

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Breast cancer affects more women than any other cancer in the world with a high mortality rate mainly related to late diagnosis and the occurrence of metastases. In vitro and in vivo experiments have shown that increased expression of the Rho kinase protein (ROCK-1) is related with tumor growth and metastasis, whereas this molecule signaling inhibition results in significant down regulation of tumor metastasis. Melatonin has several oncostatic and anti-metastatic properties, but its mechanism of action is not fully understood. The aim of this study

was to investigate the protein expression of ROCK-1 in metastatic breast cancer cells and its correlation with the potential oncostatic and oncoprotector effects of melatonin. A metastatic breast cancer cell line (MDA-MB-231) was divided into 4 groups: group I as control and with no treatment, group II treated with melatonin, group III treated with the ROCK-1 inhibitor, Y27632, and group IV treated with melatonin and Y27632. The protein expression of ROCK-1 and the proliferation marker Ki67 were verified by immunocytochemistry, and quantified by optical densitometry. Melatonin concentration of 2 mM ($p < 0.01$), 5 mM ($p < 0.001$) and 10mM ($p < 0.001$) significantly decreased the viability of MDA-MB-231 cells after 24 hours of incubation. Different concentrations of Y27632 were also evaluated and only the dose of 10 μ M was able to reduce cell viability significantly ($p < 0.05$). 10mM of melatonin and 10 μ M of Y27632, associated or not, significantly decreased the protein expression of ROCK-1 and the number of cells per cm². These drugs, when associated significantly decreased Ki67 immunocytochemical labeling. This results were validated by a migration/invasion assay. In conclusion, melatonin and Y27632 are effective drugs to down regulate in vitro metastatic breast cancer, confirming their effects of decreasing cell viability and reducing the protein expression of ROCK-1 and Ki67 in these cells. Melatonin, in particular, appears to be more efficient when associated with ROCK-1 inhibitor. Financial support: FAPESP

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Lysophosphatidic acid induces reactive oxygen species generation through protein kinase C in PC-3 prostate cancer cells.

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Prostate cancer is one of the most frequently diagnosed cancers in males. PC-3 cell is a popular cell model for investigating late prostate cancer behavior and finding a useful treatment toward it. Lysophosphatidic acid (LPA) is a lysophospholipid regulates multiple cell processes including proliferation, migration and other aggressive cell behaviors in cancer. In our previous study, we demonstrated that LPA enhances VEGF-C expression in PC-3 cell through activating the generation of reactive oxygen species (ROS), which is known important in the development of many different cancers. Using flow cytometry, we measured the relative ROS levels in PC-3 cells. LPA triggers ROS generation within ten minutes. This ROS production can be suppressed by antioxidant, N-acetylcysteine. By using LPA_{1/3} antagonist Ki16425, we confirmed that LPA induced ROS is mediated through LPA_{1/3}-dependent pathway. Moreover, pretreatment with PKC inhibitors can block the ROS generation by LPA treatment. Overall, we first demonstrated that LPA induces ROS generation in a PKC dependent manner in PC-3 prostate cancer cell line.

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Sodium butyrate induced cellular senescence and inhibited invasion of cancer cells with distinct mechanism.

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Sodium butyrate (SB) -C-4 saturated fatty acid present in the human bowel membrane in quite high concentration (2 mM) as food metabolites. Here, we focused on the role of SB on cancer cell growth, motility and invasion. SB inhibited the cellular growth, motility and invasiveness in a dose-dependent fashion in both human fibrosarcoma (HT1080) and glioblastoma (A172) cell lines. SB also affected the morphology of the HT1080 and the A172 cells, namely spread out with marked stress fibers bridging focal adhesions. Phosphorylation levels of focal adhesion kinase (Y577 and Y397 sites) were increased but that of myosin light chain (S19 site) was not

altered. All of these biological effects of SB were reversible, and recovered after withdrawal. In addition, HT1080 cells and A172 cells treated with SB showed positivity for senescence-associated beta-gal (SA-beta gal) staining with elevated expression levels of p53 and p21 proteins in a time and dose-dependent manner. By contrast, mRNA level of p21 was sharply declined after the treatment with SB. Knocking down of p21 protein expression by siRNA reversed both the growth inhibition and positivity for SA-beta gal staining but not the inhibition of invasiveness. Further, proteasome inhibitor, MG-132 decreased the cellular growth and inhibited motility and invasiveness of the HT1080 and A172 cells, and showed similar all these biological effects of SB. Finally, intrathecal administration of SB effectively inhibited the invasion of rat mammary cancer cells (Walker256) into the brain and elongated the animal survival in vivo. Collectively, SB inhibited growth with induced cellular senescence, inhibited invasion by distinct mechanism, and thus would be a good candidate for anti-invasive therapy without severe adverse effects.

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Capsaicin suppresses the migration and invasion of cholangiocarcinoma cell by downregulation of matrix metalloproteinase-9 expression via AMPK activation.

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Cholangiocarcinoma is one of the most difficult malignancies for cure. An important prognostic factor for cholangiocarcinoma is metastasis, which precludes curative surgical resection. The development of metastasis requires the movement and invasion of cancer cells from the primary tumor into the surrounding tissue. Recent evidence indicates that capsaicin has depression effects on cancer cell migration and invasion. Thus, we investigated the molecular mechanism of capsaicin-induced anti-migration and anti-invasion effects in HuCCT1 cells. The results of invasion, migration and gelatin zymography assay showed that the treatment of capsaicin significantly suppressed tumor migration and invasion. We further found that capsaicin reduced the PMA-induced expression of MMP-9 and MMP-2 but did not alter TIMP-1 and TIMP-2 levels in immunoblot and RT-qPCR analyses. Interestingly, capsaicin elevated the accumulation of NAD⁺ by AMPK activation in intracellular and enhanced SIRT1 activity in nucleus. In addition, capsaicin inhibited NF- κ B activity by the deacetylation of the p65 subunit of NF-kB through SIRT1 activation. These results suggest that capsaicin suppressed PMA-induced up-regulation of MMP9 by inhibition of the MMP9 transcription factor NF-kB through AMPK-NAD⁺-SIRT1 signaling cascade, and contributes to cholangiocarcinoma cell metastasis.

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Native type IV collagen induces an epithelial to mesenchymal transition-like process in mammary epithelial cells MCF10A.

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Basement membrane (BM) is a complex network of interacting proteins, including type IV collagen (Col IV) that acts as a scaffold that stabilizes the physical structures of tissues and regulates cellular processes. In the mammary gland, BM is a continuous deposit that separates epithelial cells from stroma, and its degradation is related with an increased potential for invasion and metastasis. Epithelial to mesenchymal transition (EMT) is a process by which epithelial cells are transdifferentiated to one mesenchymal state, and is a normal process during embryonic development, tissue remodeling and wound healing, as well as it has been implicated during cancer progression. In breast cancer cells, native Col V induces migration and gelatinases secretion. However, the role of native Col IV on the EMT process in human

mammary epithelial cells remains to be investigated. In this study, we demonstrate that native Col IV induces down-regulation of E-cadherin expression, accompanied with an increase of Snail1, Snail2 and Sip1 transcripts. Native Col IV also induces an increase in N-cadherin and vimentin expression, an increase of MMP-2 secretion, the activation of FAK and NFκB and cell migration and invasion in MCF10A cells. In summary, these findings demonstrate, for the first time, that native Col IV induces an EMT-like process in MCF10A human mammary non-tumorigenic epithelial cells.

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The Synthetic Triterpenoid Acetylenic Tricyclic Bis-(Cyano Enone) Affects the Cytoskeleton in Cell Migration and Epithelial-to-Mesenchymal Transition.

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During metastasis tumor cells undergo an epithelial to mesenchymal transition (EMT), where cell-cell junctions dissolve and actin stress fibers are formed. This transition unmask the migratory and invasive potential of the tumor cells to migrate to distant organs to establish secondary tumors. Cell migration is initiated by the asymmetrical localization of polarity proteins towards the leading edge, this stimulates the reorganization and polymerization of the microtubule and actin, which form protrusions in the plasma membrane to drive the cell forwards. Our lab has identified the synthetic triterpenoid, CDDO-Im, as a potent inhibitor of cell migration by inhibiting the polymerization of branched actin and disrupting the organization of the microtubule network (To et al., J Biol Chem. 2008, 2010). Recently, TBE-31 has been developed, which is a smaller 3 ring compound that contains the same active functional groups as CDDO-Im. Thus our hypothesis is that TBE-31 will also effectively inhibit the polymerization of actin and disorganize the microtubule network to inhibit cell migration and epithelial to mesenchymal transitions. TBE-31 was found to displace the polarity proteins from the leading edge of migrating cells, causing Rat2 fibroblasts to form multiple protrusions in multiple directions. The microtubule network became disorganized and the polymerization rates for tubulin and actin were decreased. These effects are believed to allow submicromolar concentrations TBE-31 to inhibit fibroblast migration by as much as 60%. Finally, the reorganization of actin and formation of stress fibers normally associated with EMT was also inhibited by TBE-31. In conclusion, TBE-31 disrupts the cytoskeleton, inhibits cell migration and preliminary data suggest TBE-31 may also hinder EMT.

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Cranberry flavonols affect human prostate cancer cell growth via cell cycle arrest by modulating expression of cell cycle regulators.

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Previously, we have demonstrated that an American cranberry (*V. macrocarpon*) extract can inhibit human prostate cancer cell growth via cell cycle arrest by modulating the expression of cell cycle regulators. This present study was designed to identify the phytochemicals present in cranberry responsible for the alterations in cell cycle regulators which occurred in response to cranberry. This current study examined the effects of a flavonol-enriched fraction (FL) from cranberry on the behavior of DU145 human prostate cancer cells in vitro. FL was characterized to contain quercetin and myricetin glycosides. FL (50 µg/mL) decreased cellular viability by ~15% post 6 hours of treatment. FL (post 6h. exposure) increased the proportion of cells in G2-M and decreased the proportion of cells in G1. These alterations in cell cycle were associated with

changes in cell cycle regulatory proteins and other cell cycle associated activities. FL (6h.treatment) decreased the protein expression levels of cyclin A & cyclin B1 and increased the expression of cyclin D1 & cyclin E. FL treatment resulted in a decrease in the protein expression levels of CDK2 & CDK4. Increased p21, p27, and p16 protein expression levels were evident in response to FL treatment, whereas a decrease in pRb p130 protein expression levels occurred with no readily apparent alteration in the levels of pRbp107 protein. These findings demonstrate that one of the classes of phytochemicals responsible for the cranberry mediated alterations in cell cycle protein expression levels are the flavonols. These findings also demonstrate that phytochemical extracts from the American cranberry (*V. macrocarpon*) can affect the behavior of human prostate cancer cells in vitro and further supports the potential health benefits associated with cranberries. [N.C.I.C.- Canadian Cancer Society, P.E.I. Health Research Program, The Cranberry Institute (Wisconsin Cranberry Board) & Telus MRFD Prostate Cancer Fund (PEI Division) funded]

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Cell and Molecular Processes Critical in Metastasis of Breast Cancer Cells are Inhibited by Specific Components of Pomegranate Juice.

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Breast cancer is the most common cancer and the second leading cause of cancer death and morbidity among women in the western world. Pomegranate juice (PJ) and a combination of three of its specific components have been shown to inhibit processes involved in prostate cancer metastasis. If this also proves to be true for breast cancer cells, these natural treatments will be promising agents against breast cancer that can serve as potentially effective and nontoxic alternatives or adjuncts to the use of conventional selective estrogen receptor modulators (SERMs) for breast cancer prevention and treatment. To test this possibility, we have used 2 breast cancer cell lines, MDA-MB-231 cells (ER-) and MCF7 (ER+), and the non-neoplastic cell line MCF10A. We show that, in addition to decreasing growth of breast cancer cells, PJ or a combination of its components Luteolin (L) plus Ellagic Acid (E) plus Punicic Acid (P) increase cancer cell adhesion and decrease cancer cell migration but do not affect normal cells. These treatments also inhibit chemotaxis of the cancer cells to SDF1 α ; a chemokine that attracts breast cancer cells to the bone. We hypothesize that PJ and L+E+P stimulate expression of genes that increase adhesion and inhibit genes that stimulate cell migration and chemotaxis to SDF1 α ; Using qPCR, we confirmed these proposed effects on gene expression and in addition we found that a gene important in epithelial-to-mesenchymal transitions is decreased. We also found that pro-inflammatory cytokines/chemokines are significantly reduced by these treatments, thereby having the potential to decrease inflammation and its impact on cancer progression. Discovery that PJ and L+E+P are inhibitory of metastatic processes in breast cancer cells in addition to prostate cancer cells indicates that they are very likely to be an effective treatment to inhibit cancer progression in general.

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2-D DIGE and siRNA to find new cancer targets.

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siRNA is a powerful tool in loss-of-function studies which generates valuable information on target validation. siRNA is highly selective and suppress gene expression in a sequence-

specific manner. These features have made it a key tool in signal transduction - a cornerstone in cancer biology. 2-D DIGE (two dimensional differential gel electrophoresis) is a fluorescence based technology with the capacity to separate thousands of proteins in one single run, with high reproducibility. This makes it a valuable tool for differential expression analyses. In this study two powerful methods, siRNA and 2-D DIGE, were combined to investigate a well-known signaling pathway, pivotal in cancer biology. Silencing of the target protein made it possible not only to identify a large number of proteins that were differentially regulated but also to resolve the time dependency of protein regulation. Thus the combined use of siRNA and 2-D DIGE made it possible to find potentially new targets in the signaling pathway.

Cancer Stem Cells

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IL-6 modulates cisplatin resistance involving stem cells in HNSCC.

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Cisplatin is one of the most effective drug in the management of patients with advanced head and neck squamous cell carcinoma (HNSCC). However, patients frequently develop resistance to cisplatin through mechanisms that are not fully understood. Here, we hypothesized that endothelial cell-initiated signaling contributes to cisplatin resistance in HNSCC. We observed that IL-6 secreted by primary human dermal microvascular endothelial cells (HDMEC) induce STAT3 and ERK phosphorylation in HNSCC. UM-SCC-22B cells exposed to 0-50 ng/ml IL-6 and 0-2 μ M Cisplatin were analyzed by western blot for STAT3, phospho-STAT3, ERK and phospho-ERK. STAT3 phosphorylation was enhanced in cells treated with IL-6, and remained unaffected by treatment with cisplatin. In contrast, ERK phosphorylation was induced by IL-6 alone, but was inhibited when cells were exposed to Cisplatin. However, ERK phosphorylation was seen when IL6 50 ng/mL was combined to Cisplatin. Accordingly to the Stem Cell Hypothesis, the slow growing cancer stem cells can scape from conventional therapy and as tumor-initiating cells establish local recurrences and distant metastases. We also looked at the stem cells proportion in HNSCC. We observed an increased percentage of ALDH+CD44+ cells when UM-SCC-22B cells were exposed to Cisplatin combined with 20 and 50 ng/ml IL-6 by flow cytometry. Sphere formation was enhanced when single ALDH+CD44+ cells were submitted to low attachment plates in appropriate medium and treated with Cisplatin and IL-6 together. The same regimen seems not affect ALDH-CD44- cells. By western blot we observed higher levels of Bmi-1 expression in cells treated with IL-6 in addition to Cisplatin and we also observed that IL-6R antibody can decrease the expression of this protein. Taken together these results suggest that endothelial cell-secreted IL-6 plays a role in the development of cisplatin resistance involving stem cells in HNSCC and IL-6 receptor can be evaluated as a new molecular target for the development of adjuvant therapy.

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Human glioblastoma cancer stem cells are sensitive to doxorubicin and temozolomide.

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Gliomas are aggressive brain tumors and the cell line U87 produces tumor-spheres enriched in cancer stem cells (CSCs) that are important for the establishment and maintenance of tumor mass. CSCs are more resistant to radio and chemotherapies than differentiated cells. Temozolomide (Tmz) is a DNA methylating agent and doxorubicin (Doxo) is a topoisomerase inhibitor. Our objective was to evaluate the effects of these drugs on CSCs derived from human glioblastoma.

We performed sphere formation assay to quantify the number of spheres, flow cytometry to measure the percentage of Oct4 and Nanog positive cells and beta-galactosidase senescence assay .

Using DMEM/FBS, Doxo 1 and 10 nM reduced the number of spheres (54.7 in control versus 2.5 and 1.8, respectively) and induced cell senescence in the cell line U87 after 7 days. Doxo 10 nM decreased the number of Nanog and Oct4 positive cells while Doxo 1 nM had no effect. Tmz 5 µM decreased the number of spheres (to 54.7 in control versus 30.4) and the CSCs markers. Using serum free medium, we observed that all treatments reduced the sphere formation after 7 days (224 in control and 110, 125 and 41 after treatment with Tmz, Doxo 1 nM and 10 nM, respectively).

Our results indicate that low doses of Doxo reduce the proportion of CSCs in glioma cultures as well as induces cell senescence. Tmz had a similar effect except that did not induce cell senescence. The response obtained using sublethal doses is an important condition to therapeutic approach, which could lead to a reduction on side effects.

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Sphere culture of murine lung cancer cell lines are enriched with cancer-initiating cells (CICs).

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Background: CICs are hypothesized to represent a unique cell population essential for the growth and maintenance of tumor. Most *in vivo* studies of CICs utilize human tumor xenografts in immunodeficient mice. These models provide limited information on the interaction of CICs with the host immune system. We hypothesize that assessing the tumorigenicity of CICs in immunocompetent syngeneic animal models will be a more meaningful approach than in immunocompromised animal models, particularly in the ability to accurately define the frequency of CICs in a cell line.

Methods: We studied the sphere forming capacity of 13 murine lung cancer cell lines. Additionally, we examined TC-1 and Lewis lung carcinoma (LLC) cells using sphere culture and a series of phenotypic and functional assays in an effort to isolate CICs. Furthermore, we compared CIC frequency of sphere-derived cells enriched for CICs compared to “differentiated” tumor cells in syngeneic and immunocompromised animals.

Results: TC-1 and a highly metastatic variant of LLC (HM-LLC) tumorspheres exhibited some characteristics associated with CICs. TC-1 tumorspheres were not enriched for expression of CD133 or CD44, putative CIC markers, nor did they demonstrate Hoechst 33342 side-

population staining or Aldefluor activity compared to adherent cells. In tumorsphere culture these cells exhibited self-renewal and long-term symmetric division capacity, expressed Oct-4, and demonstrated greater tumorigenicity in syngeneic animals compared to adherent cells. HM-LLC readily formed tumorspheres. Sphere-derived cells exhibited increased Oct-4, CD133, and CD44 expression, demonstrated a Hoechst 33342 side-population, and Aldefluor activity compared to adherent cells or a low metastatic subclone of LLC (LM-LLC). In syngeneic mice, HM-LLC sphere-derived cells required fewer cells to initiate tumorigenesis compared to adherent or LM-LLC cells. In immunocompromised mice, less than 500 TC-1 cells and less than 1,000 LLC cells were required to initiate a tumor.

Conclusions: We suggest that no single phenotypic marker can identify CICs in murine lung cancer cell lines. Tumorsphere culture may provide an alternative approach to enrich for murine lung CICs. Results of CIC frequency in immunocompromised animals become less meaningful as this interaction is not taken into account. We propose that assessing tumorigenicity of murine lung CICs in syngeneic mice better models the interaction of CICs with the host immune system, a critical component of tumor biology.

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Inhibitory effects of phylligenin on the proliferation of cultured rat neural progenitor cells.

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Neural progenitor cells (NPCs) differentiate into astrocytes, neurons and oligodendrocytes, which is controlled by various factors in brain. Recent evidences suggest that small molecules modulating the proliferation and differentiation of NPCs may have therapeutic value as well as use of chemical probes. Phylligenin is a lignan with anti-inflammatory activity that is isolated from the fruits of *Forsythia koreana*. We investigated effects of phylligenin on proliferation and differentiation of NPCs. Treatment of phylligenin decreased the number of proliferating NSCs in culture without effects on the differentiation and survival of neural cells such as neurons and astrocytes. To examine the mechanism of the decreased NSCs number, we performed FACS analysis. Proliferation of NSCs was decreased via G1-S transition block by phylligenin treatment. The anti-proliferative effect of phylligenin was mediated by the increase of p21 level. However, phylligenin did not induce apoptosis of NSCs as determined by TUNEL assay and PARP cleavage. We also found that viability of glioma cell lines such as C6 and U87MG glioma cells, but not that of primary neuron and astrocyte, was inhibited by phylligenin. These results suggest that phylligenin selectively inhibit proliferation of rapidly growing cells such as neural stem cells and glioma cells. Given that the possible role of brain tumor stem cells in the pathology of brain cancers, the inhibitory effects of phylligenin might be useful in the development of new therapeutic agents against brain cancers.

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Focal Adhesion Kinase Maintains Luminal Progenitor and Basal Mammary Stem Cell Activities with Differential Requirement of Tyrosine Kinase Function.

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Mammary stem cells (MaSCs) and progenitor cells are important for mammary gland development and maintenance, and also serve as potential cell origins of mammary cancer stem cells (MaCSCs). However, the signaling mechanisms regulating these cells and how they

contribute to tumorigenesis are not well understood. Here, we show that conditional deletion of focal adhesion kinase (FAK) in mammary epithelial cells (MaECs) during early embryonic stage significantly decreased the content of luminal progenitors (LPs) and basal MaSCs, and reduced their colony-forming and regenerative potential in a cell-autonomous manner. Further rescue experiments and creation of FAK kinase-defective (MFCKD) knockin mice unveiled that loss of FAK kinase activity in MaECs specifically impairs the proliferation of LPs and functional alveologenesis/milk production, but kinase-independent function of FAK is sufficient to support ductal invasion and basal MaSC activity. Such deficiency in LPs of MFCKD mice significantly suppressed tumorigenesis and formation of MaCSCs in a mouse breast cancer model derived from LPs. Lastly, we demonstrated that, in contrast to the universal inhibitory effect of FAK knockdown, FAK kinase blockers preferentially inhibited proliferation and tumorsphere formation of LP-, but not MaSC-like (claudin-low) human breast cancer cells. Together, these studies demonstrated intrinsic role of FAK and its kinase-dependent and -independent actions in regulation of LPs and basal MaSCs, and suggest that targeting distinct FAK function alone, or in combination, may serve as tailored therapeutic approaches for breast cancer heterogeneity.

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Neoblast Response to Carcinogen Induced Malignancy in Planarians: A Preliminary Study.

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Current thinking considers cancer a stem cell disease. But how these cells affect tumor growth, and whether cancer stem cells are native to tumors or home in on them are still open questions. To learn more about how stem cells react to carcinogens and to the presence of a growing tumor we turned to the planarian species *Dugesia dorotocephala*. Planarians have an exceptional regenerative capacity owing to their ready supply of undifferentiated stem cells or neoblasts. Furthermore, research has shown that various carcinogens can induce tumor growth in the planarian, and that these lesions can be histologically similar to those found in patients. We used a two-week exposure to a mixture of TPA and cadmium sulfate to stimulate tumorigenesis in the planarian. BrdU was used to examine the activity of mitotic or neoblastic cells in planarians exposed to the carcinogens. An analysis of the actin ultrastructure was used to study the overall cellular response of the organism to a growing tumor and to gauge the possible motility of the neoblasts. Work testing the migratory response of cells from tissue grafts of healthy worms to carcinogen exposed or tumor-bearing worms was also begun. This study will set the stage for developing an understanding of how stem cells react to and affect tumor growth.

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Crosstalk between matrix metallo-proteinases and chemokines during prostate cancer stem cells mediated metastasis.

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Although prostate cancer is now the most commonly diagnosed cancer in men, the etiology of this disease remains unclear. Recent efforts to identify and characterize prostate cancer stem cells (PCSCs) also support this hypothesis. Human prostate cancer cell sub-population that is,

PCSCs with the highest *in vitro* proliferative potential is negative for androgen receptor (AR) expression. This population also possesses CD44⁺α₂β₁^{hi}CD133⁺ and predominantly expresses the basal cell cytokeratins. Our study explored the ability of CD133-positive and CD44-positive cells (CD44⁺CD133⁺) to differentiate into prostate cancer epithelial cells bearing CD57, androgen receptor and prostate specific antigen (PSA). Prostate cancer stem cells expressed chemokines and their receptors, such as SDF1- α and CXCR4 (n=6). *In vitro* and *in vivo* investigations into the expression of matrix metalloproteinases (MMPs) showed the expression high levels of MMP-2 and MMP-9, whereas MMP-7 and MMP-13 were expressed in moderate levels, at both the levels of mRNA and proteins in prostate cancer tissue derived CD44⁺α₂β₁^{hi}CD133⁺ cells. Matrigel invasion assay and chemotaxis assay confirmed the role of above MMPs and chemokines during migration and differentiation of prostate cancer stem cells. *In vivo* metastasis models (n=6) and immuno-histochemical analysis showed the correlation between MMPs and chemokines during migration and differentiation of prostate cancer stem cells mediated metastasis.

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Restricting Brain Tumor-Initiating Cell Motility By Rewiring Cell-Matrix Mechanosensing.

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Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor and is characterized by poor survival even in the setting of surgery, radiation, and chemotherapy. While GBM tumors are very heterogeneous, recent work has shown that a specific subpopulation of tumor cells - so-called "brain tumor-initiating cells" (BTICs, also called brain tumor stem cells) - is particularly resistant to conventional treatments, is uniquely capable of initiating new GBM-like tumors following transplantation, and may directly participate in the invasive process. Thus, there is tremendous interest in identifying microenvironmental factors that regulate BTIC self-renewal and motility. Specifically, studies have shown that Rho family GTPases can influence brain tumor progression, but results from these studies have been contradictory. Here, we investigate the sensitivity of BTICs to extracellular matrix (ECM) mechanics and the role of Rho GTPases in regulating this sensitivity.

We cultured BTIC lines on laminin-coated polyacrylamide ECMs ranging in stiffness from 80 Pa to 119 kPa. We found no significant differences in spreading area, random motility speed, or proliferation as a function of ECM stiffness. Remarkably, all BTIC lines proliferated robustly, underwent rapid mesenchymal motility, and developed vinculin-positive adhesions on extremely soft ECMs (~ 80 Pa) normally regarded as non-permissive for spreading, adhesion maturation, and migration. Lentiviral transduction of BTICs with a constitutively active (CA) mutant of Rho GTPase strongly restored mechanosensitivity abrogating BTIC spreading and migration on soft ECMs of comparable stiffness to brain tissue. Administration of myosin II inhibitor blebbistatin offset CA RhoA-mediated mechanosensitivity and restored BTIC spreading and migration on soft ECMs. AFM measurements suggest that BTICs are very soft on both stiff and soft ECMs, thus failing to exhibit normal tensional homeostasis. These findings are consistent with a model in which BTICs evade the anti-tumorigenic effects of soft ECMs but may be rendered susceptible through activation of RhoA-dependent cell contractility.

These results provide the first evidence that BTICs can uniquely resist mechanical suppression of motility and other culture behaviors relevant to tumor spread, in the same way they can resist

radio- and chemotherapy. In addition, motility on ECMs of stiffness comparable to brain tissue may be restricted by forcing activation of myosin-dependent contractility, implying that proteins in this pathway may represent a novel set of therapeutic targets.

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In vitro and in vivo interaction of cdk6 and Eya2 indicate potential crosstalk between cell cycle and developmental pathways.

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We have identified a novel association of the developmentally significant protein, Eya2, with cyclin dependent kinase (cdk) 6. The protein-protein interaction was first identified in a yeast two-hybrid analysis of a human fetal brain library. Subsequent results confirm the association of cdk6 and Eya2 in GST binding assays and co-immunoprecipitations. GST binding assays demonstrated that Six4, a cofactor of Eya2, competes with cdk6 for Eya2 binding. Co-immunoprecipitation of transfected cell lysates and native immunoprecipitations also demonstrate binding of cdk6 and Eya2. Functional outcomes of this interaction are under investigation. Given that Eya and Six proteins are known to impact (breast) tumor cell invasiveness, we propose that the cdk6/Eya2 interaction affects fundamental properties of the cancer cell and may also function in normal development of the (mammary) epithelium.

Tumor Microenvironment

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Novel EGFR Pathways with Fast Kinetics Similar to a Neuron are Conserved in a Broad Variety of Cancer-Types.

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The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor and overexpression of EGFR (high-EGFR) is associated with an aggressive cancer phenotype, poor clinical prognosis, and high rates of recurrences and/or metastasis. Although a variety of cancer therapies exist, all treatments damage normal cells. Therefore, it is important to identify cancer-specific targets for future therapeutic intervention. The objective of this study was to directly measure in "real-time" EGFR membrane trafficking in epithelial cells (high-EGFR versus low-EGFR) from a broad variety of cancer-types by using membrane capacitance (Cm) measurements of cell surface area. Capacitance techniques have been used to track exocytosis and the subsequent endocytosis in neurons and endocrine cells. Our results show that EGFR-stimulation of high-EGFR cancer cells causes extremely fast changes in Cm measurements that have time-constants in the order of a few msec-secs, which is temporally consistent with the rate of the fast steps in exocytosis and endocytosis observed in endocrine cells as well as synaptic terminals. Importantly, these EGFR responses are absent in normal and non-malignant cells (low-normal EGFR levels). Moreover, these cancer-specific EGFR pathways have alternative and kinetically distinct modes of endocytosis depending on the frequency of stimulation. These EGFR responses could never be detected by the techniques and protocols routinely employed to study EGFR internalization. In this study we have obtained proof of concept for the use of the Cm measurements in cancer research: cancer-cell lines are not "professional" secretory cells and Cm measurements have never been evaluated in cancer-

cells nor explored in a cancer-relevant context. To that end, in the current study we have verified that the capacitance technique is a widely applicable method to directly visualize in cancer cells the unprecedented EGFR membrane incorporation and EGFR endocytosis that occurs in response to EGFR-stimulation. Based on our results, we propose that malignant cancers behave with a level of sophistication comparable to neurons with ultra-rapid responses to satisfy their high functional demands. Additionally, the fact that these EGFR responses are present only in malignant cancer cells suggests that a component(s) of these new pathways could be a potential target(s) for universal cancer-specific therapy. Understanding the details of the EGFR-processes in cancer versus noncancer cells will enable major advances in our knowledge of the molecular and cellular basis of EGFR contribution to cancer (supported by NIH GM68813).

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Role of tumor pericytes in regulating myeloid-derived suppressor cells (MDSC).

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Myeloid-derived suppressor cells (MDSC) are a heterogeneous population which defined as Gr1⁺/CD11b⁺ cells and dramatically expand in many pathological conditions, inflammation, autoimmune disease, infection and cancer. Recently it has been highly reported that MDSC might have a critical role on tumor malignancy by modulating tumor immunity and tumor microenvironment. However, it still remains elusive how MDSC infiltrate through tumor vessels. Previously, we have reported that pericyte deficient mice model, *pdgfb* retention motif knock out mice, *pdgfb*^{ret/ret} have less pericyte coverage in tumor vessels. In this study, we found that MDSC expansion was associated with pericyte coverage in tumor vessels. In both syngeneic mouse tumor models, LLC and B16 melanoma, pericyte deficient *pdgfb*^{ret/ret} tumorigenic mice has an increased MDSC expansion in blood and tumor as compared to control. Naïve MDSC in non-tumorigenic mice were also assessed in various organs, brain, lung, pancreas and skin, but the level of naïve mdsc was not associated with pericyte deficiency, which means that only tumorigenic MDSC expansion is correlated with pericyte deficiency in tumors. Furthermore, one of MDSC-recruiting cytokines, IL6, was also highly upregulated in blood of *pdgfb*^{ret/ret} tumorigenic mice, but the level of other candidate cytokines, IL1b, GM-CSF, G-CSF and VEGF-A was not changed. These results were confirmed by using B16-PDGF-BB tumor model, which have paracrine PDGF-BB production and recover from pericyte depletion near tumor vessel in pericyte deficient *pdgfb*^{ret/ret} tumorigenic mice. Taken together, these findings suggest that tumor pericytes regulate the recruitment of MDSC into tumor by modulating the cytokine release through tumor vessels.

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Visualizing the hypoxia selectivity of cobalt(III) prodrugs in tumor spheroids.

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Despite the clinical success of many chemotherapeutic agents, anticancer drugs are still limited by their lack of specificity which results in indiscriminate toxicity to both healthy and cancerous cells, and inadequate penetration into tumors. As a result, cancer patients experience side effects such as nephrotoxicity, emetogenesis and neurotoxicity. In an effort to improve the efficacy of anticancer drugs, tumor activated prodrugs have been designed whereby an inactive agent is degraded or metabolized at the tumor site to form the active cytotoxic species.

Cobalt(III) prodrugs have been previously investigated as chaperones for the selective delivery of cytotoxins to solid tumors.

To determine whether cobalt complexes are releasing fluorescent cytotoxic ligands in hypoxic regions, a concatamer of the hypoxia response element (HRE) from the human VEGF gene were cloned upstream of a minimal CMV promoter. DLD-1 colon carcinoma cells were genetically modified to respond to hypoxic stress in two and three-dimensional cell culture by turning on the expression of a fluorescent protein, EosFP. This study used tumor spheroids as an in vitro model to investigate hypoxia selectivity of the cobalt complexes, by correlating the fluorescence of the drug with the green fluorescent protein using confocal microscopy. The distance from which the green fluorescent proteins are expressed in the tumor spheroid is approximately 70 μm from the edge, this is comparable to the hypoxic regions of the immunolabelled mouse biopsies reported in literature [1]. Therefore, tumor spheroids are exemplary models for hypoxia. Co-localisation of the blue fluorescent cytotoxic ligands and the green fluorescent protein has shown that the cobalt(III) complexes are partially hypoxia selective.

Reference

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The N-terminal Projection Domain of the Microtubule Associated Protein Tau Inhibits TNF α signaling.

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Tumor Necrosis Factor-alpha (TNF α) is a multifunctional pro-inflammatory cytokine implicated in inducing both tumor promotion and tumor inhibition. This paradoxical effect can be partly explained by the fact that TNF α acts as a regulator of both proliferation and apoptosis; however the mechanisms and factors associated with how or whether a tumor cell responds to a microenvironment rich in TNF α have not been fully resolved. Here we provide evidence that the microtubule associate protein Tau inhibits TNF α signaling through its N-terminal domain and implicate it as a novel regulator of the cellular response to this key cytokine. Our findings indicate that over-expression of Tau in the human breast cancer cell lines MCF7, SkBr3 and in the pheochromocytoma cell line PC12 suppresses TNF α induced apoptosis evident in the control cells. TNF α induced cell apoptosis in control cells is triggered as early as 6 hrs post-treatment with hallmark features of cell shrinkage, membrane blebbing, disruption of tubulin architecture and caspase-3 activation. While over-expression of wild type full length Tau isoform in these cell lines, completely abrogates all TNF α associated apoptotic phenotypes. Aside from inhibiting TNF α induced apoptosis, Tau over-expression also interferes with TNF α induced NF κ B activation. This is evident by a significant decrease in NF κ B nuclear translocation in Tau over-expressed cells compared to controls. Suppression of TNF α signaling by Tau is not associated with its tubulin interaction since a truncated N-terminal projection domain lacking all microtubule binding domains still provides resistance to TNF α induced apoptosis and NF κ B activation. These findings lead us to conclude that the functional domain of Tau that regulates TNF α response lies within its N-terminal region and current mechanistic studies indicate this region inhibits TNF α receptor clustering, which is needed for signaling. Our data provide direct evidence that the N-terminal domain of the microtubule associated protein Tau can inhibit TNF α

signaling in tumor cells. These findings indicate a novel function for Tau and add to our knowledge of how a tumor cell responds to TNF α signaling.

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Extracellular matrix fiber alignment by stromal syndecan-1 requires $\beta 3$ integrin activity and syndecan-1 ectodomain and heparan sulfate chains.

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The cell surface proteoglycan syndecan-1 (Sdc1) is frequently induced in the stromal fibroblasts of invasive breast carcinomas. We have recently identified a correlation between stromal Sdc1 expression and extracellular matrix (ECM) fiber alignment, both *In vivo* (in human breast carcinoma samples) and *in vitro*. Bioactive, cell-free 3D ECMs derived from Sdc1-positive human mammary fibroblasts (HMF), termed ECM-Sdc1, showed an organized, aligned fiber architecture, which contrasted markedly with the random fiber arrangement seen in the ECM of Sdc1-negative HMFs (ECM-mock). We further demonstrated that ECM fiber alignment is responsible for the enhanced directional migration and invasion of breast carcinoma cells when seeded into the fibroblast-free ECM-Sdc1.

To decipher the molecular mechanisms responsible for the formation of an invasion-permissive ECM, a series of Sdc1 mutants were introduced into HMF. These include a heparan sulfate (HS) – deficient, a partial extracellular domain (ectodomain) deletion, a cytoplasmic domain (CD) deletion and a transmembrane domain (TMD) substitution mutant. We found that the ectodomain and HS chains of Sdc1 are required for the full activity of Sdc1 in regulating ECM alignment, while TDM and CD are dispensable.

Integrins are key players in the assembly of fibronectin-rich ECM and Sdc1 has been shown to be an important regulator of integrin activity. Therefore, we investigated the role of integrins in Sdc1-mediated ECM fiber alignment. We found that expression silencing of the integrin $\beta 3$ subunit by siRNA largely abolished the aligned ECM fiber architecture induced by Sdc1. Given the integrin subunit repertoire of HMF, this finding suggests that integrin $\alpha v \beta 3$ is responsible for Sdc1-dependent ECM fiber alignment and consequently the invasion-permissive properties of the ECM. Activation of the $\alpha v \beta 3$ integrin by $\beta 3$ clasp peptide increases ECM fiber alignment in the absence of Sdc1, further supporting this model. Understanding the mechanisms governing ECM organization may lead to the development of novel stroma-targeted therapy for breast cancer.

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The interplay of growth and migration of cancer cells in tumor growth dynamics and invasion.

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Tumors are a complex arrangement of tissues made up of several components, including dense masses of cancer cells and re-organized extracellular matrix (ECM). Recent studies have revealed the crucial role that extracellular matrix components have on single cancer cell behavior, but how the interaction of ECM components affects the growth dynamics of an entire tumor is not fully understood. Here, we use human derived fibrosarcoma cell aggregates in

combination with live cell imaging, cryo-stat sectioning, immunostaining, and confocal imaging to study changes in cell aggregate size, proliferation, and spatial distribution within 3 dimensional (3D) matrices. We coupled our experimental observations with a continuous approximation model to predict cell aggregate growth and cell density distribution and determine how cell interactions play a significant role in this dynamic growth. Using this model, we investigate the distinct contributions from cell migration, proliferation, cell-matrix interactions, and matrix remodeling to the aggregate progression. Coupling 3D experiments with mathematical modeling could have applications in predicting patient prognosis and in improving drug screening designs.

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Tumoral soluble factors secreted by breast cancer cell lines induce the expression of adhesion molecules in endothelial cells.

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In the last decade, it has been recognized that the tumor associated endothelial cells express a phenotype different to that expressed by normal endothelial cells. This phenotype appears to be determined by the tumoral microenvironment. The endothelial cells are part of the tumor microenvironment and also play an important role in metastasis including angiogenesis as well as intra- and extra-vascularization. These processes involve the direct interactions between cancer and endothelial cells and require the expression of cell adhesion molecules (selectins, lectins, Ig and integrins) on the endothelial surface and ligands on the cancer cells (glycoproteins, Ig and integrins). The objective of the present study was to determine the expression of adhesion molecules induced by tumoral soluble factors (TSF) secreted by breast cancer cell lines with high (ZR75.30) or low (MCF-7) metastatic potential in human umbilical vein endothelial cells (HUVECs). For this purpose we cultured primary endothelial cells from umbilical cords and evaluate its purity using the expression of von Willebrand Factor as an endothelial marker. We also used a bioplex system to determine the molecules present in the TSFs, finding that the ZR75.30-TSF was enriched with chemokines and growth factors such as: VEGF, IL-8 and G-CSF. These factors have been identified in vivo and in vitro as contributors to progression and metastasis. We also tested the effect of the TSFs on endothelial phenotype, using TNF treatment as a control for the endothelial activated phenotype characterized by the expression of cell adhesion molecules (CAMs: E-Selectin, ICAM-1 and VCAM-1) and an increase in cell adhesion. We found that ZR75.30-TSF induced the expression of the three CAMs at the mRNA and protein levels and also lead to a pro-adhesive phenotype as show by the adhesion assay, while the MCF-7-TSF had no effect on the expression of CAMs nor on cell adhesion.

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Clinicopathological significance and hormonal regulation of ADAMTS-1 in ovarian cancer.

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In ovarian cancer alterations in the extracellular environment are critical for tumor initiation, progression and intra-peritoneal dissemination. Extracellular matrix (ECM) remodeling would influence ovarian cancer behavior. ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) is a family of secreted matrix metalloproteases, playing a role on

biological processes such as inflammation, angiogenesis and development of urogenital system. Despite suggestive role of ADAMTS in tumor invasion and metastasis, effects played by these molecules in cancer progression are still controversial. Here we analyzed the effect of ADAMTS-1 influencing ovary cancer. Forty-eight human ovary tumors (10 benign, 9 borderline and 29 malignant) were retrieved from the files of the Department of Pathology, Hospital A. C. Camargo, Sao Paulo, Brazil. Tumors were classified according to the WHO Histological Typing of Tumors of the Breast and Female Genital Organs. Clinical stage was determined according to the UICC TNM (tumor, nodes, metastasis) staging system. Immunohistochemistry assessed the presence of ADAMTS-1 in these tumors. ADAMTS-1 substrates, such as proteoglycans versican, aggrecan and brevican, were also analyzed. In addition, immunostaining of TIMP-3, ADAMTS-1 potent inhibitor, was carried out. Immunohistochemistry differences were analyzed by measurements of stained areas normalized per nuclei area. Expression of ADAMTS-1 and TIMP-3 was significantly higher in ovary malignant tumors compared to benign and borderline tumors. ADAMTS substrates brevican, aggrecan and versican expression showed no differences among benign, borderline and malignant ovary tumors. ADAMTS-1 and TIMP-3 are highly expressed in malignant tumors. We may assume that inhibitor levels compensate enzyme effects, thus reducing substrate breakdown and resulting in maintenance of proteoglycan levels in malignant tumors. To further explore the role of ADAMTS-1 in ovary tumor biology, we investigated the role of well-known ovary cancer modulators, such as steroid hormones, in ADAMTS-1 expression. The cell lines NIH-OVCAR-3 (more differentiated) and ES-2 (less differentiated) were treated with different concentrations of progesterone, estrogen or testosterone. ADAMTS levels (mRNA) were analyzed by qPCR. In less differentiated ES-2 cells, ADAMTS-1 mRNA levels increased after treatment with hormones estrogen and testosterone. No differences were found in ADAMTS-1 levels were found in more differentiated NIH-OVCAR-3 cells treated by these hormones. We conclude that ADAMTS-1 protein expression increases in ovary malignant tumors, being regulated by steroid hormones estrogen and testosterone. Supported by FAPESP (2010/07699-1)

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Laminin-induced quiescence of breast epithelial cells is mediated by rapid decrease of nuclear actin.

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Cell quiescence is a central aspect of tissue specificity and its loss is one of the crucial steps in cancer progression. Whereas growth has been subject of much investigation, we still do not know how cells within the tissues become quiescent. We showed previously that signals from the extracellular matrix (ECM), in particular from laminin-111(LN1), lead to quiescence in mouse mammary epithelial cells preceded by a substantial decrease in nuclear actin (N-actin). Inhibition of this drop in the nucleus prevents quiescence. Here we investigated quiescence, a crucial event for organ homeostasis using HMT-3522, a human breast cancer progression series. We show that that LN1 treatment in HMT-3522-nonmalignant cells (referred to as S1) causes a rapid drop of N-actin levels (~30% by 30 minutes) and this is followed by a decrease in RNA Polymerase II activity (~60% by 2 hours) and cell proliferation (~60% by 4 hours). Interestingly, in HMT-3522-malignant cells (referred to as T4-2), the same treatment does not deplete N-actin nor does it induce changes in transcriptional activity and proliferation indicating that the mechanism(s) that controls quiescence induced by LN1 (and mediated by N-actin decrease) have gone awry in cancer cells. These data point to a new link between the ECM and the nucleus, and to a new explanation for why nonmalignant and malignant cells respond differently to growth and quiescence cues from the ECM, and suggest a new pathway to target for breast cancer therapy and possibly also other organs.

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Laminin-derived peptide C16 regulates gene expression and protein levels of cancer-related molecules in breast cancer cells.R. G. Jaeger¹, E. S. Santos², B. Smuczek¹, M. Paiva¹, J. J. Pinheiro³, V. M. Freitas¹;¹Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil, ²Department of Clinical Analysis, Faculty of Pharmaceutical Sciences, University of Sao Paulo at Ribeirao Preto, Ribeirao Preto, Sao Paulo, Brazil,³Department of Pathology, School of Dentistry, Federal University of Para, Belem, Para, Brazil

Human breast cancer constitutes worldwide health care problem. A complex pattern of molecular alterations is involved in tumorigenesis, conferring to transformed cells a higher proliferative potential, evasion to apoptosis, sustained angiogenesis, and capacity to invade the surrounding tissue and metastasize. Extracellular matrix molecules play important role influencing malignant behavior. An increasing number of evidences have shown that peptides derived from laminin cleavage are involved in tumor progression. We are particularly interested in peptide C16, derived from laminin-111 gamma-1 chain. This peptide increases cell migration, enhances metastasis, and promotes angiogenesis. These findings prompted us to investigate whether C16 would regulate gene expression of metastatic breast cancer cells (MDA-MB-231). Cells were treated with C16 (100 microgram/ml) for 24 hours. Cells treated by scrambled peptide C16SX served as controls. After treatment, gene expression was analyzed by microarray. Total RNA was extracted; biotin-labeled cDNA was generated and hybridized to Human Gene 1.0 ST Array (Affymetrix). GeneChips were stained and scanned. Expression Console software converted fluorescence intensities into numerical values. C16 induced differential expression of cancer-related genes (validated by real-time qPCR), such as FGFR3, SPOCK1 and GPNMB. FGFR3 encodes a tyrosine kinase receptor, which regulates various biological processes, including cell migration and angiogenesis. GPNMB encodes a transmembrane glycoprotein involved in angiogenesis and promotion of migration, invasion and bone metastasis of breast cancer cells. SPOCK1 encodes a proteoglycan (spock-1 or testican-1) associated with cell adhesion and increased migration. The importance of these molecules in tumor biology has prompted us to analyze whether C16 would modulate expression levels. MDA-MB-231 cells were treated overnight by either C16 (100micrograms/ml) or C16SX. Immunoblot showed that C16 increased the expression of GPNMB and SPOCK-1 compared to controls. We conclude that laminin-derived peptide C16 regulates gene expression and protein levels of cancer-related molecules in breast cancer cells. Support: FAPESP (2008/55847-0; 2009/04114-5)

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Externally applied forces can phenotypically revert malignant breast epithelial structures.G. Venugopalan¹, K. Tanner^{2,3}, W. Orellana^{2,3}, C. D. Reber¹, M. J. Bissell², D. A. Fletcher^{1,4};¹Bioengineering, University of California-Berkeley, Berkeley, CA, ²Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, ³Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, ⁴Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

Single breast epithelial cells embedded in three-dimensional laminin-rich extracellular matrix gels grow to form highly-organized, growth-arrested acini. Malignant cells form larger, disorganized structures, but they can be 'phenotypically reverted' into acini by treatment with pharmacological reverting agents (1). While extracellular matrix stiffness has been shown to be important in acinar morphogenesis (2), the role of external forces remains unclear. Here, we investigate the effects of external compression on acinar morphogenesis. Using an elastic chamber to transiently apply compression, we found that compressed malignant cells formed

growth-arrested, polarized acini complete with lumen, rather than the disorganized structures observed in uncompressed gels. This 'phenotypic reversion' of malignant cells under compression occurred without the use of exogenous pharmacological agents, but was eliminated by blocking E-Cadherin function. Recently, it was discovered that epithelial cells undergo coherent rotation during acinar development that ultimately controls multicellular architecture (3). This coherence was lost in the malignant phenotype but reestablished during 'phenotypic reversion' of malignant cells. In this study, time lapse microscopy of malignant cells under compression revealed that external forces induce rotation of the cells, leading to organized acini. Our findings suggest that external forces can modulate coherent rotation and cell-cell adhesion, thereby reprogramming malignant cells to re-enter the correct morphogenetic program.

- (1) Weaver et al., JCB, 1997.
 (2) Paszek et al., Cancer Cell, 2005.
 (3) Tanner et al., PNAS, 2012.

1674

Aging-related changes make mammary epithelia more vulnerable to cancer: a story of altered stem cells.

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Women over 50 years of age account for 75% of new breast cancer diagnoses, and the majority of these tumors are of a luminal subtype. Although age-associated changes, including endocrine profiles and alterations within the breast microenvironment, increase cancer risk, an understanding of the molecular mechanisms that underlie these observations is lacking. We generated a large collection of normal human mammary epithelial cell strains from women aged 16 to 91 years, derived from primary tissues, to investigate the molecular changes that occur in aging breast cells. We found that in uncultured epithelial cells and in finite-lifespan cultured cells, aging is associated with a reduction of myoepithelial cells and an increase in luminal cells that acquire phenotypes that are usually observed exclusively in myoepithelial cells in women under 30. Changes to the luminal lineage resulted from age-dependent expansion of defective multipotent progenitors that gave rise to incompletely differentiated luminal or myoepithelial cells. The aging process therefore results in both a shift in the balance of luminal/myoepithelial lineages and to changes in the functional spectrum of multipotent progenitors, which together increase the potential for malignant transformation. Together, our findings provide a cellular basis to explain the observed vulnerability to breast cancer that increases with age.

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Proteomic Study of Prostate Cancer Derived Extracellular Microvesicles for Identification of Novel Therapeutic Targets.

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Extracellular microvesicles (EMVs) contain material such as proteins that can be transferred from one cell to another, and interact with recipient cells by binding to adhesion receptors or fusing with the plasma membrane. They can be categorized into two populations: Microparticles (MPs, ≥100nm) which bud off the plasma membrane, and exosomes (≤100nm) which are formed

within the cell in multivesicular bodies. In prostate cancer, the second leading cause of cancer related death in men, EMVs have been shown to alter the tumor microenvironment by increasing motility and apoptotic resistance. Because EMVs may play a role in this disease, it is important to survey their content to identify potential players that can then be therapeutically targeted. The present study uses LC-MS/MS approaches to survey proteins present in EMVs derived from LNCaP prostate cancer cells. We first established a protocol for isolation of MPs and exosomes, confirmed effective by nanoparticle tracking and TEM analyses. Using 1D LC-MS/MS we identified 47 proteins in MPs with $\geq 90\%$ protein identification probability in three or more of the five technical replicates. Among those proteins, HSP90 (a component of the unliganded androgen receptor complex) was identified and its presence was corroborated by western blot. Other proteins detected include unconventional myosin-VI (which is important in maintaining malignant properties of prostate cancer), apoptosis inhibitor programmed cell death 6-interacting protein isoform 2, and α -catenin (which regulates prostate cancer cell proliferation by reducing β -catenin transcriptional activity and cyclin D1 levels). In addition, α -catenin plays an important role in prostate cancer because it regulates β -catenin, which in complex with protein FKBP52 plays an important role in androgen receptor signaling. The same proteomic approach will be employed in the analysis of several other prostate cancer cells, including 22RV1 and FKBP52KD. Our goal is to identify potential therapeutic targets for the prevention of late stage prostate cancer.

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Autophagy plays a major role in mediating hypoxia tolerance to the CTL-mediated lysis.

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Hypoxic tumor microenvironment has attracted much attention as a major contributor in tumor escape from immune surveillance. In this study, we showed that hypoxia-induced resistance of lung tumor cells to Cytolytic T Lymphocyte (CTL)-mediated lysis is associated with STAT3 phosphorylation (pSTAT3) on tyrosine-705 residue and autophagy induction in target cells. Interestingly, targeting autophagy significantly decreases pSTAT3 level in hypoxic tumor cells and restores their susceptibility to CTL-mediated lysis. We investigated the mechanism by which autophagy controls pSTAT3 level in hypoxic tumor cells. We showed that the decrease in the pSTAT3 level following autophagy inhibition is related to an acceleration of its degradation by the ubiquitin proteasome system in a p62/SQSTM1 dependent manner. Our results strongly argue for a role of autophagy in mediating hypoxia tolerance to the immune system. This argument was further supported by our results showing that inhibition of autophagy improves CTL-mediated tumor cell lysis in vivo. Knowing the efficiency of the vaccination with Tyrosinase-Related Protein-2 (TRP2) peptide in inducing tumor cell-specific CTLs response, we used this vaccination strategy in combination with autophagy inhibitor hydroxychloroquine (HCQ) in vivo. Our results showed an abrogation of tumor growth in B16-F10 tumor-bearing mice which were vaccinated and treated with HCQ compared to either treatment alone (vaccination or HCQ-treatment). Overall, this study establishes a novel functional link between hypoxia-induced autophagy and the regulation of antigen-specific T-cell lysis, and points to the critical role of autophagy in cancer immunotherapy. *Contributed equally to this work.

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Acinus Formation and Phenotypic Reversion Result from a Functioning Interaction Between Cells and the Basement Membrane in an Agent Based Model.

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When healthy mammary epithelial S1 cells from the human HMT-3522 cancer progression series are grown in a 3-dimensional laminin-rich gel, they form hollow spheres which resemble the milk-secreting structural units in breast tissue ('acini'). Their malignant counterpart - the T4-2 cells - do not form organized structures under the same conditions. The basement membrane (BM) and downstream signaling within the cells have been identified as crucial participants in this process: Manipulation of the signaling between the cells and the BM can induce the formation of acinus-like structures even in malignant T4-2 cells ('phenotypic reversion'). Whereas many molecular components involved in this process have been identified, a comprehensive connection of cellular and extracellular properties with acinus formation has not been established. Here, we present a model of acinus formation based on the interaction between the cells and the BM: 1. growth control by the BM regulates the size, 2. cell adhesion provides the spherical shape, and 3. anchorage-dependence leads to formation of the lumen. An agent-based model using these properties can reproduce phenotypes observed in cell culture under different conditions including acinus formation, full and partial phenotypic reversion. The phenotypic characteristics of the obtained multicellular structures correlates with the presence or loss of the three properties. When applying the same conditions of the agent-based model for an extended period of time in order to simulate cancer formation, we observe three distinct types of aberrant growth that not only differ in their cause and appearance, but also in their response to different treatments. In particular, we find that a loss of cell-adhesion alone can lead to aberrant growth that displays very poor response to treatment. This model reveals a link between molecular processes in cells and their micro-environment and the appearance and responsiveness of the multicellular structure that might explain differences in prognosis and help to steer the development of drugs for specific cancer subtypes.

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Inhibition of Glioma Cancer Cell Proliferation by High Crystallined Serum Coated Carbon Nanostructures.

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The majority of previous nano toxicity evaluations on carbon nanostructured materials focused the cellular response by altering conventional physiochemical surface properties, such as size and chemistry (functionalization). This study, however, demonstrated that the surface crystallinity of carbon nanostructures is an additional independent factor that should be considered for the inhibition of cancer proliferation without mitigating reactive oxygen species (ROS). In addition, cytotoxic evaluation of both proliferating cancer cells and fully differentiated nerve cells (i.e. non-proliferative) showed selective cytotoxicity: single walled and highly crystalline carbon nanostructures aggressively inhibited the proliferation of glioma cancer cells, but no notable cytotoxicity effects were observed on differentiated nerve cells. Although single wall carbon nanotubes tend to trigger ROS as a manner of potent triggering of pro-inflammatory responses, obtained results demonstrate that highly crystalline carbon structures can be utilized as a selective anti-proliferative agent against brain tumor cells without increasing ROS level and without significant cytotoxic effects to adjacent nerve cells.

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Study cell-cell communications across nano-passages.

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The interactions between cancer cells and nearby tissues are crucial for tumor development and metastasis. The traditional way to study the communications between different types of cells is by co-culturing them in a culture dish. In this way, cell communications take place in all directions without well-defined temporal starting points and the cell-cell contacts are established at any contact point. It is difficult to obtain clear data for analyses in such experiments.

In this work we introduce a microfluidic nano-slit device for studying the contact communication between cancer cells and fibroblasts. This device confines cell-cell contacts in predictable locations and enables easier observation of the temporal sequence of the communication events between the two types of cells. The microfluidic chip has parallel incubation channels connected by transversal slits of vertical dimension in the range of 100-500 nm. The slits prevent cells from crossing between the incubation channels while allowing them to easily communicate across the slits by chemical signals as well as by direct contacts, such as membrane nanotubes. Cell communications from all slits can be synchronized by mechanically controlled valves of the slits. Because our device confines the cell communications within a thin slit, all the contact communication events are kept in the focal plane of a high-numerical-aperture objective lens for long-term observation. Moreover, we make nano-slits of various lengths to study cell-cell communications over various length scales.

By using this nano-slit device, we observed the growth of membrane nanotubes across the slit from a lung cancer cell into the incubation channel of the fibroblasts. The protrusion rate of nanotubes was estimated to be ~4 $\mu\text{m}/\text{min}$. We also observed the transportation of microvesicles along the membrane nanotubes from a fibroblast to a cancer cell. The transport speed of these microvesicles was ~0.5 $\mu\text{m}/\text{min}$. The dynamical observations provide important information for the time scales of contact communications between cancer cells and the stromal fibroblasts. We are also trying to identify the substances inside these microvesicles. In addition, potential motor proteins transporting the microvesicles in the membrane nanotubes will also be checked.

Prokaryotic Cell Biology

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Generation of a protein gradient within a bacterial cell by the cell division regulator MipZ.

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Intracellular protein gradients are critical for the organization of eukaryotic cells, being involved in embryogenesis, morphogenesis of the mitotic spindle or cell size homeostasis. In contrast,

gradient-forming systems have long been assumed to be absent from prokaryotic organisms due to high diffusion rates of cytoplasmic proteins and the small dimensions of a bacterial cell. However, a bipolar gradient of the P-loop ATPase MipZ was found to be required for proper division site placement in the differentiating bacterium *Caulobacter crescentus*. Based on the crystal structures of the apo and ATP-bound protein and by means of mutant variants of MipZ, we dissected the role of nucleotide binding and hydrolysis in MipZ function and present a model for the establishment of the MipZ steady-state concentration gradient. Its formation is found to rely on a nucleotide-regulated alternation of MipZ between a monomeric and dimeric form. MipZ monomers interact with the polarly localized chromosome segregation protein ParB resulting in recruitment of MipZ to the polar regions. Our results suggest that the polar ParB complexes locally stimulate the formation of ATP-bound MipZ dimers. Moreover, dimers are retained near the cell poles through association with chromosomal DNA. Due to their intrinsic ATPase activity, dimers eventually dissociate into freely diffusible monomers that undergo spontaneous nucleotide exchange and are recaptured by ParB. The MipZ gradient can thus be envisioned as an asymmetric distribution of dimers that are released from a polar pool and slowly diffuse towards mid-cell. Given the marked differences in the interaction networks and diffusion rates of monomers and dimers, ATP hydrolysis promotes oscillation of MipZ between the polar ParB complexes and pole-distal regions of the nucleoid. The MipZ gradient thus represents the steady-state distribution of molecules in a highly dynamic system, providing a general mechanism for the establishment of protein gradients within the confined space of the bacterial cytoplasm.

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A mutation in *C. crescentus* MreB results in a variable width phenotype, revealing distinct MreB- and FtsZ-dependent modes of cell elongation.

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The bacterial actin homolog MreB is a morphogenetic protein that contributes to cell width control. A point mutation in *Caulobacter crescentus* MreB was investigated which confers a variable width phenotype, where cells can develop thin extensions at one or both poles and a wider region in the middle. Fluorescently-labeled mutant MreB localizes preferentially to thin regions of cells, without affecting canonical polar markers, suggesting that thin sections do not represent extended poles. Additionally, localization of the peptidoglycan machinery is not dramatically altered in these cells. Reports that FtsZ can direct the insertion of lateral cell wall independent of MreB led us to hypothesize that, in this mutant, growth in the wide regions is due to an FtsZ-dependent mechanism, while growth in the thin regions is due to an MreB-dependent mechanism. To test this hypothesis, we depleted cells of FtsZ and observed that addition in wide regions was halted while addition in thin regions continued. This supports a model where there are two distinct modes of growth: MreB- and FtsZ-directed cell elongation. Normally, both modes work synergistically but, in this mutant, MreB preferentially localizes to peripheral thin sections, leading to a functional separation of the two modes of cell elongation.

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Structure of the magnetosome actin MamK.

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Bacterial actins, in contrast to their eukaryotic homologs, are diverse in both sequence and function. Although bacterial actins share a common fold of the monomer, they assemble filaments with a surprising degree of variation in both their dynamic behaviors and filament architectures. The bacterial actin MamK is involved in the proper organization of a unique

membrane-bound organelle, the magnetosome, essential for magnetotaxis in a phylogenetically diverse group of bacteria. MamK is required to align magnetosomes along the long axis of the cell, where they serve essentially as a compass needle. MamK forms dynamic filaments *in vivo*, and these dynamics are dependent on other magnetosome-specific proteins. To better understand the mechanisms of magnetosome alignment by MamK and the basis for its dynamics we have determined the structure of MamK filaments by cryo-electron microscopy. Like actin and most bacterial actins, MamK forms a two-stranded filament. However, it is unique in that the two strands are in register, rather than staggered as in actin. This unusual arrangement suggests large-scale changes have been made in the cross-strand contacts, which may prove to be the primary driver of variation in bacterial actin filament structures. Thus, the structure of MamK serves as a framework for understanding its function in magnetotactic bacteria, as well as extending our understanding of the diversity of bacterial actins in general.

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Polymerization and Nucleotide Regulation of a Spindle-forming Bacteriophage Tubulin, PhuZ.

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Tubulins are essential for the reproduction of many eukaryotic viruses, but bacteriophage are generally assumed not to require such cytoskeletal elements. Here we show a divergent tubulin-like protein, PhuZ, encoded by bacteriophage 201φ2-1, assembles a dynamic spindle that positions phage DNA at the center of the cell. This is the first example of a prokaryotic spindle that performs a function analogous to the microtubule based spindles of eukaryotes. We solved the structure of full length PhuZ-GDP to 1.67 Å resolution, and found an extended C-terminus that we show to be critical for polymerization. Longitudinal packing in the crystal lattice appears to mimic packing observed by EM of *in vitro* formed filaments indicating how interactions between the C-terminus and the following monomer drive polymerization. We have solved structures of non-polymerizing, tail deletion mutants with GDP as well as with GTP soaked in, revealing no change in conformation. Additionally, we characterize the behavior of PhuZ filaments in limiting GTP and in the presence of excess GDP. Our findings reveal a previously unknown role for tubulins in viral function and provide important insights into the structural basis for polymer formation.

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Division site placement in *E. coli*: Dissecting functional domains of MinE.

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The Min system of *E. coli* mediates placement of the division septum at midcell. MinD and MinE form a dynamic oscillator to partition the division inhibitor MinC towards the poles to blocks aberrant polar division. MinE forms a ring-like structure at the receding edge of the MinD polar zone during oscillation, which arrests growth of the MinD polar zone passing midcell and switches into the shrinkage phase. This phenomenon underlies intricate interplays between the Min proteins and the membrane. In this study, we investigated self-assembly properties of MinE *in vitro* that may underlie the MinE-ring formation. Our results demonstrated that the N-terminal domain of MinE self-associated into protein fibrils consisted of multiple protofilaments. The

organization of protofilaments changed upon association with the membrane. Since the same N-terminal region is responsible for MinD interaction, the two functions are likely to take place at different time points or in different MinE molecules. The filamentous structure of full-length MinE showed spatial preference on the membrane, suggesting the C-terminal domain provides positional information for membrane localization. Taken together, we provided direct evidence for self-assembly of MinE and the function of the MinE C-terminal domain.

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Outer membrane composition and dynamics in *Escherichia coli*.

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The Gram-negative outer membrane (OM) of *E. coli* is a heterogenous bilayer comprising various proteins, lipids and polysaccharides. These OM components are often virulence determinants that facilitate motility, confer toxicity, mediate invasion, etc. Thus, a detailed understanding of the secretion and in situ dynamics of these features with respect to the cell envelope may offer novel targets for the development of future antimicrobials. In particular, the ability to track specific labeled components with high resolution over short time periods, as well as OM constituent partitioning over extended generational periods, will yield critical insights into the mechanisms governing OM biogenesis. However, many OM components are derived from essential pathways or are themselves essential, severely limiting classical genetic approaches. Others are products of complex biosynthetic or post-translational modification systems which precludes many of the standard labeling technologies (*i.e.* the inability to secrete *gfp*-chimeras from the cytoplasm). Here, we describe labeling technologies developed for compatibility with imaging the compositional dynamics of OM constituents of live cells. In one approach, we use a genetically-encoded polypeptide tag (*ybbR*) incorporated into a specific OM protein (LamB) which is covalently labeled with derivatized coenzyme A by action of the Sfp synthase. Preliminary data suggests new OM material is inserted as discrete puncta distributed along the cell cylinder rather than polar regions. Additionally, we describe the development of bacteriophage-derived proteins into recombinant, feature-specific markers for components of the *E. coli* OM. Not only will this approach permit specific, simultaneous observation of proteins and lipopolysaccharides, but can also be adapted to spatiotemporal dynamic studies of these major envelope features. We anticipate that these multiplexed labels will yield a more comprehensive understanding of OM biogenesis and maintenance.

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In vivo investigation of DNA replication in *Escherichia coli* using single-molecule fluorescence microscopy.

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An *Escherichia coli* cell contains a circular chromosomal DNA that is duplicated in a bidirectional manner during cell division. DNA polymerase III proteins, along with helper proteins, form a replisome complex that duplicates the DNA. Recent studies have shown that two individual replisomes initiate replication and duplicate the chromosomal DNA independently of one another. Our current knowledge of DNA replication in *E. coli*, specifically its mechanism of termination, has mainly been obtained from in vitro experiments. However, the processes that occur in living cells may differ considerably from those of the in vitro environment and can have

a significant influence on dynamics of protein function. Interestingly, it is now possible to monitor single-molecule processes in vivo using fluorescence microscopy by tagging the native proteins with fluorescent proteins. We employ single-molecule fluorescence microscopy to track the individual replisomes and investigate their dynamics during the process of DNA replication in live cells. The single-molecule sensitivity in such experiments can provide additional information to the results obtained from previous studies done by ensemble-averaging techniques. In this poster we discuss our live-cell imaging techniques, the strain creation and characterization methods employed, the experiment methodology, and the latest results obtained at the single-molecule level.

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How the rate of osmotic downshift affects E. coli survival: a study of mechanosensitive channel function.

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Bacteria have developed a wide variety of defense mechanisms that allow them to survive in fast changing environmental conditions. One such adaptation is the presence of mechanosensitive (MS) channels in the inner membrane which act as an osmotic valve preventing membrane rupture during osmotic downshift. Our understanding of the in vivo osmotic response of these channels has largely been based upon plating/colony-counting assays. In this work we use microscopy based techniques with single-cell level resolution to investigate the impact of the rate of osmotic downshift on the survival rate of various E. coli mutants, where combinations of MS channels have been deleted. In our measurements, we determine how specific MS channels types (e.g. YbdG and MscL) contribute to the survival rate and try to find the minimal number of MscL needed for survival after osmotic shock.

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Localized cell death focuses mechanical forces during 3D patterning in a biofilm.

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Self-organization of cells into structures during development is a fundamental process that occurs in many different biological systems. Over the last decades, a number of studies have shown that bacterial communities like biofilms can also exhibit extensive spatio-temporal organization, similarly to multi-cellular organisms. Unlike laboratory domesticated bacterial strains, native bacteria form biofilms typically with complex and intriguing three-dimensional (3D) wrinkle patterns. Investigating wrinkled biofilms of gram-positive bacteria *Bacillus subtilis*, we discovered a dynamic pattern of localized cell death that emerges during biofilm formation. We quantitatively and simultaneously measured cell death, movement and mechanical properties to analyze wild-type and gene deletion mutant biofilm development. The results suggest that localized cell death provides an outlet for lateral compressive forces, thereby promoting vertical mechanical buckling and 3D wrinkle formation. Guided by the principles that emerged from our findings, we were able to generate arbitrary patterns of wrinkles within a biofilm. The formation of 3D structures facilitated by cell death may underlie self-organization in other developmental systems, and could enable engineering of macroscopic structures from cell populations in a future.

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Crystal Structure of *Streptococcus pyogenes* Csn2 protein, a component of CRISPR-mediated bacterial immune system.*Y. Koo¹, D-K. Jung¹, E. Bae¹; ¹Seoul National University, Seoul, Korea*

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins constitute a microbial immune system against invading genetic elements, such as plasmids and phages. Csn2 is a Cas protein found in subtype II-A CRISPR-Cas systems, and was suggested to function in the adaptation process, during which parts of foreign nucleic acids are integrated into the host microbial genome to enable immunity against future invasion. Here, we report a crystal structure of *Streptococcus pyogenes* Csn2. The structure revealed previously unseen calcium-dependent conformational changes in its tertiary and quaternary structure. This supports the proposed double-stranded DNA-binding function of *S. pyogenes* Csn2.

1690

Elucidating phototaxis mechanism to engineer coordinated communities of cyanobacteria.*A. Chandra^{1,2}, D. Bhaya², K. C. Huang¹; ¹Bioengineering, Stanford University, Stanford, CA, ²Plant Science, Carnegie Institution for Science, Stanford University, Stanford, CA*

Many unicellular photosynthetic organisms such as cyanobacteria display oriented movement with respect to light (phototaxis), thus harnessing light energy for carbon fixation. By coupling metabolism and behavior, phototaxis allows a cell to quickly adapt to changing environmental conditions, governing the active migration of cells in response to shifting light gradients and contributing to community spatial organization. Cyanobacteria play a crucial ecological role in primary production and global biogeochemical cycling of nutrients, and have been targeted as possible biofuel sources using synthetic biology.

The phototaxis response can be categorized into three subsystems: a light-sensing module such as photoreceptors or photopigments, an intracellular regulatory signaling module, and a motility module organized around multi-functional TFP. Several photoreceptors have been characterized and are now workhorses for optogenetics, though cyanobacteria employ other light-sensing modes that have yet to be characterized. Access to a diverse collection of mutants in each of the three modules can be used to reveal the minimal set of molecular players.

In recent years, bacteriology has undergone a paradigm shift from reductionist “single-cell” biology toward the investigation of the complexity of interacting communities. We will elucidate the mechanisms underlying cyanobacterial phototaxis at the level of individual cells, allowing us to engineer coordinated communities with complex spatiotemporal movement. We will utilize an interdisciplinary genetics, microscopy, and biophysical modeling approach for:

- 1) Genetic identification of novel signaling elements that couple positive and negative phototaxis to regulate cell decision-making capabilities
- 2) Characterization of the localization and dynamics of the type-IV pilus (TFP) machinery in individual cyanobacteria and in multi-cellular communities

Experimental data will be utilized to develop quantitative models of cyanobacterial phototaxis that can identify key variables responsible for the spatial structure of multi-cellular communities and cellular decision-making. In the future, organized communities of a mixture of species could be used to perform synthetic or syntrophic functions and to model behavior in ecologically

relevant environments. This study has potential applications in the design of synthetic invasive, immunogenic biological devices and engineered environmental remediation communities.

1692

Quantitation of cell wall and outer membrane growth that indicates how to robustly build rod-like bacteria.

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In both Gram-negative and Gram-positive bacteria, a host of enzymes, collectively termed PBPs, regulate the reproducible and robust construction of the cell wall, whose mechanical integrity is crucial for viability under osmotic stress. Antibiotics that target these enzymes and their corresponding scaffolding proteins, disrupt cell wall construction, ultimately leading to mechanical failure of the cell. In this work, we use a combination of cell wall and outer membrane-specific fluorescent labeling, high resolution time-lapse microscopy, and computational image processing to characterize where, and with what dynamics, cell wall and outer membrane growth occurs in *E. coli*. These measurements allow us to partially uncover the interplay between cell shape and dynamic localization of proteins implicated in cell wall construction. Our results suggest that the presence of the cytoskeletal protein MreB is necessary for localized bursts in growth across the cell surface. We also find strong evidence that the necessary orchestration in growth between the cell wall and outer membrane is achieved by spatio-temporal coupling of the growth in each layer. We tested mechanistic models of outer membrane growth, and independently verified that it grows in uniformly random bursts on the bacterial cell surface, similar to the cell wall. Finally, quantitative tracking of cell wall growth is an effective method for the characterization of cell wall mechanical failure, and subsequent death, that results from treatment with beta-lactams or other upstream antibiotics. These techniques make new regimes available for studying the detailed dynamics of cell wall-associated proteins and their disturbance by antibiotics.

1693

Entropy-Driven Translocation of Disordered Proteins Through the Gram-Positive Cell Wall.

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Surface proteins mediate interactions between cells and their environment. In Gram-positive bacteria, proteins exposed on the cell surface must navigate tens of nanometers from their sites of secretion at the membrane through the bacterial cell wall. This problem is compounded for proteins that remain anchored to the bacterial membrane, such as the *Listeria monocytogenes* actin nucleation-promoting factor ActA, which contains a transmembrane domain near the carboxy-terminus, but interacts with host cell cytoplasmic factors at the amino-terminus. We have developed a physical model indicating that the entropic constraint imposed by a small periplasmic space could drive the translocation of an intrinsically disordered protein across a barrier with a thickness and porosity similar to the Gram-positive cell wall. We demonstrate experimentally that protein translocation depends on both the dimensions of the cell envelope and the length of the disordered protein. In addition, we show that disordered regions from nuclear pore complex proteins translocate in an entropy-dependent manner through the cell wall of *L. monocytogenes* and *Staphylococcus aureus*. These observations imply that purely physical forces can explain the translocation of certain Gram-positive surface proteins. Bioinformatic analyses suggest that entropic translocation is utilized by a variety of Gram-positive bacteria.

1694

The serine protease MamE acts as a checkpoint control protein during the development of the magnetosome organelles of magnetotactic bacteria.

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Research over the last decades or so has revealed that bacteria are not devoid of subcellular organization and that fine-tuned cellular processes govern their growth and development. Some bacteria even contain subcellular compartments that are reminiscent of eukaryotic organelles. One such organism is the aquatic bacterium *Magnetospirillum magneticum* AMB-1, which is capable of forming organelles called magnetosomes. These lipid-bounded compartments encase magnetic nano-particles that passively align the bacteria to the geo-magnetic field and make its search for optimal growth conditions more efficient. A select number of highly specialized proteins are involved in the formation of magnetosomes performing roles such as membrane remodeling, protein sorting, ion transport and mineral formation. In our group we have used several approaches to identify these factors and define the mechanisms by which they act to form an active magnetosome compartment. MamE, an HtrA protein homologue, appears to have roles in both protein sorting and biomineralization. Both in eukaryotes and bacteria HtrA proteins are highly regulated proteases that are essential for maintaining protein homeostasis after protein misfolding and aggregation. We have shown that MamE recruits proteins to the magnetosome independent of its protease activity and that its proteolytic activity is instead required for biomineralization of magnetic particles. Surprisingly, we have found that MamE acts on itself resulting in a subpopulation of the protein being processed into two distinct fragments *in vivo*. This autoproteolysis appears to be regulated since it only occurs at a specific stage in the development of the organelle and depends on appropriate environmental cues. We have thus hypothesized that MamE functions as a checkpoint control protein to link the quality of the environment to the biomineralization of magnetic particles. We are currently employing *in vivo* and *in vitro* approaches to further understand MamE function at the molecular level. Investigation of MamE function will provide missing information about control of organelle development in bacteria and add new knowledge about the regulation of HtrA proteases.

1695

YfiA is necessary and sufficient for dimerization and inactivation of ribosomes in *Lactococcus lactis*.

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When *E. coli* cells enter stationary phase the 70S ribosome dimerizes to form 100S structures, which represent the hibernation state of these translationally-inactive ribosomes. In *E. coli* the protein YfiA is known to inhibit translation, while the proteins RMF and HPF are involved in dimerizing the 70S ribosomes. The dimerization interface is between the two 30S subunits and induced by a conformational change upon binding of RMF. The binding site for RMF overlaps with for anti Shine Dalgarno sequence on the 16S rRNA and thereby inhibits translation. We have explored the formation of ribosome dimers in the Gram-positive bacterium *Lactococcus lactis*. An open reading frame annotated as *yfiA* is present in the genome of *Lactococcus lactis*, and the YfiA protein shares 34% sequence identity with orthologue of *E. coli*. In contrast to *E. coli*, *L. lactis* does not have homologues of *rmf* and *hpf*. Strikingly, a *Lactococcus lactis* strain lacking *yfiA* gene does not show ribosome dimerization; the dimerisation can be restored by

controlled in trans expression of *yfiA*. MS analysis of ribosome dimer fractions confirms that analogues of RMF and HPF are not present in *L. lactis* and that YfiA is necessary and sufficient for dimerization of ribosomes. YfiA from *L. lactis* differs from the *E. coli* orthologue by having an 72 amino acid extension, which most likely takes over the role of RMF and/or HPF and induces the dimerization interphase. Electron Microscopy experiments have been performed to characterize the ribosome dimers in *Lactococcus lactis*.

1696

Studying the positioning of replicating phage DNA by the divergent tubulin PhuZ at the single cell level.

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Bacteriophage were generally assumed not to require a filament-forming cytoskeleton during lytic growth. We have discovered a number of bacteriophage that encode highly divergent tubulin like proteins (PhuZ). We have shown that the PhuZ protein from the extremely large Pseudomonas phage 201[phi]2-1 assembles a dynamic spindle that positions replicating phage DNA during lytic growth. By developing a single infection assay to directly visualize phage DNA and PhuZ assembly in time-lapse microscopy, we have now defined some of the key steps in the phage life cycle. After injection of phage DNA, the host chromosome is degraded. The phage DNA then replicates in a single nucleoid which is positioned at midcell by PhuZ polymers. Phage DNA becomes encapsidated in viral particles which assemble in a spherical structure surrounding the centrally located phage nucleoid.

1697

Proteolysis autoregulates RNA helicase expression in response to abiotic stress.

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Bacteria respond to constantly changing environment by inducing expression of the specific proteins essential for adaptation to external stresses. Cyanobacterial RNA helicase, CrhR, is one of such proteins, whose levels are regulated in response to changes in temperature, light intensity, salt concentration or any other changes that alter redox status of the electron transport chain in the cell. RNA helicases are ubiquitous enzymes that are present in all domains of life. Their function is to modify RNA secondary structure, which subsequently can regulate RNA metabolism and gene expression. CrhR is unique RNA helicase that is capable of catalyzing ATP-stimulated RNA unwinding, annealing and also RNA strand exchange (Chamot *et.al.*, 2004). A basal level of CrhR protein is observed at 30°C, which is significantly increased at 20°C. This differential expression of cyanobacterial helicase with its ability to perform several functions indicates its important role in adjusting cell physiology to rapidly changing environmental condition such as temperature. However, the pathways controlling RNA helicase expression have not been well characterized. Here we describe experiments indicating that CrhR protein abundance is regulated by a temperature-controlled proteolytic mechanism that is CrhR-dependent. A series of *in vitro* experiments were conducted in which protein extracts from warm and cold grown wild type and mutant cells were mixed in all possible combinations and a time course of CrhR abundance was followed by western analysis. The results indicate that active proteolytic degradation machinery is present only in wild type cells at 30°C. Functional CrhR RNA helicase activity is required for either the presence or activity of the proteolytic machinery, as CrhR degradation is not observed in the *crhR* mutant. *In vivo* experiments with cells engineered to express CrhR constitutively in addition to endogenous CrhR indicate that

proteolysis is mostly functional in the first two hours upon the shift to 30°C. Following the degradation of the most of CrhR protein at 30°C its low level is further maintained by temperature regulated promoter. The data indicate that the proteolytic degradation of CrhR protein is autoregulatory, temperature dependent and essential to immediately reduce levels of CrhR protein at 30°C. Our data provide insights into the pathways that are involved in the regulation of RNA helicase expression. The results also have important implications with respect to the molecular mechanisms utilized by microorganisms to respond to abiotic stresses.

1698

Characterization of Foodborne Pathogens Bacteria Profiles.

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The cellular fatty acid profiles of eight strains of Bacillus, Staphylococcus, and Enterobacteriaceae (Escherichia coli, and Salmonella) were analyzed by comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GCxGC/TOF MS). A novel template method was developed to standardize the raw GCxGC retention data through the use of a chemical indexing mixture. Analyte retention coordinates were normalized in the primary dimension with respect to a series of n-alkanes (Kovats index) and in the secondary dimension with respect to a series of aromatic hydrocarbons (Lee index). Fatty acid profiles extracted from the templates were compared by multidimensional scaling (MDS) and principal component analysis (PCA). Differences in the profiles of Gram positive and Gram negative bacteria were observed, and a series of heterogeneous mixtures comprised of different fractions (containing one Gram positive and one Gram negative bacteria strains) were also distinguished from their homogeneous constituents.

Imaging Technologies, Single Molecule Imaging, and Super-Resolution I

1699

Two-color, 3D super-resolution imaging of bacterial protein ultrastructures with the double-helix point-spread function microscope.

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We have implemented a two-color 3D imaging approach for visualizing and colocalizing different protein ultrastructures in bacteria with a precision beyond the optical diffraction limit. Our experiment makes use of the recently introduced double-helix point-spread-function (DH-PSF) microscope and single-molecule photoblinking, which allows quantitative 3D super-resolution imaging of fluorescently tagged proteins throughout a ~2 μm axial depth of field. We apply the multicolor version of the DH-PSF methodology using genetically encoded fluorescent protein fusions in live *Caulobacter crescentus* cells and visualize the subcellular organization of critical cell-cycle dependent proteins, such as PopZ, at the cell pole. Fluorescent emission from two fluorophores is collected in two separate detection channels with negligible cross-talk between them, thus providing clear fluorophore identification. Quantitative overlay of the super-resolved 3D reconstructions obtained in these two detection channels is made possible by computational 3D image registration during post-processing. We show that the precision in distance measurements between any imaged fluorescent proteins within the same cell is chiefly limited by the localization precision of the individual single-molecule fluorophores themselves. This allows us to probe the relative subcellular organization within the bacterial cell, i.e. the size and

shape of neighboring protein ultrastructures. Furthermore, by sampling the cell surface using the PAINT technique, we can position the cell-internal fluorescent protein localizations with respect to the 3D spatial context of the cell's outer boundary.

1700

Resolving structural features in biological and biomedical imaging with STED super-resolution microscopy.

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Super-resolution optical microscopy is a rapidly emerging method that is creating multiple opportunities for biological imaging beyond the visible diffraction limit of ~250 nm. We have built a Stimulated Emission Depletion (STED) super-resolution microscope to image cells and tissue with 50-80 nm full-width-at-half-maximum resolution. Utilizing the sectioning ability of STED to reject out-of-focus fluorescence, we have probed planar cell polarity protein complexes tens of μm deep in intact *Drosophila* tissue at ~80 nm resolution (Proc. SPIE 7910:7910N-1-8).

The influence of labeling density in super-resolution microscopy studies is more critical than in diffraction-limited imaging. We have quantified labeling density effects in STED immunofluorescence imaging using the centriole as a model system (Biophys. J. 102(12):2926-2935). A key component of the microtubule organizing center in eukaryotic cells, the centriole is one diffraction limit in diameter and possesses a 9-fold radial symmetry. To our knowledge, no specific centriole component had been identified with this symmetry. We employed optimized immunolabeling to reveal that a particular centriole distal appendage protein, Cep164, localizes as 9 clusters spaced in a ring of ~350 nm diameter. The labeling density influenced the observed cluster number, size and brightness. As opposed to the averaged structure obtained from electron microscopy, the ability to simultaneously observe many single centrioles with optimized labeling allowed measurement of the distributions of behavior.

A hallmark of Huntington's Disease is the aggregation of mutant (polyQ-expanded) huntingtin (Htt) protein in neurons. Understanding the morphology of these aggregates may provide insight into the neurodegenerative mechanism. We have characterized a range of structures including inclusion bodies, fibers and perinuclear aggregates by STED microscopy of fluorescently labeled Htt-Exon1 in a neuronal cell-culture model. We found that individual fibers can be ~1-2 μm long and ~100 nm wide, while inclusion bodies often are up to ~10 μm in size. This work provides a detailed catalog of huntingtin structures for further investigations inside cells.

Our STED measurements are consistent with results from ensemble measurements by electron microscopy while avoiding the complex sample preparation required by the latter. This work shows that STED microscopy is an excellent tool for elucidating structural features in a variety of biological and biomedical applications.

1701

Enzymatic activation of nitro-aryl fluorogens in live bacterial cells for Enzymatic Turnover Activated Localization Microscopy beyond the diffraction limit.

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Many recently developed super-resolution (SR) microscopies (e.g., PALM, FPALM, STORM) based on single-molecule (SM) fluorescence require the conversion of a dark fluorogen into a bright emitter. This process allows the experimenter to control fluorophore concentration, enabling sparse sets of single-molecule fluorophores to be successively imaged and localized with spatial resolution 5-10 times smaller than the diffraction limit of ≈ 200 nm for visible wavelengths. This work uses a new strategy to control fluorescence emission for SR imaging: the enzymatic activation of a small-molecule and cell-permeable fluorogen, or Enzymatic Turnover Activated Localization Microscopy (ETALM), and demonstrates its use in live bacterial cells. Previous work using fluorogenic substrates enabled detailed SM enzymology studies, but without extracting subdiffraction information. Our study combines synthesis, bulk spectroscopic measurements, and microscopy (both *in vitro* on a surface and *in vivo* in cells). Synthesis of model compounds and optical spectroscopy identify a hydroxyl-amino derivative as the product fluorophore, which is bright and detectable on the single-molecule level for fluorogens attached to a surface. The fluorescent hydroxyl-amino derivative emits an average of 1.7×10^6 photons per SM in the aqueous surroundings of poly(vinyl alcohol). Solution kinetic analysis shows Michaelis-Menten rate dependence upon both NADH and the fluorogen concentrations as expected with fit parameters k_{cat} of 224 ± 40 s⁻¹ and K_M^{NADH} of 13 ± 3 μM and a $K_M^{\text{fluorogen}}$ of 4.9 ± 1 μM . The generation of low concentrations of single-molecule emitters by enzymatic turnovers is used to extract subdiffraction information about localizations of both fluorophores and nitroreductase enzymes in *B. subtilis* with an average of 17.9 ± 0.3 nm statistical localization precision. Together, this work shows that ETALM is a complementary mechanism to activate SM fluorescence to image beyond the diffraction limit in biological systems.

1702

Noninvasive Imaging of Three-Dimensional Dynamics in Thickly Fluorescent Specimens Beyond the Diffraction Limit.

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Understanding of cell activities often requires live fluorescence imaging with high spatial and temporal resolution. However, 4-D (space and time) fluorescence imaging is challenging, due to tradeoffs between spatial resolution, temporal resolution, photobleaching and photodamage. Here, we report a 4-D fluorescence imaging technique with spatial resolution beyond the diffraction limit and temporal resolution at the level of seconds per 3D volume which induces minimal photobleaching and sample damage. The method (Bessel SR-SIM) combines ultra-thin planar illumination produced by scanned Bessel beams with super-resolution structured illumination microscopy. We demonstrate high speed super-resolution Bessel SR-SIM from single cell to multicellular length scales, including: cell membrane and actin cytoskeleton dynamics in cancer cells, fibroblasts, and *D. discoideum*, *in vivo* karyotyping and tracking of mitotic mammalian cell kinetochores and chromosomes from prophase to anaphase; and development within *C. elegans* and *Drosophila* embryos. Furthermore, by combining Bessel SR-

SIM with two-photon excitation, the imaging capability can be extended to large multicellular samples such as *C. elegans* larval worms and the adult brain of *Drosophila*. In comparison with widely adopted spinning-disk confocal imaging and other live super-resolution imaging techniques on samples at different scales, Bessel SR-SIM shows significant advantages in balancing spatial resolution, imaging speed, photobleaching and sample damage, especially on thickly fluorescent specimens.

1703

Probing neural circuitry in the retina with molecular specificity through serial section reconstruction and stochastic optical reconstruction microscopy (STORM).

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One of the main challenges in biology is to understand how the molecular structure and function detected at the micro-scale leads to an observed phenotype at the cellular and higher order macro-scales. While conventional light microscopy allows for both molecular specificity and comparative studies using several spectrally separated probes, its use is precluded in the investigation of most molecular scale interactions due to the technique's resolution barrier imposed by the diffraction limit of light. Conversely, serial section electron microscopy allows for nanometer resolution throughout a sample volume, but is limited in its power to probe molecular interactions due to its monochromatic nature. Within the past decade, several super-resolution fluorescence microscopy techniques, including stochastic optical reconstruction microscopy (STORM), have succeeded in circumventing the diffraction limit of light microscopy, thereby allowing for the unprecedented visualization of molecular organization in a wide range of systems from bacterial chromosomes to neuronal synapses. Here, we combine STORM with ultra-thin serial section reconstruction across hundreds of sections of neuronal tissue to achieve truly volumetric imaging at a molecular scale with four spectrally distinct probes. By utilizing the transgenic YFP-H mouse line, which labels a sparse population of ganglion cells within the retina, in combination with membrane markers necessary for proper serial section alignment, we demonstrate nearly complete reconstructions of the dendritic arbors of retinal ganglion cells (~100µm in diameter) and their cell bodies with a lateral resolution of 20-30nm and an axial resolution dictated by our section thickness (50-70nm). By also probing against targets of both the presynaptic cytomatrix (Bassoon, Munc13, ELKS, and Piccolo) and the postsynaptic density (Gephyrin) within these reconstructions, we are able to investigate the distribution and organization of both excitatory and inhibitory synapses within the volume as a whole, as well as identify those synapses which are specifically associated with the YFP+ neuron of interest. Through this combination of ultra-structural information and molecular specificity, we are able to probe both structure and function over four orders of magnitude and begin to explore how the organization and integration of these competing signals leads to the emergence of an observed functional output at the level of the entire neuron.

1704

Probing the Nuclear Pore Complex Architecture by Super-Resolution Microscopy.

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The nuclear pore complex (NPC) is the exclusive gateway for transport between the nucleus and the cytoplasm, including both proteins and RNAs. As a giant macromolecular complex (~1000 Å in size), the NPC represents a great challenge to current structural biology methods. Here we demonstrate that super-resolution microscopy can resolve individual nucleoporins

(nups) in the yeast NPC, which provides structural insights into the architecture of the NPC in situ. Specifically, we use stochastic optical reconstruction microscopy (STORM), which localizes individual fluorescent molecules in a structure by stochastically switching them between a fluorescent and a dark state. STORM has demonstrated a three-dimensional localization precision of ~20 nm.

A key challenge for precisely determining nup positions is to label them specifically in a cell with bright fluorescent dyes. To address this issue, we have explored the enzymatic approach, by fusing the target nups to a small enzyme which can covalently link to a fluorescently labelled small molecule substrate. Among the available enzymatic tags, we have discovered that the widely used SNAP-tag interferes with the attached cyanine dye, reducing the fluorescence signal as well as increasing photobleaching. We have identified HALO-tag as a complimentary approach.

The enzymatic approach allowed us to label and image single nucleoporins in fixed yeast cells and to measure the radial positions of 10 nups in the NPC. We believe that this approach can be generally applicable to many other molecular complexes, especially those difficult to be purified and/or reconstituted *in vitro*.

1705

Ultra-High Resolution Imaging Reveals Formation and Preponderance of neuronal SNARE/Munc18 Complexes in situ.

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Membrane fusion is mediated by complexes formed by SNAP-receptor (SNARE) and Sec1/Munc18-like (SM) proteins, however the precise modes of molecular interactions between SM and SNARE proteins and when and how these complexes assemble inside the cell is presently unclear. Here we describe an improved fluorescence nanoscopy technique that can achieve effective resolutions of up to 7.5 nm FWHM (3.2 nm localization precision), limited only by stochastic photon emission from single molecules. We use this technique to dissect for the first time inside a cell, the spatial relationships and molecular interactions between the essential components of the membrane fusion machinery, the neuronal SM protein Munc18-1 and SNARE proteins syntaxin-1 and SNAP-25. Strikingly, we observed nano-scale clusters consisting of syntaxin-1 and SNAP-25 that contained pools of associated Munc18-1. Rescue experiments with syntaxin-1 mutants revealed that Munc18-1 recruitment to the plasma membrane depends on the Munc18-1 binding N-terminal peptide of syntaxin-1, and occurs through interactions with the open conformation of syntaxin-1 that is permissive to SNARE-complex assembly. Extensive complimentary biochemical experiments further corroborate our imaging results. Our data provide unexpected insights into the spatial organization and association of Munc18-1, SNAP-25 and syntaxin-1 on the neuronal plasma membrane, and suggest that recruitment of an SM protein to an on-pathway tri-partite SM - syntaxin - SNAP-25 complex could be a general mechanism for setting the stage for SNARE-mediated membrane fusion reactions. Our high-resolution imaging approach provides a framework for investigating interactions between the fusion machinery and other sub-cellular systems in situ.

1706

Techniques for reducing background fluorescence for single molecule imaging of processive myosins: linear Zero Mode Waveguides (ZMW) and Convex Lens Induced Confinement (CLIC).

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It is often the case that enzymatically useful concentrations of fluorescent species cause a high enough background that it is difficult to resolve single fluorescent molecules. For example, with total internal reflection fluorescence (TIRF) microscopy, single molecules can only be detected in the presence of an approximately 100 nM concentration of fluorophores, whereas many biological interactions require significantly higher concentrations. We present the application of two techniques, linear Zero Mode Waveguides (ZMW) and Convex Lens Induced Confinement (CLIC) for increasing that concentration in studies of processive myosins V and VI. Both techniques limit the excitation volume to a region significantly closer to the coverslip surface than is possible with TIRF. ZMWs use small holes in an aluminum film to confine laser excitation to a region very close to the coverslip surface [1]. We demonstrate a rectangular ZMW geometry that allows the observation of long (~1 micron) processive runs of myosin molecules. We show that actin can be inserted into linear ZMWs by polymerizing filaments inside the waveguides, and that myosin V will walk processively along these actin filaments. CLIC mechanically confines fluorescent molecules to a thin region between two glass surfaces [2]. We demonstrate a new version of this technique that produces confinement within a flow cell device, allowing for easier implementation and smaller sample volumes. We show that myosin VI can walk processively on actin in the CLIC device at a high degree of mechanical confinement (<50 nm). It is likely that these techniques will have many wider applications to observing enzymatic reactions with other single molecule systems, such as other processive motors, including myosins, kinesin, and dynein. An important future application of these technologies is in imaging single fluorescently labeled nucleotide molecules interacting with motors as they undergo processive motion in order to probe chemomechanical coupling and mechanisms of gating. Increasing the possible concentration of fluorescently labeled nucleotide would be a very valuable tool for such experiments.

[1] Levene, et al. (2003). *Science*, 299, 682–6.

[2] Leslie, et al. (2010). *Anal Chem*, 82(14), 6224-9.

1707

High-resolution protein localization in yeast using correlative super-resolution and electron microscopy.

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Specific recognition signals that govern quality control within the secretory pathway provide a basic framework for determining trafficking mechanisms of recombinantly expressed GPCRs. Using high-resolution correlative microscopy approaches, we have investigated processes that regulate the heterologous expression of two human adenosine receptors, hA2aR and hA3R, and engineered chimeras of these two receptors in yeast, *Saccharomyces cerevisiae*. To

improve functional production (i.e. ligand-binding yields indicative of active receptors) of GPCRs, we assessed protein structure/function through rational engineered chimeras. By implementing DNA recombination strategies combined with high-resolution imaging techniques, we have determined the intracellular localization of heterologous GPCRs in yeast compared to endogenous organelle markers at high spatial resolution using the super-resolution techniques Structured Illumination Microscopy (SIM), Fluorescence-Photoactivation Localization Microscopy, (F-PALM) and direct stochastic optical reconstruction microscopy (dSTORM). Specifically, we investigated discrete subpopulations of tagged proteins using correlative transmission electron microscopy and scanning electron microscopy with dSTORM (AlexaFluor® 488 and AlexaFluor® 633) and F-PALM (photoconvertible GFP variant mEos2). We will describe optimized protocols using cryo-fixation of high-pressure frozen freeze-substituted yeast cells followed by various epoxy and lowicryl resin infiltration regimen. The goal of this work was to obtain the best morphological preservation at the electron microscopy level while at the same time maintaining optimal fluorescence signal for super-resolution imaging, thus facilitating the identification of sub-cellular organelles and compartments where fusion proteins localized.

1708

Color-shifting FRET sensor for detection of phagocytic phase of apoptosis.

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Apoptotic cell clearance is a collaborative cellular event where the corpses of cells which committed suicide are rapidly eliminated by phagocytosis. This phagocytic reaction is critical for normal apoptosis completion. Its disturbance can promote self-immunization, inflammation and the release of viral or tumor DNA. Therefore it is essential to have a specific assay capable of detecting such an engulfment. To solve this problem, we developed the first FRET sensor which selectively detects apoptotic cell engulfment in cell suspensions and cultures. The sensor signals its detection of apoptotic cell engulfment by shifting color of its fluorescence within minutes after addition to cells.

The new assay is useful in studies where detection and quantification of apoptotic clearance is essential, such as cancers, inflammation, infection and auto-immune disorders.

1709

Imaging protein-protein interaction within neurons *in situ* through FRET quantification of GFP lifetime.

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Molecular signaling results from direct associations of proteins, which in turn are highly sensitive to their immediate molecular environment of a given cell as well as to various surroundings of the organisms. In the past decade, the networks of interacting proteins have been systematically catalogued with yeast-two-hybrid and co-affinity purification analyses. These assays address exclusively protein associations and are amenable to high-throughput screens. They are, however, designed to yield molecularly amplified digital output with little information on restrictions in time and space imposed from the dynamic yet complex contexts of living organisms. Moreover, conducted in an either non-native or isolated unicellular environment, positive and negative protein interactions demonstrated might not reflect those that would occur in the native cellular environment within intact animals. In contrast, the iSPIN project takes a

novel approach to characterizing the protein-protein associations through direct visualization *in vivo* (see www.ispinproject.org). The project adopts distance-dependent Förster resonance energy transfer (FRET) as its quantification principle. It also combines the molecular genetics to systematically tag diverse proteins with genetically encoded green fluorescent protein (GFP). Positive evidence for the nanometer-scale association by signal partner proteins can be captured *in vivo* using a diffraction-limited fluorescent microscope that is capable of FRET quantification. Compatible with live imaging within multicellular model organisms such as *Drosophila*, the iSPIN project is capable of evaluating the influence of native molecular environment from specific cellular contexts on the protein-protein interaction networks throughout the dynamic development of animals under normal and medically relevant conditions. Here, we describe the fluorescent lifetime imaging microscope (FLIM) tuned specifically for quantifying GFP lifetime in three-dimensional (3D) biological samples and present a proof-of-concept demonstration of nanometer-scale physical association occurring between two known signal partner proteins, Cdc42 (cell division control protein 42 homolog) and WASp (Wiskott–Aldrich Syndrome protein), within the molecular environment of intact neurons in the model organism. We propose that FRET quantification of GFP lifetime could serve as a basis for characterizing dynamic interactions among these and other proteins directly within native molecular environment *in situ*.

1710

Measuring actin flow in cell protrusion in 3D.

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Cells generate forces through the cytoskeleton that are transmitted to the extracellular matrix via cell adhesion in order to adhere and migrate. Though cellular force generation has been studied in detail in 2D environment, less is known about cytoskeleton dynamics of cells embedded in natural 3D matrices. Fluorescent Speckle Microscopy (FSM) has been used to capture high-resolution images of actin turnover dynamics within live cells in 2D. However, this method is not applicable when cells are in 3D environment due to lower resolution and signal level.

In this work we developed a new method to capture actin flow in 3D with high spatial-temporal resolution. nSPIRO and correlation spectroscopy techniques were combined to show the directional flow of actin in 3D live cell. MDA-MB-231 cells with actin-GFP expression were cultured in type I collagen. The laser bin was oscillating back and forth at nearby two cross sections on cell protrusion, while scanning in orbit manner at each plane. The orbit radius was modulated in order to detect actin flow in the center of the protrusion. The intensity profiles from two positions were then cross-correlated.

We found that actin in cell protrusion in 3D is relatively stable. Most of the measurement showed no correlation of two positions while some showed directional flow near protrusion surface. The actin flow rate of cell protrusion in 3D measured using this method is in the order of seconds, which is comparable to 2D results using FSM. With this method, we are able to visualize and quantify actin flow in 3D without the need of special speckle vectors and with high spatial-temporal resolution.

1711

Using a novel genetic tag compatible with electron microscopy to determine ultrastructural localization of ciliopathy associated proteins.

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Primary cilia are a microtubule based organelle that function in a sensory capacity and can be found on almost all cell types. This organelle is important for a copious amount of critical signaling events during many developmental processes. Ciliopathies are diseases caused by the dysfunction of primary cilia and result in severe developmental defects. Many ciliopathy proteins localize to a region at the base of the cilium called the transition zone (TZ). The TZ is thought to be important for the establishment of the cilium as a unique signaling center due to its ability to control protein access to the cilium, possibly via the unique structures of this region. Some of the proteins that cause ciliopathies such as Nephronphthisis and Meckel Gruber Syndrome are known to localize to the TZ and form two distinct yet genetically interacting complexes. Loss of these proteins in *C. elegans* has shown ectopic entry into the cilium of non-ciliary proteins indicating a breakdown of the specialized ciliary barrier. Based on the loss of critical architectural components in TZ mutants it is hypothesized ciliopathy proteins comprise these important structures. Ultrastructural localization of TZ ciliopathy proteins and their contribution to these structures will be determined using *C. elegans* and mammalian cells expressing Nephronphthisis and Meckel Gruber associated proteins tagged with a novel genetic tag (miniSOG), visible through electron microscopy. This will expand our understanding of where these proteins are localized and the role they play in ciliary gating, disease, and developmental processes.

1712

A hybrid confocal & atomic force microscope for probing biophysical properties of migrating cells.

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Directed cell motility plays a central role in a variety of biological processes including development, wound healing, inflammation, and metastasis. The process of cell crawling is highly complex, and occurs with spatiotemporal coordination between components of the cell molecular machinery. In order to better understand the biophysical mechanism of cell protrusion, we have built a hybrid AFM / confocal microscope that allows us to image the cytoskeleton of individual migrating cells and at the same time measure cell mechanical properties during lamellipodial extension or retraction.

We apply contact AFM techniques in conjunction with the recently developed Bottom Effect Cone Correction to the Sneddon model for estimating the Young's modulus in different regions of crawling or retracting cells, and in particular in very thin regions such as the cell lamellipodium. Moreover, we use non-contact frequency modulated atomic force microscopy to calculate cellular stiffness and viscosity. Stiffness measurements on transfected PtK-1 cells reveal that the cell stiffens as it retracts while the lamellipodium is softest during the extension phase. Measurements on cells transfected with fluorescent constructs allow us to focus our measurements on specific cell structures, such as focal adhesions or stress fibers.

1713

Time-resolved nanometer scale AFM imaging of antimicrobial peptide activity on live *Escherichia coli* cells.A. Slade¹, J. H. Kindt¹, S. C. Minne¹; ¹*Bruker Nano Inc., Santa Barbara, CA*

Understanding drug-membrane interactions is crucial to drug research and development. Bacterial membranes have a much more complex structure than mammalian cell membranes. As such, knowledge of bacterial membrane composition and organization, as well as characterization of the molecular-level responses to drug interactions, is critical to the development and assessment of effective drug formulations. Cellular drug responses involve highly dynamic processes. However, the ability to image live cells with nanometer resolution on timescales relevant to dynamic cellular events has proven challenging. The ability of atomic force microscopy (AFM) to obtain three-dimensional topography images of biological molecules with nanometer resolution and under near-physiological conditions remains unmatched by other imaging techniques. However, with traditional AFM systems, the typically longer image acquisition times required to obtain a single high-resolution image (~minutes) has limited the ability to investigate dynamic biological processes. While recent years have shown significant progress in the development of high-speed atomic force microscopy (HS-AFM), the nature of the instrumentation that has been developed has several drawbacks in specimen size, requiring small scan sizes and flat sample surfaces. As such, the majority of biologically-related HS-AFM studies have concentrated on imaging single biomolecules with little focus on using HS-AFM to examine cellular processes. With the rapidly growing antibiotics crisis, antimicrobial peptides (AmP) are increasingly being investigated as therapeutic alternatives. Key to their success is an understanding of the mechanisms by which AmPs interact with the cell membrane and facilitate cellular death. Using HS-AFM, we have obtained the first high-resolution time sequence images of the native structure of a bacterial outer membrane, obtained directly on the surface of live *Escherichia coli* cells. The increased time resolution of HS-AFM allowed us to observe dynamic changes in the nanoscale structure of the outer membrane in direct response to the AmP CM15, at timescales relevant to the mechanism of AmP-induced cell death. The results of these HS-AFM studies have provided the first opportunity to resolve the dynamics of AmP-mediated cell death in a native cell membrane environment in real-time and with nanoscale resolution.

1714

RNA sampling and analysis with single living cells using Atomic Force Microscopy.M. Tom-Moy¹, K. Bernick¹, A. Sridhar¹, K. Pekrun¹, A. Yamada¹; ¹*Agilent Laboratories, Agilent Technologies, Santa Clara, CA*

The ability to sample specific biological molecules from individual, live cells without disruption of their cellular environment presents new opportunities in cell biology measurements. In order to achieve this sampling, we have adapted a combined AFM and fluorescence microscope to visualize and manipulate single, living cells for the extraction of cellular material such as RNA, protein and protein-RNA complexes. Using an AFM probe functionalized with streptavidin on an Agilent 6000 AFM, we have extracted beta-actin mRNA from a single living rat fibroblast. Beta-actin mRNA was visualized by biotinylated Cy5-labeled oligonucleotide-based FISH probes, introduced into living cells by cell penetrating peptides. Live cell imaging of these cells confirmed that probes successfully hybridized to target RNA first at 4 hours with optimal signal at 24 hours. The AFM probe was inserted 2 micrometers into the cell for 5 minutes before being retracted. Cell membrane puncture by the AFM tip was confirmed by using the force-curve feature of the AFM. After collection of the RNA inside the living cells, the retracted tip was washed extensively and analyzed by PCR for the presence of beta actin mRNA signal. Specific controls confirmed the positive PCR signal for beta actin RNA was from the single cell that the

probe penetrated and not from other external sources such as the media, other cells, or non-specific binding to the AFM tip. We are currently extending this method to the detection of various genes in other cell types, such as stem cells and induced pluripotent cells. The ability to measure gene expression in single cells, as we show here, will provide a more accurate representation of cells in a population, permitting a more complete picture of the expression networks that underlie cell and tissue function.

New Technologies for Cell Biology II

1715

Visualization and analysis of fluorescent labels in energy filtered TEM.

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Precise localization of functional proteins in tissue and cellular samples is frequently used for biological and medical applications. For localisation a variety of specific fluorescent markers is available in light microscopy. Linking fluorescent light microscopical information to high resolution, ultrastructural data obtained by transmission electron microscopy (TEM) is a demanding task and the identification of labeled molecules at nanometer scale is still a challenge.

Energy filtered TEM (EFTEM) offers both: high spatial resolution and additional analytical imaging modalities for distinguishing materials by their spectral characteristics of electronic interactions. In EFTEM electron energy loss spectra (EELS) can be recorded as 3D image data and thus it is possible to visualize and localize different materials by their specific excitations of valence electrons.

We show that fluorescent labels such as quantum dots (Qdots) can be localized and identified by analyzing their characteristic energy loss in the low energy region (bandgap and plasmon excitations) using EFTEM. This method has the potential to visualize functional labels: It allows collecting spectral information with spectral energies from 1 - 3.5 eV energy-loss corresponding to light-optical absorptions, to the UV optical range (> 3.5 eV) up to plasmonic interactions (up to 30eV). Using both a Carl Zeiss LIBRA 200[®] MC equipped with an aberration corrected in-column omega filter, a monochromated electron source (CEOS, Heidelberg) and a Carl Zeiss LIBRA 120[®] PLUS energy filtered images from 0 eV to 30 eV energy-loss were acquired. Spectra were extracted from the 3D EFTEM data cube for each image pixel and classified via Multivariate Statistical Analysis (MSA) [1]. Data and a comparison of results for different acceleration voltages are presented. Ultrathin sections of muscle tissue labeled with Qdots were studied. We were able to distinguish Qdots and heterochromatin based on their specific energy loss signals.

Results from our experiments and previously published data indicate that the described method can be used to visualize Qdots in EFTEM by characteristic energy losses in the low loss region. We conclude that Qdots have the potential for multimodal analysis in the field of correlative microscopy. The possibility of directly detecting Qdots and fluorescent markers in EFTEM allows a combination of functional and ultrastructural studies by using a single instrument.

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[1] Pfanmüller et al., *Nano Lett.* 2011, 11, 3099 - 3107.

1716

A simple imaging-based method to characterize protein-protein interactions *in vivo*.

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Over the last decades there has been an explosion of new methodologies to study protein complexes. However, most of the approaches currently used are based on *in vitro* assays (e.g. NMR, X-ray, EM, ITC etc). *In vivo* studies are often limited to stable complexes or they cannot be used to quantitatively characterize the properties of the complex. Additionally, these approaches are often performed in a context far from the normal physiology of the target proteins (e.g. yeast two-hybrid method). Methods such as FRET or FCCS allow a more quantitative analysis of protein-protein interactions (PPIs) at conditions closer to normal physiology, but are technically challenging and therefore difficult to apply to high-throughput studies.

Here we present PICT (“PICT is Imaging of Complexes after Translocation”), an approach that provides a very simple readout for the analysis of direct and indirect PPIs in living cells, including transient interactions. This approach is based on the rapamycin-induced heterodimerization of the FRB and FKBP domains. The FKBP domain is fused to an anchor protein that has a distinct and stable localization. The protein of interest (“bait”) is fused to the FRB domain. Prey proteins, tagged with GFP, will co-translocate with the bait to the anchor sites upon addition of rapamycin if they interact with the bait. Consequently, PPIs are encoded as a change in localization of GFP-tagged prey proteins.

We show the potential of PICT by analysing different protein complexes in the yeast *Saccharomyces cerevisiae*. For example, we characterized the PPIs within the Ste5-Ste11-Ste50 complex of the pheromone-activated Mitogen-activated protein kinase (MAPK) cascade. We also combined PICT and FRAP methods to quantitate the transient interaction between the endocytic proteins Syp1 and Ede1.

In summary, PICT is a novel approach to study PPIs *in vivo*, follow their behaviour upon different perturbations and facilitate their characterization with additional methods such as FRAP. Importantly, due to its simple microscopic readout, PICT can be easily automatized for high-throughput screens.

1717

High-throughput real-time analysis of fluctuation in cell oxygenation using a fluorescent intracellular oxygen-sensitive probe.

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Tissue and cell oxygenation is of critical importance to a large variety of disease states and adaptive responses and as researchers strive for more biologically relevant *in vitro* models, the ability to both control and monitor this parameter is of growing importance. Here we describe a novel plate-reader based method of monitoring cell oxygenation using an intracellular oxygen-sensitive probe and apply the method to both 2D and 3D cultures generating real-time oxygenation profiles in oxygen scale. The data generated using a plate reader equipped with an atmospheric control unit reveals that significant differences can exist between the oxygen concentration that pertains at the cell monolayer and the applied ambient oxygen concentration,

and that this difference is dependent on both the applied oxygen concentration, cell confluence and the basal metabolic activity of the test cells. Data is also presented illustrating that the degree of monolayer hypoxia can be modulated by altering the oxygen permeability of the test plate thereby allowing more precise control of cell oxygenation. The impact of metabolic stimulation and inhibition is also examined as is the potential for multiplexing with additional informative endpoints such as fluorometric assessments of mitochondrial membrane potential, reactive oxygen species and glycolytic flux measurements thereby providing a more complete measure of monolayer hypoxia as well as a real-time measure of the metabolic implications of stress imposed.

1718

Measuring Barrier Tissue Integrity with the Organic Electrochemical Transistor.

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The ability to measure paracellular transport provides a wealth of information about barrier tissue function, and disruption or malfunction of the structures involved in transport through barrier tissue is often indicative of toxicity or disease. The use of barrier tissue models in combination with different measurement technologies has hitherto been hampered by invasive, expensive, slow to respond and difficult to implement methods.

To respond to this challenge, we have integrated the organic electrochemical transistor (OECT) with both epithelial and endothelial cells to model both the gastrointestinal barrier tissue layer, and the blood brain barrier respectively, as a means of assessing barrier tissue function. The ability of organic electronic materials to interface with biological systems has been much vaunted of late, with examples showing the elegant use of these materials to deliver neurotransmitters in vivo, control cell adhesion and migration and measure neuronal activity in vivo. With the unique ability of organic electronic materials to conduct both electronic and ionic carriers, they act as an ideal platform for the integration of electronic and biological systems. The OECT has been recently employed as a sensor for DNA, enzymes and cell attachment. The OECT is particularly suited for sensing due to inherent amplification of signal provided by the transistor.

In this study we demonstrate the ability to measure minute variations in paracellular ionic flux induced by toxic compounds in real time, with unprecedented temporal resolution and extreme sensitivity. Addition of concentrations down to 1 mM hydrogen peroxide, in complex cell media, to Caco-2 epithelial cell layers, was detected within 30 seconds. Our results are validated by comparison with existing methods used to evaluate barrier tissue layers, including immunofluorescence of tight junction proteins, a permeability assay using a fluorescent tracer (Lucifer Yellow) and a commercially available TER measurement system (Cellzscope, Nanoanalytics).

The integration of commercially available cell culture filters with the OECT demonstrates a sensing device in a format that is compatible with low cost manufacturing techniques, of great importance for high throughput screening. This work constitutes an elegant example of harnessing the power of engineering for relevant research in biological sciences. Future development of this technology will have implications for toxicology, drug development, reduction of animal use by enabling in vitro models, infectious disease diagnostics and basic research on the molecular biology and electrophysiology of barrier function.

1719

Long-term simultaneous dual measurement of electrophysiological properties and mechanical responses of cardiomyocytes using on-chip extracellular field potential recording and real-time optical image analysis.

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Quantitative evaluation of mechanophysiological responses in cardiomyocytes has becoming more important to acquire more precise prediction of cardiotoxicity, especially relationships between mechano-chemical coupling in their electrophysiological responses during their adaptation process is one of the important topics of remodeling of cardiomyocyte networks in heart. Hence, for the evaluation of correlation between mechanophysiological effects and electrophysiological effects of drugs, we have developed an on-chip simultaneous dual measurement system that records the extracellular field potentials and the contractile motions in lined-up cardiomyocyte network with non-invasive/destructive measurement for long-term experiment. Cardiomyocytes were seeded in rectangular microchambers fabricated on the multi electrode array (MEA) chip for the field potential recording. To detect contractility of cardiomyocytes, the cells were labeled with the polystyrene beads attached on the surface of target cells and the motions of beads were acquired and analyzed using a newly developed wider depth of field optics equipped with 1/100 s high-speed digital camera. Electrophysiological properties [interspike intervals and field potential durations (FPDs)] and mechanophysiological properties [displacements, velocities, and direction of displacements of beads] were obtained simultaneously using real time processing system module. In the presence of verapamil, typical L-type calcium channel blocker, beating rate of cardiomyocytes decreased accompanying with shortening of FPD and its fluctuation increase, and contractility was weakened significantly with its fluctuation increase. Correlation of fluctuations of the FPD and force generation (the direction of displacement) was also examined and observed, and confirmed that the increase of both of fluctuation reflects the increase of inhomogeneity of tension generation of cardiomyocytes in the network according to the increase of uncertainty of electrophysiological responses. The results indicate that the fluctuation of force generation should also become an effective biomarker of cardiotoxicity risk more correctly in drug development, also might be used for the clarification of mechanisms especially for the adaptation process of cardiomyocytes from the viewpoint of spatiotemporal mechanoelectrophysiology.

1720

Surface Modification of a Reconfigurable Co-Culture Device to Assess the Effect of Substrate Stiffness and Cell-Cell Interactions.

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Co-culture technology allows one to understand how one cell type affects another. There is a need to improve existing technologies to allow researchers to better understand what factors affect cell behavior, particularly for stem cell differentiation. Recently, a novel reconfigurable co-culture device was developed which allows a user to study physical cell-cell interactions and paracrine effects in an organized fashion. This device is an interlocking pair of silicon combs that allow the user to study organized co-culture in either "contact" or "gap" configurations (Hui, E et al. PNAS 2007). Our lab has shown that this tool is powerful, particularly for determining how mature cells affect progenitor cell differentiation (Rao et al., Biomedical Microdevices, accepted). Stem cell fate can be driven by the presence of other cell types and also by

changing the stiffness of the substrate that these stem cells feel. As a result, by adding a tunable surface stiffness to the device we show a synergistic effect when co-culturing at a specific stiffness. The surface of the device was treated for covalent attachment of polyacrylamide using slightly modified protocols (Tse et al. PNAS, 2007). Device pairs were locked in contact mode. Acrylamide, bisacrylamide and initiator were well mixed, added to the surface, and allowed to spread evenly with consistent height using coverslips. PA was photo polymerized by UV light for 10 minutes. AFM measurements were carried out by established methods (Engler et al. Cell 2006). Sulfo-Sanpah was used to attach 10 μ g/mL fibronectin. Adipose derived stem cells (ASCs) were used to test the ability of this technology to demonstrate that both co-culture and substrate serves synergistically to drive a myogenic lineage. Cells were seeded on individual comb halves, adhered, and placed together in a gap configuration. ASCs were cultured with ASCs or C2C12s on either the silicon combs or the combs coated with a 11kPA PA gels (4 groups total). The cultures were then stained with MyoD (an early myogenic marker), or ASC RNA was isolated, and qPCR normalized to GapDH was performed. MyoD was detected in all cultures except group 1. Myogenin was consistently upregulated on 11 kPA and in co-culture with C2C12s compared to other cultures. Likewise, Desmin showed increased gene expression in this group compared to ASCs on gel only or ASCs co-cultured with C2C12s on silicon ($p < 0.05$). By using ASCs as proof of concept, we have demonstrated that we can develop a novel technology that can parse out whether substrate stiffness, cell-cell contact, and cell-cell paracrine effects cause changes in differentiation.

1721

Monolayer Stress Microscopy: Uncovering inter- and intra-cellular forces in cell sheets.

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In wound healing, tissue growth, and certain cancers, the epithelial or endothelial monolayer sheet expands. Within the expanding monolayer sheet, motion of the individual cell is strongly guided by physical forces imposed by adjacent cells. This process is called plithotaxis and was discovered using Monolayer Stress Microscopy (MSM). MSM rests upon certain simplifying assumptions concerning boundary conditions and cell material properties, however. To assess their validity and quantify associated artifacts, we relaxed these assumptions and then used rigorous numerical and experimental analysis. For several commonly used experimental monolayer systems, we show that the simplifying assumptions lead to errors that are quite small. Moreover, characteristic features of intercellular stresses that underlie plithotaxis are largely unaffected. Finally, stresses due to out-of-plane tractions have little effect upon in-plane stresses. Taken together, these findings define the broad limits of applicability of Monolayer Stress Microscopy.

1722

Polyacrylamide scaffolds for studying cellular responses to substrate stiffness in three dimensions.

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Recent developments in two-dimensional (2D) culture substrates with tunable stiffness and patterned adhesion ligands have demonstrated that biochemical and mechanical cues regulate the biological functions of living cells. We have extended these cell culture platforms into three dimensions (3D), as in complex biological systems, by producing highly ordered scaffolds of polyacrylamide coated with extracellular matrix proteins. We characterized important

parameters for ordered polyacrylamide scaffolds of tunable stiffness. We then grew individual fibroblasts in the identical pores of our scaffolds, examining cellular morphological, cytoskeletal, and adhesion properties. In a 3D scaffold, these cells sensed the surrounding stiffness as a value between the stiffness of bulk polyacrylamide and a scaffold. Many cells exhibited features similar to those of cells cultured in conventional 3D matrices. The cells were often elongated, with actin filaments concentrated at the cortical region and only forming thin stress fibres. We also observed the surprising phenotype of thick stress fibres similar to fibres detected in a 2D system, but these fibres did not occur ventrally. The rich variety of morphologies and anchoring strategies assumed by cells growing on our tunable 3D polyacrylamide scaffolds demonstrate the richness of cell-microenvironment interactions when cell adhesions are not confined to 2D surfaces.

1723

High content profiling of cellular invasion in 3D cell culture models.

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To-date, most invasion or migration assays use a modified Boyden chamber-like design to assess migration as single-cell or scratch assays on coated or uncoated planar plastic surfaces. Here, we describe an easy-to-use, 96-well, microplate-based, high-content, three-dimensional (3D) cell culture assay capable of assessing live kinetics and molecular signatures of cellular invasion. This assay will help to investigate aberrant motility processes of cells harvested from diseases like fibrosis, metastatic cancers, or chronic inflammatory states. Additionally, our technique is suitable for high-throughput pharmacological screening of novel compounds regulating invasive and migratory pathways, and is applicable to a wide spectrum of cells from different origins. Thus, we were able to demonstrate significant effects on the invasion capacity by comparing the adenocarcinomic human alveolar basal epithelial cell-line A549 (invasion of 1% of seeded cells) to the lung fibroblast cell-line Mlg 2908 (invasion of 9% of seeded cells). Treatment of Mlg 2908 fibroblasts with epidermal growth factor (EGF) even augmented the invasiveness of these cells (invasion of 18% of seeded cells). These results were further corroborated by live-cell imaging of invading fibroblasts. We could also monitor and measure significant changes in cell shape, cell surface area, cell volume and cell sphericity. By using DNA microarrays and qRT-PCR we observed a highly significant deregulation of various genes and proteins involved in migration in 3D collagen embedded fibroblasts. Furthermore, our method is easily expandable, as using a 3D collagen gel of different elastic moduli or adding additional ECM components like hydroxyproline or elastin might increase the versatility in the readout.

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3D Angiogenesis Assay with Concurrent Imaging and Multiplexed Biomarker Identification.

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It has been shown that angiogenesis is an important player in carcinogenesis and cancer cell metastasis, and thus is a promising target for anti-cancer drugs. Increasing evidence has shown that the conventional 2D tube formation assay on endothelial cells is inadequate to demonstrate clinical relevancy. In this study, we have adopted a commercially available microfluidic device which integrated 3D cell culture and continuous perfusion of reagents into a high throughput/content screening compatible format to develop a more physiologic-relevant angiogenesis assay. Since the reagents are continuously perfused through the 3D cultured

cells, this assay utilizes both imaging on the cell bodies as well as sampling of the flow-through volumes concurrently to correlate 3D sprout formation with multiplexed secreted biomarker identification to enable multi-facet profiling of the angiogenesis process. Using human umbilical vein endothelial cells (HUVEC) cultured in both 3D fibrin gel and traditional 2D format, the cells were exposed to various concentrations of known inducer PMA and inhibitor suramin, with regular culture medium as the control. Imaging of the sprout formation in 3D was conducted every 24 hours, with 50 μ L of the flow through volume collected at the same time for multiplexed biomarker identification with Luminex assay in order to quantify the secretion of Angiopoietin-2, Endothelin-1, Endoglin, FGF-1, IL-8, HB-EGF, PLGF, and VEGF-C. We observed that when grown in the absence of fibrin gel, the cells assumed a monolayer morphology with no noticeable angiogenesis activity, while in the fibrin gel, HUVEC exposed to pro-angiogenic factors for 24 hours arrested growth and exhibited sprouting behavior characteristic of angiogenesis, also evident by the biomarker assay. Sprouts were readily visualized under phase contrast imaging on an inverted microscope. Here, we have established a unique long-term perfusion 3D angiogenesis assay also with the co-culture potential that may improve drug screening and biological insight into this fundamental process.

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Micro-devices for imaging of cells and model organisms.A. Groisman¹, E. Gutierrez¹; ¹UC San Diego, La Jolla, CA

The imaging of model organisms and cells presents a variety of challenges. Long-term, high-resolution imaging of non-adherent cells (yeast, lymphocytes, oocytes) requires some degree of confinement and protection of cells from unwanted displacement by flow of the surrounding medium that may result from convection, motion of the microscope stage, or medium exchange. *C. elegans* worms are difficult to image, because they are constitutively motile and grow nearly 100-fold during their development. Zebrafish embryos are difficult to position with an optimal orientation because of their irregular shapes. Here we present different micro-devices for imaging of yeast, primary lymphocytes, *C. elegans* eggs and larval worms, and zebrafish embryos. Devices for yeast, *C. elegans* eggs, and lymphocytes have arrays of micro-wells at the bottoms of larger wells, which are accessible to pipetting. Yeast are mixed with low-melting agarose that solidifies in thin layers in micro-wells, both immobilizing yeast cells for high-resolution imaging and making them accessible to rapid and easy medium exchange. *C. elegans* worms carrying permeable eggs are loaded into a large well, where the worms are cut with a scalpel releasing the eggs. The eggs are then moved into micro-wells, where they are mechanically protected from flow. Therefore, the permeabilized eggs remain motionless, while the medium is exchanged (by aspirating the old medium and dispensing a new one), and can be imaged at a high-resolution. Primary human CD4⁺ T lymphocytes loaded with a pipette into small wells settle by the gravity into finger-like micro-channels, where their motion is restricted to nearly one-dimensional manifolds. The device is used to image the expression of fluorescent proteins caused by HIV-infection at a single-cell resolution over a 2-3 day interval at several different conditions in parallel. Micro-well devices for zebrafish embryos enable well-defined orientation of the embryos for prolonged high-resolution imaging of specific parts of their body. Finally, a microfluidic device enables longitudinal imaging of larval and adult *C. elegans* worms with periodic complete immobilization for collecting stacks of high-resolution confocal images in different regions of interest.

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Innovative "Confocal SEM" 3D microscopy in a biological context.

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Over the last decade electron microscopy techniques have evolved from delivering 2D to 3D information with a main focus on the architecture of organelles on a subcellular level. These techniques have yielded important new insights in intracellular architecture and connectivity of organelles.

However, understanding complex biological systems requires knowing how this high resolution 3D Electron microscopy data correlates with the situation in-vitro, or even better in-vivo, in a relevant biological system.

To obtain more complete and meaningful information about the biological processes within a cell developments have taken place to combine light microscopy data with electron microscopy data.

The main challenge we face today is not only the acquisition and the presentation of the data (the amounts of data are vast) but also the extraction of valuable information from this data. In the field of image processing the first important steps towards automated segmentation are made. The success of automatic segmentation algorithms will be a critical next step in the near future of large volume 3D electron microscopy data acquisition. What has recently become clear is that the quality of both automated and manual segmentations are very dependent on the raw data quality that comes directly from the EM. In TEM, as well as in SEM, the data quality in the image plane (X,Y) is typically good enough for 2D segmentation, but when moving into the 3rd dimension the data quality in the Z plane is determined by tilt limitations in the TEM when tomography is used. In an automated SEM approach the data quality is limited to the smallest section that can be cut before the next image is acquired. Hence both in SEM approaches as well as in TEM approaches, data quality is mainly limited in the Z-plane. Here we explore the possibility to obtain isotropic 3D electron-imaging information down to the membrane level.

In this work we will present two practical parts: first we elaborate on how light and electron microscopy domains are correlated in an easy and intuitive way to determine the area for high resolution 3D data acquisition; second we elaborate on how an innovative technique is used to collect the required high resolution 3D in this particular specimen location. We use multi-energy SEM image acquisition and deconvolution techniques to reveal the sub-surface 3D structure in a biological relevant area, hence obtaining the highly desired isotropic data quality.

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Endogenous protein tagging for fluorescence studies and identification of protein-protein interactions by High Throughput.

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Tagged proteins represent a powerful tool to study cell signaling cascades, protein-protein interactions and disease-related mechanisms in eukaryotic cells. Until now, it has been time-consuming and laborious to generate fluorescence-tagged cell lines that stably express tagged genes at or near physiological levels.

Here we present a powerful pipeline that enables us to tag nearly every gene of interest in a fast and reliable manner using bacterial artificial chromosomes (BACs). The most important

advantages of using BACs are that cell-type specific processing, regulation, and splicing of the gene of interest are maintained within the stable cell line. This is typically not included when using cDNAs. Furthermore, BACs containing a tagged target gene can be transfected into multiple human and mouse cell lines including tumor cell lines, embryonic stem cells and human neural stem cells.

The resulting stable transgenic cell lines then enable molecular characterization of for example unknown genes that have been identified in RNAi and proteomic screens. The lines expressing the tagged protein can be used in a variety of applications e.g. in fluorescence studies, by mass spectrometry to identify protein-protein interactions, and rescue experiments to monitor the specificity of an RNAi treatment (Ref.).

To increase the throughput of our pipeline we are currently building a customized automated cell culture robot that will enable us to increase our transfection ability by about 5 times. With this improved pipeline we plan to further increase our valuable resource of stable transgenic BAC cell lines, currently standing at 3000 lines.

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Expanding applications of BioID: A new screen for candidate protein interactions.

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Identification of protein-protein interactions is critical to the study of cell biology. Current methods to screen for these protein interactions, such as yeast-2-hybrid or complex purification, can present certain limitations. As a result, investigators often expend resources pursuing false-positives and/or miss relevant interactions. In attempt to overcome some of these limitations and provide an alternative approach to screen for protein interactions we have generated a method that is fundamentally different. Called BioID for proximity-dependent biotin identification, this method is based on fusion of a promiscuous biotin ligase to a protein of interest. When expressed in the presence of excess biotin, the BioID-fusion protein biotinylates vicinal proteins in live cells. This biotinylation permits the selective isolation and identification of these proteins. The net effect is an ability to generate a history of protein proximity in live cells that can be used as a screen for biologically relevant candidate protein interactions. Our initial use of BioID was with the A-type lamins, constituents of the nuclear lamina. Here we report new applications of BioID in different subcellular compartments and/or with a variety of proteins. We have utilized BioID with an integral membrane protein, oriented either towards the cytoplasmic/nuclear compartment or within the endoplasmic reticulum. BioID has also successfully been used in the mitochondrial matrix and applied to well-defined stable protein complexes of the nuclear pore complex. These studies considerably expand the applications of BioID and have refined our understanding of this new method.

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Integration of Automated Live Cell Perturbation and Quantitative Time-Lapse Imaging via a Multi-Well Microfluidic Perfusion System.

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Dynamic live cell assays offer better understanding of cellular behavior, however control and monitoring of living cells is limited by the technical challenge of performing precise perturbations without interfering with the microenvironment and/or removing cells from the analysis platform. CellASIC has recently developed microfluidics-based systems for perfusion-based microenvironment control and live cell analysis, integrating temperature, gas, cell loading and fluid control. This study integrated CellASIC's ONIX system with automated time-lapse microscopy and quantitative image analysis to create a state of the art platform for dynamic live cell analysis. All cell loading, culture and drug treatment steps were automated via the CellASIC ONIX system; thus cell culture and perturbation could be performed while the plate was on an automated microscope stage. We used HeLa cells expressing Cdt1-Germinin fluorescent ubiquitination-based indicator (FUCCI) fusion proteins as dynamic cell cycle sensors to perform quantitative analysis of the kinetics of cell cycle progression in asynchronous controls and cells synchronized by thymidine block (S-phase); and serum removal (G0/G1 block). Using the FUCCI system cells in G1 display red fluorescence; green in S, G2 and M phases; during G1/S transition both red and green are observed. Thus, dynamic cellular color change from red-to-yellow-to-green represents progression through the cell cycle. Automated time lapse brightfield and fluorescence images were acquired for up to 96 hours for multiple stage positions. For quantitative analysis of cell images, open-source CellProfiler software was used to perform cell segmentation and quantify object counts and intensities. We demonstrate that with this system, cell loading, culture and drug treatments are readily automatable, enabling multi-well cell perturbation and multi-well time-lapse imaging of cells in situ without the need to remove the plate from the microscope stage. Using CellProfiler software we quantified % of cells at each cell cycle phase over time in asynchronous and synchronized cell populations, enabling dynamic tracking of cell cycle progression, arrest, and release in response to each perturbation. This study provides proof of concept that integration of the CellASIC microfluidics platform with automated microscopy and image analysis enables programmable cell loading, perturbation and analysis over time in a physiologically relevant microenvironment, facilitating novel assay designs and hands-free execution. By integrating and automating precise cellular environment control and quantitative imaging a wide range of advanced cell biology applications become possible, enabling significantly improved research tools to investigate the complex and dynamic behaviors of living cells.

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Optogenetic Protein Clustering Allows Modular and Tunable Control of Diverse Signaling Cascades in Living Cells.

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Protein clustering is known to play an important role in cellular signal transduction for diverse classes of proteins, but its study is made difficult by a lack of effective methods to modulate a protein's oligomeric state within a living cell. The best current tools require the addition of an exogenous molecule to multimerize a transgene product, but these approaches suffer from poor spatial control and lack the capability for dynamic regulation. Optogenetic techniques have in recent years demonstrated the ability to modulate protein activity with spatiotemporal precision, though these methods have been limited to individually engineered proteins or protein dimers. In this work, we present a genetically encoded photoactivatable protein capable of clustering in a light dependent manner, and we demonstrate its utility in activating a diverse set of signaling pathways with high spatiotemporal resolution.

Cryptochrome2 (Cry2) is a light sensitive protein that has been reported to form light-dependent, oligomeric "photobodies". We have demonstrated for the first time that these photobodies can be formed inducibly, reversibly, and tunably in mammalian cells in response to blue light. We first established the utility of this capability by photoactivating the β -catenin pathway, and as a result can achieve a higher transcriptional response than obtained even with the natural ligand Wnt3a. We also used Cry2 clustering to robustly and dynamically activate the Rac1 and RhoA pathways with light. While Rac1 oligomerization has been reported to increase enzymatic activity in *in vitro* assays, notably this is the first report of activating Rac1 in living cells through inducible oligomerization. Furthermore, in creating a photo-activatable RhoA, to our knowledge this is the first demonstration of clustering as a mode of activation for this well-studied protein.

Light-induced clustering of target proteins may allow biological researchers to pattern signaling activity onto cells and tissues with unprecedented ease, and may allow systems biologists to interrogate β -catenin, Rac1, and RhoA signaling network responses to quantitatively controlled and time-varying signaling inputs. More generally, the ability to abstract Cry2-mCherry as a "plug-and-play" clustering module will enable scientists to rapidly expand the optogenetic toolbox and, provided that at least one node of a signaling network involves clustering, endow numerous other signaling pathways with photocontrol.

1731

Biochemical approach combined with immunometric tools to enable tracking metal uptake fluctuations in bacteria.

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A lot of attention has been drawn in the recent years to devising novel tools for quantitative analysis of chemicals associated with bacterial metabolism. Metal ions, often with toxic properties, are amongst these constituents that heavily affect viability of cells. Bacteria evolved intricate mechanisms of feedback, where specific protein transporters eject excessive amounts of contaminant out of the cell. The fluctuations are to restore balance before life processes suffer dent. To measure how strong adaptive aptitude of cells is we can track changes in their

microenvironment. While traditional analysis would involve costly equipment and people trained to operate it, alternative routes has been under investigation and expanding rapidly. Having studied successful cases, where elements such as mercury, cadmium or led were subject to determination, our laboratory has started a project thought to enable analysis of nickel in complex matrices, like food extracts or expansion media. Trace nickel does enrich bacterial media as an ingredient of yeast extract as well as a product of micro-corrosion in lab tools and equipment made of metal. A biosensor was composed of antibodies isolated from sera of rabbits immunized with protein complex bovine serum albumin-nickel and protein-chelate conjugate bovine serum albumin-dimethylglyoxime-nickel. Applied onto immobilized nickel-EDTA antigen and followed by anti-rabbit enzymatically labeled secondary antibody, the biosensor was shown to have potential in metal determination in the range of micrograms per milliliter. With our immunoassay being a biochemical test that checks for the presence and concentration, we can surpass conventional means of analyses for the benefit of conducting real-time reliable examinations at relatively low cost. The project is innovative as to date there is scarce literature on that type of detection with nickel as a central point to the research and none considers its prospective use in evaluation of bacterial fitness.

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Cyclic Di-Nucleotide Detection: The Development of RNA Sensors for Bacterial Second Messengers.

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Cyclic di-nucleotides have recently emerged as an important class of second messengers in bacteria. In particular, c-di-GMP has been shown to regulate diverse cellular processes, such as biofilm formation, virulence, and cell cycle progression. Here we present a simple gel mobility shift assay that allows for quantitation of c-di-GMP from cell extracts. Furthermore, we have exciting preliminary results on the development of an *in vivo* fluorescent biosensor for c-di-GMP based on the Spinach aptamer. The ability to quantitate c-di-GMP both *in vitro* and *in vivo* will assist ongoing efforts to elucidate the signaling pathways regulating this second messenger.

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Monitoring Alterations in RNA Levels of Living Cells Using a Novel RNA Detection Technology.

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The ability to determine the expression level of a given gene and more importantly to monitor changes in its expression due to stimulation or inhibition allows researchers to elucidate the complex regulatory networks inside of living cells. Unfortunately, current technologies require fixation, transfection, or cell lysis in order to determine RNA expression levels. These techniques alone can alter gene expression making monitoring a specific RNA problematic.

The ideal solution should be non-toxic, transfection free, allow for detection of the RNA target of interest, and then leave the cell unharmed and unchanged. Only then would the detection be based on normal cellular activity and not artifacts created solely by the detection method. Since the cells would remain unharmed they could be used for downstream experimentation following their original profiling.

We have developed an innovative technology that enables researchers to specifically detect RNA in living cells, while at the same time reducing the numerous hands-on steps and pain points associated with traditional RNA-based assays. It will allow the researcher the freedom to design and carry out improved gene expression experiments.

1734

A systematic genome-wide knockout generation and analysis of zebrafish protein-coding gene function.

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Recent advances in sequencing technology have opened the age of personalized genomics but even within the existing annotated vertebrate genomes less than half of the identified genes have been assigned a function. Detailed investigations involving model organisms have played a fundamental role in connecting genotype to phenotype but if we are to realize the dream of personalized medicine we will first require a functional analysis of all genes. The ability to thoroughly investigate the role of a gene in various biological processes greatly depends on loss of function analysis which has traditionally been carried out, in vertebrates, on a gene-by-gene basis.

The zebrafish is a genetically tractable organism with its external, transparent and rapidly developing embryo offering an *in toto* model of the complex cellular processes required for the formation of all major vertebrate organs. In recent years it has moved beyond being a model of early development and has become a first stop choice for human geneticists aiming to assign function to human orthologs or clinicians hoping to develop a model for a particular disease.

Enabled by a completed and annotated zebrafish reference genome sequence, high-throughput sequencing and efficient chemical mutagenesis, we describe the active Zebrafish Mutation Project (ZMP) at the Wellcome Trust Sanger Institute which aims to identify and phenotype disruptive mutations in every zebrafish protein-coding gene.

Thus far we have identified potentially disruptive mutations in more than 30% of all of the 26,000 known zebrafish protein coding genes. We have developed a multi-allelic phenotyping scheme to efficiently assess the effects of each allele during embryogenesis and have analyzed the phenotypic consequences of more than 1000 alleles. Our phenotyping scheme is also adaptable to phenotypic analysis beyond embryogenesis and we are currently expanding our analysis to encompass a wider array of phenotypes as we set forth to fully functionally annotate the zebrafish genome. A further goal is to place the rich collection of knockouts we are generating in the hands of specialists capable of carrying out detailed functional analysis. To this end, all knockout alleles and data are immediately made available to the community as we produce them via our website http://www.sanger.ac.uk/cgi-bin/Projects/D_erio/zmp.

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RapR analogs of Src, Fyn, Yes and Lyn: new tools for activation of specific kinase isoforms *in vivo* demonstrate specific roles for isoforms in cell motility.

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The Src family of kinases (SFK) is comprised of nine highly homologous members that participate in a diverse spectrum of signaling pathways, regulating proliferation, differentiation, cell migration, adhesion, and survival. Similarities in their structure and regulation have made it difficult to separate the roles of each family member. To identify unique functions for individual SFKs, we constructed rapamycin-regulated (RapR) analogs of Src, Fyn Yes and Lyn, enabling each isoform to be activated specifically and independently. The RapR approach (Karginov *et al.* 2011) takes advantage of the rapamycin-induced interaction between an engineered iFKBP domain and an FRB domain. The iFKBP domain is inserted into kinases at a conserved position in the catalytic domain, rendering the kinase inactive. The RapR kinase is expressed in living cells together with FRB. Addition of rapamycin or its nonimmunosuppressive analogs to the extracellular medium causes heterodimerization of the RapR-kinase with FRB, activating the kinase. This technology has proven broadly applicable, and to date has been used for Fak, Src, Fyn, Yes, Lyn, Pak1 and p38 MAPK. Morphological changes and changes in SFK distribution induced by activation of Src and Fyn were quantified using new computational methods. The methods use successive frames from time lapse movies of live cells to quantify key features of cell movement including the “uniformity” of cell spreading and the rate of cell edge change. The morphological dynamics are represented as a trajectory in the space of key parameters that characterize cell movement at each time point. The results of this analysis demonstrate that Src induces spreading followed by polarized movement, while Fyn generates uniform spreading, with each behavior occurring with well-defined kinetics following activation. Furthermore, activation of Src led to its redistribution from the perinuclear region to the cell periphery while Fyn remained more uniformly distributed throughout activation. Mutations were introduced into the RapR-SFKs to examine how these kinases are regulated and determine the role of cellular localization in their function. When RapR-Fyn could not be palmitoylated, it showed a distribution like RapR-Src and upon activation generated morphological changes like those produced by RapR-Src. Swapping of functional domains (SH3 and SH2) generated effects on both localization and polarized movement. Differences in focal adhesion dynamics driven by Src versus Fyn activation were quantified using a previously described method (Berginski *et al.* 2011). Our results provide a mechanistic model for the distinct morphological changes induced by each kinase.

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Increasing protein expression to 1g/L in the next generation mammalian transient protein production system: Expi293™.

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Transient expression provides a rapid, flexible and economical protein production alternative to the time consuming and costly process of generating and selecting stable high-expressing cell lines. However, there is a growing demand for higher protein yields necessary to feed the increasing needs of pharmaceutical research. This study shows how redesigning the transient

expression model enabled the superior protein yield and reproducibility typically obtained with stable cell lines while maintaining the simplicity and ease of use associated with current transient expression systems. The combination of a unique high-capacity media, a new 293F cell variant, and the ExpiFectamine293™ transfection reagent and enhancer, together enable the new Expi293™ system to achieve expression levels approaching and exceeding 1 gram per liter of protein within 7 days. We present data demonstrating the ability of the new media to support the increased growth rate, transfection and higher levels of protein productivity of the new Expi293F™ cell line. In some cases, expression of both IgG and non-IgG proteins were increased up to 10-fold over levels obtained with the FreeStyle™ 293 expression system. Proteins produced in the Expi293™ system exhibit biologically relevant target recognition, binding and native pharmacokinetic performance. The system is scalable, and as a result of the high yields, enables the use of microplates for high throughput applications. The system has been demonstrated to maintain high yields with a wide variety of proteins and applications including lentiviral and membrane protein production. By enabling the generation of substantially higher levels of protein in smaller culture volumes, the Expi293™ system represents a powerful, economical and unique expression technology for research and the production of therapeutic candidate proteins.

Physical Cell Biology and Tissue Engineering

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Embryonic Airway Peristalsis: the Mechanical Framework.

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The developing lung is a fluid filled, at times almost glandular structure that undergoes cyclic rounds of epithelial branching under cues from the surrounding mesenchyme and its derivatives. Some of these cues are mechanical, so full understanding of lung morphogenesis requires study of the mechanics of the developing lung. Fetal studies have revealed that the liquid filled developing airway is at a positive pressure relative to the amniotic fluid (from which it is distinct) and that this is maintained by net secretion by the lung. However the developing lung is also subject to a range of mechanical interactions including not only diaphragmatic 'breathing movements' but also the effects of propagating airway peristalsis. In this study, we have used measures and estimates from developing embryonic lung to build an in silico model of propagating waves of ASM-mediated airway peristalsis traveling to a closed distal end.

We can report a hitherto unexpected finding that airway peristalsis can be expected to regulate the speed of diffusion of luminal macromolecules in particular. Secondly, and perhaps contrary to expectations, proximal fetal airway occlusions, as used in human fetuses with congenital diaphragmatic hernia, would not appear to unduly affect the distal effects of airway peristalsis at least in larger airways. Third, airway peristalsis provides a synchronized mechanosignal to the airway distal to the point of peak contraction. Fourth, tissue stretch in the airway stalk is unaffected by luminal liquid viscosity, but flow sensing in that region would be predicted based on the model showing flow reversal in that area. Fifth, fluid velocities measured by the movement of luminal debris can yield straightforward surrogate information on fluid shearing. However, shearing of the epithelium is negligible which means there may be an absence of epithelial shear sensors at points they might otherwise have been expected. More generally, we find that tube size and its closed-end nature can affect a range of features that would be of

direct relevance from cancer angiogenesis and ureteric bud development to development of the major exocrine glands. In the latter, our prediction of enhanced diffusion for macromolecules may presage an exquisite physiological role for peristalsis in closed-end systems.

1738

Intrinsic, temporal mechanical oscillations of COS-7 cells.

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Cellular oscillations are a natural phenomenon reflecting the dynamic nature of cytoskeletal and other cellular components. Recently, low frequency periodic oscillations of the mechanical properties of epithelial cells were measured using the atomic force microscope (AFM). For example, AFM nanoindentation was used to measure temporal changes in bronchial epithelial cells and in cerebral endothelial cells. For both cell types, periodic, low frequency oscillations with periods between 1 and 2 minutes were demonstrated. Nanoindentation was also utilized to measure temporal changes of the Young's modulus of vascular smooth muscle cells from both young and aged monkeys. This study revealed that the elasticity of these cells oscillates and those oscillations have pronounced periodic components whose frequencies increase with aged. However, these studies measure elasticity by repeatedly indenting the cell, a procedure which may be perturbing the cells and biasing the dynamics of the cytoskeleton in ways that are not clearly understood.

In this study, we extend the investigation of periodic oscillations to a different type of cell and aim to better understand the phenomenon by introducing a second, complimentary method of observation. In addition to the standard AFM nanoindentation methods, we also passively observe the elastic fluctuations of the cells while the AFM probe is in static, constant force contact with the cell. In these experiments we used COS-7 cells, fibroblast-like cells derived from monkey kidney cells. The experiments reveal that the elasticity of COS-7 cells also fluctuates and there are strong indications of periodic components at frequencies in the range observed in the previously investigated cells. The frequencies observed by the two methods are similar and the differences can be explained by the differing mechanics of the observations. We describe the methodologies of data acquisition and analysis and possible sources of such behavior.

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Estimation of forces driving a cytoplasmic flow in the *C. elegans* embryo using data assimilation.

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Cytoplasmic streaming is a collective flow of the cytoplasm observed in many eukaryotic cells. Cytoplasmic streaming can be observed in *Caenorhabditis elegans*, a widely used model organism, shortly after the fertilization. This streaming is important to establish anterior-posterior axis of the embryo. In a previous study, we succeeded in quantifying the velocity distribution of the cytoplasmic streaming in the *C. elegans* embryo. We also constructed hydrodynamic simulation model that well reproduces the velocity distribution observed in the cell [1]. Here we report that, using a statistical method called data assimilation, we estimated the distribution of forces generated by molecular motors to drive the cytoplasmic streaming. The spatial distribution of forces required to reproduce the streaming was not proportional to the velocity distribution: strong forces were estimated to be generated even at region where the streaming is

slow. In the presentation, we will discuss possible causes of this non-linearity between the driving forces and resultant flow velocity.

[1] Niwayama, Shinohara, and Kimura. *Proc. Natl. Acad. Sci. USA* **108**, 11900-11905 (2011)

1740

Deformability Cytometry: High-Throughput Single-Cell Mechanical Measurements for Mechanophenotyping.

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There is growing evidence that cell deformability (i.e., the ability to change shape under load) is a useful indicator of cell state and may be a label-free biomarker of metastatic potential, cell cycle stage, degree of differentiation, and leukocyte activation. Clinically, a measure of metastatic potential could guide treatment decisions; a measure of degree of differentiation could prevent transplantation of undifferentiated, tumorigenic stem cells in regenerative therapies; and a measure of leukocyte activation could be employed to monitor progression of HIV or transplant rejection. In order for deformability measurements to be clinically valuable given the heterogeneity of complex biological samples, there exists a need for a high-throughput automated assay of these mechanical properties. We have developed a robust method for obtaining high-throughput single-cell mechanical measurements (~2000 cells/sec—comparable to flow cytometry). The method employs inertial focusing for uniformly positioning cells in flow, hydrodynamic stretching in an extensional flow, high-speed imaging, and automated image analysis to extract a size and deformability parameter. Thus far we have demonstrated the method's ability to distinguish mouse and human embryonic stem cells from their differentiated progeny, cancerous cells from benign mesothelial cells within pleural fluids, and activated white blood cells from resting white blood cells. For hundreds of thousands of cells, in this work we show that mechanical state as measured by our system can effectively discriminate between populations of leukocytes. In contrast to resting granulocytes and mononuclear blood cells, which we have measured to be rigid, both myeloid (HL60) and lymphoid (Jurkat and H9 lymphoma) leukemic cell lines cells are highly deformable. The differentiation of these cells, for example differentiation of HL60 by all-trans retinoic acid, greatly reduces their deformability. On the other hand, activation events such as stimulation with mitogenic compounds increase the deformability of primary blood cells. Both in immune activation and hematological malignancy, large scale cellular changes (e.g., relative nuclear size and structure and reorganization of intermediate filaments) are apparent along with the molecular changes that are usually assayed. These changes affect mechanical properties, and the accessibility and ease of their measurement will make them useful for monitoring disease and efficacy of therapy, drug screening, and biophysical studies.

1741

A geometry-dependent slow feedback from cell shapes to the spatial patterning of PIP3 / Rac1 / actin.

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The spatial patterns of cell shapes are critically important in many biological processes. The current theory is that cells use biochemical cues-induced patterning of signaling molecules (time scales: seconds - minutes) to guide cell shape patterning. However, how the patterns of cell shapes influence signaling molecule dynamics and how fast such influences are remain to be characterized. Using fabricated environments and quantitative image analysis, we show that in the absence of biochemical cues, non-uniform cell shapes can spontaneously emerge without

preceding patterning of signaling molecules including PIP3 and active Rac1. These spontaneously emerged shapes can guide the patterning of PIP3, active Rac1, and actin filaments which in turn stabilize the cell shapes. Moreover, the rate of shape-guided patterning (time scales: ~ hours) is much slower than that of biochemical cues-induced patterning. Numerical simulations show that shape-guided patterning can be simply a result of the boundary effect of non-uniform shapes, without the involvement of any molecular feedback circuits. We suggest that cell shapes can influence the dynamics of PIP3 / Rac1 / actin, after cells are committed to specific spatial patterns for a long period of time. The large gap of response times between biochemical cues-induced patterning and shape-guided patterning might allow cells to respond to fast environmental changes under minimal interferences from previously-developed cell shapes.

1742

Changes in Cellular Mechanical Properties Following Cytoskeleton Components Disruption.

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An unsolved problem in cellular biology is how the individual cytoskeleton components behave to permit or impair the transmission of the mechanical stimuli and how the magnitude of forces affects cellular functions. To address this gap, numerical simulation can be helpful to describe the contribution of the cytoskeleton to cell physiology. We propose a new 3D finite element model of a single adherent cell to study the role of different components of the cytoskeleton depending on the external stimuli and how these contribute to cell integrity. The model includes actin cortex, actin fibers and microtubules in a continuum cytoplasm and nucleus. Physiological values of prestress are simulated due to 24% pre-stretching of actin bundles. The cell model was compressed and stretched with a bead on the top of the cell simulating different single-cell stimulating techniques. Numerical simulations were combined with force-indentation measurements using atomic force microscopy (AFM) of U2OS GFP-actin cells and NIH-3T3 cells for model validation and further prediction of mechanical properties of different cell types. Our findings show that under a compressive load simulating AFM, the actin cortex and microtubules are the major components to resist compression. During stretching, simulating magnetic twisting cytometry (MTC), cell stiffness is mainly dependent on the stress transmission between actin bundles and microtubules. Therefore, the developed finite element model of the organization of the cell allows us to interpret and compare AFM and MTC for cytoskeleton disruption that characterizes biophysical changes associated with cytoskeleton functions. Visible differences in the distribution of actin in 3T3 and U2OS cells were observed before and after actin disruption with cytochalasin-D. A thicker network of actin at the cortical level was observed for the 3T3 cells compared to the U2OS before disruption. Treatment of 3T3-NIH cells with cytochalasin-D disrupted mainly the cortical network while the same concentration of drug disrupted the entire structure of F-actin in the U2OS cells. We found numerical differences in the structure of actin cortex and actin bundles that we were able to relate with different stiffness of the cells tested with AFM and therefore, predict whole cell strains for different cell lines. The model identifies which cytoskeleton components determine the physical properties of adherent cells and target cellular mechanosensitivity depending on cytoskeleton mechanical properties and on the type of external forces sensed by cells. This study illustrates that considering one elasticity to describe cellular mechanical properties on a whole cell basis is not sufficient, but we also need to consider variations of specific intracellular mechanical properties.

1743

Stretch Modulation of Cardiomyocyte Conduction.*E. Pfeiffer¹, J. Stowe¹, K. McNall¹, J. Tan¹, A. McCulloch¹; ¹Bioengineering, UCSD, La Jolla, CA*

Cardiac volume overload and acute deformations have been linked to increased incidence of arrhythmia and sudden cardiac death, possibly due to proarrhythmic conduction slowing with tissue stretch. Both conduction velocity increases and decreases have been reported under mechanical loading *ex vivo*. These changes have been attributed to stretch modulation of ion channels, cell-cell junctions, cell capacitance, and properties of the interstitium.

To separate interstitial effects from intrinsic changes in myocyte conduction, neonatal murine cardiomyocytes were cultured on micropatterned stretchable substrates for optical mapping of excitation conduction velocity. A homogeneous anisotropic biaxial strain field of up to 14% in the primary direction and 3.6% in the secondary direction was applied to these substrates for five minutes, where the primary direction of stretch was oriented either parallel or perpendicular to the longitudinal axis of the aligned cell culture. Reversibility was demonstrated by measurements recorded five minutes following load removal.

When the primary direction of strain was oriented parallel to the longitudinal cell culture axis, longitudinal conduction slowed to $72\% \pm 5\%$ of 264 mm/s, the baseline velocity along the longitudinal axis of the cell culture, and transverse conduction to $80\% \pm 4\%$ of 141 mm/s, the baseline along its transverse axis ($n=5$, mean \pm SEM). Conduction velocities before and after loading were significantly different by paired t-test, $p = 0.0064$ and $p = 0.0144$ respectively. Five minutes after removal of stretch, conduction velocities recovered to $90\% \pm 8\%$ and $114\% \pm 18\%$ respectively. A repeated measures ANOVA of the effect of the three loading conditions on longitudinal conduction detected significant slowing with loading ($F(2,4)=5.671$, $p=0.0293$). These observations of conduction slowing with stretch are in line with our previous observations in isolated and perfused rabbit hearts, in which conduction velocity slowed by $12 \pm 3\%$ with volume inflation, and recovered to $95 \pm 1\%$ of the initial value of 30.8 ± 4.7 cm/s following unloading ($n=5$, mean \pm SEM) (Mills 2008; Sung 2003).

Progressive conduction slowing occurred isotropically with biaxial stretch above 7% Lagrangian strain in the primary direction and 1.8% in the secondary direction, oriented along either axis of the cell culture. Lower stretch magnitudes resulted in conduction increases to 125-150% of initial conduction velocities. Both low-stretch speeding and high-stretch slowing continue after treatment with cation-selective mechanosensitive channel inhibitor GsMTx-4.

1744

Analyzing Contractile Functions of Gene Targeted Cardiomyocytes.*S. Lange¹, X. Zhang², J. Chen¹; ¹School of Medicine, UC San Diego, La Jolla, CA, ²ACEA Biosciences, San Diego, Cambodia*

Gene targeted mice are widely used model systems for human diseases and tools to investigate functions of a gene/protein in an *in vivo* context. One example of a widely studied animal model for cardiomyopathy is the muscle lim protein (MLP) knockout mouse. MLP mice develop a severe form of dilated cardiomyopathy (DCM) within 2 months after birth and exhibit many of the characteristic pathological hallmarks displayed by human DCM patients.

Despite its wide use in cardiovascular science, the MLP knockout mouse is just one example of a gene targeted mouse model that exhibits alterations to cardiac functions, with many other gene knockout models in need of better characterization on the physiological, histological and biochemical level. Although cardiac function of gene targeted animals can be assessed *in vivo*

through echocardiography analysis, or on the organ level by Langendorff systems, investigating contractile parameters on the cellular level is usually limited by small sample size and high variability: e.g. electrophysiological studies of single cardiomyocytes using patch clamp techniques, measurement of calcium transients in single cells, or force measurements on isolated, skinned cardiac myocytes.

We are taking advantage of the Roche/Acea xCelligence cardio-system to characterize contractile parameters of neonatal mouse cardiomyocyte cultures derived from gene knockout models in a high throughput, high replicate fashion. Data gathered from these experiments are a novel tool to further characterize pathological alterations in the contractile behavior of these gene targeted cardiomyocytes. Moreover, the treatment of cardiomyocytes derived from disease models with small molecule inhibitors/activators may help to elucidate the function of cardiac signaling pathways, optimal drug concentrations and effects on contractile functions.

1745

Single cell mechanics in the immortalization process investigated by atomic force microscopy.

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Mechanical properties of single cells are closely related to various cell functions. Previous studies^[1] reported that the stiffness of metastatic cancer cells was softer than the benign cells, suggesting that mechanical properties of cells changed depending on the degree of various cell transformations. In general, normal cells have a finite life span so that they do not undergo spontaneous immortalization in culture. However, fibroblast cells from rat or mouse have the ability to multiply indefinitely in the process of transformations of growth (Phase II), senescence (Phase III) and immortalization (IM) and consequently the cells overcome the senescence. In this study, we measured the number distribution of complex shear modulus, G^* , of rat fibroblast cells during the transition from Phase II to IM by atomic force microscopy with a cell microarray substrate. We observed that the frequency dependence of G^* of cells (N~100) in different phases commonly exhibited a single power-law behavior, which was well fitted to the structural damping model^[2].

The mean and standard deviation of the power exponent α and Newtonian viscous damping coefficient μ were different among these phases. The cells in phase II had a smaller α , than those in other phases, indicating that the former cells exhibited a solid-like behavior compared with the latter. Moreover, the standard deviation of α for the cells in phase III was broader than that in other phases. The mean of μ significantly increased for the cells in the process of transformation. For the treatment of cells with cytochalasin D, which disrupts actin cytoskeleton by filament capping, α increased, and the increased α was independent of the phase.

[1] S. E. Cross et.al., Nat. Nanotechnol. 2, 780 (2007) [2] B. Fabry et.al., Phys. Rev. E., 68, 041914 (2003)

1746

Measuring intracellular viscoelastic properties of human mammary epithelial cells by organelle tracking.

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We employ organelle tracking as a minimally perturbing method for measuring viscoelastic properties of live human mammary epithelial cells (HMEC's). From the mean-square displacements (MSDs) and radii of these organelles, we compute the viscoelastic moduli using a generalization of the Stokes-Einstein equation. We first validated our method by tracking and

analyzing the motion of beads in 2 mg/mL xanthan, a polysaccharide with similar viscoelastic properties to those of PC12 cytoplasm, and obtaining expected viscoelastic moduli over a range of frequencies. With this confirmation, we tracked the Brownian motion of fluorescently labelled peroxisomes and lysosomes in live HMEC's for 100 s at a rate of 100 fps. From our analysis of peroxisome motion, we obtained a curve for the viscoelastic moduli which exhibited shear-thinning over four orders of magnitude in frequency. We also observed a crossover in the relative contributions from the storage and loss components of the moduli; at low frequencies the viscous component is the predominant contributor to the complex viscoelastic modulus, while at higher frequencies the elastic component predominates. The mechanics of a cell, including its viscoelastic properties, affect its signaling, cytoadherence, migration, and metastatic potential, and thus may be influential in cancer development. In this vein, we plan to compare observed viscoelastic properties of normal HMEC's with two transformed, increasingly tumorigenic HME cell lines. We expect that a greater understanding of the connection between cell mechanics and cancer will pave the way for novel approaches to treatment.

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1747

Cell Adhesion on Nanometer Scale Protein Patterns with Micrometer Scale Spacings

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Surface patterning techniques are proving to be tremendously useful for cell biology applications. Electron beam lithography (EBL) is a high resolution, flexible direct patterning method used in semiconductor industry. We have previously developed an EBL based patterning approach which directly uses proteins as EBL resists on silicon surfaces. However, silicon is not transparent and thus not suitable for many light microscopy techniques. On the other hand, indium tin oxide coated glass (ITO-glass) is suitable for light microscopy due to its transparent nature; it is also conductive and can thus be used for EBL. Our results show that nanometer scale patterns of the cell adhesion promoting protein fibronectin (FN) can be fabricated on ITO-glass surfaces. We show that using lines in the design and choosing micrometer scale step sizes, protein nanopatterns can be fabricated with the added advantage of significantly reduced exposure times (from days to minutes). We determine that a minimum dose of 50 pAs/cm is required for pattern formation and FN nanodot radius increases linearly from 118 ± 5 nm to 326 ± 9 nm as dose increases from 212 to 1000 pAs/cm. In addition, we test the effect of micrometer scale spacing between and size of protein nanopatterns on cell adhesion. We show that cells respond to protein nanopatterns on ITO-glass by forming pattern specific focal adhesions. While cells do not form focal adhesions on FN nanodots with radius of 100 nm, they do so on FN nanodots with radii of 200 and 400 nm. Furthermore, number and maturation of focal adhesions is inversely correlated with spacing between nanodots namely two, four and eight microns.

1748

Tracking cytoplasmic flow during development and regeneration of *Stentor coeruleus*

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The mechanisms that determine the shape of cells remain largely unknown. The giant ciliate *Stentor* has a complex structure that can regenerate after perturbations. *Stentor* morphogenesis faces many of the same problems as development of multicellular embryos, raising the question of whether gradients of diffusible morphogens may play a role in spatial patterning. The times-scale required to establish such a gradient, and the sensitivity of this gradient to external input and its stability in space and time impose contradictory constraints on the rheological properties of the cytoplasm, with the former requiring the fluidity typical of a low Reynold's number material and the later requiring the viscosity typical of a high Reynold's number material. *Stentor coeruleus*, one of the largest known unicellular ciliates measuring in at millimeters in length, is capable of complete regeneration of its asymmetric and highly complex structure from a cell fragment over minutes as long as one of its nuclei remains intact. Its ability to do so implies that it has found a way around the conundrum, and the molecular mechanism by which this is done is not well understood. In a series of experiments conducted during the Physiology Course at the Marine Biological Laboratory, we studied the cytoplasmic streaming of *Stentor* treated with various drugs targeting the cytoskeleton including AMP-PNP, paclitaxel, blebbistatin, and cytochalasin D, and compared with the wild-type. Using Particle Image Velocimetry (PIV) techniques implemented in MatLAB, we extracted local flow vectors over time inside *Stentors* immobilized in 4% methylcellulose and visualized by DIC microscopy. The flow in the main body of the animal is circumferential in the dorsal-ventral plane and can travel across the cell in minutes, whereas the flow in the tail is highly linear. None of the drug treatments lead to statistically significant changes in the flow patterns, flow velocities, and length scales of flow persistence across both time and space. Preliminary data on real-time cytoplasmic flow of *Stentor* right after a clean cut divided the cell into two halves show that the cytoplasmic flow changes immediately, not only preventing the cytoplasm from leaking out of the gap in the membrane, but also actively flowing back towards the center of the cell.

1749

Functional interplay between cell cycle and cell phenotypes.

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Cell cycle distribution of adherent cells is typically assessed using flow cytometry, which precludes the measurements of cell phenotypes and their cycle phase in the same environment. Here we use a microscopy system to simultaneously and quantitatively analyze the cell-cycle phase of thousands of cells and their associated cell properties. This assay demonstrates that population-averaged cell phenotypes can be written as a linear combination of cell-cycle fractions and phase-dependent phenotypes. By perturbing cell cycle through forced cell synchronization and inhibition of cell-cycle regulators or changing nuclear morphology by depletion of structural proteins, our results reveal that cell cycle regulators Cdk4/6 and structural proteins Nesprins and Lamin A/C significantly interfere with each other's *prima facie* functions. This study introduces a high-throughput method to simultaneously measure cell cycle and

phenotypes at single-cell resolution and highlights the functional interplay between cell cycle and cell phenotypes.

1750

Thermorheology of living cells.

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Within reasonable temperature ranges, many biological functions are known to undergo modulations, like myosin motor activity, CO₂ uptake of cultured cells or sex determination of several species. As mechanical properties of living cells are considered to play a key role for plenty of cell functions - ranging from stem cell differentiation to cancer progression - it is surprising that only little is known on how their rheology is affected by temperature. This might be due to the fact that a variation in temperature triggers complex regulatory processes and alterations of the molecular architecture of cells. Rheological studies done so far have in common that temperature was varied exclusively on long timescales where the physical response of cellular matter is overlaid by complex biological alterations. Using an Optical Stretcher, we utilize laser induced heating to vary temperature on a millisecond timescale, while simultaneously probing the mechanical response of cells to optical force. In contrast to temperature variations on long timescales, milliseconds are probably too short for cells to react on a regulatory level. Surprisingly, we find that time-temperature superposition - a simple scaling concept frequently used in polymer physics - sufficiently describes the observed shift of creep compliance of whole living MCF10A breast epithelial cells upon temperature changes. From the found scaling factors, we derive the activation energy for the viscous flow to be on the order of 80kJ/mol, while it is not necessary to make any assumption about a specific viscoelastic model. Furthermore, we compare effects of short time temperature variations to those induced by temperature changes on long time scales and conclude that differences arise from cellular adaptation to temperature. Short time temperature variation is a powerful approach to explore pure physical responses and to separate them from those caused by biological adaptation. This could help to bridge the gap between the rheology of living matter and polymer physics. Furthermore, the revealed strong temperature dependence of the mechanical properties of cells has important implications for other studies on cells and cell mechanics.

1751

Insights into cytoplasmic rheology gained from modeling cellular blebbing.

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Blebbing occurs when the cytoskeleton detaches from the cell membrane, resulting in the pressure-driven flow of cytosol towards the area of detachment and the local expansion of the cell membrane. Recent experiments involving blebbing cells have led to conflicting hypotheses regarding the timescale of intracellular pressure propagation. The interpretation of one set of experiments supports a poroelastic cytoplasmic model which leads to slow pressure equilibration when compared to the timescale of bleb expansion. A different study concluded that pressure equilibrates faster than the timescale of bleb expansion. To address this, a dynamic computational model of the cell was developed that includes mechanics of and the interactions between the intracellular fluid, the actin cortex, the cell membrane, and the cytoskeleton. Results show the relative importance of cytoskeletal elasticity and drag in bleb expansion dynamics. Our results also show that a poroelastic model of the cytoplasm can explain experimental data and support the hypothesis that pressure equilibrates relatively fast.

1752

Interaction between Lung Cancer Cell and Myofibroblast Mediated by Cyclic Tensile Strain.

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Myofibroblasts (MFs) play important roles in the development and metastasis of tumors by exchanging major growth factors with the cancer cells. It is also well known that the phenotype of MFs is subject to the stimulations of mechanical tension. Therefore, it is intriguing to understand how mechanical tension is involved in cancer metastasis by mediating the activities of MFs. This topic is of special importance for lung cancers because cells near the lung keep experiencing cyclic tensile strain (CTS) along with breath. However, to quantify the degree of mechanical strain on the MF and to measure the migration ability of cancer cells in vivo are both difficult.

In this work we used a two-layer microfluidic cell-culture chip to introduce CTS on MFs, and characterized the migration speed of lung cancer cells under the influence of the conditioned medium of MF without and with the CTS. A 0.1-mm-thick PDMS film between the top and bottom layers of this culture chip was used as the stretchable substrate for the MF and the valves controlling the flows of the media. For better cell adhesion, we coated the surface of culture chambers with fibronectin. With an injected liquid volume of 50 μ l into the chamber under the PDMS film, the strain on this film was nearly 10%.

The cell lines used in this work were human lung cancer cells (CL1-0) and lung fibroblasts (MRC-5). Cells in the chip were cultured in Leibovitz L15 medium. Before the experiments, the MRC-5 cells were activated to be MF by 10 ng/ml transforming growth factor- β 1 (TGF- β 1). The activation was confirmed by measuring the α -smooth muscle actin (α -SMA), a major marker for MFs, in fixed cells. We measured that the expression level of α -SMA in the MRC-5 cells was increased for 80% by TGF- β 1.

We employed 0.27 Hz, 5-10% CTS on the MFs for 6 hours and let the conditioned medium of the MFs flow to the chamber of cancer cells. The migration speed was calculated from the displacements of cell centroids in the 6 hours. We found that the conditioned medium of MFs effectively increased the cancer cell motility, while the CTS significantly reduced the ability of MFs to enhance the migration speed of cancer cells. In the cell culture chip we also verified that the effect of CTS on the MF could be compensated by interleukin-1 β . This result suggests that mechanical stimulations are involved in cancer metastasis through the modification of the phenotype of MF.

1753

How growth cone leading edge dynamics alters with substrate adhesion.

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While growth cones move on two-dimensional substrates their leading edge alternates between rapid phases of protrusion and retraction. Actin polymerization underneath the plasma

membrane, that switches stochastically between On and Off states and a steady retrograde actin flow were found to be the driving forces of leading edge fluctuations.

According to the adhesion clutch hypothesis a growth cone can only move forward if increased adhesion slows down retrograde flow and thus faster polymerization leads to net protrusion. What is still missing in this picture is how the stochastic nature of the leading edge contributes to growth cone advancement and how these dynamics are coupled to the substrate. Therefore, we measured the actin dynamics of retinal ganglion cell growth cones on substrates with specific (Laminin 1) and unspecific binding (poly-D-lysine) and compared retrograde flow velocity, protrusion and retraction speed of actin polymerization as well as stochastic switching times between protrusion and retraction events. We find that retrograde flow and retraction velocities are much faster for growth cones on poly-D-lysine, but protrusion velocity is similar for poly-D-lysine and Laminin. Analysis of stochastic switching show that alterations between protrusion and retraction events happen much faster on poly-D-lysine than on Laminin. These findings may lead to a deeper understanding on how the stochastic nature of growth cone actin dynamic can be classified in terms of growth cone motility.

1754

Precise assembly of multiscale and multicomponent 3D tissues.

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The spatial arrangement of multiple cell types in three dimensions contributes to the functional properties of human tissues. To better understand how the arrangement of cells in a tissue affect the tissue's function, we have developed a bottom-up strategy for building components of the human mammary gland that are suitable for 3D in vitro culture and live cell imaging. The method provides 10 μm resolution for the positioning of individual cells in the x and y directions and across distances greater than 1 cm. Using this approach, we are probing the role of microenvironment in driving the self-organization of luminal and myoepithelial cells in the human mammary epithelium, testing the capacity of epithelial tissues to buffer for cell-to-cell variability in growth rates during morphogenesis, and assessing how the spatial distribution of activated signaling nodes such as Ras affect multicellular behaviors that contribute to the form and function of a tissue.

TUESDAY, DECEMBER 18

Conventional Myosins

1755

Regulation of the actin-activated MgATPase activity of *Acanthamoeba* myosin II by phosphorylation of serine 639 in motor domain loop 2.

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Acanthamoeba myosin II (AMII) is a typical Class II myosin with a pair of identical 1509-residue heavy chains and two pairs of light chains. The C-terminal non-helical tailpiece that begins with Pro1483 contains 27 residues with a pattern of four continuous repeats of XXSXR. Previous studies identified that at least three of these serines were phosphorylated *in vivo* and *in vitro* and phosphorylation correlated with almost complete inactivation of actin-activated MgATPase activity. Several lines of evidence suggested that filamentous forms of AMII were required for the full activity. To understand the fundamental mechanisms we re-investigated these interesting issues by using recombinant AMII expressed in SF-9 cells. Here we show that two non-filamentous forms of AMII, heavy meromyosin and myosin subfragment, are very active and down-regulated by phosphorylation like full-length AMII. By mass spectroscopy, we show that Ser639 in loop 2 of the motor domain, in addition to the four serines in the non-helical tailpiece but no other residues, is phosphorylated by a partially purified kinase *in vitro* and Ser639 is also phosphorylated in inactive endogenous myosin isolated from the amoebae. Mutation of Ser639 to Ala in both subfragment 1 and full-length myosin does not affect actin-activated MgATPase. Phosphorylation of the serines in the non-helical tailpiece or their mutation to Glu or Asp does not affect the activity of S639A mutants. Conversely, S639D mutants of both subfragment 1 and full-length myosin are inactive irrespective of the phosphorylation state of the serines in the non-helical tailpiece. To our knowledge, *Acanthamoeba* myosin II is the first example of regulation of myosin actin-activated MgATPase activity by phosphorylation of loop 2. We further demonstrate by electron microscopy that phosphorylation of the serines in the non-helical tailpiece or their mutation to Glu alters the lateral association of AMII dimers in tetramers and octomers irrespective of mutation or phosphorylation of Ser639.

1756

Dictyostelium MHCK D exhibits lamellipodial localization in chemotaxing cells and is required for normal slug migration.

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Contraction-dependent processes such as cytokinesis and cell migration rely on the proper assembly and localization of myosin II bipolar filaments. In *Dictyostelium* cells, as well as in mammalian cells, myosin II filament disassembly can be driven by phosphorylation of the myosin II heavy chain (MHC) "tail". MHC phosphorylation in *Dictyostelium* is catalyzed by at least three kinases (MHCK-A, -B, and -C) that share homologous alpha-kinase catalytic and WD-repeat domains. In the current study, we examined the cellular and biochemical characteristics of another *Dictyostelium* alpha kinase, MHCK-D, that possesses the same domain organization as the other MHCKs; and thus is predicted to function in a similar manner. We report here that over-expression of MHCK-D slows suspension growth, with cells becoming large and multinucleated over time; a phenotype consistent with a role for MHCK-D in driving myosin II filament disassembly. Moreover, *in vitro* kinase experiments revealed that MHCK-D

phosphorylates MHC to a stoichiometry of ~0.6 mol/mol MHC and in manner that leads to myosin II filament disassembly. RT-PCR analysis of expression revealed that MHCK-D, unlike the other MHCKs, is expressed only during *Dictyostelium* multicellular development (peak expression at ~16h). We also found that in live *Dictyostelium* cells GFP-tagged MHCK D exhibits robust and persistent localization to the leading edges of cellular extensions. By contrast, a truncation of MHCK-D lacking the WD-repeat domain localizes mainly to the cytoplasm, suggesting that the WD-repeat domain is involved in the targeting the kinase to cellular protrusions. Comparisons of *Dictyostelium* slug phototaxis between wild-type and *mhkD*-null cells revealed that the absence of MHCK-D leads to increased slug migration. Taken together, our results demonstrate that MHCK-D is a bone fide myosin II heavy chain kinase in *Dictyostelium* that is likely to function in myosin II filament turnover at the leading edges of migrating cells, and that MHCK-D plays a critical role in facilitating the highly complex process of multicellular slug migration.

1757

UCS protein Rng3p stabilizes myosin-II motor activity to initiate actomyosin ring assembly in fission yeast.

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UCS proteins have been proposed to operate as co-chaperones that function with Hsp90 during the de novo folding of myosin-II motors. We show that the fission yeast UCS protein, Rng3p, is recruited to pre-assembled actomyosin ring structures and promotes ring assembly. Isolation of Myo2p in the absence of Rng3p function demonstrated that it is not required for Myo2p folding or actin binding but is essential for its ATPase activity and actin filament motility. A mutant form of Myo2p, hypersensitive to reduced Rng3p function (Myo2-E1p), binds actin filaments yet lacks motor activity and exhibits conformational instability. Rng3p is specific to Myo2p, however we could artificially target Rng3p to myosin-I patches using a point mutant that destabilizes the motor. We propose a novel role for UCS proteins in myosin stabilization and activation that responds to the accumulation of a critical concentration of motors. We predict this role is independent of de novo folding involving Hsp90 .

1758

Genetic Replacement of Nonmuscle Myosin (NM) IIA with NM IIC1-GFP In Vivo.

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The purpose of this study is to learn whether NM IIC1 can functionally replace NM IIA, during mouse development. Alternative splicing of pre-mRNA encoding NMHC IIC generates several isoforms. An alternative exon encoding 8 amino acids can be incorporated near the ATP binding site at amino acid 227 to form NM IIC1. A second alternative exon encoding 41 amino acids can be incorporated near the actin binding site at amino acid 636 to form NM IIC2. Because NM IIC1 is the most abundant form of NM IIC expressed in a variety of tissues such as liver, kidney, testes, brain, and lung, we chose NM IIC1 to replace NM IIA in vivo. Previous work has shown that ablation of NM IIA results in lethality in mice at E6.5, prior to gastrulation, due to abnormalities in the visceral endoderm (Conti et al. JBC 279:41263, 2004). To replace NMHC IIA with NMHC IIC1 in the mouse, homologous recombination was used to ablate NMHC IIA by inserting the cDNA encoding NMHC IIC1-GFP into the first coding exon of the Myh9 gene to produce A^{C⁻GFP}/A⁺ mice. We have obtained heterozygous mice without apparent abnormalities however, the breeding of heterozygous mutant mice does not produce live homozygous A^{C⁻GFP}/A^{C⁻GFP} mice. Homozygous embryos die at approximately E10.5 after gastrulation, showing

that NM IIC1-GFP, by restoring a normal visceral endoderm, can support gastrulation. At both E9.5 and E10.5, $A^{C^*-GFP/A^{C^*-GFP}}$ embryos are markedly smaller than wild-type littermates and are developmentally delayed. Cell proliferation is decreased while large numbers of apoptotic cells are found in homozygous embryos at these stages, indicating that the cause of embryonic lethality is related to cell death. Explants from the E9.5 embryos were cultured and MEF cell migration from the explants was recorded by time lapse microscopy. Compared to wild-type MEFs $A^{C^*-GFP/A^{C^*-GFP}}$ MEFs display an increased speed of outgrowth. Furthermore the $A^{C^*-GFP/A^{C^*-GFP}}$ MEFs are polarized, containing both larger ruffling lamellipodia and longer filopodia. Immunostaining shows that NM IIC1-GFP can be incorporated into stress fibers in $A^{C^*-GFP/A^{C^*-GFP}}$ MEFs. However these stress fibers were fewer and less well-organized compared with wild-type cells. Focal adhesions in $A^{C^*-GFP/A^{C^*-GFP}}$ MEFs are also fewer in number and smaller in size. These results indicate that although NM IIC1, which is not normally expressed in mice until E11, can restore a normal visceral endoderm at E6.5 it can't replace NM II-A's function in focal adhesion formation and maturation.

1759

Myosin IIA regulates the cellular architecture of podocyte.

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Podocytes are characterized by a large cell body and numerous foot processes. Cell body and primary processes contain microtubules and intermediate filaments, while foot processes have only actin filaments. Cytoskeletal organization in podocyte is a key factor for maintaining its cell shape. Especially, actin cytoskeleton may play a crucial role in the regulation of foot process structure, because the reorganization of actin-binding proteins causes the effacement or retraction of foot processes. Point mutations in MYH9, the gene encoding nonmuscle myosin heavy chain II-A (NMHCII-A), develop progressive glomerulosclerosis. However, the roles of myosin IIA in kidney are not elucidated. Our preliminary study showed that podocytes specifically express myosin IIA, but not myosin IIB in glomerulus. Immunoelectron microscopy of myosin IIA resulted that myosin IIA is distributed in the cytoplasm of cell body and primary processes. Specialized localization is seen on the actin arc shown at the boundary between cell body and foot processes. The signals for myosin IIA were dramatically decreased in PAN nephrotic kidney, in which the morphological changes of podocytes including the effacement of foot processes were seen. In addition, myosin IIA was dephosphorylated under the nephrotic condition. We next examined the role of a cytoskeletal motor protein, myosin IIA in cell process formation by using immortalized rat podocyte cell lines which we have originally established. These cell lines express all of cytoskeletal proteins seen in vivo podocyte including alpha-actinin 4, vimentin and nestin intermediate filaments. Cytochalasin D treatment induces the rearrangement of vimentin and nestin intermediate filaments and cell process formation through the membrane retraction. Cell processes induced by cytochalasin D treatment contain bundled intermediate filaments never seen in the resting cells. Myosin IIA is located at the cell margin with actin filaments in resting cells, but the main signals for myosin IIA are colocalized with vimentin filaments after stimulation. Myosin IIA is also seen at the base between neighboring cell processes with actin filaments, suggesting that the membrane retraction is achieved by the mechanical force of actomyosin. Treatment of blebbistatin, myosin II inhibitor reduced the morphological change of cultured podocytes induced by cytochalasin D. Myosin IIA silencing using siRNA is associated with significantly reduced cell process formation and inhibition of intermediate filament bundling. In conclusion, myosin IIA is expressed in podocyte and could play an important role in maintaining the structural integrity of podocytes.

1760

Role of Nonmuscle Myosin IIB in Cancer Cell Invasion through 3-Dimensional Matrix.

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Tumor metastasis is a complex process that involves the migration of a tumor cell from the primary tumor to distant organs. A major component to cellular migration is the motor protein nonmuscle myosin II (NMII). NMII is expressed in mammalian cells to regulate cellular structure and cytoskeletal dynamics. The three nonmuscle isoforms (IIA, IIB, and IIC) are expressed in tissue and cell type specific patterns. Treatment of the murine mammary gland epithelial cell line, NMuMG, by transforming growth factor β -1 (TGF β 1) induces an epithelial to mesenchymal change in morphology. During this transition, NMIIB is dramatically upregulated at both mRNA transcript and protein levels. This observation led our group to investigate if NMIIB is required for the invasion of tumor cells through a dense 3D collagen matrix.

We used a macrophage-stimulated migration assay, where the tumor cells enter a paracrine loop with macrophages to enhance the upward migration through a three-dimensional collagen gel. NMIIB knockdown in human breast carcinoma cells (MDA-MB 231) reduced tumor cell invasion by 85% compared to control. Similarly, the murine mammary tumor cell line (4T1) exhibited a 47% reduction in invasion in NMIIB knockdown cells compared to control.

To better understand the mechanism of which NMIIB knockdown inhibits migration, we are examining the contribution NMIIB plays in both the generation of traction stress to the extracellular environment as well as other aspects of cellular organelle organization and polarity. Knockdown of NMIIB in 4T1 cells, reduced the average traction stress of a cell 86% compared to scramble control ($p < 0.05$). Knockdown of NMIIA, an abundant isoform in 4T1 cells failed to reduce the traction stress. Also, we are generating a collection of fluorescent protein-tagged organelle and nuclear markers to assess the role of NMIIB in cytoskeletal organization and cell polarity during 2D and 3D migration.

These data suggest that NMIIB plays an important role in tumor metastasis and invasion as by regulating a cell's interaction with its extracellular environment. Further studies are being pursued to identify mechanisms and possible therapeutic targets.

1761

C2 insert containing isoform of Nonmuscle Myosin II-C is necessary for neuritogenesis of Neuro-2a cells.

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Nonmuscle myosin IIs (NM IIs) are ubiquitously expressed throughout the entire mammalian organism and play distinct roles in cell division, adhesion, and migration. Three isoforms of NM II: namely II-A, II-B and II-C are found in vertebrates. Recently, it has been shown that the heavy chain of NM II-C undergoes alternative splicing at loop1 and loop2 of the head region, and gives rise to four possible isoforms: NM II-C0, -C1, -C2, -C1C2. C2 insert containing isoforms NM II-C2 and NM II-C1C2, are specifically expressed in mouse and human brain, and their ATPase and in vitro motility activities are independent of myosin light chain phosphorylation. However, the functional role of these alternatively spliced isoforms is unknown. Here, we report for the first time that the expression of the C2 insert containing isoform, NM II-C1C2 is inducible in Neuro-2a cells during differentiation both at mRNA and protein levels. Immunoblot and RT-PCR analysis reveal that expression of NM II-C1C2 peaks between days 3-

6 of differentiation. Localization of NM II-C2 and -C1C2 in Neuro-2a cells suggest that C2 insert containing isoforms are localized in the cytosol and along the neurites, specifically at adherence point to substratum. Inhibition of endogenous NM II-C1C2 using siRNA decreases the neurite length by 43% compared with control cells treated with nonspecific siRNA. Time lapse image analysis reveals that neurites of C2-siRNA treated cells have a net negative change in neurite length per minute, leading to reduction of overall neurite length. During the neuritogenesis, NM II-C1C2 can interact and colocalize with β 1-integrin in neurites as assessed by immunoprecipitation and immunofluorescence studies. Altogether, these studies indicate that NM II-C2 may be involved in stabilizing neurites by maintaining their stable structure at adhesion sites

1762

Myosin II isoforms self-organize characteristic contractile units in actomyosin bundles.

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Diverse myosin II isoforms regulate contractility of actomyosin bundles in disparate physiological processes by variations in both motor mechanochemistry and the extent to which motors are clustered into thick filaments. While the role of mechanochemistry is well appreciated, the extent to which thick filament size regulates actomyosin contractility is unknown. Here we studied the contractility of minimal actomyosin bundles formed in vitro by mixtures of F-actin and thick filaments of non-muscle, smooth and skeletal muscle myosin isoforms with varied thick filament size. The diverse myosin II isoforms guide the self organization of distinct contractile units within in vitro bundles with measured shortening rates similar to those of in vivo myofibrils and stress fibers. The frequency of self-organized contractile units along the bundle is proportional to the thick filament size, resulting in a bundle shortening rate that is proportional to thick filament size. These experiments provide insight into the regulation of smooth muscle contractility where thick filament size is dynamically regulated. We develop a model that describes our data, providing a framework in which to understand how diverse myosin II isoforms regulate the contractile behaviors of actomyosin bundles found in muscle and non-muscle cells.

1763

F-actin Buckling Coordinates Contractility and Severing in a Biomimetic Actomyosin Cortex.

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Myosin II motors drive contractility of the cortical actin network, enabling shape change and cytoplasmic flows underlying diverse physiological processes ranging from cell division and migration to tissue morphogenesis. Yet, despite its importance, the mechanisms that describe contractility and the generation of mechanical forces within the cortex are not well understood. Here we recapitulate contractility in vitro, through the development of a minimal model of the cell actomyosin cortex by coupling a two-dimensional, cross-linked F-actin network decorated by myosin thick filaments to a model cell membrane. Myosin motors generate both compressive and tensile stresses on F-actin and consequently, induce large bending fluctuations. Over a large range of conditions, we show the extent of network contraction corresponds exactly to the extent of individual F-actin shortening via buckling. This demonstrates an essential role of buckling in facilitating local compression to enable mesoscale network contraction of up to 80% strain. Portions of F-actin with a radius of curvature \sim 300 nm are prone to severing and thus, compressive stresses mechanically coordinate contractility with F-actin severing, the initial step

of F-actin turnover. Finally, the F-actin curvature acquired by myosin-induced stresses can be further constrained by adhesion of the network to a membrane, accelerating filament severing but inhibiting the long-range transmission of the stresses necessary for network contractility. Thus, the extent of membrane adhesion can regulate the coupling between network contraction and F-actin severing. These data demonstrate the essential role of the non-linear response of F-actin to compressive stresses in potentiating both myosin-mediated contractility and filament severing. This may serve as a general mechanism to mechanically coordinate contractility and cortical dynamics across diverse actomyosin assemblies in smooth muscle and non-muscle cells.

1764

Tension-sensing by myosin motors as a function of duty-ratio and network stiffness

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Motor proteins are critical to cells in processes such as cellular contractility, motility, cell division, and intracellular transportation and compartmentalization. Thus detailed simulations of these processes require motor representations that can be “benchmarked” to encapsulate key features of the biochemical and mechanical motor characteristics. Here we explore the feasibility of a simple two-spring motor model: we assign a measured crossbridge stiffness, powerstroke conformation change, and biochemical transition rates such that each individual *in silico* motor is a close abstraction (in these key features) of the real thing. We gain assurance in our model by mimicking experimental assays that measure the ability of myosin heads to translate actin filaments, both as a function of myosin density and load on the filament. Finally, we assemble these individual myosin motors into minifilaments to explore the fundamental units of actomyosin contraction. We discover that different isoforms of myosin—which may differ from each other mainly in duty-ratio—can serve to amplify or distribute load. This “tension-sensing” (or lack thereof) may be an important driver of the geometry and organization of contractile structures.

1765

Defining myosin relay domain interactions in muscle by mutation suppression.

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The relay domain of myosin is proposed to be a key communicator element that interacts a) within the main body of the motor to sense the nucleotide state and b) with the converter domain to drive the power stroke and muscle contraction. We tested specific residues involved in these proposed interactions by engineering mutations at sites modeled to contact the relay domain and then making putative compensatory mutations within the relay domain. The mutant myosins were expressed transgenically in *Drosophila* indirect flight muscles, which allow assessment of myosin function *in vitro* and *in vivo*. We mutated residue R714 near the SH1-SH2 helix in the main body of myosin. We found that R714E myosin ATPase is reduced by ~80% and that no *in vitro* motility is detected. R714E organisms show myofibril assembly defects that are exacerbated with age and these organisms cannot fly. Putative compensatory mutation E499R in the relay domain improves ATPase activity so that there is only ~35% reduction compared to wild type. Further, R714E/E499R myosin moves actin filaments at ~30% of wild-type velocity. Normal myofibril assembly is restored and myofibrils are more stable, but no flight ability is present. We next studied mutation R759E in the converter domain. It depresses myosin ATPase activity, *in vitro* motility and flight ability. Although myofibrils

assemble normally, they degenerate as flies age. We inserted lysine mutations at putative interacting relay domain loop residues I508, N509 or D511 in an attempt to suppress the R759E mutant phenotypes. N509K dramatically rescues muscle structure and function, with significant increases in ATPase rates and in vitro motility. In contrast, D511K has no effects on R759E myosin and muscle, whereas the I508K/R759E double mutant myosin has no ATPase activity or in vitro motility. Overall our compensatory mutation approach shows that residue R714 in the main body of the myosin motor interacts with residue E499 of the relay domain and that relay residue N509 interacts with converter domain residue R759. These interactions are critical for normal ATPase activity, in vitro motility, myofibril assembly and/or stability and flight muscle function. Thus the specific residue contacts identified permit the myosin relay domain to communicate information between the nucleotide binding pocket and the converter domain and are essential for progress through the mechanochemical cycle that powers muscle contraction.

Dynein

1766

A 3.3-Å structure of the dynein motor domain.

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Dyneins are minus end directed microtubule motors which transport many intracellular cargos, are necessary for mitosis and allow the beating of cilia and flagella.

Dyneins are large multi subunit complexes with molecular weights in the megadalton (MDa) range. The heavy chain of dynein contains a 300 kDa motor domain, which is present in all members of the dynein family. The motor domain is comprised of a ring of six " ATPases Associated with diverse cellular Activities " (AAA+) domains. Hydrolysis of ATP at the AAA+ domains causes the relocation of the linker domain to generate the force which is required for the movement of dynein. It is not known how the linker interacts with the ring during the ATP hydrolysis cycle. We use X-ray crystallography and subsequent biochemical experiments to gain insight into this mechanochemical cycle.

We determined a 3.3-Å crystal structure of the *Saccharomyces cerevisiae* cytoplasmic dynein motor domain, crystallized without nucleotide. In this state the linker interacts with a conserved site on AAA5, which mutagenesis experiments show is functionally necessary. Nucleotide soaking experiments show that the main ATP hydrolysis site (AAA1) is in a low-nucleotide affinity state and gives insight to the nucleotide interactions of three other sites (AAA2, AAA3 and AAA4).

1767

How Dynein and Microtubules Rotate the Nucleus.

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In living cells, a fluctuating torque is exerted on the nuclear surface but the origin of the torque is unclear. In this study, we found that the nuclear rotation angle is directionally persistent on a time scale of tens of minutes, but rotationally diffusive on longer time scales. Rotation required the activity of the microtubule motor dynein. We formulated a model based on microtubules undergoing dynamic instability, with tensional forces between a stationary centrosome and the nuclear surface mediated by dynein. Model simulations suggest that the persistence in rotation angle is due to the transient asymmetric configuration of microtubules exerting a net torque in

one direction until the configuration is again randomized by dynamic instability. The model predicts that the rotational magnitude must depend on the distance between the nucleus and the centrosome. To test this prediction, rotation was quantified in patterned cells in which the cell's centrosome was close to the projected nuclear centroid. Consistent with the prediction, the angular displacement was found to decrease in these cells relative to unpatterned cells. This work provides the first mechanistic explanation for how nuclear dynein interactions with discrete microtubules emanating from a stationary centrosome cause rotational torque on the nucleus.

References:

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Effects of Molecular Motors on Microtubule Mechanics.

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In order to determine forces on intracellular microtubules, we measured shape changes of individual microtubules following laser severing in bovine capillary endothelial (BCE) cells. Surprisingly, regions near newly created minus ends increased in curvature following severing, while regions near new microtubule plus ends depolymerized without any observable change in shape. With dynein inhibited, regions near severed minus ends straightened rapidly following severing. These observations suggest that dynein exerts a pulling force on the microtubule which buckles the newly created minus end. Moreover, the lack of any observable straightening suggests that dynein prevents lateral motion of microtubules. To explain these results, we developed a model for intracellular microtubule mechanics which predicts the enhanced buckling at the minus end of a severed microtubule. Our results show that microtubule shapes reflect a dynamic force balance, in which dynein motor and friction forces dominate elastic forces arising from bending moments. A centrosomal array of microtubules subjected to dynein pulling forces and resisted by dynein friction is predicted to center on the experimentally observed timescale, with or without the pushing forces derived from microtubule buckling at the cell periphery.

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Structural basis for microtubule binding and release by dynein.

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Cytoplasmic dynein is a large molecular motor responsible for nearly all minus-end-directed microtubule-based transport in cells. Movement by all motors involves coordinating cycles of track binding and release with cycles of force-generating nucleotide hydrolysis. For the well-studied myosin and kinesin motors, track binding and nucleotide binding occur within close proximity; in contrast, these sites are separated by 25 nm in dynein. A major question in the field is how coordination between these distant sites is achieved. We have obtained a sub-nanometer-resolution structure of dynein's microtubule-binding domain bound to microtubules by cryo-electron microscopy and used molecular dynamics to arrive at a pseudoatomic model of

the interaction. We have identified the structural rearrangements within the microtubule-binding domain that communicate microtubule binding to the rest of the motor as well as specific interactions used by dynein to tune its affinity for the microtubule. We used single molecule motility experiments to probe the functional role of these interactions, and provide evidence that cytoplasmic dynein has been selected for sub-maximal processivity. The importance of a finely tuned motor is supported by our finding that dynein-powered nuclear oscillations in yeast are altered in strains expressing highly processive mutant motors.

1770

Characterization of dynein by single-molecule investigations in vivo.

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Cytoplasmic dynein is a motor protein that exerts force on microtubules and in doing so, drives a myriad of intracellular activities including mitotic spindle positioning and chromosome movements in meiotic prophase. To generate these forces, dynein needs to be anchored, with the anchoring sites being typically located at the cell cortex. The key question is: By what mechanism do single dyneins accumulate at sites where they can generate large collective forces? Here, we directly observe single dyneins in fission yeast, which allowed us to identify the main steps of the dynein binding process: (i) from the cytoplasm to the microtubule, and (ii) from the microtubule to the cortical anchors Mcp5. We uncovered that dyneins on the microtubule move, surprisingly, either in a diffusive or a directed manner, with the switch from diffusion to directed movement occurring upon binding of dynein to the cortex. This was further confirmed by abolishing the cortical-binding ability of the anchor (Mcp5-PHΔ). We were thus able to force dynein on the microtubule bound to Mcp5-PHΔ to switch to directed movement, providing further evidence to the idea that dynein is 'activated' upon binding to its anchor. This dual behavior of dynein on the microtubule, together with the two steps of binding, constitute the mechanism by which dyneins find cortical anchors in order to generate large-scale movements in the cell.

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Cytoplasmic Dynein Uses Its Two Motor Domains To Crosslink And Slide Anti-parallel Microtubules.

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Cytoplasmic dynein is the predominant minus-end directed microtubule motor protein in cells. Dynein performs a myriad of functions in addition to canonical transport of vesicular cargo, including the organization of microtubules within the mitotic spindle. How dynein performs such non-canonical functions is unknown. Here we demonstrate using *in vitro* and *in vivo* assays that dynein utilizes its two motor domains to crosslink and slide anti-parallel microtubules. *In vitro*, both native dynein purified from brain tissue, as well as a minimal GST-dimerized construct lacking the tail domain and accessory subunits are capable of crosslinking microtubules, suggesting the motor domains alone are sufficient for this activity. Addition of ATP causes sliding of overlapped anti-parallel microtubules, demonstrating that dynein can slide microtubules independently of any other factors. Single molecule imaging reveals that individual dynein motors encountering an anti-parallel microtubule overlap often pause for prolonged periods of time and frequently reverse direction, suggesting that the two motor domains can bind both microtubules in the overlap simultaneously. *In vivo*, dynein produces an inward force in the mitotic spindle that antagonizes the kinesin-5-dependent outward sliding

force. We show using RNAi-rescue experiments that a minimal, tailless dynein dimer is sufficient for this activity of dynein, indicating that sliding of anti-parallel microtubules within the spindle can be driven by the action of the dynein motor domains alone. Our results identify an unexpected new mode of dynein driven motion and shed light on the mechanisms cells use to organize the microtubule network.

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Cytoplasmic Dynein Steps Through Uncoordinated Action of its Two Heads.

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Cytoplasmic dynein is a homodimeric AAA+ motor that transports a multitude of cargos towards the microtubule minus end. How the two catalytic head domains interact and move relative to each other during processive movement remains poorly understood. We have tracked the relative positions of both heads with nanometer precision and directly observed that the heads move independently along the microtubule. The heads remain widely separated and the stepping behavior of the heads varies as a function of interhead separation. Consistent with a lack of tight coordination, only one active head is sufficient for processive movement and the active head drags its inactive partner head forward. These results show that dynein is the first dimeric motor that moves processively without interhead coordination, a mechanism fundamentally distinct from the hand-over-hand mechanism of kinesin and myosin.

1773

Testing the tug-of-war model of bidirectional transport by measuring molecular motor forces *in vivo*.

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Molecular motor proteins are responsible for force generation in myriad cellular processes. Much of our understanding of how motors function has benefited from force measurements and manipulation at the single molecule level *in vitro*. We report on novel optical trapping methodology capable of precise *in vivo* stall-force measurements of *individual* endogenous cargoes hauled by molecular motors in their native environment. We present unprecedented stall force histograms of motor-driven lipid droplets in *Drosophila* embryos. Force measurements show that equal numbers of kinesin-1 and cytoplasmic dynein haul each cargo in opposite directions. Critically, by measuring cargo dynamics in the optical trap, we find that there is memory: it is more likely for a cargo to resume motion in the same direction -rather than reverse direction- after the motors transporting it detach from the microtubule due to the trap force. This suggests that only motors of one polarity are active on the cargo at any instant in time and is not consistent with the tug-of-war models of bidirectional transport where both polarity motors can bind the microtubules at all times. We further use the optical trap to measure, *in vivo*, the detachment rates from microtubules of kinesin-1 and dynein-driven lipid droplets. Unlike what is commonly assumed, we find that dynein's but not kinesin's detachment time *in vivo* increases with opposing load. This suggests that dynein's interaction with microtubules behaves like a catch bond and further argues in favor of a regulatory model for bidirectional transport.

1774

Dynein from *C. reinhardtii* translocates microtubules from conspecific axonemal tubulin faster than from porcine brain tubulin.

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Eukaryotic cilia are motile organelles whose regular beating propels cells through fluids and moves fluids past cells. They contain a highly conserved structure, the axoneme, consisting of a "9 + 2" array of nine microtubule doublets surrounding a central pair of singlet microtubules, dynein motor proteins, and accessory proteins. How the microtubules, motors, and accessory proteins give rise to the periodic beat is not known. However, recent studies have suggested that the mechanochemical properties of dynein may be sufficient to achieve coordination. Because the action of dynein requires it to interact with microtubules, it is likely that its mechanochemical cycle is dependent on specific dynein-tubulin interactions. It is known that axonemal microtubules are enriched in certain tubulin isotypes and post-translational modifications over non-axonemal microtubules. We asked if the mechanochemical and biophysical properties of axonemal dynein depend on the source of the microtubules.

We compared the gliding motility of microtubules polymerized from tubulin purified from *Chlamydomonas* axonemes and porcine brains over surfaces coated with *Chlamydomonas* axonemal dynein. We found that the translocation speed of microtubules polymerized from *Chlamydomonas* tubulin was 1.6 times faster than porcine brain microtubules. We also found that the saturating mean gliding velocity was reached with shorter *Chlamydomonas* microtubules than with porcine microtubules. These results show that the source of tubulin does affect the biophysical properties of motor proteins. Modeling these results further suggested that tubulin source affected dynein-microtubule affinity and the kinetics of its powerstroke.

These preferences may be attributable to differences in primary structure or post-translational modification of the tubulin. We further investigated some of the commonly occurring post-translational modifications in our gliding assays. The results additionally are suggestive of the roles these modifications may play in axonemal motility.

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The Minimal Requirements for Dynein Processivity and a Force-Dependent Gating Mechanism.

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Cytoplasmic dynein drives minus-end directed motility along microtubules. In contrast to other cytoskeletal motors, the detailed mechanism of dynein processivity and force generation remains unclear. By engineering the mechanical, catalytic, and geometric properties of the dynein motor domain we show that neither a rigid linkage between monomers nor dimerization between N-terminal tail domains is essential for processive movement. Instead, dynein processivity minimally requires the linker domain of one active monomer to be attached to an inert microtubule tether retaining only the dynein microtubule binding domain. The release of a dynein monomer from the microtubule can be mediated either by nucleotide binding or external load. Using optical trapping we find that nucleotide dependent microtubule release is inhibited when force is applied to the linker domain. Force dependent release is strongly asymmetric, with faster release towards the minus-end. On the basis of these measurements, we develop a model that describes the basis of dynein processivity, directionality and force generation.

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A novel function for Dynein in the plus-end transport of a localized mRNA.

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The proper functioning of most eukaryotic cells requires the establishment and maintenance of polarity. The *Drosophila* oocyte uses mRNA localization to establish polarity and hence is an excellent model to study these processes. In particular, *bicoid* mRNA localizes to the anterior margin of the oocyte, *gurken* mRNA localizes to the dorsal-anterior corner, and *oskar* mRNA localizes to the posterior pole. The spatial restriction of these mRNAs and subsequent protein products is essential for patterning the embryo. The localization of all three mRNAs requires intact microtubules, and presumably, microtubule-based motor proteins. For instance, loss-of-function mutants in Kinesin heavy chain (Khc), the motor subunit of Kinesin-1, results in *oskar* mRNA delocalization. Furthermore, the vast majority of *oskar* mRNP particles are non-motile in *khc* mutants. However, a small percentage of *oskar* particles display active and directional motility in *khc* nulls. These results suggest that although Khc is essential for *oskar* mRNA localization, another motor might also participate in the transport step. Consistent with this notion, published results indicate that Dynein heavy chain (Dhc), the motor subunit of the Dynein complex, co-localizes with Khc and *oskar* mRNA at the posterior pole. Additionally, we found that the targeted mis-localization of *oskar* mRNA to the center of the oocyte resulted in a similar re-distribution of Dhc and Khc. We therefore hypothesized that Dynein and Kinesin are present in a complex with *oskar* mRNA and that both motors are required for its efficient transport. In order to test this hypothesis, we specifically depleted Dhc in the germline using shRNA mediated knock-down. This strategy enabled us to bypass the requirement for Dynein in oocyte specification, a limitation that had previously restricted the analysis of Dynein function in mRNA localization. Germline-specific depletion of Dhc severely compromised the localization of *oskar* mRNA. In addition to *oskar* mRNA, the posterior localization of Khc was also affected. Based on these results, we conclude that the plus-end directed transport of *oskar* mRNA by Khc requires Dynein. Our results provide the first evidence that *oskar* mRNA localization requires the concerted activities of opposite polarity motors.

1777

Linking dynein mutations to crystal structures and cell phenotypes.

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Even with recent advances in the dynein heavy chain (DHC) crystal structure, the complex organization of the DHC has made it difficult to understand the dynein mechanochemical cycle. To further probe dynein structure/function relationships, we utilized forward and reverse genetics in the ascomycete fungus *Neurospora crassa*. *N. crassa* has a haploid genome and dynein is not an essential gene, making genetic studies straightforward to perform. Using a forward genetic screen we isolated over 30 spontaneous missense mutations spanning almost all the domains of the DHC gene. In addition, we reverse engineered mammalian neurodegenerative mutations into the DHC. The effect of DHC mutations on dynein localization was probed by replacing the native dynein intermediate chain (DIC) with a fluorescently tagged DIC. Live-cell imaging studies on strains carrying DHC mutations revealed the presence of at least six major classes of dynein localization phenotypes. Endomembrane cargo transport is altered to different extents in the different classes of DHC mutant strains. To further understand the mechanisms by which the mutations affect dynein function, mutations located distantly from

the enzyme active site were chosen for in vitro characterization. Studies on purified dynein from these strains revealed novel mechanisms of dynein regulation. We identify several interesting correlations between the location of individual mutations within the dynein structure and the specific cellular defects observed in vivo. The linkage between defects in particular areas of the DHC 3D structure with cellular mislocalization phenotypes indicate that the individual DHC mutations entrap the motor at particular stages of the dynein mechanochemical and intracellular transport cycles.

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The Dynein Inhibitor Ciliobrevin D Does Not Inhibit T Cell Microcluster Movement.

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The contact area between a T cell and an antigen presenting cell (APC) is organized into a bull's eye arrangement of segregated concentric regions, collectively known as the immunological synapse (IS). The IS serves as the structural basis of signaling and secretion between the T cell and APC. The center area of the IS, termed the central supra-molecular activation cluster (cSMAC), is marked by the accumulation of T cell receptor (TCR) microclusters (MCs). We recently showed that the centripetal movement of TCR MCs to the cSMAC is driven entirely by a combination of actin polymerization driven actin retrograde flow in the dSMAC (lamellipodial actin network) and actomyosin II driven actin arc contraction in the pSMAC (lamellar actin network). Saito and colleagues have, however, reported that the microtubule dependent transport of TCR MCs driven by cytoplasmic dynein contributes significantly to the centripetal movement of TCR MCs. Recently, a very effective membrane-permeable small molecule inhibitor of cytoplasmic dynein called Ciliobrevin was described. Here we show that the kinetics of centripetal TCR MCs movement are normal in Ciliobrevin-treated cells, suggesting that their movement is indeed largely, if not entirely, driven by actin-dependent mechanisms. Finally, we show that Ciliobrevin D is cytotoxic to Jurkat T cells when the cells are imaged with blue light, such as when imaging GFP-chimeras, but not when imaging with green light and higher wavelengths, representing a cautionary tale for other investigators interested in using Ciliobrevin to inhibit dynein in living cells.

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Dynein Phosphorylation Involved in Adenovirus Attack and Host Defense.

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Viruses have evolved to successfully exploit physiological host cell processes for efficient infection. Critical entry barriers for DNA viruses and retroviruses are the plasma membrane and the dense cytoplasm, which limits virions to sub-diffusional motility and attenuates nuclear targeting. Human adenovirus 5 (Ad5) binds to cell surface receptors (CAR and integrins), activating PKA and other protein kinases, and then enters the host cell by endocytosis. Following endosomal escape, Ad5 requires dynein-mediated transport to reach the nuclear envelope (NE) and complete the infectious cycle. Ad5 recruits dynein through a direct interaction between its hexon subunit and the dynein IC and LIC1 subunits. We recently found PKA phosphorylation of LIC1 to be essential for this interaction and for virus transport to the nucleus. However, why host activation of PKA should serve to enhance virus transport is unclear. To test for protective cellular effects of PKA phosphorylation, we tested for changes in microtubule organization and the behavior of a range of vesicular structures, including mitochondria, the Golgi apparatus, multi-vesicular bodies, early endosomes, and lysosomes/late

endosomes (lyso/LEs). We observe dramatic specific dispersal of lyso/LEs within 15-30 min of infection using lysotracker, GFP-Rab7, and GFP-NPC1 as markers, but no effect on microtubules or other vesicular organelles. Lyso/LE dispersal is blocked by RILP overexpression, and by pharmacological PKA or integrin inhibition. We identify a single, novel PKA site in LIC1, which is required for both Ad5 transport to the nucleus and lyso/LE dispersal as judged by use of phosphomutant and phosphomimetic LIC1 constructs. These results suggest a novel host defense mechanism involving centripetal lyso/LE transport to intercept incoming virus. Ad5 may, in turn, have evolved a compensatory mechanism for enhanced transport to the NE. Our results also identify what seems to be an important PKA target involved in regulating bidirectional organelle transport, a result with important implications for a general understanding of transport regulation.
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Force up-regulation in dynein driven lipid droplets of cos1 cells.

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Most intracellular organelle transport is along the microtubules and is carried out by a combination of kinesin variants and dynein. A key question in transport regulation is how the system deals with various impediments to motion, to avoid or minimize subsequent potential traffic jams. The force adaptation—evident within ten seconds, complete within thirty—improved the ability of LDs to escape the trap between three and four-fold. To date, there has been no suggestion that cargos can sense or respond to opposition to motion. However, at a larger scale there is a well-known biological precedent called the Starling's law¹. It is about the adaptive behavior that the heart undergoes where the more the heart is stretched, the stronger the cardiac muscle contracts. The adaptation could involve the activation and inactivation of motors wherein 'inactive' motors could be rapidly turned on, as needed, if signaling controlled the reactivation process. For kinesin, such inactivation/reactivation could occur via a newly discovered tail-independent pathway², by head-tail binding³, or by cofactors that directly inhibit the motor domain. For dynein, in vitro evidence shows the motor is inactive when bound only to NudE, and this inhibition is reversed when also bound to Lis1⁴. During the development of a new system to measure forces powering lipid droplets in cells, we were surprised to find that there was not a well-defined stalling force. That is, when we attempted to stall moving cargos, the majority stopped initially, but most escaped from the optical trap on subsequent attempts. We will be presenting the data showing that droplet force production adapts to overcome obstacles to advancement.

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2 Xu, J. et al. Casein kinase 2 reverses tail-independent inactivation of kinesin-1. *Nature communications* 3, 754, doi:10.1038/ncomms1760 (2012).

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1781

Heterologously Expressed BicD2 is Sufficient to Drive Apical Interkinetic Nuclear Migration in Developing Rat Brain.

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Radial glial progenitor cells (RGPCs) are precursors for the majority of neurons in the developing neocortex. The RGPCs undergo bidirectional cell cycle-dependent nuclear oscillations known as interkinetic nuclear migration (INM). Defects in INM contribute to brain developmental disorders, but the purpose and mechanism of this behavior remain under investigation. Our previous work revealed that KIF1A mediates basal nuclear migration during G1 whereas cytoplasmic dynein mediates apical nuclear migration during G2 (Tsai et al. Nat. Neurosci. 2010, 13:1463-71). Mitosis then occurs exclusively at the ventricular surface of the developing brain. These results provide a mechanism to account for alternating basal and apical nuclear transport. How cell cycle regulation of this behavior is controlled, however, remains largely mysterious. We have found that factors responsible for G2-specific recruitment of dynein to the HeLa cell nuclear envelope - BicD2, Nup133, and CENP-F - are required for apical nuclear migration in embryonic rat brain (Nayak et al., *MBoC*. 22 [abstr. 47] 2011). In utero electroporation of shRNAs for these factors arrested this process and further revealed that BicD2 is required earlier during G2 than Nup133 and CENP-F. These results support our hypothesis that dynein acts from the nuclear envelope during apical migration, which we have now further confirmed by immunohistochemical colocalization of dynein and BicD2 to the nuclear rim of RGPCs in situ. To test further the relative roles of BicD2 vs. Nup133-CENP-F in apical migration, we constitutively targeted BicD2 to the NE using a BicD2-KASH fusion protein. Nuclei of transfected RGPCs dramatically accumulated at the ventricular surface of the brain, suggesting that BicD2 can be sufficient for dynein recruitment to the nuclear envelope and apical migration. Expression of BicD2 alone rescued the late apical nuclear migration arrest we observe in RGPCs subjected to Nup133 RNAi. Our results indicate that a pool of nuclear envelope-associated cytoplasmic dynein drives apical INM, and that G2-dependent dynein recruitment to this site is not only necessary, but also sufficient to control G2 INM. Supp. by NIH HD40182.

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Molecular adaptations in dynein for generation of large forces inside cells.

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The unit generator of force inside living cells is often a motor protein that exerts a tiny force of 1-10 piconewtons. However, many processes in cells require much larger forces and must therefore rely on motors working in a team. How collective force is generated by such teams of motors is fundamentally important, but has evaded understanding. To investigate this, we develop a new model system for optical trapping directly inside living cells that overcomes certain caveats and technical hurdles with earlier in vivo force measurements. This allows us to measure the function of endogenous cellular motor-assemblies with a precision comparable to in vitro bead assays.

We make the paradoxical observation that weak dynein motors team up to generate very large forces whereas stronger kinesins fail as a team. We show that dynein's improved teamwork arises from this motor's unique ability to vary stepsize in response to load, i.e. to shift gears (Mallik et al, Nature 2004). Each dynein motor in a team speeds up or slows down, depending

on the load it experiences. Dyneins therefore bunch closer together when hauling load, and consequently share load better to work efficiently as a team. We provide multiple independent lines of experimental evidence to support this hypothesis, and bring out precise mechanistic details of this process.

These results connect single-molecule properties of motors to their biologically relevant cellular functions, and suggest that nature has designed different motor proteins to adapt them for specific functions. More specifically, our work shows why dynein, the only known geared motor, needs a gear inside cells.

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Regulated inhibition of dynein motility along astral microtubules ensures unidirectional spindle movement along the polarized cell axis.

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Cytoplasmic dynein and kinesin transport a multitude of cargos toward the opposite microtubule ends in the cell. Whether and how these motors are differentially regulated along microtubules are poorly understood. In yeast, we have proposed a regulated-offloading model to explain how dynein powers the transport of the nucleus into the mother-bud neck via microtubule sliding along the cell cortex: the dynein regulator She1 limits dynein offloading by gating the recruitment of dynactin to the astral microtubule plus end, a prerequisite for offloading to the cortex. However, whether She1 subsequently affects cortically anchored dynein activity during microtubule sliding is unclear. Using single-molecule motility assays, we show that She1 strongly inhibits dynein movement along microtubules, acting directly on the motor domain in a manner independent of dynactin. She1 has no effect on the motility of either Kip2, a kinesin that utilizes the same microtubule track as dynein, or human kinesin-1, demonstrating its specificity for the dynein motor. At single-molecule resolution, She1 binds tightly to and exhibits diffusional behavior along microtubules. Diffusive She1 collides with and pauses motile dynein motors, prolonging their attachment to the microtubule. Furthermore, Aurora B/Ipl1 directly phosphorylates She1 and this modification appears to enhance the diffusive behavior of She1 along microtubules and its potency against dynein. In cells, She1 dampens productive microtubule-cortex interactions specifically in the mother compartment, thereby polarizing spindle movements toward the bud cell. Our data reveal how inhibitory microtubule-associated proteins selectively regulate motor activity to achieve unidirectional cargo transport, and demonstrate a direct link between cell cycle machinery and dynein pathway activity.

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Cortical dynein is critical for proper spindle positioning in human cells.

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Correct placement of the mitotic spindle is a fundamental process for defining cell division axis. Studies in several cellular systems revealed that cytoplasmic dynein together with evolutionarily conserved cortically anchored ternary complex (LIN-5/GPR-1/2/G α in *C. elegans* and NuMA/LGN/G α in human cells) is required for spindle positioning, but their relationship is not completely understood. By analyzing fixed specimens on fibronectin-coated coverslips and conducting live imaging experiments, we uncover that balance levels of ternary complex components are critical for dynein-dependent spindle positioning in non-polarized HeLa cells and *C. elegans* embryos. Intriguingly, by employing mutant versions of G α lacking myristoylation

signal, we establish that dynein is needed at the cortex to direct spindle positioning. Importantly, we identified a region within NuMA that mediates its interaction with dynein motor complex. By targeting this region to the plasma membrane, we demonstrate that the mere presence of dynein at that location is sufficient to direct spindle oscillations independent of ternary complex in HeLa cells. Overall, our findings support a model in which the balanced proportion of ternary complex serves to anchor dynein at the plasma membrane to direct spindle positioning.

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Attenuation of Dynein-Mediated Spindle Elongation Ensures Proper Force Generation During Anaphase.

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The segregation of sister chromatids during anaphase ensures that each resulting daughter cell possesses one set of chromosomes. This process is mediated by the mitotic spindle, which consists of microtubules, microtubule-associated proteins (MAPs), and molecular motors. It is currently unclear how these motors and MAPs generate an appropriate output of force to separate the spindle poles and thus achieve accurate chromosome segregation. Here, we use live-cell imaging to show that budding yeast deficient for She1 (*she1Δ*), a well-characterized inhibitor of dynein pathway function, exhibit significantly faster pole separation than wild-type cells. Combining *she1Δ* with mutations in either of the two kinesin-5 motors (*kip1Δ* or *cin8Δ*) did not restore the rate of pole separation to wild-type levels, suggesting that neither of these motors is responsible for the fast anaphase phenotype. Consistent with this hypothesis, we find that recombinant She1 has no effect on the *in vitro* motility of Cin8-3GFP. However, we found that *she1Δ* cells that were also deleted for the dynein heavy chain (*she1Δ dyn1Δ*) exhibited anaphase kinetics that were in fact restored to wild-type levels, suggesting that dynein is responsible for the fast anaphase phenotype. We also find that loss of dynein alone (*dyn1Δ*) has no effect on anaphase kinetics, indicating that dynein may normally be prohibited from participating in this process in wild-type cells. To determine whether hyperactivity of dynein can account for this phenotype independent of She1 dysfunction, we enhanced dynein function by overexpressing Pac1/LIS1, which increases the frequency and the level of dynein at the cell cortex. We found that overexpressing Pac1 in wild-type cells phenocopied the fast pole separation observed in *she1Δ* cells. Previous studies suggest that *she1Δ* cells exhibit a chromosome segregation defect, which may be a consequence of the rapid spindle elongation observed here. Thus, our data suggest that cortical dynein can contribute to anaphase forces, but its activity is prevented by regulated cortical targeting and She1-mediated inhibition to effectively attain an appropriate rate of spindle elongation. (*These authors contributed equally to this work)

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Motor Neuron Disease-Associated Mutations in p150^{Glued} Disrupt the Initiation of Retrograde Axonal Transport.

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Increasing evidence suggests that defects in axonal transport may be a primary cause of neurodegenerative diseases. p150^{Glued} is the major subunit of dynactin, a complex that functions with dynein in minus-end-directed microtubule transport. Different mutations within the p150 CAP-Gly microtubule-binding domain cause distinct autosomal dominant neurodegenerative syndromes, termed Hereditary

Motor Neuropathy 7B (HMN7B) and Perry Syndrome. HMN7B-associated mutations introduced into *Drosophila Glued* using homologous recombination generate a partial loss-of-function allele (Gl^{G38S}) with impaired neurotransmitter release at the neuromuscular junction (NMJ) and adult-onset locomotor dysfunction. Interestingly, Gl^{G38S} animals accumulate endosomes within swollen terminal boutons of the NMJ, and this phenotype is due to a neuron autonomous loss of *Glued* function. Surprisingly, the anterograde motor kinesin and retrograde motor dynein colocalize on these expanded terminal endosomes, and genetic interactions studies show that kinesin and p150 function synergistically at the NMJ. Live imaging analysis of vesicle transport along axons and at the NMJ suggests that this phenotype is not due to a generalized defect in axonal transport, but rather a specific defect of dynactin in coordinating initiation of retrograde transport at microtubule plus ends of the distal-most NMJ boutons, called terminal boutons. Together, these data suggest that the presynaptic retractions that occur early in motor neuron degenerative diseases begin with an inability to initiate retrograde axonal transport at terminal boutons. We are currently investigating: (1) How p150 affects synaptic vesicle cycling and neurotransmitter release, (2) How HMN7B and Perry mutations differentially affect p150 function in axons and synapse, and (3) Modifiers of HMN7B-mediated axonal degeneration.

1787

Dynactin contributes to formation of a septin barrier in the midbody and is required for timely abscission.

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Dynactin is a critical component of the cytoplasmic dynein motor that acts as a cargo adaptor and processivity enhancer. We showed previously that dynactin's "pointed end complex" subunits, p27 and p25, help target dynactin to early and recycling endosomes. Cells depleted of p27 and p25 show no obvious structural defects in their mitotic spindles and complete karyokinesis normally, but they do exhibit profound delays in cytokinetic abscission. Dynactin is not essential for MKLP-1 or Aurora B kinase targeting to the central spindle or midbody. However, microtubule growth patterns and the localization of the microtubule cross-linker, PRC1, in the cytokinetic bridge are affected in cells depleted of dynactin subunits. Our data suggest that dynein/dynactin based events are required for assembly of a physical barrier in the midbody region that is necessary for timely abscission. In keeping with this, we found septin accumulation in the midbody to be significantly reduced in cells depleted of dynactin subunits. These results are consistent with a model in which septin is carried into the cytokinetic bridge via dynein/dynactin-based movement of recycling endosomes.

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Probing the subunit interactions in the dynactin shoulder/sidearm.

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Dynactin is a multi-subunit complex that acts as a cargo adaptor and processivity enhancer for the microtubule-based motors cytoplasmic dynein and kinesin-2. Biochemical analysis of the dynactin complex indicates that it is composed of three sub-structures: the shoulder/sidearm, the Arp1 minifilament and the pointed end complex. The shoulder/sidearm, which consists of p150^{Glued}, dynamitin and p24, binds to cytoplasmic dynein via the p150^{Glued} subunit. We aim to understand the protein-protein interactions within dynactin's shoulder/sidearm, as well as how the shoulder/sidearm anchors to the Arp1 minifilament. To this end, we have taken advantage of the insolubility of bacterially expressed p24 to probe the interactions among the shoulder/sidearm subunits. In the presence of either dynamitin or p150^{Glued}, p24 can be renatured by step-wise dialysis out of denaturant, whereas it is insoluble on its own under the same conditions. Using a series of fragments containing its predicted α -helical domains, we

have been able to refine the portions of p24 necessary for complex formation. AA 1-67 of p24 form a complex with AA 127-1048 of p150^{Glued}. AA 114-178 of p24 interacts with both the N-terminal and C-terminal halves of dynamitin, suggesting it binds both parts of the molecule. AA 33-67 of p24 interacts with just the C-terminal half of dynamitin (AA 213-406). Taken together, these results indicate that the extreme N-terminus of p24 binds p150^{Glued} and the C-terminus of p24 interacts with dynamitin.

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Production of recombinant cytoplasmic dynein complexes.

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Movement is one of the defining characteristics of life at the cellular level. Cytoplasmic dynein is one of the motors that power this movement. It carries a wide range of cargos and plays a crucial role during cell division. The largest component of the dynein motor complex is the dynein heavy chain, which consists of a head and a tail domain. The head contains the motor domain, which powers movement utilizing the energy from ATP hydrolysis. Although the dynein motor domain generates the force needed for movement, it depends heavily on the tail domain to function as a processive motor that can transport cargo. The heavy chain tail serves as a scaffold for a number of additional dynein subunits. The intermediate chain and light intermediate chain bind directly to the heavy chain tail, whereas light chains bind to the intermediate chain. Together these accessory chains and the heavy chain make up the dynein tail complex that provides the site of cargo and adaptor protein interaction. Unlike other motors, only one cytoplasmic dynein exists, which suggests that it has unique mechanisms for controlling cargo specificity. In budding yeast (*Saccharomyces cerevisiae*) dynein is not essential, which has allowed us to engineer yeast strains that express tagged dynein complexes at very high levels. Furthermore we use fed batch fermentation to grow large quantities of these strains allowing us to scale up protein production. Our preliminary data indicate that we are able to produce large quantities of complete dynein complexes as well as separate dynein tail complexes obtained by truncating the heavy chain gene. We have been able to confirm the functionality of the full length complexes using single molecule TIRF microscopy assays. Furthermore, we have confirmed that the dynein tail complexes are well behaved and complete using both mass spectrometry and gel filtration analysis. Taken together, the tools that we have developed to date will help us to uncover the mechanisms underlying of dynein's cargo specificity.

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Polymerase Chain Reaction Procedures for the detection of Human T-Cell Leukemia Virus Type 1.

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Human T-Cell Leukemia Virus Type 1 (HTLV-1) affects an estimated 10-20 million people worldwide. It is believed to be the causative agent of adult t-cell leukemia (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). To initiate a successful infection, HTLV-1 must bind to the cell, penetrate into the cytosol, deposit its genome into the host cell cytoplasm and progress to the nucleus in order to replicate. Previous studies have shown that HTLV-1 infection is dependent upon interactions between the virus and host cell cytoskeleton. Researchers have illustrated that HTLV-1 intracellular movement is dependent upon microtubules. Our laboratory has found evidence that HTLV-1 interacts with a microtubule-based motor protein called cytoplasmic dynein. Therefore, we have formulated the hypothesis that cytoplasmic dynein is required for HTLV-1 replication. To investigate this, we will use

polymerase chain reaction (PCR) method. Initially, we purified an HTLV-1 DNA plasmid called delta X-M. This plasmid contains two of the three HTLV-1 viral genes. To determine if delta X-M was purified we performed restriction enzyme digest with Bam H1. Gel analysis indicated the presence of two expected band sizes of 4315 and 6633 base pairs. Next, we performed PCR using the purified delta X-M plasmid. Gel electrophoresis analysis demonstrated that a region of HTLV-1 Pol gene was amplified. Collectively, the results support the notion that PCR is an effective method to study HTLV-1 replication. Currently, we are employing siRNA technique to knock-out cytoplasmic dynein. This approach will help us determine whether cytoplasmic dynein plays a significant role in HTLV-1 replication process.

Actin and Actin-Associated Proteins III

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Formin INF2 localizes to focal adhesions and is critical for coordination of adhesion and contraction.

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Focal adhesions (FAs) are mechanosensitive complexes that form the physical and signaling link between the extracellular matrix and the actin cytoskeleton and are critical to driving cell migration. During migration, FAs form at the cell leading edge then undergo force-dependent maturation during which they elaborate a dorsal stress fiber to allow integration of force transmission across the cell. The formin family of actin nucleators is thought to be critical to FA maturation and formation of dorsal stress fibers. A proteomic analysis of FAs from our lab revealed two formin family members as FA components. We investigated the role of one of these, the formin INF2, in assembly and maturation of FAs and their associated stress fibers in mouse embryonic fibroblasts. We found that green fluorescent protein-tagged INF2 associated with FAs, partially co-localizing with known adhesion proteins including paxillin, VASP, and vinculin. INF2 localized to the rear of paxillin-marked FAs, partially co-localizing with the growing stress fiber. We utilized siRNA-mediated knockdown of INF2 to determine its role in FA and stress fiber formation and dynamics. Knockdown of INF2 had no effect on localization of paxillin, vinculin and VASP to FA, however had major effects on FA morphology. Compared to control, in cells with reduced INF2, FAs were smaller, more numerous, and failed to elongate, indicative of a possible maturation defect. Examination of the actin cytoskeleton in cells with reduced INF2 showed that although both stress fibers and dorsal arcs were present, INF2 knockdown cells contained fewer dorsal stress fibers, and actin bundles in both stress fibers and arcs were wavy and disorganized. To determine the requirement for INF2 in cell migration, we tracked migration of individual cells. INF2 knockdown cells also exhibited migration defects migrate displaying an abnormal "jumping" motility. These results suggest that INF2 participates in coordinating adhesion and contraction during cell migration by integrating FA maturation and stress fiber formation.

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Single actin filament imaging studies of the *Drosophila* formin Cappuccino.

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The *Drosophila* formin Cappuccino (Capu) is essential for body axis establishment in the fly oocyte. When Capu is mutated, a diffuse cytoplasmic actin mesh is absent during mid-oogenesis, a condition that is correlated with premature cytoplasmic streaming and a loss of cell polarity. Capu has two mammalian homologues, Fmn1 and Fmn2, the latter of which is essential for construction of a cytoplasmic actin network in the mouse oocyte. Since the first *in vitro* studies of formin/actin interactions, a common theme has emerged: while formins build F-actin through highly conserved domains, there is a great deal of diversity in activities across the seven families of mammalian formins. These activities include processive elongation; filament nucleation, capping, and severing; intra- and inter-molecular regulation; microtubule binding; and F-actin bundling. We are interested in understanding the biochemical details of Capu family formins in order to better understand the nature of the actin-based structures that they build *in vivo*. Toward that end, we have used total internal reflection microscopy to study the effects of Capu-CT (including the FH1 and FH2 domains through the C-terminus) on actin filament growth. Similar to other formins, Capu and Chickadee (Chic, *Drosophila* profilin) accelerate the rate of filament elongation three- to four-fold over the rate of actin alone. This rate acceleration is only observed with Chic and not seen when Capu is mixed with human or yeast profilins. In the absence of Chic, Capu does not change the rate of filament growth. The C-terminal tail (~30 aa) of Capu has been identified as a crucial region for actin filament nucleation, regulation by the WH2-based nucleator Spire, microtubule association, and autoinhibition. We find that it also plays a role in filament elongation. When the tail of Capu-CT is truncated, the elongation rate of filaments from Chic-actin is unchanged, but the processivity of Capu-CT is compromised. We are currently using mutagenesis and chimeric formin constructs to understand the nature of the interaction between the tail and the elongating filament.

1793

Cell-free analysis of the curious regulation of actin dynamics by the formin INF2.

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INF2 is a formin protein that catalyzes both the polymerization and depolymerization of actin filaments. In cells, INF2 localizes in a splice variant-dependent manner to either the endoplasmic reticulum (ER) or the cytoplasm, and regulates mitochondrial or Golgi dynamics, respectively. Mutations in INF2's Diaphanous Inhibitory Domain (DID), a key auto-inhibitory region for other formins, have been identified in two diseases: a kidney disorder called Focal Segmental Glomerulosclerosis (FSGS) and the neuronal disease Charcot Marie Tooth Disease (CMTD). The placement of these mutations suggests regulatory abnormalities in these diseases. My project targets the unusual regulation of INF2. In cells, INF2 is largely inactive for actin assembly, and this regulation depends upon auto-inhibitory interaction of DID with the C-terminal Diaphanous Auto-regulatory Domain (DAD). When purified INF2 is tested, however, the DID/DAD interaction does not inhibit actin polymerization. These results suggest that the DID-DAD interaction is important for INF2 regulation, but requires additional factors for robust inhibition. We term this regulation "facilitated auto-inhibition". To identify factors acting in facilitated auto-inhibition, I have developed a cell-free assay that monitors INF2-mediated actin polymerization activity on ER membranes. Alone, these membranes have a low ability to polymerize actin, but are strongly activated by a poly-clonal antibody against the DID. Addition of an antibody against the FH2 domain of INF2 abolishes polymerization, suggesting the

polymerization is INF2 dependent. I use this system to test the relevance of protein and non-protein co-factors in INF2 facilitated auto-inhibition.

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Involvement of formins in myosin-X driven filopodia formation.

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Filopodia are dynamic cellular protrusions composed of actin filament bundles. They have been found to be important in many cellular activities including cell migration, neuronal growth cones dynamics, cell-cell junction formation and others. Unconventional myosin-X (Myo10) is a powerful inducer of filopodia formation and elongation once it undergoes over-expression (Berg & Cheney, 2002, Nature Cell Biology 4, 246). Live-cell imaging shows that Myo10 is present at the filopodia tip as a bright puncta and remains there as the filopodia extend and retract. The exact mechanism, by which Myo10 induces filopodia formation and elongation remains unclear. Formins are a family of actin nucleators, which also promote the elongation of actin filaments. A few members of the formin family have been found in filopodia, though the exact role they play in filopodia dynamics remains controversial. Here we present that Myo10 induced filopodia formation is highly dependent on formins. We found at least two formins, Dia1 and Dia2, were present in filopodia of HeLa cells with over-expression of myosin 10. SMIFH2, a small molecule inhibitor of formin homology 2 domains, dramatically decreased the number and length of Myo10 induced filopodia and in higher doses lead to the disappearance of Myo10 from the filopodia tip. Applying low doses of SMIFH2 caused changes in the dynamics of Myo10 enriched puncta at the filopodia tip. We observed numerous Myo10 positive patches being pinched out of the myosin 10 puncta at the filopodia tip and moving rearward towards the cell body at rates corresponding to the retrograde flow of actin in filopodia. Forward motion of Myo10 puncta observed in control conditions was completely blocked by SMIFH2. Furthermore, we demonstrate that the morphological phenotype observed after SMIFH2 exposure is consistent with the phenotype observed after specific short-hairpin (sh) RNA-mediated down regulation of human Dia2 gene. Ectopic expression of mouse Dia2, which is insensitive to human shRNA knockdown, rescued the Myo10 induced filopodia formation similar to the control phenotype. We hypothesize that Myo10 functions in concert with formins in filopodia formation and maintenance.

1795

Mechanistic Studies of *Drosophila* Spir, a WH2-Nucleator.

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In the past few decades research has shed light on how nucleators contribute to the initiation of actin polymerization. Specifically we have learned a great deal about the Arp2/3 complex and formins, but there is still little understood about the third and newest class, WH2-nucleators. Beyond the fact that they use multiple actin-monomer-binding-WH2 domains to initiate actin filament assembly, a common theme is that an extra motif or domain is needed. To better understand the mechanism of WH2-nucleators we are using *Drosophila* Spire (Spir), as a model. We found that two WH2 domains connected by a specific linker motif, called linker-3, are sufficient for nucleation. Spir has a WH2 cluster consisting of four WH2 domains that are ~20 amino acids long, tethered together by short linker regions, the last one being linker-3. We took a reductionist approach to studying how Spir nucleates actin filaments by first examining the affect of each individual WH2 domain on actin. We observed large differences in affinity for actin monomers (30 nM - 1 μ M), as well as distinct effects on spontaneous actin polymerization. To

our surprise, the amount each WH2 domain inhibited spontaneous actin polymerization did not correlate with the affinity of the WH2 domains for actin monomers. Next, we worked with more complex constructs trying to incorporate what we have learned. The latter half of Spir's WH2 cluster (C3D) has roughly half the nucleation activity as the full cluster. We will refer to this as wildtype activity. We have created chimeras, consisting of different WH2 domains connected by linker-3 to identify how domain-order affects nucleation activity. Briefly, placing the third WH2 domain of Spir (WH2C) N-terminal to linker-3 produced the strongest nucleators, similar to the wildtype construct. In fact, a construct with reversed order (D3C) had no nucleation activity. Finally, we found that only two point mutations were sufficient to convert a moderate strength nucleator (W3D) to wildtype activity levels. Continuing this work will help us refine our model of the mechanism of WH2-nucleators, which play important roles in various processes such as oogenesis and neuronal development.

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Interactions between microtubules and the *Drosophila* formin Cappuccino.

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Formin family actin nucleators are potential coordinators of the actin and microtubule cytoskeletons; in addition to nucleating actin filaments, all formins tested to date can bind and/or bundle microtubules *in vitro*. To gain a more detailed mechanistic understanding of formin-microtubule interactions and formin-mediated actin-microtubule crosstalk, we studied microtubule binding by Cappuccino (Capu), a formin involved in regulating actin and microtubule organization during *Drosophila* oogenesis. We found that the C-terminal half of Capu (CapuCT, containing the FH1, FH2, and tail domains) binds taxol-stabilized microtubules with submicromolar affinity. Removal of the ~30 aa C-terminal tail dramatically reduces CapuCT's ability to bind microtubules. This sequence is sufficient for microtubule binding, albeit with micromolar affinity, suggesting that both the tail and upstream regions in CapuCT are required for high-affinity binding. Interestingly, the microtubule binding stoichiometry of the tail is approximately twofold higher than that of CapuCT, and both constructs exhibit reduced binding stoichiometry to microtubules at higher ionic strengths. Attempting to further map a microtubule-binding motif within the tail domain, we found that CapuCT-microtubule binding decreased approximately linearly as the tail was successively truncated. Furthermore, no single point mutation within the tail had a marked effect on microtubule binding. Together, these results suggest that microtubule binding by the tail domain is mediated by nonspecific charge-based interactions. To investigate the role of Capu in coordinating the actin and microtubule cytoskeletons, we next asked whether microtubules affect CapuCT's actin polymerization activity: indeed, they potently inhibit CapuCT's actin polymerization activity *in vitro*. We then mutated two conserved actin binding residues in CapuCT's FH2 domain (I706A and K856A) and tested the ability of these mutants to bind microtubules. While the I706A mutation had minimal effects on CapuCT-microtubule binding, the K856A mutation increased microtubule binding stoichiometry approximately twofold to a stoichiometry similar to that of the tail. Together, these findings fit our model that specific residues within FH2 are important for microtubule binding, perhaps orienting the formin dimer on the microtubule lattice, while the highly charged tail confers avidity through non-specific ionic interactions. Loss of FH2 domain binding may reveal additional microtubule-tail binding sites, thereby increasing binding stoichiometry. Microtubules could inhibit CapuCT's actin polymerization activity by preventing actin from binding to the FH2 and tail domains, which are both necessary for robust actin nucleation activity.

1797

The formin FMNL3 induces filopodia that act in cell-cell adhesion.*T. J. Gauvin¹, H. N. Higgs¹; ¹Biochemistry, Geisel School of Medicine at Dartmouth, Hanover, NH*

FMNL3 is a vertebrate formin that bundles actin filaments *in vitro*, in addition to the common formin activities of enhancing actin nucleation and allowing extended actin filament elongation. Constitutively active FMNL3 constructs produce abundant filopodia when over-expressed in multiple cell types. In the current work, we show that the role of endogenous FMNL3 is to produce filopodia, since siRNA-mediated FMNL3 suppression results in a 2-fold filopodial decrease in U2OS cells. FMNL3-induced filopodia enrich at new cell-cell contacts. FMNL3 suppression results in cell-cell adhesion defects in U2OS cells moving as a sheet, with a 3-fold increase in the occurrence of gaps between cells and in the lifetime of these gaps. Given the effect of FMNL3 on cell-cell adhesion, we investigated its localization and dynamics during the process in more detail. By immunofluorescence microscopy, endogenous FMNL3 localizes to filopodial tips and also to diffraction-limited (<300 nm) cytoplasmic puncta. Two lines of evidence suggest these puncta are microtubule-associated. First, the puncta are dispersed by nocodazole (microtubule depolymerization) but not Latrunculin B (actin depolymerization). Second, the puncta appear to align along microtubules in fixed cell images. Upon cell-cell contact, FMNL3 rapidly enriches at the nascent contact site, with decreased enrichment as the contact matures. Active Cdc42 or Rac1 (but not RhoA or RhoC) also induce FMNL3 translocation to the leading edge. To monitor FMNL3 dynamics, we developed an internal GFP-fusion construct, since both N- and C-termini serve important functions that are disrupted by tagging. Live-cell microscopy confirms that the intracellular FMNL3 puncta are associated with microtubules, and also shows that they are capable of rapid translocation along these microtubules. At the cell surface, FMNL3-labeled filopodia are highly dynamic, with lifetimes < 30 sec. Upon cell-cell contact, filopodia stabilize dramatically as the tips adhere to the neighboring cell. Based on these results, we propose a model whereby FMNL3 is sequestered on cytoplasmic vesicles that are microtubule-tethered. During cell migration, these vesicles are delivered to the leading edge, where FMNL3 is activated by Cdc42 to promote filopodial growth. Upon cell-cell contact, these filopodia stabilize, allowing cadherin-mediated adhesion.

1798

Arp2/3-dependent mechanism of filopodium formation by FMNL3: cellular and biochemical evidence for convergent elongation.*E. G. Heimsath, Jr. ¹, H. N. Higgs¹; ¹Biochemistry, Geisel School of Medicine at Dartmouth, Hanover, NH*

Filopodia are finger-like cellular protrusions involved in a myriad of processes, including chemosensing, cell migration, and cell adhesion. These structures are made up of bundled actin filaments oriented with their barbed ends toward the distal tip. To assemble filopodia, the activities of at least four components are needed: an actin nucleator, a factor that enables filament elongation in the presence of capping proteins, a filament bundling factor, and a factor that tethers filaments to the plasma membrane. In principle, the formin FMNL3 conducts all four of these activities, since N-terminal myristoylation localizes it to the plasma membrane, it can bundle actin filaments, as well as perform the common formin activities of increasing nucleation and preventing capping protein-mediated termination of elongation. Constitutively active constructs of FMNL3 induce potent filopodial assembly in multiple cell types. Curiously, however, FMNL3 constructs that are poor nucleators (but retain elongation and bundling activities) are equally capable of filopodia assembly. These results prompted us to probe the mechanism of FMNL3-mediated filopodial assembly in more detail.

Two general models have been proposed for filopodial assembly. In the “Tip Nucleation” model, a formin (or other nucleator) concentrates at the plasma membrane, nucleates actin filaments *de novo*, and then maintains elongation, causing protrusion and deformation of the membrane. Subsequent recruitment of the bundling protein fascin results in filopodial maturation. In the “Convergent Elongation” model, filopodia originate from an Arp2/3-derived lamellipodial dendritic network of branched filaments. In this Arp2/3-dependent mechanism, a membrane-bound formin (or other elongator) outcompetes capping protein for pre-existing filament barbed ends, facilitating synchronized elongation. Subsequent filopodial convergence occurs through fascin-mediated bundling.

Our data support a convergent elongation model for FMNL3-mediated filopodial assembly. Through Arp2/3 inhibition studies using the small molecule CK666 or shRNA-mediated suppression, we demonstrate in Jurkat pre-B leukemic cells that FMNL3-mediated filopodial assembly requires Arp2/3 complex. Using TIRF microscopy and a purified protein system including actin, profilin, capping protein, Arp2/3 complex, and fascin, we show that both nucleation-competent and nucleation-compromised FMNL3 constructs support bundling of Arp2/3 complex-mediated dendritic networks. Arp2/3 complex and FMNL3 are the key factors in this system, the former to nucleate filaments and the latter to allow elongation. Fascin enhances filament bundling, however is not sufficient to form filopodia either biochemically or in cells. We propose that FMNL3’s bundling activity facilitates initial convergence of Arp2/3 complex-initiated filaments, followed by bundle stabilization by fascin. Further experiments with VASP test its potential role in this process. We will relate these studies to cellular studies in our laboratory linking FMNL3-generated filopodia to cell-cell adhesion.

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Autoregulation of the formin Cappuccino in the absence of canonical autoinhibitory domains.

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Formins are a conserved family of proteins known to enhance actin polymerization. Five of the seven groups of metazoan formins are regulated by autoinhibitory interactions between the C-terminal diaphanous autoregulatory domain (DAD) and the N-terminal diaphanous inhibitory domain (DID). Based on the lack of DID/DAD sequence homology the formin family of formins was not predicted to be autoregulated. Previously published data for the *Drosophila* formin Cappuccino (Capu) is consistent with this prediction (Rosales-Nieves et al., NCB 2006). However, the mammalian ortholog of Capu, Formin-1, is autoinhibited (Kobielak et al., NCB 2004). We, therefore, re-examined Capu and found that it is, in fact, autoinhibited. Pyrene actin polymerization assays and TIRF single filament assays show that the N-terminal half of Capu (Capu-NT) potently inhibits nucleation and binding to the barbed end of elongating filaments by the C-terminal half of Capu (Capu-CT). Furthermore, Capu-NT was able to dissociate barbed end bound Capu-CT from actin filaments. We mapped the interacting regions. Within Capu-NT we identified residues 80-222 as the Capu Inhibitory Domain (CID). Hydrodynamic analysis indicates that Capu-NT is a dimer, similar to the N-termini of other formins. A dimerization domain is within residues 222-321. These data combined with circular dichroism suggest that the CID is structurally distinct from previously described DID domains. Within Capu-CT, the Capu-tail, C-terminal to the FH2 domain, was necessary to bind CID. Interestingly, the Capu-tail

also binds to the Spir-KIND domain (Pechlivanis et al., JBC 2009; Vizcarra et al., PNAS 2011). None of the single Capu-tail point mutations sufficient to abolish the Capu-tail/Spir-KIND interaction affect the Capu-tail/CID interaction, suggesting that Spir-KIND and CID bind to Capu-tail differently. Future work will be aimed at understanding how Capu autoinhibition fits with known regulatory interactions with Spir.

1800

The formin INF2 severs actin filaments through a fundamentally different mechanism from cofilin: relating biochemical function to cellular activity.

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Formins are a class of proteins that accelerate actin nucleation, then influence filament elongation rate by remaining at the barbed end. INF2 is a biochemically unique mammalian formin in that it accelerates both actin polymerization and depolymerization. Importantly, mutations in INF2 lead to the kidney disease focal and segmental glomerulosclerosis (FSGS) and the neurological disorder Charcot-Marie Tooth Disease (CMTD). I am elucidating the molecular mechanism of INF2's unique depolymerization activity, using TIRF microscopy and other biochemical assays of actin dynamics. Prior work in our lab has shown that depolymerization requires both the FH2 and WH2/DAD sequences, and occurs in two steps: a) a severing step, which requires phosphate release from the actin subunits; and b) a depolymerization step, which requires the WH2/DAD. My work reveals the following mechanistic features of INF2-mediated severing/depolymerization. First, rapid severing occurs throughout the length of the filament and not progressively from the barbed end. This suggests that INF2 binds filament sides prior to severing and accelerates phosphate release of filaments since it is capable of severing filament segments less than 50s after monomer addition. Second, INF2 can bind filament sides that are both phosphate-bound and phosphate-free, but with different stoichiometries. Additionally, INF2 can alter filament flexibility in the phosphate-free state. These results suggest that phosphate release causes a conformational change to filament-bound INF2, allowing severing. Lastly, INF2 bound at the barbed end causes catastrophic disassembly of short filaments. Based on these results, I postulate that both filament side binding and barbed end binding by the FH2 domain changes filament architecture, which promotes severing and changes monomer dynamics at filament ends. I postulate further that the WH2/DAD enhances severing by insertional binding between actin subunits in the filament. These results contrast in several aspects with the severing mechanism employed by cofilin. This mechanistic investigation of INF2 severing/depolymerization activity gives insight on recent cellular results from our lab, which reveal a role for INF2-mediated actin dynamics in fission of both Golgi and mitochondria.

1801

Functional analysis of the N-terminal extension of troponin I muscle contraction and organization in *C. elegans* striated muscle.

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Troponin is a complex of three proteins [troponin I (TNI), troponin T (TNT), and troponin C (TNC)], which functions as an actin-linked calcium switch for muscle contraction. Calcium binding to TNC induces a large conformation change of TNI; TNT mediates the structural change to the tropomyosin-actin complex. However, the precise mechanism of the troponin regulation is not completely understood. TNI is at the core of the troponin complex, and its

elongated structure allows interaction with multiple molecules including TNC, TNT, and actin. The central region of TNI plays a major role in inhibition of actin-myosin interaction in the absence of calcium and is conserved among TNI proteins from different species. However, in invertebrate TNI proteins, N-terminal extensions of variable lengths are present, but their function remains unclear. Vertebrate cardiac TNI also has an N-terminal extension that modulates contractility in a PKA-phosphorylation dependent manner, while vertebrate skeletal TNI lacks an N-terminal extension. To better understand the function of the N-terminal extension of TNI, we used the nematode *Caenorhabditis elegans* as a model system. The body wall muscle of *C. elegans* is obliquely striated muscle and uses troponin as a major actin-linked regulatory system for muscle contraction. *C. elegans* has four TNI genes, and all TNI isoforms have N-terminal extension of ~30 amino acids. Among them, *unc-27* plays a major role in contractile regulation in the body wall muscle. *unc-27(e155)* is a putative null allele, and *unc-27(e155)* homozygotes showed impaired contractility and disorganization of sarcomeric actin filaments. Transgenic expression of GFP-tagged wild-type UNC-27 protein rescued both muscle contractility and sarcomeric actin organization. In contrast, GFP-UNC-27 lacking the N-terminal 29 amino acids failed to rescue the contractility defect but was able to rescue sarcomeric actin organization. Disorganized sarcomeric actin filaments in *unc-27* mutants are most likely caused by excessive and uncontrolled actin-myosin interaction, in the absence of TNI. Therefore, our results suggest that UNC-27 lacking the N-terminal extension inhibits actin-myosin interaction, thereby rescuing the actin organization, but fails to release the inhibitory state. Thus, in *C. elegans* muscle, the N-terminal extension of TNI appears to play an essential role in regulating muscle contractility.

1802

A troponin-T mutation initiates cardiac and skeletal myopathy due to impaired thin filament regulation in *Drosophila melanogaster*.

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Muscle contraction results from an orchestrated series of molecular events that permit transient interactions between myosin containing thick filaments and actin containing thin filaments. The high affinity of myosin for actin suggests that without regulation, muscle would remain in a continuous state of contraction. Contractile regulation of striated muscle is predominately achieved by Ca²⁺-dependent activity of the thin filament troponin-tropomyosin complex. Lesions in various subunits of the complex cause dysregulation of force production and myopathy. For example, at least 5 point mutations in a span of 10 amino acids (from positions 130 – 139) in human cardiac troponin T (cTnT) are associated with distinct cardiomyopathic responses. We have examined the consequences of an amino acid substitution at the end of this well-conserved region of TnT on different muscle types from *Drosophila melanogaster* up101 mutants. Fly hearts were surgically exposed. Cardiac performance and morphology were assessed using direct immersion DIC optics, high speed video imaging and motion analysis. Relative to control, up101 hearts displayed a phenotype reminiscent of human restrictive cardiomyopathy. Diastolic and systolic dimensions and percent fractional shortening were significantly reduced. Furthermore systolic intervals were significantly prolonged. This suggests thin filament dysregulation, excessive periods of force production and diastolic dysfunction. To characterize the molecular phenotype and fundamental cause of cardiac remodeling we isolated thin filaments from fly indirect flight muscles (IFM). The constitutively expressed mutation results in flightlessness due to severe IFM hypercontraction and destruction within hours of adulthood. Electron microscopy and three-dimensional reconstruction of IFM thin filaments revealed Ca²⁺-dependent tropomyosin strand movement, typical of control filaments,

was not a general feature of the TnT mutants. The vast majority of Ca²⁺-free mutant thin filaments exhibited tropomyosin associated with the inner domain of successive actin monomers, distal to known myosin binding sites, and are unlikely to sterically prevent contraction. We are currently performing in vivo genetic interaction screens to identify second site mutations critical for interplay among the mutant troponin-tropomyosin complex and actin. We aim to elucidate genetic modifiers and to suppress the myopathic phenotypes to better understand thin filament-mediated contractile regulation in health and in disease.

1803

Insights into post-translational modification of Profilin-1.

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Profilin-1 (Pfn1) is a small actin-monomer binding protein that plays an essential role in actin cytoskeletal control in cells. In addition to actin, Pfn1 also interacts with phosphoinositides and a myriad of proteins bearing poly-L-proline motifs. The mechanisms by which ligand associations of Pfn1 are regulated are not well understood. We postulated that ligand interactions of Pfn1 are fine-tuned by post-translational modifications. By two-dimensional (2D) gel electrophoresis analyses of HEK293 cell lysate, we have identified up to 5 different charged forms of Pfn1 in vivo. Among these, the most stoichiometrically abundant form of Pfn1 appears to be in a phosphorylated state. Mutagenesis studies further revealed that the major in vivo phosphorylation site is not the previously speculated S137 residue in its C-terminal polyproline motif-binding region. These data demonstrate that the bulk of cellular Pfn1 exists in phosphorylated state. (This study is supported by a grant from the National Cancer Institute of NIH 2R01CA108607-06 to PR).

1804

GSK3 Phosphorylates the Actin-Regulating Protein CAP1 (adenylyl Cyclase-Associated Protein 1).

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Background: The actin cytoskeleton is crucial for many important cellular functions such as cell morphogenesis, cytokinesis, cell polarity and motility. Understanding how cells signal to the actin cytoskeleton may also shed light on the interplay between signals leading to morphological and proliferative transformation in cancer. For example, cofilin is an example that links cell signaling to the actin cytoskeleton because it is phosphorylated by LIM kinase to control actin dynamics. CAP (adenylyl Cyclase-Associated Protein) is a conserved key actin-regulating protein that functions with cofilin to regulate actin dynamics through two independent mechanisms. We have recently identified mammalian CAPs as phospho-proteins, and we aimed to unravel mechanisms of regulation of CAP1 by phosphorylation.

Methods and Results: We identified 9 phosphorylation sites on mouse CAP1 through a proteomics approach. Results from stable expression of point mutants that mimic both phosphorylated and dephosphorylated residues in NIH3T3 cells suggest that Ser36 and Ser307/Ser309 are regulatory sites since expression of the phosphomimetic mutants led to distinct actin cytoskeleton phenotypes. We developed phospho-specific antibodies against the Ser36 and Ser307/Ser309 sites, and found that inhibitors of GSK3 (Glycogen Synthase Kinase 3) reduces phosphorylation at the Ser307/Ser 309 sites. Kinase assays using synthetic peptides

narrowed the site to Ser309; Thr314 appears to serve as the priming site (motif: S/TxxxxS/T). Furthermore, whereas in untreated cells CAP1 partially localizes to cell peripherals, treatment with GSK3 inhibitor caused CAP1 to lose this localization and the cells lost cell polarity as well. We also screened physiological stimuli that may regulate CAP1 phosphorylation, and found that Ser309 phosphorylation is reduced in cells undergoing dynamic actin rearrangement, in contrast the phosphorylation is elevated in quiescent cells and cells cultured in suspension. GSK3 has been shown to play roles in cell migration and polarity, and our studies suggest that CAP1 may mediate some GSK3 signals.

1805

Mutation of the Tlr1999 GAF domain from the cyanobacterium *Thermosynechococcus elongatus* to obtain red-fluorescence for possible use in cellular tagging.

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Fluorescent tagging is a very effective method to label proteins. GFP (green fluorescent protein) is the most widely used fluorescent tag, but as it is green fluorescent, the tags cannot be visualized beyond a certain depth into cells or tissues. Red fluorescent light travels farther, thus scientists can observe a wider range and depth by microscopy of tissue. There are red fluorescent tags currently available on the market, but we are engineering a shorter amino acid sequence to lessen the possibility of interference with other processes in living cells. The Spiller Lab is working with the Class II GAF domains of five distinct cyanobacteriochrome genes from the *T. elongatus* genome and has found that when mutated, these genes no longer perform blue/green photoconversion and instead they fluoresce near infrared light. Analysis of Tlr1999 cyanobacteriochrome in particular appears promising in its ability to fluoresce efficiently. It is in our interest to shorten the amino acid sequence of the tags, so a 176 amino acid truncation of the GAF region is being investigated for the behavior of its unmutated form to allow for investigation of the properties of its mutated form. The mutated form of Tlr1999 contains a point mutation of cysteine to aspartate performed to prevent photoreversibility (ensuring light absorption and then fluorescence in the longer wavelength red spectrum). Our ultimate goal is to bioengineer a small, red fluorescent tag (SmuRF) that can be used to visualize the lymphocyte cytoskeleton interacting with green fluorescent labeled HIV. Visualizing the details of the HIV infection process with advanced microscopy techniques may allow researchers to obtain insight and progress towards a cure.

1806

Mutant alpha-crystallin inhibits lens growth by perturbing lens cell-cell alignment and cell elongation.

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The mechanism for how the α A-Y118D crystallin mutant causes a unique postnatal lens growth defect remains unknown. We hypothesized three possible explanations for the postnatal growth defect in mutant α A-Y118D lenses: 1) increased epithelial cell apoptosis, 2) decreased epithelial cell proliferation, or 3) failed fiber cell elongation. Results showed that α A-Y118D lenses exhibited no increase in normal levels of lens epithelial cell apoptosis. BrdU labeling revealed no difference in epithelial cell mitotic index between P7 wild-type (WT) and mutant lenses. In contrast, by P21, mutant lens epithelial cells had about half the normal mitotic index compared to WT lens epithelial cells. The decrease in mitotic index in mutant lenses suggests that the α A-

Y118D mutant protein affects epithelial cell proliferation. Imaging of GFP-positive whole lenses displayed perturbed fiber cell-cell alignment and aberrant or delayed fiber cell elongation in α A-Y118D mutant lenses from mice older than three weeks. To further investigate the mechanism for how α A-Y118D mutant crystallin protein might cause these cellular defects, we determined the protein distributions of WT α A, α A-Y118D mutant, WT α B in cultured primary epithelial cells isolated from WT and α A-Y118D mutant lenses. Co-labeling with cytoskeletal proteins, we found that WT α A and α B crystallin proteins are associated with F-actin filaments in WT cultured lens epithelial cells. In contrast, in the cultured α A-Y118D mutant lens epithelial cells, WT α B remains associated with F-actin while α A-Y118D shows no association with F-actin. Thus, α A-Y118D probably affects F-actin in some way to perturb fiber cell-cell alignment and fiber cell elongation in mutant lenses.

1807

The GAF only domain TePixJ from Cyanobacteriochrome of *Thermosynechococcus elongatus* BP-1 transfected into mammalian cells for enhanced in vivo microscopy in the near-infrared spectrum.

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Cyanobacteriochromes are widely occurring photoreceptors in photosynthetic bacteria that respond to all wavelengths of the visible spectrum. The diversity of two cyteines photocycles of cyanobacteriochromes has been recently demonstrated. *Thermosynechococcus elongatus* BP-1, a thermophilic cyanobacterium, have several cyanobacteriochromes whose ability to fluoresce far-red light after a single mutation to the second cysteine in their GAF domain. In this study we engineered a short, near-infrared fluorescent protein from isolated GAF domain of TePixJ (TII0569) in class II cyanobacteriochromes, incorporating phycocyanobilin as the chromophore. The fluorescent protein has excitation and emission maxima of 647 and 660 nm, respectively, a quantum yield of 0.081, and consists of 174 amino acids, which is much smaller than previously characterized Infrared Fluorescent Proteins. To examine its function in fusion proteins, we constructed a plasmid using an actin vector as a model and transfected mammalian cells for in vivo imaging. Visualization of transfected lymphocyte cells suggests promising success of labeling the actin cytoskeleton with near-infrared fluorescence. Future goal of our project is to construct plasmids using a tubulin vector and other actin vectors as models, and use the red-labeled lymphocyte cells to study virus infection processes, in collaboration with partners at the Center for Biophotonics Science and Technology (CBST-UC Davis) and UC Davis Department of Biochemistry.

1808

Cooperative G-actin binding regulates shuttling of the RPEL protein Phactr1 to control PP1 activity and actomyosin assembly.

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Actin-binding proteins play crucial roles in the regulation of actin dynamics and assembly of F-actin structures involved in cell migration, adhesion and metastasis. We previously established that G-actin binding controls the subcellular localization and activity of the MRTF family of

transcription co-activators. The regulatory RPEL domain of MRTFs contains three copies of the G-actin binding RPEL motif. Signal-induced depletion of the G-actin pool reduces G-actin-RPEL interaction, causing nuclear accumulation and activation of MRTFs. In contrast, the Phactr family of novel PP1-binding proteins each contain four RPEL motifs, but whether G-actin can control their function and how they regulate PP1 activity is unclear. In this study we analyze the role of actin-Phactr interaction in control of Phactr localization and interaction with PP1.

Activation of Rho-actin signaling in fibroblasts induces nuclear accumulation of Phactr1, but not other family members. Actin binding by the three C-terminal RPEL motifs is required for Phactr1 cytoplasmic localisation in resting cells and its nuclear accumulation is importin α - β -dependent. Moreover, G-actin and importin α - β compete to bind NLSs associated with the N- and C-terminal RPEL motifs and all four motifs are required for the inhibition of Phactr1 nuclear accumulation by elevated G-actin.

We report the structure of a Phactr1 G-actin•RPEL domain complex with three G-actins around the crank-shaped RPEL domain forming a closed helical assembly. Their spatial relationship is similar to our previously described pentavalent MRTF-A G-actin•RPEL domain complex, suggesting conserved cooperative interactions between actin•RPEL units organize the RPEL domain. We identified novel contacts with neighbouring actins and show that secondary contact residues of the N-terminal RPEL motif are required for actin-mediated inhibition of Phactr1 nuclear import. This strongly suggests that those contacts govern formation of multivalent actin•RPEL assemblies.

We show that G-actin and PP1 bind competitively to the Phactr1 C-terminal region. Expression of Phactr1 C-terminal RPEL mutants that cannot bind G-actin induces actomyosin foci, which occur in a PP1-dependent manner. Finally, in CHL-1 metastatic melanoma cells, Phactr1 exhibits actin-regulated subcellular localization and is required for stress fibre assembly, motility and invasiveness. These data reveal a novel role for Phactr1 in actomyosin assembly and suggest that Phactr1 G-actin sensing allows its coordination with F-actin availability.

1809

Probing the functional roles of caldesmon using phosphomimetic peptides.

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The interaction between the actin-binding protein caldesmon (CaD) and actin is regulated by Erk- and Pak-mediated phosphorylation at sites in the C-terminal region where the major actin-binding sites reside. To probe the roles of CaD and its phosphorylation in vivo, we have designed three phosphomimetic peptides: ErkA, corresponding to the CaD sequence flanking the Erk-phosphorylation sites with the two Ser residues replaced by Ala; PakA, corresponding to the CaD sequence flanking the Pak-phosphorylation sites with the two Ser residues replaced by Ala; and ErkD, same as ErkA except that the Ser residues are replaced by Asp. All peptides were labeled with FITC and coupled to a Tat-tag at the N-termini. Overnight incubation of these CaD peptides with tissues or cells attained high loading efficiency as evidenced by the incorporation of the FITC fluorescence. We have performed force measurements on intact rat mesenteric arteries incubated with CaD peptides to see how the contractile properties are perturbed. Tension was measured with phenylephrine stimulation. For ErkA/PakA-loaded tissues, the same force level was obtained as the control (buffer alone), but the relaxation was slower. For the tissue incubated with ErkD peptide, the kinetics was more similar to the control. The observed effect of the peptides (ErkA+PakA) was reminiscent of the results with smooth muscle CaD-null tissues, suggesting that, as shown by the in vitro experiments, filament-bound

smooth muscle CaD is displaced by ErkA/PakA, but not by ErkD. In another experiment peptide-loaded human metastatic breast cancer cells MDA-MB231 were tested in nude mice through subcutaneous injection. It was found that ErkA and PakA effectively suppressed tumor development. How these peptides interfered with tumor growth awaits further investigation, but these pilot experiments demonstrated the feasibility and usefulness of synthetic decoy peptides in the studies of CaD's functions. Supported by a grant from NIH (HL92252).

1810

Toxoplasma gondii Actin Assembles via Isodesmic Polymerization

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The protozoan parasite *Toxoplasma gondii* relies on actin polymerization for motility and host cell invasion. Despite this strict requirement for actin filaments, *T. gondii* actin (TgACTI) remains largely unpolymerized *in vivo* and recombinant TgACTI only forms short filaments *in vitro*. TgACTI has several amino acid substitutions when compared with muscle actin, and our previous work showed that these changes significantly diminish the lateral contacts and interactions within the actin filament. The net result is that the two strands of the filament assemble with less cooperativity and not surprisingly this has significant effects on the polymerization kinetics. Here we show that the highly unusual characteristics of TgACTI result from isodesmic polymerization rather than the nucleation-elongation kinetics of conventional eukaryotic actins. TgACTI polymerization kinetics lack a lag phase and critical concentration, and all of the results from dynamic light scattering, dilution induced depolymerization, and sedimentation can be fit using a single pair of kinetic rate constants. These findings expand the repertoire of how actin functions in cell motility and offer clues about the evolution of self-assembling, stabilized protein polymers.

Regulation of Actin Dynamics II

1811

Drosophila nurse cell dumping reveals a novel interaction between prostaglandin signaling and Fascin.

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Prostaglandins (PGs), lipid signals produced downstream of cyclooxygenase (COX) enzymes, are known to regulate actin cytoskeletal dynamics. However, the mechanisms by which PGs mediate this remain unknown. *Drosophila* nurse cell dumping, an important process in oogenesis, provides a model for studying how PG signaling affects actin remodeling. This process requires active remodeling of the actin cytoskeleton to allow the nurse cells to squeeze their cytoplasmic contents into the growing oocyte. Using this model, we have previously shown that PGs are required for actin remodeling during nurse cell dumping, and that Pxt is the *Drosophila* COX-like enzyme. A screen utilizing our *in vitro* follicle maturation assay (Spracklen, Meyer, and Tootle, unpublished data) identified the actin bundling protein Fascin (Singed in *Drosophila*) as a putative downstream target of PG signaling. Fascin has previously been shown to be required for actin filament bundle formation during oogenesis and nurse cell

dumping. Here, we show that fascin and pxt mutants display similar actin remodeling defects in nurse cells. Reduced Fascin levels enhance the nurse cell dumping, and thus actin remodeling, defects of both COX inhibition and reduced Pxt levels. Reduced Fascin levels synergize with reduced Pxt levels to affect actin filament bundle formation, cortical actin structure, and membrane integrity in nurse cells. Additionally, over-expression of Fascin in the germline suppresses the effects of COX inhibition and loss of Pxt. Importantly, Fascin levels, both mRNA and protein, are not affected by alterations in PG signaling. These data indicate that Fascin activity is a downstream target of PG signaling in nurse cell dumping. Understanding the regulation of Fascin by PGs has relevance to cancer cell migration and metastasis, where independent studies have implicated critical roles for both PGs and Fascin. Current efforts focus on determining the mechanism by which PGs regulate Fascin in both *Drosophila* and human breast cancer cells. In *Drosophila* follicles, we find that Fascin localization, both nuclear and cytoplasmic, is altered by loss of PGs. We are also assessing the roles of PKC, Rho, and Rac as potential downstream effectors of PGs in the regulation of Fascin. These studies will advance our understanding of mechanisms controlling Fascin, which is becoming an attractive target for cancer therapeutics.

1812

Calling the Shots: Prostaglandins Temporally and Spatially Regulate Actin Remodeling During *Drosophila* Nurse Cell Dumping.

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Prostaglandins (PGs), small bioactive lipids that act as autocrine/paracrine signaling molecules, mediate numerous physiological and cellular processes including actin cytoskeletal remodeling. However, the exact mechanisms by which PG signaling regulates actin remodeling remain largely unknown. Previously, we have shown that PGs regulate actin cytoskeletal dynamics during *Drosophila* nurse cell dumping, providing a novel, developmental model in which to study PG-dependent actin remodeling. Lasting just thirty minutes, *Drosophila* nurse cell dumping is a highly dynamic process requiring spatial and temporal coordination of active actin cytoskeletal remodeling in order for the nurse cells to supply their cytoplasmic contents to the oocyte. Recently, we have found that loss of Pxt, the *Drosophila* COX-like enzyme, results in aberrant actin filament and extensive actin aggregate formation prior to the onset of canonical actin remodeling during nurse cell dumping. We are taking advantage of this temporal misregulation of actin remodeling to identify actin binding proteins and regulatory molecules acting downstream of PG signaling to tightly control the onset of actin remodeling. Specifically, we are using immunofluorescence to identify factors that co-localize to these aberrant, early actin structures and genetic interaction studies to identify factors that suppress or enhance aberrant, early actin structure formation in *pxt* mutants. By combining correlative microscopy with robust genetic analysis, we can begin to elucidate the mechanisms by which PG signaling temporally regulates actin cytoskeletal remodeling. Actin remodeling is tightly temporally regulated both during development and adult homeostasis. Temporal misregulation of actin remodeling directly contributes to the development and progression of multiple cardiovascular diseases, including developmental defects, heart attack, stroke, and cardiomyopathies. Understanding the molecular mechanisms by which PGs tightly regulate actin remodeling during nurse cell dumping may provide insight into the conserved mechanisms by which PG signaling temporally regulates actin remodeling.

1813

Endothelial cell responses to fluid shear stress and inflammatory mediators.S. N. Farwell¹, L. J. Lowe-Krentz¹; ¹Biological Sciences, Lehigh University, Bethlehem, PA

Vascular endothelial cells (ECs) are constantly exposed to fluid shear stress (FSS), chemical stresses (such as inflammatory cytokines, etc.), and immune cell trafficking. ECs must therefore respond to physical and chemical cues. Three key signaling systems involved in this regulation are the actin cytoskeleton, adherens junctions, and immune cell recruitment. Together, these modulate important downstream signaling induced by FSS and/or inflammation. However, studying these interactions *in vivo* is difficult and current *in vitro* models seldom mimic *in vivo* conditions or include FSS and chemical stressors. ECs initially respond to both FSS and inflammatory stressors with actin realignment, barrier changes, and immune cell recruiting; over time, FSS is athero-protective while inflammatory stress results in EC layer damage. In this study, we employ a FSS system to compare both primary large vessel and microvessel EC responses to FSS, with or without inflammatory stress, to static conditions. Our laboratory has established that high FSS (>15 dyn/cm²) induces an actin remodeling process beginning shortly after FSS exposure. We now show that FSS also induces an interaction between actin and β catenin in large vessel EC barriers after 90 minutes. TNF α induces actin remodeling within 30 minutes and barrier localization of β catenin after 60 minutes in static culture, similar to FSS effects. In microvessel ECs, actin remodeling and barrier stabilization in response to high FSS are similar, but appear somewhat delayed compared to large vessel cells. When challenged with TNF α under FSS, actin stabilization is hindered and barrier integrity is lost, as evidenced by a reduction in β catenin. In static culture, both EC types express the immunological signaling marker ICAM-1 in vesicles around the nuclear membrane. Under FSS, ICAM-1 translocates to the surface and cell-cell junctions around 30 minutes in large vessel ECs. Some ICAM-1 remains in cytoplasmic vesicles for several hours. In these same cells, 2 hours of static TNF α treatment results in ICAM-1 translocation to the cell surface. Unlike large vessel ECs, when microvessel ECs are challenged with TNF- α under FSS, surface ICAM-1 is upregulated quickly but does not localize to junctions. Short-term FSS and TNF α stimulation induce quite similar actin remodeling, β catenin localization, and ICAM-1 expression in both EC types. How long-term FSS contrasts with TNF α to result in vastly different effects remains an important question. Support from NIH award HL54269 to LLK and the BDSI funded by an HHMI undergraduate education grant to Lehigh University is gratefully acknowledged.

1814

Turnover of focal adhesions required for efficient cell migration is regulated by parallel actin bundles.N. Elkhatib¹, M. Neu¹, C. Zensen², K. M. Schmoller², D. Louvard¹, A. R. Bausch², D. M. Vignjevic¹; ¹Institut Curie, Paris, France, ²Technische Universität Muenchen, Garching, Germany

Cell initiates migration by extending membrane protrusions which are stabilized by focal adhesions (FAs) that link the actin cytoskeleton to the underlying extracellular matrix. A stress fiber is a bundle of actin filaments with mixed polarity cross-linked with α -actinin and myosin II. It is not clear how tensile forces, generated by actomyosin contraction of stress fibers, can strengthen FAs at the cell front and disassemble FAs at the cell rear allowing cell migration. Using PA-GFP-actin we found that the distal part of the stress fiber anchored to the FA has a different actin organization, dynamics and function than the proximal part. It is a non-contractile bundle composed of parallel actin filaments cross-linked by fascin and deprived of myosin II. Fascin-depleted cells were more contractile and their adhesions were thinner and longer

suggesting that myosin II is more active. Indeed, myosin II ATP-ase and in vitro motility assays showed that fascin inhibits myosin II motor activity possibly through the competition for binding to actin filaments. The turnover of FAs in fascin-depleted cells was slower and using FAs disassembly assay, we found that fascin is required for FAs disassembly after microtubule re-growth. Based on those data we propose a following model: at the cell leading edge, the distal non-contractile bundle of a stress fiber indirectly strengthens FAs providing a necessary power to resist actomyosin contractile forces. However, at the cell rear, de-bundling of the stress fiber's distal part is necessary for efficient FAs disassembly. Thus, actin bundling by fascin could determine the fate of adhesions under force.

1815

Switching contractility in active actin networks by pH.

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The interplay of local force generation by myosin-II motor filaments and crosslinking of actin filaments is essential for various cellular processes ranging from cell division to tissue formation. While recently the local organization principles in such active gels have been discovered, the regulation process of the active behavior still remains to be investigated. In vivo, cytoskeletal processes are controlled by various biochemical pathways. Whereas such biochemical signal cascades are intrinsically highly specific but rather slow, variation of pH might provide a fast alternative, which could trigger a rapid cytoskeletal response. Here we show that the contractility of active actin networks is tightly controlled by the local pH. The pH dependent intrinsic crossbridge strength of myosin-II can be employed to sharply switch the actomyosin network from contractile to non-contractile by a change in pH of only 0.1. The specific type and concentration of cross-linking protein used allows to sensitively adjust the range of pH where contraction occurs, which can recover the behavior found in *Xenopus laevis* oocyte extracts. Controlling the contractility of cytoskeletal structures by only a small variation in pH provides a new mechanism which can be expected to have broad implications in our understanding of cytoskeletal regulation.

1816

Molecular mechanism of Rickettsia Sca2-mediated actin assembly.

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Many bacterial pathogens utilize the host cell actin cytoskeleton during invasion or to propel their movement within and between infected cells. Often, pathogens resort to mimicry for such activities, by expressing proteins that adopt the core functions of some key actin cytoskeletal components. In so doing, pathogens tend to bypass the intricate regulatory networks and interactions characterizing eukaryotic proteins, offering a rare opportunity to dissect their functions within a simplified system, with implications for our understanding of pathogenicity and the eukaryotic actin cytoskeleton alike. Rickettsia Sca2 is an outer membrane protein that promotes actin filament nucleation and barbed end elongation and has been implicated in the formation of the actin comet tails that promote Rickettsia motility. Although these activities have suggested that Sca2 functions by mimicking eukaryotic formins, the actual mechanism of actin assembly by Sca2 remains a mystery. Particularly, the lack of sequence similarity between

Sca2 and eukaryotic formins suggests a fundamentally different mechanism of nucleation, unlike those described so far for eukaryotic or bacterial nucleators. The passenger domain of *Rickettsia conorii* Sca2 (the species studied here) consists of 1482-aa and comprises a repeat of three WH2 domains toward the middle of the polypeptide, flanked by two Pro-rich sequences, which are often implicated in binding of profilin. Here, we have used a battery of biophysical approaches, including fluorescence, TIRF microscopy, ITC, x-ray crystallography and SAXS to determine the structural and functional bases for nucleation and elongation by Sca2. We show that Sca2 can be conceptually described as consisting two major functional domains, with one domain being primarily responsible for strong actin filament nucleation and the other domain involved in barbed end processivity. We have determined the atomic structure of the domain responsible for processivity, revealing a new fold and a unique mode of actin filament barbed-end tracking, as also confirmed by structure-based mutagenesis. In summary, the results suggest that *Rickettsia* Sca2 has adopted a novel mechanism of actin nucleation and elongation, which involves both mimicry of eukaryotic proteins and new adaptations without any parallel in the eukaryotic kingdom.

1817

Eph/ephrin signaling is essential for precise cell-to-cell alignment during lens formation.

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Mammalian lenses are avascular and transparent organs made up of bulk elongated fiber cells covered by a monolayer of epithelial cells on the anterior surface. Equatorial epithelial cells elongate to form peripheral differentiating fibers that are precisely overlaid onto previous generations of fiber cells. Organization and tight packing of fiber cells is required to establish high refractive index and to maintain lens transparency. Disruption of Eph/ephrin signaling leads to cataracts in human and mouse lenses. We have investigated the roles of Eph/ephrin during lens development by using ephrin-A5 and EphA2 knockout (-/-) mice. Lens phenotypes of ephrin-A5(-/-) and EphA2(-/-) mice were examined, and confocal images of GFP-positive lens epithelial and fiber cells were collected. Cell-cell junctions, cell adhesions and cytoskeletal structures were studied by immunohistochemistry. Ephrin-A5(-/-) lenses often develop anterior cataracts associated with clusters of anterior epithelial cells undergoing epithelial-to-mesenchymal transition (EMT), and changes in E-cadherin and β -catenin staining further indicate that cell-cell junctions are compromised in these cells. Unexpectedly, anterior epithelial cells of EphA2(-/-) lenses show no obvious morphological changes, but equatorial epithelial cells fail to align into organized meridional rows. Immunostaining reveals that EphA2, β -actin, total and phosphorylated cortactin and phosphorylated Src are localized at cell membranes and are enriched at the vertices of wild-type hexagonal equatorial epithelial cells organized into meridional rows. In disorganized EphA2(-/-) equatorial epithelial cells, β -actin and total cortactin display abnormal clusters at the cell membrane, and there is a loss of phosphorylated cortactin and Src in these cells. EphA2 is crucial for lens equatorial epithelial cell alignment by regulating the phosphorylation of Src kinase and cortactin to recruit actin to cell vertices to form and align hexagonal cells into meridional rows. Actin dynamics control EphA2-mediated cell-cell packing of lens equatorial epithelial cells, and actin cytoskeleton changes probably lead to abnormal cell packing of EphA2(-/-) epithelial cells that result in misaligned fiber cells, causing subsequent changes in the refractive index of EphA2(-/-) lenses. The phenotypic difference between ephrin-A5(-/-) and EphA2(-/-) lenses suggests that these two molecules are not a receptor-ligand pair in the lens.

1818

Perinuclear actin rim assembly upon mechanical stimulation.

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The ability of cells to sense the physical microenvironment and respond to mechanical cues is critical for their functions and survival. Actin cytoskeleton, connecting focal adhesions to the nucleus, plays a central role in cellular mechanotransduction. However, questions remain about how extracellular force is transmitted to actin, and how actin behaves upon a transient force application. Recently we have found that an intense mechanical stimulation by pushing the cell at periphery with micropipettes causes immediate actin cytoskeleton reorganization, namely, an actin rim incorporated with α -actinin is quickly assembled at the cytoplasmic face of the nuclear envelope, which is detectable by phalloidin staining and live cell imaging using lifeact and F-tractin. The intensity of the nuclear rim reaches to its maximum in around 10-20 seconds and then recovers to the state before perturbation in another 40-60 seconds. The cells are able to respond to repeated stimulations. Blocking actin polymerization with Cytochalasin D eliminates the force-induced actin assembly, suggesting an important part of actin polymerization in this process. However, neither Arp2/3 inhibitor nor formin inhibitor SMIFH2 so far has precluded this phenomenon. Removing extracellular calcium by EGTA totally blocks the actin rim assembly, indicating the key role of calcium signaling. Furthermore, when adding thapsigargin or calcium ionophore A23187 that increases cytoplasmic calcium to the cell, a transient perinuclear actin rim shows up, same as induced by mechanical stimulation. Visualizing via an indicator Rhod-3, the intensity peak of calcium is a few seconds prior to that of actin. In summary, the force-induced perinuclear actin rim assembly is based on actin polymerization and regulated by calcium influx.

1819

Cell plasticity is tightly linked to elastic stresses in the cytoskeleton.

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Cells show pronounced non-linear visco-elastic and visco-plastic properties under large deformations and forces. These properties are important for protecting the cell against mechanical damage. We used a high-force magnetic tweezer setup to deliver unidirectional forces of up to 30nN with high precision to fibronectin-coated magnetic 5 μ m beads bound to cell surface adhesion receptors. To probe cells with bidirectional forces, the cell culture plate was placed on a rotational/translational stage such that the magnetic bead remained at a constant distance to the magnetic tweezer tip after a 180° rotation. Bead displacements were measured during application of force steps (creep response) and after the force was removed (recovery response). With increasing force magnitude, the cells stiffened, and the recovery became increasingly incomplete, indicating the emergence of plastic behavior. This plasticity was a constant fraction (~20%) of the total bead displacement. The plastic behavior is attributable to a buildup of excess slack in the cytoskeletal fibers; when the force direction was suddenly reversed, the beads jumped by twice the slack length in the opposite direction. The creep and the recovery response were fully characterized by a simple power-law vs. time with only two force-dependent parameters (viscoelasticity and slack). Our results show that plastic energy dissipation during large cell deformations is tightly linked to elastic stress dissipation and provides additional protection against mechanical damage.

1820

Regulation of actin dynamics by cross-linking molecules.

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The ability of cells to control the internal dynamics of actin filaments plays a crucial role in many cellular processes, including cell motility and cell division. We demonstrate that spontaneous fragmentation is the crucial process in the comparably slow steady state dynamics of in vitro actin filaments. Yet, in vivo, actin turn-over is enhanced by many different regulator proteins. Simultaneously, cells need mechanisms to locally stabilize actin filaments against induced depolymerisation in order to maintain certain actin structures for longer times. Using biochemical and fluorescence microscopy techniques we demonstrate that employment of cross-linking and bundling proteins not only gives cells a powerful tool to govern the architecture of cytoskeletal systems, but also allows for a drastic stabilization against internal kinetics by suppressing disassembly of cytoskeletal filaments. Even the actin depolymerizing factors are not sufficient to disintegrate highly cross-linked actin networks unless molecular motors are used simultaneously.

1821

The specific actin bundling properties of alpha-actinin are tailored for cytokinesis in fission yeast.

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During cytokinesis, the assembly and constriction of the contractile ring are mediated by the coordinated action of diverse actin-binding proteins, which nucleate, bundle, sever, and move along actin filaments. Alpha-actinin SpAin1 is the primary actin bundling protein for cytokinesis in fission yeast *Schizosaccharomyces pombe*. SpAin1 has a similar domain organization as animal isoforms, with an actin-binding domain followed by spectrin repeats that facilitate dimerization. Our goal is to biochemically elucidate the actin binding and bundling properties of SpAin1, and determine whether the specific properties are physiologically important for contractile ring function. Using bulk sedimentation assays and TIRF microscopy observation, we determined that SpAin1 binds and bundles actin filaments of mixed polarity. However, compared to animal alpha-actinins, SpAin1 bundles actin filaments with significantly lower efficiency. Because SpAin1 contains only two spectrin repeats for dimerization whereas animal alpha-actinins have four, we hypothesized that weak dimerization efficiency may contribute to SpAin1's weak bundling activity. We constructed various hybrid versions of SpAin1 by replacing its central dimerization domain with spectrin repeats from human alpha-actinin or commonly used synthetic dimerization peptides. As expected, replacement of the central dimerization domain enhanced SpAin1's F-actin bundling efficiency. We then examined whether changing SpAin1's actin filament bundling efficiency affects cytokinesis by expressing the hybrid versions of SpAin1 in cells. Fission yeast cells carrying the most efficient hybrid version of SpAin1 displayed severe cytokinesis defects including a high percentage of cells stalled in cytokinesis with abnormal rings, and/or excessive ring materials. We conclude that precise control of actin filament bundling activity is critical for proper ring dynamics. The spectrin repeats of SpAin1 make it a unique bundling factor with inefficient crosslinking efficiency, suitable for contractile ring dynamics in fission yeast. Excessive actin filament bundling might crosslink filaments so tightly that myosin fails to slide filaments during both assembly and constriction of the contractile ring.

1822

RhoD regulates cytoskeletal dynamics via the actin nucleation-promoting factor WHAMM.

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RhoD is a member of the classical Rho GTPases sub family within the Ras superfamily. It has a role in the regulation of actin seen as the formation of filopodia and the bundles of actin filaments. Additionally, RhoD also plays an important role in endosome trafficking. The Rho GTPases are vital for fundamental cell processes such as cell motility, cell contraction, cell division and cell migration.

Here, we show that RhoD has a role in the organization of actin dynamics that is different from the better-studied members like Cdc42, RhoA and Rac1. We have identified the Filamin-A binding protein, FILIP1 and the nucleation-promoting factor, WASP homolog protein associated with actin, membranes and microtubules (WHAMM) as RhoD-interacting proteins. We show that FILIP1 and WHAMM belong to a group of proteins that possess similarities in their domain organization. Although both FILIP1 and WHAMM were found to possess a RhoD-binding capacity, they showed unique role in the control of the actin filament system. In agreement with the distinct function of these RhoD effectors, FILIP1 links to actin filaments via FLNa, whereas WHAMM acts through its C-terminal Arp2/3-binding motifs. In accordance with a key role of the RhoD in cytoskeletal dynamics, RhoD and its effectors were found to control vital cytoskeletal-driven cellular processes most notably cell attachment and cell migration.

1823

The interplay between SCAR (WAVE) and WASP in actin-based motility.

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The WASP family of proteins recruit and activate the Arp2/3 complex to promote actin polymerization. In particular, WASP family member SCAR (or WAVE) underlies protrusion (or 'pseudopod') extension during cell migration. Although the simple amoeba *Dictyostelium discoideum* is in many ways an ideal model for the study of cell migration, the limited but undeniable ability of the *Dictyostelium scar* null to extend pseudopodia and migrate has been a continued source of frustration and contention. Recently our lab has demonstrated that WASP is unexpectedly able to compensate for the loss of SCAR, adopting very different temporal and spatial dynamics compared to its normal behavior in wild-type cells (Veltman, D. M., *et al.* 2012. J. Cell Biol.)

A *Dictyostelium wasp* knockout has been unobtainable thus far and many people have assumed WASP must be essential for viability. We have now disrupted *wasp* by homologous recombination both alone and in combination with *scar*. We first confirmed that WASP is not required for normal cell migration in wild-type cells and instead predominantly localizes to sites of clathrin-mediated endocytosis. When visualized by TIRF microscopy, clathrin internalization is severely impaired in the *wasp* null and arrested clathrin pits accumulate on the plasma membrane. During cytokinesis, these trapped pits are swept into the forming cleavage furrow, which bulges and often fails to resolve. Our evidence suggests that the defective internalization of coated pits in the *wasp* null results in an impaired ability of the cell to remodel its plasma membrane, a process necessary for successful cleavage furrow formation and cytokinesis.

The loss of either SCAR or WASP alone has negligible effects on growth, however the loss of both has dire consequences. The double *scar/wasp* null has a severe defect in cell migration and is reminiscent of the recently published Arp2/3 depleted cells in morphology (Wu, C., *et al.* 2012. *Cell* & Suraneni, P., *et al.* 2012. *J. Cell Biol.*)

Having demonstrated that SCAR and WASP are each capable of driving actin-based protrusion and cell migration, we shall present the detailed analysis of cells that lack one or both of these proteins.

1824

Regulation of actin polymerization during clathrin-mediated endocytosis by SLAC, a complex between Sla1 and Las17.

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An important step during clathrin-mediated endocytosis is the polymerization of branched actin filaments nucleated by the Arp2/3 complex. Actin polymerization generates the necessary force to drive vesicle internalization. The homolog of the human Wiskott Aldrich syndrome protein (WASp), Las17, is the strongest Arp2/3 complex activator in yeast cells, it is not regulated by auto inhibition, and is present at sites of endocytosis 20 seconds before actin polymerization is detected. It is unclear how Las17 is inhibited during its initial 20 seconds of recruitment to sites of endocytosis limiting actin polymerization to late stages. In this study we demonstrate that Las17 and the clathrin adaptor Sla1 are co-recruited to endocytic sites in a pre-formed complex where Las17 is kept inhibited. Here we show that the Sla1- Las17 interaction is multivalent, direct, and strong, and is mediated by Las17 proline-rich sequences that simultaneously resemble class I and class II poly-proline motifs (class I/II). The mechanism by which Sla1 inhibits Las17 activity depends on the class I/II poly-proline motifs and is based on competition between Sla1 and monomeric actin for binding to Las17. Mutations in the Las17 class I/II poly-proline motifs that impede the interaction with Sla1 disrupt its ability to inhibit Las17 activity in *in vitro* pyrene-actin polymerization assays. In live cells, the same mutations uncoupled the recruitment of Sla1 and Las17 to endocytic sites, and altered the dynamics of actin polymerization. In addition, live cell imaging demonstrated that the interaction between Sla1 and Las17 is important for both bulk and clathrin-mediated endocytosis. Together, this study advances our understanding of how Sla1 and Las17 regulate actin polymerization during clathrin-mediated endocytosis (SLAC) as components of a stable complex.

1825

The rhomboid protease Rbd2 regulates the actin cytoskeleton during clathrin-mediated endocytosis.

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Clathrin-mediated endocytosis (CME) is the main port of entry into yeast cells. This pathway is highly conserved and is critical for cell homeostasis. Defects in CME have been linked to several diseases including cancer, diabetes and cardiovascular disease.

The budding yeast, *Saccharomyces cerevisiae*, has served as an invaluable organism in which to analyze the ordered recruitment, activity and disassembly of endocytic proteins during CME. In yeast, actin polymerization is required to reshape the plasma membrane for endocytic vesicle formation, and a burst of actin assembly accompanies endocytic vesicle internalization. Despite

its key role in CME, only a limited understanding of the molecular signals that regulate the actin cytoskeleton currently exists.

Recent, large-scale screens identified several physical interactions between the relatively unstudied putative rhomboid protease, Rbd2, and known actin regulators including Las17 (yeast WASP), Sla1, Bzz1 and Lsb3/4. Rhomboid proteases are multi-pass, transmembrane proteins that regulate a variety of cellular events through the limited proteolysis of target substrates. Consequently, we tested the function of Rbd2 as a new molecular player in CME. Rbd2 affects endocytic protein lifetimes and morphology in yeast, with the most dramatic effects observed on proteins of the actin cytoskeleton. Rbd2 appears to be associated with one or more membrane compartments, and Rbd2-GFP comes in close contact with endocytic sites late in the pathway, coinciding with the initiation of actin polymerization. Yeast genetics combined with live-cell imaging demonstrate that actin polymerization is initiated earlier in an *rbd2* null strain, and suggest that Rbd2 negatively regulates actin polymerization upstream of the Las17 regulator, Bzz1. A catalytic mutant of Rbd2 phenocopies the *rbd2* null strain, indicating that Rbd2's protease activity is critical for its function during CME. Because rhomboid proteases are highly conserved, our research is expected to aid our understanding of actin regulation during CME in more complex eukaryotes.

1826

The analysis of the roles of the Arp2/3 complex activators in the endocytic actin patch assembly in fission yeast.

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In yeast and other organisms, endocytosis is dependent upon Arp2/3 complex-mediated actin assembly into endocytic actin patches, a highly organized process involving signaling molecules and regulatory proteins. The goal of this study is to determine the roles of three Arp2/3 complex activators Pan1, Myo1, and Wsp1, which bind Arp2/3 complex via their Central-Acidic (CA) domains, in stimulating actin patch assembly in fission yeast. To assess the contribution of these proteins to actin assembly, we performed quantitative image analysis of actin patch dynamics by tracking the GFP-tagged actin binding protein fimbrin Fim1 in *pan1*, *myo1* and *wsp1* mutants with deleted CA domains. In *myo1*ΔCA, like in the wild type cells, most patches internalized and patch dynamics was the same as in the wild type strain. In *pan1*ΔCA, most patches internalized but accumulated 2.3 times more actin and at the faster rate than in the wild type cells. In *wsp1*ΔCA, the majority of the patches failed to internalize, indicating failed endocytosis, and the rate of patch assembly and the total actin accumulation in the patch were decreased compared to the wild type cells. Next, we used genetic crosses and tetrad analysis to determine which of the CA domains of the Arp2/3 complex activators are essential for cell viability. We observed that combinations of *myo1*ΔCA with *pan1*ΔCA and *myo1*ΔCA with *wsp1*ΔCA resulted in viable cells, while the combination of *wsp1*ΔCA and *pan1*ΔCA mutants was synthetically lethal. This suggests that activation of the Arp2/3 complex by Wsp1 alone or Pan1 alone but not Myo1 alone is minimally sufficient for cell viability. Imaging Fim1-mGFP in cells with viable combinations of ΔCA mutations revealed that both the *myo1*ΔCA *pan1*ΔCA cells and the *myo1*ΔCA *wsp1*ΔCA cells contained actin patches, indicating that activation of Arp2/3 complex by either Wsp1 or Pan1 alone suffices to promote actin patch assembly. However, given the patch internalization defect in *wsp1*ΔCA but not in *pan1*ΔCA or *myo1*ΔCA mutants, we concluded that Wsp1 is the primary Arp2/3 complex activator at the endocytic sites, minimally sufficient to support actin assembly, cell viability, and endocytic internalization.

1827

Mechanism of Activation of the Arp2/3 Complex by Nucleation Promoting Factors.

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Arp2/3 complex is a ubiquitous actin filament nucleator comprised of seven proteins, two of which (Arp2 and Arp3) are related to actin. By itself Arp2/3 complex is inactive. It is activated by nucleation-promoting factors (NPFs), proteins that contain a C-terminal WCA sequence featuring binding sites for both actin (W) and Arp2/3 complex (CA). By bringing together actin and Arp2/3 complex and inducing a conformational change within Arp2/3 complex itself, NPFs catalyze a reaction that leads to the formation of a new actin filament branch bound at 70° angle to the side of a pre-existing (mother) filament. It is generally believed that the activation mechanism involves a conformational change within Arp2/3 complex that brings the two Arps close to one another, analogous to two parallel actin subunits of the actin filament. The two Arps, together with the actin monomer(s) delivered by NPFs, form a polymerization 'seed'. However, the exact mechanism of activation remains unknown, and there is disagreement as to which subunits of Arp2/3 complex interacts with NPFs and how. Currently, there are two competing models of activation. The first model is based on small angle X-ray scattering (SAXS) of Arp2/3 complex in complex with the WCA region of the NPF N-WASP and actin. According to this model only one NPF binds Arp2/3 complex and delivers the first actin monomer of the branch at the barbed end of the Arp2 subunit of Arp2/3 complex. The second model assumes that two NPF molecules are required for optimal activation, with the first actin monomer being delivered at the barbed end of Arp3, followed by binding of a second actin monomer (contributed by the second NPF) at the barbed end of Arp2. Of note, the studies in support of the latter mechanism do not directly analyzed binding of NPF carrying actin to Arp2/3 complex. Here, we present a study of the polymerization mechanism of the Arp2/3 complex that takes into consideration this critically missing factor, by using isothermal titration calorimetry (ITC) and fluorescence to investigate the interaction of NPFs carrying actin with Arp2/3 complex. Based on the results we propose a new model of Arp2/3 complex activation that reconciles the two previous competing models.

1828

Ena/VASP synergizes with the Arp2/3 complex via a molecular hand-off mechanism during actin-based motility.

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To investigate how actin is assembled for cell migration, we study an in vivo model for a lamellipodial type of cell motility: migration of the epidermal cells on the surface of the embryo during the ventral enclosure event of *Caenorhabditis elegans* embryogenesis. Enclosure is governed by the Arp2/3 complex and its activator, WAVE, a related protein, WASP, and Ena/VASP protein. Here we show that VASP's capacity to bind F-actin and profilin is key for its function in ventral enclosure. We use an in vitro motility assay to dissect the mechanism of action of VASP, and discover that VASP is recruited directly by Arp2/3 complex activators to potentiate actin assembly. Our data is consistent with a molecular "hand-off" mechanism whereby the Arp2/3 complex activator positions Ena/VASP so that the nascent barbed end issuing from the Arp2/3 complex branch is passed directly to Ena/VASP for protection against capping protein and retention at the surface, thus increasing the growth of the actin network and enhancing cell motility.

1829

Cell cycle regulation of actin cable organization and polymerization in *Saccharomyces cerevisiae*.

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Changes in actin cytoskeleton organization during the cell cycle underlie such processes as cell polarity development and cytokinesis. However, little is known about the mechanisms by which actin nucleation, filament organization and dynamics are regulated during the cell cycle to coordinate and arrange polarized cell processes. Using the yeast *Saccharomyces cerevisiae*, we have begun to address these mechanisms.

Having previously reconstituted Arp2/3-mediated branched actin network assembly on surfaces of micro-beads in yeast extracts, we have now successfully reconstituted formin-mediated actin cable polymerization in yeast cell extracts. Intriguingly, we found that actin cable formation is highly cell cycle regulated and dependent on Cdk1, which is different from Arp2/3-nucleated actin assembly. To identify the proteins that contribute to cell cycle regulated actin cable assembly, we performed comprehensive investigations by mass spectrometry of the actin cables polymerized in the cell extracts. We identified about 90% of the known actin cable regulatory proteins and a number of other subgroups of proteins including uncharacterized proteins. We found that one previously uncharacterized protein from our proteomic study associates with the polarisome, a structure found at sites of formin-mediated actin nucleation, and we showed that this protein functions as a potential actin filament-capping protein. Interestingly, in addition to this newly identified protein, several polarisome proteins are highly phosphorylated by Cdk1 with cyclin specificity. Consistently, both biochemistry and cell biology methods show Cdk1 kinase activity plays an important role in regulating actin cable assembly and organization. Finally, we also found evidence that such cell cycle regulation is conserved for yeast and vertebrate formin proteins.

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1830

Dueling nucleation factors: Competition between formin and Arp2/3 complex for actin monomers.

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Cells simultaneously assemble and maintain differentially organized actin filament networks with specific architectures involved in diverse processes such as endocytosis, motility, polarization, cytokinesis. Actin filament organization is initially established by mechanistically distinct nucleation factors. Arp2/3 complex directs the formation of a branched dendritic network. Formin generates straight actin filaments. These nucleators are in competition for actin monomers, where formins have a disadvantage due to significantly lower cellular concentrations. Our hypothesis is that the small actin binding protein profilin allows formin to successfully compete with Arp2/3 complex. To test our hypothesis, we determined the contribution of profilin to actin-generated networks *in vivo* in the unicellular fission yeast

Schizosaccharomyces pombe utilizing genetics, chemical-genetic and live-cell imaging methods. Then, we reconstituted nucleator competition between the Arp2/3 complex and formin *in vitro* with 'bulk' pyrene actin assays, and by visualizing mixtures of formin- and Arp2/3 complex-nucleated actin filaments with Total Internal Reflection Fluorescence (TIRF) microscopy. We investigated a temperature sensitive profilin *cdc3-124* mutant. After 4 hours at the semi-permissive temperature of 33.5°C, the lack of functional profilin causes defects in 95% of formin Cdc12-dependent contractile rings. However, contractile ring defects are significantly reduced (60%) when Arp2/3 complex is inhibited via the small molecule inhibitor CK-666. We hypothesize that diminishing Arp2/3 complex decreases actin consumption in patches, liberating the remaining profilin-actin for assembly into formin-mediated structures. *In vitro* TIRF studies revealed that Arp2/3 complex-mediated branch density decreases in presence of profilin, favoring formin for the utilization of actin monomers. This study indicates that a primary role of profilin is to allow formin to efficiently compete with an excess of Arp2/3 complex for actin consumption.

1831

Determination of the molecular basis for formin specification of profilin isoforms and its importance for fission yeast viability.

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Profilin stimulates rapid formin-mediated actin elongation by simultaneously binding both to the proline-rich formin FH1 domain and actin monomers. We found that formins variously utilize different profilin isoforms. The molecular basis for this isoform specificity does not stem solely from differences in profilin's affinity for the FH1 domain, suggesting that profilin's interaction with actin and/or the formin FH2 domain might also be critical. Interestingly, there is a direct correlation between profilin's ability to be efficiently utilized by the fission yeast cytokinesis formin Cdc12 *in vitro* and its ability to allow fission yeast cells to grow. We hypothesize that formin utilization of profilin-actin explains why profilin is required by fission yeast.

To test these hypotheses, we mutated a "suboptimal" profilin that is utilized poorly by a formin into an "optimal" profilin that can be utilized well by the formin. We are taking advantage of two important facts: (1) The fission yeast cytokinesis formin Cdc12 efficiently utilizes fission yeast profilin (SpPRF), but does not efficiently utilize budding yeast profilin (ScPFY), and (2) ScPFY does not complement a fission yeast profilin temperature sensitive mutant. Libraries of ScPFY mutants were prepared that systematically converted residues in ScPFY to match that of SpPRF. We identified a ScPFY mutant that has mutations in 9 amino acids that complements the fission yeast profilin-ts strain and is utilized significantly better by Cdc12 *in vitro*. Biochemical characterization revealed that the only biochemical parameters changed between the ScPFY mutant and wild-type profilin is its utilization by Cdc12 and slightly stronger PIP2 binding. The mutations lie in the actin-binding region of profilin, which also interacts with the formin FH2 region. We therefore propose a mechanism of specificity whereby the formin interacts with profilin at the barbed end of the actin filament, which helps release the profilin for subsequent cycles of filament elongation.

1832

The Diaphanous-related formin dDia1 of *Dictyostelium discoideum* regulates cell migration.

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Formins are large multi-domain proteins that drive actin polymerization via de novo nucleation and elongation of unbranched filaments. The *Dictyostelium discoideum* genome project revealed that this organism expresses 10 different formins, from which nine belong to the Diaphanous-related formin (DRF) family. DRFs act as downstream effectors of Rho family GTPases, which in turn are major regulators of the actin cytoskeleton. Here, we focus on the DRF dDia1 (also known as formin F) from *Dictyostelium discoideum*. To explore its physiological role, we created null mutants by gene disruption or cell lines that overexpress dDia1. Analyses by time lapse phase-contrast microscopy revealed that random cell migration in dDia1 overexpressing cells was compromised. In contrast, loss of dDia1 led to a more than ~2-fold increase in cell speed. To ascertain the underlying cause of the motility phenotype, we analyzed the subcellular distribution of constitutive active dDia1 by indirect immunofluorescence microscopy. Notably, dDia1 was found to be accumulated evenly throughout the entire pseudopodium (the equivalent of the lamellipodium of mammalian cells) suggesting that it controls pseudopodial microfilament architecture to regulate cell migration. To biochemically analyze this formin in vitro, several C-terminal FH1FH2 constructs were expressed in *E. coli* and purified. Initial experiments employing actin pyrenyl assays and in vitro TIRF microscopy revealed that in absence of profilin dDia1 is a rather weak nucleator. Consistent with the interaction of all three profilin isoforms with the proline-rich FH1 region of dDia1 in Yeast two hybrid assays, recombinant profilin I-III accelerated formin-mediated actin filament barbed end elongation two to three fold in the TIRF microscopy assay. Interestingly, we frequently observed dissociation of the formin from fast growing barbed ends. These findings are consistent with dilution-induced depolymerization assays in the presence or absence of dDia1 FH1FH2 suggesting that dDia1 is weak capper when compared to heterodimeric capping protein.

1833

Towards the better muscles in *Caenorhabditis elegans*: A tale of two formins.

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Even though extensive studies have examined the structure of striated muscle actin cytoskeleton, not much is known about the mechanism of how actin filaments are incorporated into the sarcomere, the smallest unit in striated muscle organization. The barbed ends of actin filaments are anchored at the Z-lines that define the sarcomere ends, where net polymerization of actin filaments occurs favorably. It is known that formins, one of the highly conserved and most widespread families of actin nucleating factors (ANFs), have unique association with the barbed ends of action filaments.

In this study, we have shown that two nematode formins, CYK-1 and FHOD-1, work hand in hand to promote the growth of the contractile lattice and maintain the proper organization of the *C. elegans* striated muscle system. Through combination of genetic and microscopy methods, we have shown that depletion of either formin during larval development of *C. elegans* caused the stunted growth of contractile muscle lattice, and depletion of both formins severely reduced the lattice size and number of striations per muscle cell. Moreover, the near complete RNAi-

induced depletion of CYK-1 in *C. elegans* adults caused results ranging from the partial loss of Z-line-associated actin filaments to collapse of the entire muscle lattice.

In summary, our study provides the first *in vivo* genetic evidence of the existence of sarcomere-associated formins and their importance in growth and maintenance of the striated muscle contractile lattice.

1834

Biochemical Characterization of the *Drosophila melanogaster* Ena/VASP Enabled and the formin Diaphanous.

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Formins and Ena/VASP family proteins stimulate the production of long-straight actin filaments. The general actin assembly properties of formins have been well established, revealing a common general mechanism whereby formins remain processively associated with the rapidly elongating barbed while directing the addition of 1000s of profilin-actin subunits before dissociating. Various formin isoforms have significantly different actin assembly rate constants, which tailors them for different cellular roles. Ena/VASP family proteins are less well understood. In solution they may remain processively associated with the elongating barbed end for the addition of only ~40 actin monomers and/or they may be highly processive when clustered on a surface. Both the formin Diaphanous (Dia) and the Ena/VASP family protein Enabled (Ena) assemble actin for the production of filopodia at the leading edge of *Drosophila melanogaster* cells. To better understand the general mechanism of how Formins and Ena/VASPs drive the rapid assembly of long-straight actin filaments, we have biochemically characterized the actin assembly properties of both Dia and Ena using bulk and single filament single/dual color TIRF microscopy. Dia behaves like a canonical formin, nucleating actin and processively adding profilin-actin at high rates to the actin filament barbed end. Surprisingly, in solution Ena also increases the elongation rate while remaining processively associated with the barbed end during the addition of ~1000 actin monomers. When at higher concentrations, Ena also nucleates and bundles actin filaments. The biochemical analysis of mixtures of Ena and Dia may help determine why cells possess two similar actin assembly factors for the production of filopodia.

1835

Bud6 coordinates polarized actin cable assembly and maintenance of an ER-membrane diffusion barrier through its mechanistically distinct interactions with two formins.

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Cell polarity in *S. cerevisiae* depends on formin-mediated nucleation of actin cables required for transport of secretory vesicles to the bud tip, and on septin structures at the bud neck, which are required to maintain membrane diffusion barriers between the mother and bud. We recently showed that the polarity protein Bud6 serves as an actin nucleation-promoting factor (NPF) for one of the two yeast formins, Bni1, located at the bud tip [1]. Other groups have shown that Bud6 is essential for maintenance of an endoplasmic reticulum (ER) membrane diffusion barrier at the bud neck [2], but whether there is any connection between these two functions of Bud6 has remained unknown. Here, we explored the potential role of Bud6 in regulating the bud neck formin, Bnr1, and whether there is any connection between its roles in actin regulation and maintenance of membrane diffusion barriers. Genetic analyses using *bud6* alleles defective in NPF activities in a *bni1Δ* background showed that Bud6 indeed serves as an NPF for Bnr1 in

vivo. Further, a purified C-terminal fragment of Bud6 (550-788) directly bound to Bnr1 and potently stimulated its actin nucleation activity in vitro. Strikingly, inclusion of adjacent sequences (489-549) masked Bud6 NPF effects on Bnr1, but not Bni1. Using endogenously-expressed Bud6-GFP, we isolated and identified by mass spectrometry a novel in vivo binding partner of Bud6, which we named Bud6-interacting ligand (Bil1). Bil1-GFP co-localized with Bud6-RFP at the bud neck, and depended on BUD6 for its localization. Purified Bil1 bound directly to the regulatory sequence in Bud6 (489-549) and unmasked Bud6's NPF effects on Bnr1. Genetic analysis showed that Bil1 is required for Bnr1-dependent actin cable assembly. To address whether Bud6 NPF effects are involved in maintaining the diffusion barrier at the neck, we used FLIP (fluorescence loss in photobleaching) to compare diffusion rates of Sec63-GFP, an integral ER membrane protein, in wild-type and bud6 mutant cells. This analysis revealed that mutants impaired in Bud6 NPF activity are as defective in maintaining the mother/bud ER diffusion barrier as *bud6Δ*. Taken together, our results demonstrate that through a Bil1-regulated mechanism Bud6 promotes Bnr1-mediated actin cable assembly at the neck, and that this function is closely integrated with its role in maintaining the mother/bud ER membrane diffusion barrier.

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Single Molecule Imaging of APC, mDia1, and EB1 Coordinated Effects on Actin Filament Assembly and Actin-Microtubule Crosstalk.

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Mounting evidence suggests that the process of actin assembly in cells is catalyzed not by individual actin-nucleating proteins, but by sets of interacting proteins that form multi-component nucleation and elongation machines. We have begun to dissect the mechanism by which three interacting mammalian proteins, adenomatous polyposis coli (APC), the formin mDia1, and microtubule end-binding protein EB1, control the initiation and elongation of actin filaments, and coordinate actin and microtubule (MT) dynamics. By combining single-molecule TIRF microscopy with reconstituted actin polymerization, we defined an actin assembly "rocket launcher" mechanism mediated by mDia1 and APC. Fluorescently-tagged mDia1 and APC molecules formed dimer:dimer nucleation complexes that bound to actin monomers, leading to efficient formation of filaments even in the combined presence of the nucleation-suppressor profilin and filament capping protein CapZ. Formation of the nucleation complex required a direct interaction between APC and the C-terminal tail region of mDia1. At the onset of filament elongation, the mDia1-APC complex separated, resulting in mDia1 translocating at the growing barbed end and leaving APC behind at the pointed end. More recently, we have found that the APC-binding partner EB1 strongly inhibits APC nucleation activity, and we are currently testing its effects on mDia1-APC. Finally, to understand how this triad of interacting proteins coordinates actin and MT dynamics, we co-reconstituted actin polymerization and microtubule dynamic instability in two different colors in the same reaction. This system enables simultaneous analysis of the individual and combined effects of APC, mDia1, and EB1, each of which binds to MTs, on the spatial and temporal dynamics of the two polymer systems. Our preliminary data suggest that EB1 directs APC to MT tips, and that APC physically connects growing actin filaments with MTs. Combined with single-molecule imaging, this novel

experimental setup will provide unprecedented functional and mechanistic insights into the cellular mechanisms controlling actin-MT cross-talk.

Microtubules and Associated Proteins

1837

CAMSAP2 and 3 cooperate to organize non-centrosomal microtubules and in turn regulate organelle assembly.

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Microtubules are one of the major components of the cytoskeleton, and play important roles in various aspects of eukaryotic cellular structure and functions, such as intracellular transport, organelle assembly, motility, and cell division. Animal cells contain two populations of microtubules, centrosomal and non-centrosomal. Centrosomal microtubules radially grow from the centrosome, with their minus ends anchored to the γ -tubulin ring complex. On the other hand, non-centrosomal microtubules are produced by various mechanisms, and their minus ends are located in the cytoplasm. In mammalian epithelial cells, their major microtubules are not anchored to the centrosome. Where they are nucleated and how their minus ends are stabilized are, however, little understood. Recent studies identified a novel protein, termed Nezha or CAMSAP3, that binds to the minus end of non-centrosomal microtubules. Moreover, two other proteins related to CAMSAP3, CAMSAP1 and CAMSAP2, were reported. However, their biological functions remain undetermined. Here we found that CAMSAP3 and CAMSAP2 cooperate to organize non-centrosomal microtubules in human Caco2 epithelial cells. We found that these two molecules cluster together at the minus ends of non-centrosomal microtubules, and cooperate to sustain their plus-end growth. Depletion of these molecules affected the pattern of microtubule assembly, causing a dramatic increase of centrosomal microtubules, and this effect was maximized by co-depletion of them. In CAMSAP-depleted cells, the Golgi apparatus was fragmented, and early endosomes displayed an altered distribution. Further analysis suggested that the ER-Golgi membrane trafficking was impaired in the absence of CAMSAPs. From these observations, we concluded that CAMSAPs play a key role in maintaining a population of non-centrosomal microtubules, and this population of microtubules is important for proper organelle assembly.

1838

Stathmin regulates mitotic entry in HeLa cells by controlling activation of both Aurora A and Plk1.

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Microtubules have been an effective target for chemotherapeutics. However, microtubule disruptors also damage healthy tissues and not all cancer cells die during the mitotic arrests they induce; instead, they can exit without cytokinesis and continue to cycle as aneuploid cells. Improvements in current therapies would allow cancer cells to be selectively targeted without induction of further mutations. Loss of a microtubule regulator, stathmin, has been shown to slow the growth of certain cancer cells, accompanied by an accumulation of cells with 4N DNA content and an increase in cell death. We have shown previously by long term imaging that HeLa cells depleted of stathmin by siRNA had an increased interphase duration of about 5 hours in asynchronous cell populations, an increased interval from thymidine release to mitosis and

increased populations of interphase cells staining positive for cyclin B, cdk1Y15 or TPX2. From these data we hypothesized that stathmin depletion inhibited mitotic entry. We have now traced the delay to decreased activation of Aurora A kinase (AURKA) at the centrosome and Polo-like Kinase 1 (PLK1) on chromatin by measuring levels of phosphorylated (active) kinase by quantitative immunofluorescence. AURKA and PLK1 activation is reduced 50% and 70% respectively compared to control siRNA treated cells. By tracking live cell fates, we find that inhibition of Aurora A with selective inhibitor S1451 at 300nM resulted in a mitotic delay without a change in interphase timing. Inhibition of PLK1 with selective inhibitor BI2536 at 0.8nM caused a small interphase delay without a change in mitosis timing. Remarkably, treatment with both S1451 and BI2536 yields a long interphase delay of about 9 hours, which mimics loss of stathmin. To address how stathmin affects activation of AURKA and PLK1, we performed *in vitro* reactions with purified proteins. We discovered that AURKA phosphorylates stathmin at S16 and 63 and autoactivation of PLK1 is inhibited by stathmin, revealing a functional interaction between these proteins. It is possible that stathmin directly regulates activation of these two kinases. Alternatively, we have previously demonstrated that depolymerization of microtubules abrogates the stathmin depletion induced G2 delay, indicating that microtubules function as a relay signal. Therefore, stathmin influences mitotic entry either directly or via microtubules by controlling activation of key cell cycle enzymes.

1839

Stu2p acts as a weak microtubule polymerase and a rescue factor *in vitro*.

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Stu2p is a member of the Dis1/XMAP215 family of polymerases and is required for spindle orientation and metaphase chromosome alignment, and for anaphase B spindle elongation. Despite Stu2p having a phenotype suggesting that it promotes microtubule growth, studies till now with purified proteins indicate only that Stu2p antagonizes growth. To investigate the effect of Stu2p on microtubule dynamics *in vitro*, we purified recombinant Stu2p from insect cells and studied its effect in a total-internal-reflection fluorescence (TIRF) microscopy assay: GMPCPP-stabilized microtubules (seeds) were immobilized on the glass surface and varying amounts of fluorescently labeled porcine-brain tubulin, GTP and Stu2p proteins were added to the chamber. Images were acquired using time-lapse microscopy. In the presence of tubulin, Stu2p increased the microtubule growth rate, though to a lesser extent than XMAP215. Stu2p had no effect on the catastrophe frequency, but significantly increased the rescue frequency. In conclusion, Stu2p shares functional properties with its metazoan homolog XMAP215, though its polymerase activity is weaker. These properties go some way to explaining the phenotypes of the Stu2p-depleted cells.

1840

The dynein regulator, Pac1p/LIS1, interacts with a STUbL.

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The function of Pac1p, the yeast homologue of Lis1, is closely associated with the minus-end directed motor protein, dynein. Mutations in Lis1 result in Miller-Dieker lissencephaly, a developmental brain syndrome caused by defects in neuronal migration. As part of the dynein pathway in yeast, Pac1p is important for recruiting dynein to the plus end of the microtubule. Dynein is subsequently "off-loaded" to the cortex where it pulls on cytoplasmic microtubules to move the mitotic spindle across the bud neck, a key step in spindle positioning. Although Pac1p plays a vital role in microtubule function, little is known about how it is regulated. Sumoylation is

a post-translational modification that covalently attaches the Small Ubiquitin-like Modifier (SUMO) protein to target substrates. Whereas sumoylation regulates many cellular processes, such as cellular transport, protein stability, and transcription, it has only recently been shown to regulate spindle positioning. Using a two-hybrid assay, Pac1p interacted with *SMT3*/SUMO and other key players of the sumoylation system. Ubiquitin-Like Protein-1 (Ulp1) is a protease that specifically cleaves Smt3p from its protein conjugates. Using a temperature-sensitive allele of *ULP1*, Pac1p displayed an accumulation of higher molecular weight bands, suggesting that SUMO can accumulate on Pac1p. In contrast to ubiquitination, sumoylation does not directly target its substrates for degradation. However, SUMO-Targeted Ubiquitin Ligases (STUbLs) can recognize a sumoylated substrate and promote its degradation by poly-ubiquitinating it. By two-hybrid analysis, Pac1p interacted with the STUbL enzyme Nis1p/Ris1p and the SUMO isopeptidase, Wss1p. Strains deleted for *RIS1* or *WSS1* displayed an accumulation of higher molecular weight Pac1p conjugates. Pull-down assays suggest that Pac1p is modified by both ubiquitin and SUMO. Modification of Pac1p was also increased by deletion of the dynein regulator, *SHE1*; and *KAR9*, protein required for correct positioning of the mitotic spindle. These findings also suggest that She1p is a novel inhibitor of Pac1p ubiquitination. This work has implications for the regulation of dynein's interaction with various cargoes, including its off-loading to the cortex. We suggest a novel model in which Pac1p is regulated via STUbLs.

1841

Molecular Determinants for Regulation of Microtubule-Severing by Katanin.

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Microtubules (MTs) are dynamic cytoskeletal polymers that form complex and highly organized arrays essential for cell motility, morphogenesis, and division. MT-severing is a reaction that generates an internal break in the MT polymer and catalysis of MT-severing is required for the proper formation and function of MT-based arrays in a wide range of eukaryotic cells. The MT-severing enzyme Katanin is a conserved heterodimeric ATPase that severs and disassembles MTs, but the regulatory mechanisms that control MT-severing by Katanin are poorly defined. To better understand the regulation of Katanin, we conducted a structure-function study of Katanin in cultured *Drosophila* cells using a combination of high-throughput microscopy and live-cell imaging. We identified regions of the Katanin catalytic subunit that regulate its stability and MT-severing activity in cells and that are important for association with the Katanin regulatory subunit. We also identified regions of the Katanin regulatory subunit that regulate the MT-severing activity of the Katanin catalytic subunit and that are important for association with the Katanin catalytic subunit. By expressing differentially tagged Katanin catalytic and regulatory subunits alone or together in cells, we discovered that efficient MT-targeting by Katanin required both subunits. Furthermore, we found that the dynamic localization of Katanin on MTs was dependent on the ATPase activity of the Katanin catalytic subunit. These results indicate that both Katanin catalytic and regulatory subunits contribute to the activity and dynamics of Katanin in living cells.

1842

Myosin II Co-Chaperone UNC45A is required during natural killer (NK) cell functions.

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Natural killer (NK) cells are a component of the innate immune system whose functions is the lysis of virally infected and tumor target cells. Receptor-mediated recognition of target cells by NK cells triggers an orchestrated motion of microclusters toward the center of the contact area between effector and target cell (NKIS). This movement is associated with centripetal F-actin flow and polarization of the microtubule organizing center (MTOC) and perforin-containing

granules(1). Because polarization and exocytosis of lytic granules are necessary for NK cell cytotoxicity, understanding their mechanism of regulation is crucial for the understanding of physiological and pathological processes including autoimmune diseases and cancer. UNC45A is a member of the UCS family of proteins that interact with myosins to ensure their proper activity during cytokinesis, cell motility and contraction, as well as organelle trafficking within the cellular compartment(2). The myosin motor protein, myosin IIA (MYH9) has been recently shown to be recruited at the NKIS site and have a crucial role during re-direction and fusion of membrane fusion of lytic granules and NK cell cytotoxicity(3, 4). We and others have shown that myosin IIA functions are tightly regulated by the expression levels of its co-chaperone UNC45A(5, 6). Therefore, we sought of determining whether the myosin II co-chaperone UNC45A plays a role during NK cell functions. Herewith we report that : a) UNC-45 is expressed in primary NK cells and NK cell lines where it localizes and interacts with myosin II and Hsp90, b) UNC-45 genetic silencing results with impaired cytotoxic in NK cell lines and primary NK cells, c) UNC-45 plays a crucial role during release of lytic granules in NK cells. To our knowledge, this is the first report linking myosin II co-chaperone UNC45A function to natural killer cell activation.

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1843

TIP150 molecular delineation, function, and regulation in cell migration.

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The microtubule cytoskeleton orchestrates the cellular dynamics and plasticity that underlies cellular behavior such as cell cycle, morphogenesis and migration. Microtubule plus-end-binding proteins regulate the dynamic properties of microtubules during cell migration. Our recent study revealed that TIP150 is a novel plus end tracking protein interacting with EB1 and MCAK in vitro and in vivo (*EMBO Reports*. 2009. 10:857-65). We have also shown that TIP150 is essential for chromosome segregation, and governs proper kinetochore-microtubule attachment. It is, however, unclear whether TIP150 serves a functional role in cell migration when microtubule polymerization and depolymerization are spatiotemporally regulated, and how does the interaction with EB1 facilitate this dynamics process. We hypothesize that TIP150 functionally serves a role in cell migration and facilitates cellular dynamics during its migration. To delineate the molecular function of TIP150 and its regulation in cell migration, a combination of biophotonic analyses with RNA interference and chemical inhibitors was employed.

Immunofluorescence reveals that TIP150 localizes to EB1 in interphase cells. We speculate that phosphorylation disrupts the electrostatic interaction between EB1-TIP150 via hydrogen bonds between arginine of TIP150 and glutamate residues in the C-terminal tail of EB1.

1844

Dissecting the nanoscale distributions and functions of microtubule-end-binding proteins EB1 and ch-TOG in interphase HeLa cells.

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Recently, the EB1 and XMAP215/TOG families of microtubule binding proteins have been demonstrated to bind autonomously to the growing plus ends of microtubules and regulate their behaviour in in vitro systems @ (Bieling et al., Nature, 2007, 45, 1100-5; Brouhard et al., Cell, 2008, 132, 79-88). However, their functional synergy or redundancy in cells remains obscure.

Last year, we reported that ch-TOG is localized at more distal along the microtubules than EB1 in interphase HeLa cells using high-resolution SIM (structured-illumination microscopy) technique. Since then we carefully evaluated the SIM images and analysed large number of microtubules, and concluded that the ch-TOG accumulation sites protrude ~100 nm from the EB1 comets. Over-expression experiments showed that they bind to microtubule tips in a noncompetitive manner, confirming that they recognize distinct regions at the ends of microtubules. While both EB1 and ch-TOG have similar effects on microtubule plus end dynamics and additively increase microtubule dynamicity, only EB1 exhibits microtubule-cell cortex attachment activity. These observations indicate that EB1 and ch-TOG regulate microtubule organization differently through the distinct portion of microtubule plus ends.

1845

Systematic analysis of the dynamic EB1 +TIP-interactome in budding yeast.

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The highly dynamic microtubule (MT) cytoskeleton plays many essential roles in chromosome segregation, intracellular transport and cell morphology. MT dynamics and attachment to cellular structures is regulated by a wide plethora of plus-end tracking proteins (+TIPs) that specifically interact with growing MT plus ends. The majority of +TIPs are recruited to MT ends by interacting with the End Binding (EB) family members. These are autonomous +TIPs considered to be at the “core” of the +TIP interaction network. Association of proteins to the highly conserved EBs is mediated by two distinct structural features; one involves the interaction between EB’s hydrophobic C terminal cavity with a short linear (S/T)x(I/L)P (SxIP) peptide motif in its partner and the second involves EB’s C terminal acidic and aromatic tail binding to CAP-Gly domains.

Recent advances in the field have greatly increased the number of known mammalian EB interactors and thereby the complexity of the EB1 +TIP-interactome. It has thus become difficult to dissect the specific roles of individual +TIPs in modulating MT function, in addition little is known about how the composition of the +TIP network is established and regulated both in time and space.

To circumvent the problem of increased mammalian complexity and to characterize +TIP networks in a genetically tractable organism, we use the budding yeast *S. cerevisiae* to systematically analyze the changing composition of the EB1 +TIP-interactome over the cell cycle.

By combining a bioinformatics approach to search the budding yeast genome for SxIP-motif containing proteins and a biochemical method in which EB1 interacting proteins are purified from defined cell cycle stages and analyzed by quantitative mass spectrometry, we have identified known EB1 interaction partners, such as Ipl1, Kar9 and Stu2, validating our approach. In addition, we have identified novel EB1 interactors that display differential EB1 affinities over the yeast cell cycle. We are currently using a combination of cell biology, biochemistry and fluorescent microscopy techniques to unravel the cell biological significance of these interactions as well as their structural basis.

1846

The critical role of EB1 and dynein in three-dimensional cell migration.

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Cell migration is important for various biological processes including embryogenesis, wound healing, inflammatory response, and cancer cell metastasis. Cell migration requires synchronization of many events such as polarization of signaling molecules, orientation of microtubule organizing center towards the direction of migration, and importantly the rapid reorganization of actin and microtubule cytoskeleton. Numerous studies have focused on elucidating the role of actin in cell migration; however, recent studies have shown that stabilization of microtubules plays a pivotal role in this process. Most of these studies have been performed in cells migrating on flat two-dimensional (2D) substrates, but cell migration *in vivo* predominantly occurs through 3D matrices and not much is known about the role of microtubule and microtubule-binding-proteins, EB1 and LIC2 in 3D motility. Here we report that both microtubule organization and dynamics are critical for 3D cell migration. Moreover, we show that destabilization of microtubules by depletion of EB1 and LIC2 attenuates cell migration by reducing protrusion activity.

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GTSE1 is a Microtubule Plus-end Tracking Protein that Regulates EB1-dependent Cell Migration.

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The regulation of cell migration is a highly complex process that is often compromised when cancer cells become metastatic. The microtubule cytoskeleton is necessary for cell migration, but how microtubules and microtubule-associated proteins regulate multiple pathways promoting cell migration remains unclear. Microtubule plus-end binding proteins (+TIPs) are emerging as important players in many of cellular functions, including cell migration. Here we identify a novel +TIP, GTSE1, that promotes cell migration. GTSE1 accumulates at growing microtubule plus ends through interaction with the EB1 +TIP. The EB1-dependent +TIP activity of GTSE1 is specifically required for cell migration, as well as for microtubule-dependent

disassembly of focal adhesions. GTSE1 protein levels determine the migratory capacity of both nontransformed and breast cancer cell lines. In breast cancers, increased GTSE1 expression correlates with invasive potential, tumor stage, and time to distant metastasis, suggesting that misregulation of GTSE1 expression could be associated with increased invasive potential.

1848

Clasp1 is essential for neonatal lung function and survival.

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CLIP-Associating Proteins (CLASPs) are microtubule plus-end-tracking proteins with an important role in the regulation of microtubule dynamics and involved in chromosome segregation during mitosis. In *Drosophila* and yeast, CLASP loss-of-function resulted in the generation of mitotic abnormalities, with subsequent aneuploidy, indicating that this protein is essential for cell division and viability. In *C.elegans*, RNAi of the orthologue *cls-2* yielded embryonic lethality. In mammals, including humans, two paralogues, CLASP1 and CLASP2, exist, which prompted us to further investigate their individual roles. For that, we successfully generated *Clasp1* knockout (KO) mice through homologous recombination in embryonic stem cells. Our data demonstrate that targeted disruption of *clasp1* gene not only causes intrauterine growth restriction, as revealed by low body weight at birth, but also leads to early neonatal lethality as a consequence of an acute respiratory failure, with the KO animals displaying cyanotic features shortly after being born. Histological analyses of the KO animals proved lack of inflation in the lungs without any obstruction of the respiratory tract being observed. Our immunohistochemical results suggest a delayed fetal lung maturation characterized by decreased sacculation that, however, does not impair the presence of surfactant. Importantly, protein expression results show *Clasp1* presence in the lungs throughout embryonic development. Altogether, our findings reveal a novel and critical role for the plus-end tracking protein *Clasp1* in fetal growth, as well as proper lung development and respiratory function in a mammalian model system.

1849

Molecular mechanism of cooperative microtubule stabilization by microtubule plus-end tracking proteins CLASP2 and EB1.

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Microtubules (MTs) are highly dynamic polymers that are essential for physiological events such as cell shape remodeling and intracellular transport. They possess a structural polarity, with fast growing plus-ends directed toward the cell periphery and minus-ends anchored at the centrosome. The plus-ends of MTs display alternating phases of growth and shrinkage, which is called dynamic instability. Growing plus-ends are composed of a layer of GTP-tubulin that stabilizes the MT structure, whereas the rapid depolymerization of the filament is attributed to the loss of the "GTP-cap."

A large number of MT-associated proteins (MAPs) contribute to the dynamics of MTs. The plus-end tracking proteins (+TIPs), which specifically localize to the MT plus-ends, directly regulate the MT behavior and are thought to link the MT-ends to the various cellular structures. One of the +TIPs, CLASP proteins, are members of the XMAP215/Dis1 family in the MAPs and work as stabilizing factor of MTs. While XMAP215 directly binds to the tubulin dimer and functions as a MT polymerase, CLASPs rather work as a rescue factor and suppress the catastrophic events of MTs. MT-binding regions are located in the N-terminal half, composed of two tubulin-binding

TOG-like domains and the Ser-rich (SR) region. EB1, known as a hub protein of the +TIPs at the plus-end, binds to the internal motif of the SR region, indicating that CLASPs associate with MTs in two distinct manners; MT lattice-binding through the MT-binding domains and the plus-end tracking via EB1. In addition, it has been known that the serine/threonine protein kinase GSK3 β is involved in the regulation of CLASP-mediated MT dynamics. The putative phosphorylation sites are located in the vicinity of the EB1 binding sites, suggesting that the post-translational modification affects both to CLASP- and EB1- mediated MT stabilization.

In order to understand the molecular mechanism of both CLASP2-binding to MTs and MT regulation by EB1 via CLASP2, we have determined the crystal structures of two MT-binding domains in CLASP2. Both structures form six HEAT-repeats known as a TOG domain. The analysis of GTP hydrolysis by tubulin showed that the CLASP2 and EB1 complex influence MT stabilization *in vitro*. We will discuss the molecular mechanism of MT stabilization which may affect cell migration and chromosomal segregation.

1850

EB1 and XMAP215 recognise structurally and functionally distinct binding sites at growing microtubule ends.

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Growing microtubule ends serve as transient binding platforms for essential plus end binding proteins (+TIPS) that regulate microtubule dynamics and their interactions with cellular substructures. The core of the +TIP network is formed by end binding proteins (EBs) that autonomously bind microtubule ends and recruit a diverse family of other +TIPs, which enable microtubule ends to participate in a variety of processes. Another prominent autonomous microtubule end tracking protein is the processive microtubule polymerase XMAP215, the *Xenopus* chTOG ortholog, which is able to increase microtubule growth speed up to 10-fold *in vitro* by increasing the association rate of tubulin. It is an open question of whether these two proteins bind to the same or different binding sites. It is clear that EBs recognise hundreds of binding sites located between protofilaments in an extended region at growing microtubule ends. Most likely, GTP hydrolysis is involved in the generation of the high affinity conformation of these sites. Strikingly, the majority of the extended EB binding region is lost before onset of microtubule depolymerisation (catastrophe), suggesting an interesting mechanistic link with the phenomenon of dynamic instability of microtubules. The nature of the XMAP215 binding site is less well understood. Here, we addressed the question of whether the two end trackers EB1 and XMAP215 potentially bind to sites at different positions at the microtubule end.

Using simultaneous two-colour TIRF microscopy, we imaged *in vitro* reconstitutions of purified microtubule end tracking proteins. Using automated image analysis, we investigated the spatial characteristics of the fluorescence signals of EB1-GFP and XMAP215-GFP at the end of microtubules grown in GTP. Averaged fluorescence signal profiles provided high-resolution insight into the distributions of the different +TIP binding sites. In contrast to the extended nature of the EB1 binding region covering several 100 nm at the microtubule end, the binding sites of XMAP215 fell into the region of a diffraction-limited spot, demonstrating that these two proteins recognise different features at growing microtubule ends. An interesting difference between these sites is that the XMAP215 binding sites remain present at an almost constant level throughout the dynamic cycle of the microtubule, in clear contrast to the EB sites. Therefore, the different features recognised by EB1 and XMAP215 likely reflect different sub-regions at the microtubule plus end with different functions in dynamic instability.

1851

Microtubule stability mediated by new mammalian STOP domain family members.*I. A. Onyeneho¹, T. Stearns¹; ¹Stanford University, Stanford, CA*

Stable Tubule Only Polypeptide or STOP proteins are a class of microtubule-associated proteins that are unique in that they have been shown to protect microtubules from depolymerization under destabilizing conditions. To date, Map6 and the related protein Map6 domain containing 1 (Map6D1) are the only known mammalian STOP domain-containing proteins. However, through a recent screen identifying genes upregulated during ciliogenesis in multiciliated tracheal cells, we have identified two new uncharacterized mammalian STOP domain-containing proteins, which we have named Map6-like 1, and Map6-like 2 (Map6L1, Map6L2). Though Map6 has not been previously shown to localize to the primary cilium and centrosome, we sought to assess the ability of these new proteins to stabilize microtubules and their potential function at the cilium and centrosome. Proteins related to Map6L1 and Map6L2 are found in all vertebrates as well as *Drosophila* and *Chlamydomonas*. GFP tagged Map6L1 localizes to the centrosome and the primary cilium, and overexpressed protein stabilizes microtubules to cold treatment and anti-microtubule drugs in cells. Studies are currently in progress to establish specific microtubule binding domains and other protein-protein interactions. Though the similarity of Map6L1 and Map6L2 to Map6 is distant, both Map6L1 and Map6L2 contain extensive repeat sequences much like Map6, and we are currently investigating the role of this repeat structure in the function of the protein.

1852

MAP65-1/Ase1 increase microtubule flexibility.*D. Portran¹, M. Zoccoler¹, J. Gaillard¹, V. Stoppin-Mellet¹, E. Neumann², I. Arna³, J-L. Martiel¹, M. Vantard¹; ¹Institut de Recherches en Technologies et Sciences pour le Vivant, Grenoble, France, ²Institut de Biologie Structurale, Grenoble, Grenoble, France, ³Institut de Neurosciences, Grenoble, Grenoble, France*

Microtubule (MT) bundling plays a key role in the formation of functional MT arrays in numerous eukaryote cells. Although detailed mechanisms responsible for the formation of these self-organized bundles are not well understood, observations in living plant cells showed that physical collisions between MTs/small bundles at angles up to 40° result in their co-alignment and zippering. As MTs are rigid structures with a persistence length up to 1 μm and as they have to be locally deformed to co-align at such angles, we investigated whether MT cross-linkers that belong to the conserved MAP65 family (plant MAP65-1 and yeast Ase1) could manipulate MT flexural rigidity. We have reconstituted bundle arrays of growing MTs in the presence of MAP65-1/Ase1 *in vitro*. We observed that these two orthologous MAP65 induce the co-alignment and bundling of antiparallel MTs that encounter at angles up to 45°. During collision, MTs were deformed to amplitude that suggests a fine-tuning of their stiffness. To measure the rigidity of growing MTs in the presence of MAP65-1/Ase1, we developed an *in vitro* assay based on hydrodynamic flux and a biophysical model. We demonstrated that MAP65-1/Ase1 drastically increased the flexibility of both, single MTs and MT bundles (persistence lengths of single MTs in presence of MAP65-1 and Ase1 are 4 and 3.2 times smaller than that of single naked MTs). The MT binding domain of MAP65-1/Ase1 specifically mediates their softening effect. These data provide new insights on how these MT bundlers, by modifying MT mechanical properties control the issue of MT-MT encounters resulting into the spontaneous formation of self-organized ordered MT arrays.

1853

Formation of a parallel microtubule array by MAP4 is required for muscle cell differentiation.

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During muscle cell differentiation, myoblasts elongate and fuse into syncytial myotubes. Microtubule integrity and proper regulation of microtubule dynamics are essential to support these morphological changes. Myogenesis is accompanied by the remodelling of the microtubule cytoskeleton, involving disassembly of the centrosome and, crucially, the alignment of microtubules into a parallel array along the long axis of the cell. We show that microtubules in undifferentiated myoblasts are highly motile, and show bidirectional sliding movements that are largely driven by dynein. In contrast, microtubules are long-lived and relatively static in myotubes, suggesting that microtubules might be heavily crosslinked to provide physical support to these elongated cells.

Here, we identify a previously uncharacterised isoform of microtubule-associated protein MAP4, oMAP4, as a microtubule organising factor that is crucial for myogenesis. We show that oMAP4 is expressed upon muscle cell differentiation and is the only MAP4 isoform that is essential for normal progression of the myogenic differentiation programme. Depletion of oMAP4 impairs cell elongation and cell-cell fusion, causes a delay in centrosomal protein reorganisation and expression of sarcomer components. We find that oMAP4 is required for parallel microtubule organisation in muscle cells by preventing microtubule-microtubule sliding. Consistently, purified oMAP4 aligns dynamic microtubules in vitro. We propose a model in which zippering of microtubules by oMAP4 drives formation of a parallel microtubule network that supports myogenesis.

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Testing a Neuroprotectant for Improving Memory in Aged Rats.

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The aim of this research project was to test the effect of Z-phenylalanyl-alanyl-diazomethylketone (PADK), a proof-of-concept lysosomal modulator, in aged rats. This small molecule has gained key attention due to the many studies showing its ability to enhance lysosomal enzyme levels and produce protective clearance of PHF-tau (hyper-phosphorylated tau) and A β 42 (Amyloid beta) in the brain. Both PHF-tau and A β 42 have been thought to contribute to Alzheimer's disease (memory loss). Aged Fischer and Long Evans rats provide a natural model of aging and cognitive decline and may be more translatable to humans, as compared to transgenic mouse models used previously to assess lysosomal modulators – the mouse models focus only on one particular aspect of the disease. Two different tests were performed, the Spontaneous Alternation Behavior (SAB) Experiment and the Passive Avoidance Experiment, to demonstrate how age and the administration of PADK affects two different types of rats who are of different ages. The SAB experiment indicates that there was an age effect among the Young Long Evans and the Old Long Evans, substantiating indications that with age comes cognitive decline of the memory in both animals and humans. In the Passive Avoidance test, PADK was proven to work on the Fischer rats. The initial findings indicate that PADK-treated rats display upgraded passive avoidance behavior as compared to aged rats that received drug delivery vehicle only.

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Small molecules synthesized by *Aspergillus nidulans* inhibit Tau aggregation.

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Alzheimer's disease (AD) is the most common form of dementia, affecting millions of people every year. The treatments currently available in the field are limited either to improving the cognitive abilities for a short period of time or are controversial. They do not modify or reverse disease progression. In AD the microtubule-associated protein tau forms insoluble intracellular aggregates in the brain that correlate with dementia and neurodegeneration. Therefore anti-aggregation compounds inhibiting formation of these aggregates are seen as potential therapeutics for AD. In a previous study, 200000 compounds were screened for tau aggregation inhibitor activity and some compounds belonging to the class of anthraquinones were found to be effective [Mandelkow, E. *et.al.* (2005). *Journal of Biological Chemistry* **280** (5): 3628-3635]. *Aspergillus nidulans* has the ability to synthesize many natural products including anthraquinones as secondary metabolites through different biosynthetic pathways. We therefore tested 17 compounds synthesized by *Aspergillus nidulans* for their activity against tau aggregation *in vitro*. 10 compounds out of 17 showed a decrease in tau polymerization activity in a dose-dependent fashion in *in vitro* assays. The compounds affected the length and number of tau filaments formed as well as the total amount of polymerization. The most efficacious compounds were tested in *in vitro* reactions to determine whether they interfere with tau's normal function of stabilizing microtubules (MTs). While there was variability among compounds, the majority did not completely inhibit tau stabilization of MTs. These results will be further evaluated in various cellular systems and animal models. This study demonstrates that *Aspergillus nidulans* metabolites may provide a rich source of potential therapeutic lead compounds that inhibit tau aggregation without compromising its normal cellular functions of stabilizing microtubules. Supported by grant P01 GM 084077 and by the H. L Snyder foundation.

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A Novel Tauopathy Model Utilizing *Dictyostelium discoideum*.

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Alzheimer's disease is a chronic, progressive brain disorder, affecting approximately 35 million people worldwide. Pathologically, Alzheimer's disease is characterized by the accumulation of two types of brain lesions: senile plaques and neurofibrillary tangles. Neurofibrillary tangles are found within neurons and are formed by the aggregation of paired helical filaments, in which the main component is tau, a microtubule associated protein. The mechanisms by which tau aggregates into filaments remains uncertain, but studies have shown that phosphorylation and proteolysis by calpain are major contributors to tau-mediated toxicity. Cleavage of tau by calpain is thought to result in a highly toxic form of tau called the 17kD fragment. In this study, we are using *Dictyostelium discoideum* as a novel tauopathy model to investigate how these post-translational modifications of tau lead to its cellular toxicity. *Dictyostelium* cells were transformed with plasmids to express wild-type (tau^{WT}) and mutant forms of human tau (phosphorylation-incompetent, tau^{AP}; calpain-resistant, tau^{CR}; tau 17kD fragment, tau¹⁷). The constitutive expression of tau^{WT}, tau^{CR}, and tau¹⁷ was sufficiently toxic to induce cell death. However, tau^{CR} expression appeared to be slightly less toxic than expression of tau^{WT} or tau¹⁷. Expression of tau^{AP} was not toxic to *Dictyostelium* wild-type cells. These results suggest that phosphorylation

plays an important role in tau toxicity. Because the expression of tau under a constitutive promoter rapidly induces cell death, we are now using an inducible vector system in which transcription of the tau gene can be reversibly turned on with the addition of doxycycline. Thus far, tau^{WT} has been successfully cloned into the inducible vector. Once all of the inducible vector plasmids are constructed, we will characterize the effect of tau and mutant tau expression on *Dictyostelium* survivability and cell division. Development of *Dictyostelium* as a tauopathy model may provide a unique system for high-throughput screening of new therapeutic molecules for the treatment of Alzheimer's disease.

1857

α -tubulin K40 acetylation is dispensable for brain development but required for epithelium morphogenesis in the cornea.

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Acetylation of lysine 40 of α -tubulin is a highly conserved post-translational modification that predominates in long-lived microtubules such as those found in axons or cilia. Although α -tubulin K40 acetylation is highly correlated to microtubule stability, the causal link between acetylation and stability is still elusive and the functional importance of tubulin acetylation remains unclear. Protozoa lacking α -tubulin K40 acetylation display no overt phenotypes and their cilia appear normal while nematodes lacking α -tubulin K40 acetylation have impaired mechanosensation.

In mammals, α -tubulin K40 acetylation was previously shown to play a role in axonal polarization and in cortical neuronal migration. It is thus expected that α -tubulin K40 acetylation plays a major role in brain development. To assess the contribution of acetylated tubulin to neural development in vivo, we genetically ablated the enzyme responsible for α -tubulin K40 acetylation, α TAT1, in mice. Surprisingly, α TAT1-deficient mice are fertile and viable and do not present any obvious morphological or behavioral defect. Furthermore, although α TAT1-deficient mice appear completely devoid of acetylated tubulin, their overall brain anatomy was indistinguishable from their wild-type littermates, and the number and length of their cilia were unaffected.

A remarkable pattern of acetylated tubulin is found in the adult corneal endothelium, the innermost cellular layer of the cornea. This epithelium is composed of a highly ordered array of hexagonal cells where the acetylated tubulin is restricted to a perinuclear ring of microtubules. Interestingly, in absence α TAT1, cell shape, nuclei position and global tubulin organization are disrupted in the corneal endothelium, suggesting a role for α TAT1 in corneal endothelium morphogenesis.

Finally, to address the long-standing question of whether α TAT1 confers stability to microtubules, we studied microtubule stability in α TAT1-deficient embryonic fibroblasts. Upon nocodazole treatment, fewer resistant microtubules were found in absence of α TAT1.

Collectively, these results demonstrate that, as is the case in nematodes and protozoa, α TAT1 is the major α -tubulin K40 acetyltransferase. Surprisingly, and despite the fact that a large proportion of α -tubulin is acetylated at K40 in the brain (27% by our measurements), α TAT1 is dispensable for mammalian neural development. However, our work suggests that α TAT1 participates in the establishment or the maintenance of epithelial morphology in vivo through its role in stabilizing microtubules.

1858

Klp59C and fidgetin: two microtubule disassembly proteins that help clear damaged regions of neurons.J. Tao¹, M. Rolls¹; ¹Penn State, University Park, PA

A neuron extends one axon and several dendrites far from its cell body, leaving these extremely thin processes subject to various insults, including acute injury and neurodegenerative diseases. Both axons and dendrites are able to initiate a rapid intrinsic program to clear the severely damaged part in a short period of time. Microtubule (MT) fragmentation has been observed in both axon and dendrite degeneration. However, little is known about the intracellular mediators responsible for the physical degradation of the MTs in the degenerating axons or dendrites. In this study, we used a pulsed UV laser to induce a small-scale injury on the axon or dendrite of the ddaC neuron in the *Drosophila* larva. Through a genetic screen of MT depolymerizing or severing proteins, we found that decreasing Klp59C protein level by RNAi or a genetic deficiency delayed clearance of the distal part of injured axons or dendrites by 3 to 6 hours. To test whether reduction of Klp59C was likely to delay clearance through alterations in microtubules we tracked EB1-GFP comets after axon and dendrite injury. Comets were still observed at 1.5h or 4.5h post severing in transected Klp59C RNAi axons and dendrites, respectively, times at which MT dynamics are no longer seen in control neurons. This result suggests that Klp59C is activated shortly after axon or dendrite injury to disassemble MTs. In contrast to the general role of Klp59C in all types of neuron degeneration, we found that fidgetin is specifically involved in injury-induced dendrite degeneration. In fidgetin RNAi and mutant neurons, the onset of beading of injured dendrites was significantly delayed compared to controls. The timing of axonal clearance after injury was not, however, altered in these genetic backgrounds indicating that the role of fidgetin is more specific than that of Klp59C. We conclude that Klp59C acts as general microtubule destabilizer after injury of axons or dendrites whereas fidgetin acts specifically in dendrite degeneration. Moreover, the identification of these two factors supports the idea that microtubules are actively disassembled during neuronal degeneration, and that this disassembly is required for normal timing of axon or dendrite clearance.

1859

What makes the Kinesin like protein (KLP) 11/11 chimeric motor nonprocessive?S. Koesem¹, Z. Oekten¹; ¹TUM, 85748 Garching, Germany

Homodimeric kinesin motors power intracellular transport processes in most eukaryotic cells. Most kinesins involved in long-range transport are processive and can take multiple steps without dissociating from their microtubule track. For homodimeric kinesins, this is achieved by coordinating the ATPase activities of the catalytic head domains to ensure the attachment of at least one motor head to its track during the stepping cycle. The kinesin-2 class is unique among all double-headed kinesin motors because it combines two distinct polypeptide chains to function as a transport motor. Recently, by means of *in vitro* assays we could show that the kinesin-2 heterodimer KLP11/KLP20 of the *C. elegans* sensory cilium works in a processive manner. Intriguingly, processivity in the KLP11/KLP20 heterodimer is brought about by a combination of the nonprocessive subunit KLP11 with the processive KLP20 subunit, respectively. Consequently, chimeric constructs KLP20/20 and KLP20/11 (swapped head positions) were processive, whereas KLP11/11 resulted in a nonprocessive motor. To obtain a better understanding of why the KLP11/11 combination results in a nonprocessive motor, we have designed various truncated constructs, lacking the native tail domains to eliminate a putative inhibitory interference of the native tail domains in the motor's activity. Furthermore, we

introduced flexible residues, namely two, four and eight Glycine and Serine repeats between the head domains, with the aim to probe for possible steric hindrance which may prevent the KPL11/11 motor from taking consecutive steps. The motor heads were artificially zipped together by a stretch of the leucine zipper motif, GCN4, which has a strong propensity to form a coiled-coil. To follow and analyze those constructs by single molecule TIRF microscopy (under zero load conditions), all constructs were C-terminally tagged with a GFP. Interestingly, we observed no processive movement with the KLP11/11 motor domains when zipped together with the GCN4. However, processivity was reconstituted with the constructs containing the flexible head extensions. Taken together, the here presented findings suggest that relieving the steric hindrance indeed is sufficient to turn the KLP11/11 motor combination processive.

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PKC and the Cytoskeletal Scaffold.

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Protein Kinase C (PKC) is a common enzyme found in mammalian cells and is known to be associated with the regulation of many cellular activities such as secretion, proliferation and gene expression. Recent research has shown that PKC provides regulation for the stability of cytoskeletal elements such as microtubules to influence the establishment of polarity, growth cone formation, MTOC orientation and structural support for vesicle transport and other cellular activities. This introduces a new paradigm of thinking that there may be two levels of regulation which would include not only the direct signal transduction mechanism but also a system of scaffold regulation that is independent of the signaling cascade. Within this concept, inhibition of a scaffold mechanism would result in the mistaken interpretation that the signaling mechanism was being directly affected. In this study we are pursuing the theory that PKC isotypes are key signaling factors that are required to maintain the cellular scaffold system. FRET studies have identified close molecular proximity between PKC and the structural cytoskeletal elements such as alpha and gamma tubulin in both the cytoplasmic microtubule arrays and as well as associated with the mitotic spindle. Using SH-SY5Y neuroblastoma cells and various biochemical approaches we identified that disruption of microtubule polymerization results in an increase in various PKC isotype concentrations within the cell and upon restoration of the scaffold in the presence of a PKC zeta pseudosubstrate inhibitor, partial recovery occurred but there was also an increase in the concentration of other PKC isotypes. No recovery was seen when all isotypes of PKC were inhibited which suggests that there is a priority of PKC expression where some isotypes such as PKC zeta are predominate but is supplemented by other isotypes in changing cellular conditions. Furthermore, studies of vesicle and mitochondrial transport that requires a scaffold system showed the same disruption in the event of interrupted microtubule polymerization as compared to the same affect induced by PKC inhibition. These results support the theory that PKC has a role in maintaining a cytoskeletal scaffold system in the cell such that it helps maintain the spatial and temporal positioning of signaling elements but its function is separate from the signal transduction cascade.

1861

Purification and biochemical analysis of recombinant type I β -tubulin.

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A large number of biochemical studies on tubulin have been performed using authentic tubulin proteins purified from mammalian brain or cultured cells. However, since these tubulins are mixtures of several tubulin isoforms, there are limitations in analyzing isoform specific properties and mutations. To address this bottleneck, we have developed a new method for purification of recombinant type I β -tubulin as a functional heterodimer with endogenous α -tubulin from HeLa cells. BioEaseTM (Invitrogen) tagged type I β -tubulin was stably expressed in HeLa cells using an episomal vector system. Then, we purified functional tubulin heterodimers by a cycle of polymerization/depolymerization followed by affinity chromatography. The resulting tubulin was >95 % pure and competent for microtubule assembly in vitro. In addition, drug-binding analysis by SPR (Surface Plasmon Resonance) showed that our recombinant β -tubulin bound to a tubulin-binder chemical probe (biotinylated eribulin) in a dose-dependent manner. In conclusion, recombinant β -tubulin molecules purified by our method are useful for cell-free biochemical studies.

1862

The role of tubulin acetylation in the maintenance of epithelial cell polarity.

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The post-translational modification of tubulin subunits in microtubules is thought to provide directional cues to molecular motors so that cargoes may be efficiently delivered to specific sub-cellular domains. The precise delivery of cargoes is particularly important for the establishment and maintenance of epithelial cell polarity as different sets of proteins are needed in the apical and basal domains. Previously, we have shown that two tubulin post-translational modifications, acetylation and detyrosination, define functionally different populations of microtubules in epithelial cells. We also showed that there is a switch in the predominant form of tubulin modification from detyrosination to acetylation as cells become polarized, and that the two populations of modified microtubules are localized to distinct regions of MDCK cells at each stage of polarization. If these modified microtubules do provide the directional cues for polarized cargo traffic, then the establishment and maintenance of cell polarity may depend on this modified tubulin network. Here we have focused on the role of tubulin acetylation. Tubulin is acetylated by cellular tubulin acetyltransferases, including MEC-17, and deacetylated by tubulin deacetylases, including HDAC6. We have hyperacetylated the microtubule network by inhibition or knockdown of HDAC6 and hypoacetylated the microtubule network by overexpression of HDAC6 or knockdown of MEC-17. We then examined the trafficking of an apical cargo, the p75 neurotrophin receptor, as well as the localization of other apical markers such as ZO-1, E-cadherin, and par6 in order to determine whether the establishment and/or maintenance of epithelial cell polarity depends upon the proper balance of tubulin acetylation and deacetylation.

1863

Changes in the post-translational modification of tubulin in response to ATP- depletion.

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Prior research has shown that ATP depletion in many cell types causes the formation of microtubules (MTs) which are more resistant to microtubule depolymerizing agents and more heavily detyrosinated. We investigated the relationships between ATP depletion, microtubule stability, and tubulin post-translational modifications in MDCK cells during the process of polarization. ATP depletion was achieved by treatment with 20 mM sodium azide and 10 mM 2-deoxyglucose; stable microtubules were identified by their resistance to 10 μ M nocodazole. We found that upon ATP depletion, there was an increase in stable MTs in both 2D-polarized (subconfluent monolayers) and 3D-polarized MDCK cells, and an increase in both tubulin detyrosination and acetylation. The majority of the stable MTs showed acetylation and/or detyrosination. This was surprising in part because we had previously shown that 3D-polarized cells have little detyrosinated tubulin. The pattern of acetylation was altered as well. Instead of small, discontinuous patches of acetylated tubulin, many MTs were acetylated along their entire lengths. Immunoblotting of cell lysates showed that in both 2D- and 3D-polarized cells, detyrosinated tubulin increased 4-5 fold. Acetylation increased substantially, as well. A sudden drop in ATP is one of several stressors known to activate adenosine-monophosphate kinase (AMPK), an energy-sensing kinase thought to be one of the master regulators of metabolism. Our preliminary experiments show that inhibition of AMPK partially blocks the increase in detyrosinated and acetylated microtubules induced by ATP depletion. In summary, ATP depletion leads to a global increase in both the acetylation and detyrosination of MTs, which overrides the normal pattern of these modifications that is established as MDCK cells acquired a polarized morphology. And preliminary results suggest that activation of the AMPK pathway can modulate tubulin acetylation and detyrosination. Supported by NSF Advance Grant 0820032 (BD) & American Cancer Society RSG-10-245-01-CSM. (LAL).

1864

Redundancy ensures microtubule plus-end targeting of MCAK in meiosis.

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Female meiotic divisions segregate chromosomes using mechanisms similar to mitosis despite a much greater volume of cytosol. Because of this scaling challenge, special adaptations may aid the proper formation of meiotic spindles. We have tested the importance of the direct interaction between microtubule end-binding (EB) proteins and MCAK, a tip-tracking, microtubule-depolymerizing Kinesin-13, in the context of *Xenopus laevis* egg extracts. We mutated the EB-binding motif SxIP (SKIP in MCAK) to SKNN, and purified GFP-tagged wild-type and mutant proteins from SF9 insect cells. The SKNN mutation did not impair MCAK's catalytic activity. As expected, the mutation did disrupt binding of EB1 to MCAK as evaluated by in vitro pulldown. However, when added to egg extracts depleted of endogenous MCAK, MCAK-SKNN did not show full loss of tip-tracking. Although lattice binding of MCAK-SKNN was highly enhanced relative to its wild-type counterpart, a small amount continued to co-localize with EB1 and to tip-track. MCAK-SKNN was competent to rescue depletion of endogenous MCAK as evaluated by its ability to form bipolar spindles and the steady-state length of those spindles. Our results suggest that, in contrast to published results for mitotic tissue culture systems, MCAK can target plus-ends effectively in *Xenopus* egg extracts despite loss of direct

EB binding. We are working to determine the mechanism by which MCAK targets plus-ends without direct EB binding.

1865

Novel alleles of genes required for organelle distribution and motility in *Aspergillus nidulans*: a whole-genome sequencing approach.

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Many organelles are transported long distances along microtubules in most eukaryotic organisms. To identify novel factors required for microtubule-based organelle distribution and motility, we performed a screen in the filamentous fungus, *Aspergillus nidulans*. As a model system, *A. nidulans* combines some of the advantages of yeast molecular genetics with the long-range microtubule-based transport of metazoans. Using whole-genome sequencing, we found a number of single nucleotide polymorphisms (SNPs) that resulted in misdistribution of peroxisomes, endosomes, or nuclei. Some of these SNPs were novel alleles of cytoplasmic dynein, dynactin, Lis1, and kinesin-1. Here, we characterize the transport defects in these novel mutants and report methods for using whole genome sequencing as a tool in mutagenesis studies in *A. nidulans*.

Centrosomes II

1866

CEP215 interaction with pericentrin is critical for centrosome maturation during mitosis.

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The microtubule-organizing activity of the centrosome oscillates during the cell cycle, reaching its highest level at mitosis. At the onset of mitosis, the centrosome undergoes maturation, which is characterized by a drastic expansion of the pericentriolar matrix and a robust increase in microtubule-organizing activity. We previously revealed that PLK1 phosphorylation of pericentrin is a critical step for centrosome maturation. A number of the pericentriolar matrix proteins such as CEP192 and GCP-WD are recruited to the centrosome once pericentrin is phosphorylated by PLK1. On the other hand, CEP215 is also required for PCM accumulation for centrosome maturation. Depletion of CEP215 results in reduction of the PCM proteins at spindle poles. Among the PCM proteins, pericentrin was significantly reduced at spindle pole in CEP215-depleted cells. In fact, CEP215 physically interacts with pericentrin. Based on the results, we propose that CEP215 interaction with pericentrin is critical for centrosome maturation during mitosis.

1867

The centrosome regulates the Rab11-dependent recycling endosome pathway at appendages of the mother centriole.

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The recycling endosome localizes to a pericentrosomal area through microtubule-dependent transport. The observation that the mother centriole appendage protein, centriolin, interacts with Sec15, an effector of the recycling endosome component, Rab11-GTPase, suggests an interaction between centrosomes and endosomes. For the first time we show that the recycling

endosome associates with the centrosome, more precisely, with mother (older) centriole appendages. The mother centriole appendage proteins, centriolin and cenexin/ODF2, regulate the association of the endosome components Rab11, the Rab11 GTP-activating protein Evi5, and the exocyst at the mother centriole. We have developed an in vitro method for reconstituting endosome protein complexes onto isolated centrosomes. This in vitro method for reconstituting proteins has a number of applications from understanding the hierarchy of protein association at the centrosome to understanding microtubule nucleation. Our experiments illustrate that purified GTP-Rab11 but not GDP-Rab11 binds to mother centriole appendages in the absence of membranes. Furthermore, centriolin depletion displaces the centrosomal Rab11 GAP, Evi5, and increases mother-centriole-associated Rab11; depletion of Evi5 has the same effect on Rab11 localization. This suggests that centriolin localizes Evi5 to centriolar appendages to turn off centrosomal Rab11 activity. Depletion of centriolin or cenexin disrupts recycling endosome organization and function suggesting that mother centriole proteins can regulate Rab11 localization and activity at the mother centriole through Evi5 and the exocyst.

1868

Structural analysis of Polo-Like Kinase-4's Cryptic Polo Box reveals a novel pair of tandem Polo Box Domains required for centriole assembly.

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Centrioles are barrel-shaped, microtubule-based structures responsible for nucleating polarized microtubule arrays. Importantly, the number of centrioles dictates the capabilities of these organelles: two centrioles form a centrosome, and two centrosomes are needed for formation of the bipolar mitotic spindle. Centriole biogenesis via duplication is cell cycle-controlled to prevent cells from developing multipolar microtubule spindles, a situation that promotes chromosomal instability. A key "licenser" of centriole duplication is Polo-Like Kinase-4 (Plk4), a polo kinase family member that localizes to the site of nascent centrioles in M/G1. Plk4's activity is tightly controlled through trans-autophosphorylation that primes SCF-mediated ubiquitination and degradation. Other polo kinase family members (Plks1-3) contain two polo box domains (PBDs), which are used to regulate kinase activity and localization. Conversely, Plk4 is thought to contain only one PBD in addition to a unique, conserved central region called the "Cryptic Polo Box," or CPB. Plk4's CPB was found to bind the centrosomal scaffolding protein Asterless, though the field of centriole biology currently lacks a mechanistic understanding of how Plk4 employs the CPB to achieve centriole duplication licensing. In this study, we employed x-ray crystallography to determine the structure of Plk4's CPB region to a resolution of 2.3 Å. Surprisingly, the structure reveals two tandem, homodimerized polo boxes, henceforth called PB1-PB2, that form a winged structure unique among the known PBD structures. Thus, Plk4 contains not one, but three PBDs: PB1-PB2 that compose the CPB plus the C-terminal PB, PB3. No other polo kinase family member contains a trimeric PBD array, suggesting that the conserved Plk4 PBDs play unique mechanistic roles in Plk4's function. Our cellular localization studies show that the PB1-PB2 homodimerized cassette is required for robust centriole targeting and Asterless binding. Together, our results represent a paradigm shift in the structural classification of polo kinase members: Plk4 contains a distinctive polo box architecture that facilitates oligomerization (requisite for trans-autophosphorylation) and centriole targeting, determinants that facilitate a single cell cycle centriole duplication licensing step.

1869

Phosphorylation of centrosomal proteins in *Drosophila*.*J. Baumbach¹, P. Conduit¹, J. Raff¹; ¹Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom*

The centrosome is the major microtubule-organizing center of almost all animal cells and it helps to set up the mitotic spindle. It consists of a pair of centrioles surrounded by the pericentriolar material (PCM), which is a protein meshwork harbouring proteins that have the ability to nucleate and organize microtubules. At the beginning of mitosis, the amount of PCM that is organized by the pair of centrioles increases dramatically, which results in an increase in microtubule-organizing activity and therefore promotes efficient mitotic spindle assembly. By comparing the centrosomal and cytoplasmic fractions of centrosomal proteins we show that many centrosomal proteins are phosphorylated when they are at the centrosome as opposed to when they are in the cytoplasm. It is assumed that phosphorylation activates these proteins and is needed for their localization to the centrosome. Using a phosphoproteomic approach, we have identified phosphorylation sites in Centrosomin, Asterless and DSpd-2, which are key proteins of the PCM recruitment pathway in *Drosophila*. Using a phosphospecific antibody we show that a phosphorylation site in Asterless is specifically phosphorylated only in mitosis. We are currently analyzing the biological functions of the phosphosites obtained by testing different phosphomutant constructs in the mutant backgrounds.

1870

Nek2 stabilizes β -catenin through its interaction with known regulatory GSK3 β sites in β -catenin.*B. Mbom¹, K. Siemers¹, W. Nelson¹, A. Barth¹; ¹Stanford University, Stanford, CA*

Beta-catenin is a multifunctional protein with critical roles in cell-cell adhesion, Wnt-signaling and bipolar spindle formation. Whereas the roles of β -catenin in adhesion and Wnt-signaling have been extensively studied, the mechanism(s) by which β -catenin promotes bipolar spindle formation is poorly understood. The centrosomal NIMA-related protein kinase 2 (Nek2) binds and phosphorylates β -catenin and stimulates centrosome separation. Nek2, like β -catenin, promotes bipolar spindle formation. Using in vivo and in vitro approaches, we analyzed the effects of Nek2 on β -catenin stability. Nek2 phosphorylates the same regulatory sites as GSK3 β in the N-terminus of β -catenin as well as additional sites. Nek2 inhibits β -catenin ubiquitylation by preventing binding of the E3 ligase β -TrCP and promotes β -catenin stability independent of its kinase activity. These results indicate that although Nek2 protein is required for β -catenin stability, its kinase activity is not. Taken together, these results allow us to further elucidate the key role of Nek2 and β -catenin in regulating centrosome cohesion during the cell cycle.

1871

The autoregulated instability of Polo-like kinase 4 limits centrosome duplication to once per cell cycle.*A. J. Holland¹, D. Fachinetti¹, Q. Zhu², M. Bauer³, I. Verma², E. Nigg³, D. Cleveland¹; ¹Ludwig Institute For Cancer Research, University of California, San Diego, La Jolla, CA, ²Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, CA, ³Biozentrum, University of Basel, Basel, Switzerland*

Centrioles are microtubule-based structures that contribute to the formation of the centrosome, the cells major microtubule-organizing center. Centriole duplication occurs once per cell cycle and accurate control of centriole number is critical for the maintenance of genomic integrity.

Centriole duplication is controlled by the conserved kinase Polo-like kinase 4 (Plk4) and overexpression of Plk4 promotes centriole overduplication and cell transformation. We have shown that Plk4 regulates its own stability by phosphorylating itself to promote its degradation by the proteasome. Using conditional gene targeting we now demonstrate that this autoregulated instability is required to control the abundance of endogenous Plk4 and limit centriole duplication to once per cell cycle. Preventing Plk4 autoregulation leads to centrosome amplification, activation of p53 and loss of cell proliferation; moreover, suppression of p53 allows the proliferation of cells carrying amplified centrosomes. We conclude that Plk4 autoregulation forms an essential pathway to guard against genome instability by limiting centrosome duplication to once per cell cycle.

1872

Polo activity controls centrosome maturation in *Drosophila*.

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Centrosomes in mitosis mature through the addition of pericentriolar material (PCM) the site of MT-nucleation and anchoring. Mitotic centrosomes control the bipolar spindle formation by organising the MT-cytoskeleton and by the creation of spindle and astral MTs. Astral MTs contact the cell cortex and position the mitotic spindle according internal and external cues. How PCM recruitment is initiated and modulated in mitosis is not well understood. Our findings show that Polo kinase phosphorylation of the major *Drosophila* PCM protein centrosomin (Cnn) is key for PCM nucleation on top of centrioles when cells enter mitosis. Constant Polo phosphorylation is needed to maintain Cnn at the centrosome. Cnn phosphorylation only takes place on the centriole scaffold. Cnn is phosphorylated at serine 567. Mutation abolishing phosphorylation results in a strong reduction of the PCM. In contrast, mutation of serine 567 mimicking constitutive phosphorylation is very toxic and induces PCM formation during all cell cycle stages. Ectopic expression of active polo to interphase centriole induces PCM recruitment and aster formation. Thus, phosphorylation of Cnn by Polo in mitosis is both needed to initiate and maintain PCM in *Drosophila*.

1873

Mechanistic Insight and Regulatory Control of the Microtubule Organizing Center γ -tubulin small complex (γ -TuSC).

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Mitotic spindle formation into a bipolar structure is a critical control point for chromosome segregation. Distinct protein complexes at microtubule minus ends at the spindle pole form a microtubule organizing center (MTOC) complex. The γ -tubulin small complex (γ -TuSC) forms the direct link to growing microtubules and is embedded in a larger macromolecular structure, the γ -tubulin ring complex (γ -TuRC). We combine recent structural insights of γ -TuSC and GCP4 with our detailed genetic analysis, site-directed mutagenesis, cross-species functional studies and biochemical purification and nucleation assays to provide novel insights into MTOC nucleation and regulation. Included in our analysis are mechanistic details on γ -TuSC protein functional conservation and divergence as well as γ -TuSC regulation by a subset of Kinesin-14 kinesin-like protein (Klp) members. We have identified and refined key domain elements in the fission yeast Kinesin-14 Pkl1 Tail that drives targeting and regulatory interference at poles (TRIP). The TRIP domain is conserved within a subset of Kinesin-14 Klps from yeast to man and distinct from the previously identified microtubule targeting elements found in other Kinesin-14 members such as *Drosophila* Ncd. Our analysis supports application of the recent Kollman-

Agard structure as a general eukaryotic model, but also reveals species-specific protein constraints in the γ -TuSC between yeasts, or yeasts and man. We propose a model and supporting evidence for Kinesin-14 regulation of γ -TuSC during spindle assembly. Our findings have broad application towards a general understanding of cellular MTOC machinery and reiterate the flexibility of Kinesin-14 members to localize to multiple spindle compartments and participate in a diversity of cellular roles. Natural cellular machines more than ever are providing tools and inspiration for biomimicry and for resolution of societal grand challenges. This includes nanomanufacturing at the sub-50nm level. The studies here not only provide biological information that is expected to help expand targets for cancer therapeutics but also advance nanodesign principles. We are utilizing knowledge from cellular systems along with the purified MTOC complex, selected Klps and tubulin assembly dynamics to assemble complex microtubule-based structures that can inform us on trainable self-assembly platforms with regulatable nanonetworks.

1874

CEP120 interacts with CPAP and regulates centriole elongation.

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The centrosome is the primary microtubule-organizing center, which is composed of two orthogonally arranged centrioles surrounded by the pericentriolar proteins. Centriole duplication begins with the formation of a single procentriole next to the pre-existing centriole and requires the assembly of centriolar microtubules. We (Tang et al., Nat. Cell Biol. 2009) and others (Kohlmaier et al., Curr. Biol. 2009; Schmidt et al., Curr Biol. 2009) previously reported that CPAP is involved in the process of procentriole elongation and controls centriole length. Here, we show that CEP120 is a CPAP-interacting protein, which plays a critical role in centriole elongation. Overexpression of CEP120 induces supernumerary centrioles and promotes the formation of abnormally long microtubule-based structures. Confocal immunofluorescence analysis showed that these elongated structures contain stable microtubules and several bona fide centriolar proteins, including STIL, SAS6, centrin, CP110 and CPAP. Further electron microscopy analysis showed that these extra-long structures are similar to that of CPAP-induced elongated centrioles. Intriguingly, we found that CEP120 directly interacts with CPAP, which was demonstrated by co-immunoprecipitation, GST pulldown, and yeast two-hybrid assays. Depletion of CEP120 inhibits CPAP-induced centriole elongation and results in a failure to localize CPAP, but not hSAS6 or STIL, to the centrioles. Similarly, CPAP depletion also blocks CEP120-induced centriole elongation and perturbs CEP120 targeting to the centrioles. Further analysis shows that CEP120 contains a microtubule-binding domain and a self-dimerization domain. Overexpression of either a CEP120-K76A mutant that fails to bind to microtubules or CEP120-E831P that disrupts the CEP120 dimerization activity significantly suppressed the formation of elongated centrioles. Together, our results indicate that CEP120 is a novel positive regulator that interacts with CPAP to promote centriole elongation and both the intrinsic microtubule binding and the dimerization activity of CEP120 are essential for centriole elongation.

1875

Two distinct functions for one kinase: casein kinase 1 δ regulates actin-mediated endocytosis and microtubule dynamics.

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Across diverse eukaryotic organisms, actin assembly facilitates clathrin-mediated endocytosis while microtubule dynamics underlie mitosis. We found that the budding yeast casein kinase 1 δ , Hrr25, associates with and regulates both systems. Hrr25 is recruited to endocytic patches early in the pathway by the C-terminus of the yeast Eps15-like protein, Ede1, where it phosphorylates Ede1 and several other endocytic proteins. An analog-sensitive allele was created and used to show that Hrr25 kinase activity is required for the kinase's recruitment to endocytic sites and to control endocytic patch turnover. Furthermore, our results in mammalian cells demonstrate likely conservation of casein kinase 1 δ functions in higher organisms. In addition to co-purifying with Ede1, Hrr25 co-purifies with several spindle pole body proteins. In contrast to recruitment to endocytic patches, recruitment of Hrr25 to spindle pole bodies was not dependent on Ede1 or on Hrr25 kinase activity. Hrr25 phosphorylates several spindle pole body proteins and its activity plays a critical role in regulation of microtubule assembly nucleated at spindle pole bodies in vivo. Ongoing studies are aimed at elucidating the detailed mechanisms and specific in vivo phosphorylation targets underlying Hrr25's regulatory functions in endocytosis and mitosis.

1876

Understanding the function of centrosomes during asymmetric divisions in the interfollicular epidermis.

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Centrosomes, the major microtubule organizing centers of cells, are crucial for the formation of a bipolar spindle and for ciliogenesis in many organisms. Recent work emerging from invertebrate studies has potentially uncovered a novel role for centrosomes in cell fate determination. However, the mechanism by which centrosomes contribute to cell fate remains a mystery, although the regulation of asymmetric divisions is strongly implicated. Additionally, the presence of extra centrosomes has long been hypothesized to contribute to tumorigenesis. However, in light of research suggesting that tumorigenesis in the context of centrosomal dysfunction does not result from genetic instability, and that flies with extra centrosomes have defects in asymmetric divisions of neuroblasts, it remains untested whether the presence of extra centrosomes alters asymmetric divisions in the mammalian context. Thus, we are exploring the role of centrosomes in the development of the murine epidermis, a tissue in which asymmetric divisions are demonstrated to promote stratification and differentiation. By utilizing a mouse model which conditionally over-expresses Plk4, a kinase required for initiation of centrosome duplication, we are inducing the formation of extra centrosomes specifically in the epidermis. Our initial findings include increased cell death and disruption of keratin expression within the tissue, which suggest defects in proliferation and homeostasis. To further address the source of these errors, we are determining whether the presence of extra centrosomes affects asymmetric divisions, proliferation, and differentiation in ongoing experiments.

1877

Asymmetry in centrosome activity is generated by blocking centrosome maturation.D. A. Lerit¹, N. M. Rusan¹; ¹Cell Biology and Physiology Center, National Institutes of Health, Bethesda, MD

As the microtubule-organizing centers (MTOCs) of most eukaryotic cells, centrosomes polarize cells and model tissues. Comprising an inner core of two centrioles surrounded by a cloud of pericentriolar material (PCM), centrosomes break the symmetry of cells by generating specialized cellular structures and localized enrichments of microtubules. Studies of centrosome activity within stem cells suggest asymmetries inherent to the centrosomes themselves may direct cellular symmetry-breaking events. For example, localization of a single centrosome defines the apical-basal polarity axis of neuroblasts (NBs), stem cells of the *Drosophila* larval brain. In NBs, the duplicated interphase centrosomes display a striking enrichment of centrosome protein content and MTOC activity at one dominant centrosome that remains tethered to the apical cortex. In contrast, the second centrosome fails to mature until just before mitotic onset, when the second MTOC appears at a distant basal site. How asymmetries in centrosome protein localization and activity are regulated is unknown. Using mutant analysis, we have identified a novel mechanism required to establish centrosome asymmetry. Our data indicate that *Drosophila* Pericentrin-like protein (D-PLP) functions to establish asymmetry in centrosome activity during interphase and is required for centrosome segregation before mitotic onset. Quantitative analysis indicates D-PLP affects the localization of several key PCM proteins known to regulate centrosome maturation. We show D-PLP blocks recruitment of the master regulator of centrosome maturation, Polo kinase, to the second (basal-fated) centrosome of interphase NBs. Likewise, loss of D-PLP results in ectopic enrichment of γ -Tubulin to the second centrosome, producing two equally active interphase MTOCs. Such centrosomes fail to segregate, resulting in two proximal MTOCs during nuclear envelope breakdown. These data suggest NBs selectively block the maturation of the basal-fated centrosome until mitosis to permit proper centrosome segregation. Our model demonstrates, for the first time, that the mechanism of centrosome maturation includes the removal of negative regulators of PCM recruitment.

1878

Measuring centriole length: size matters!S. Montenegro Gouveia^{1,2}, M. Bettencourt Dias^{1,2}; ¹Gulbenkian Institute of Science, Oeiras, Portugal, ²Marine Biology Laboratory, Woods Hole, MA

The centrosome is the primary microtubule-organizing centre (MTOC) in animal cells regulating cell motility, adhesion and polarity in interphase and organizing spindle poles during mitosis. Despite their essential functions, centriole biogenesis is still poorly understood. Centriole biogenesis is first seen during S phase of the cell cycle by the appearance of procentrioles that elongate as the cell cycle progresses to reach nearly full length in mitosis. Most mature centrioles in a healthy organism have similar length, yet very little is known on how centriole length control is exerted.

Our goal is to investigate centriole biogenesis, in particular centriole elongation, its kinetics and regulation. We ask a fundamental question: How is mature centriole length achieved? To tackle this question we are reconstituting centriole elongation in vitro in the context of xenopus egg extracts. We are using procentrioles isolated from mammalian GFP-tagged centrin cell line and monitor their elongation by TIRF live imaging. One of the great advantages of the xenopus in vitro system is the fact that we can manipulate easily its protein content by antibody depletion or in vitro mRNA transcription. This system allows us to express in the extract our fluorescent proteins of interest, in a wild type or depletion background, in order to better visualize and

understand centriole elongation. In addition, we are taking advantage of PLK4 centriole amplification in *Xenopus* extracts to induce MTOC assembly. Together, these approaches will allow us to study how different centriolar players interact with each other and microtubules to promote and control centriole length.

1879

MEI-1/katanin is required for epithelial polarization.

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The centrosome is the major microtubule organizing center (MTOC) in dividing cells and in some post-mitotic, differentiated cells. In other cell types, however, MTOC function is reassigned from the centrosome to non-centrosomal sites. We are using *C. elegans* intestinal cells to analyze how MTOC function is reassigned to the apical surface of epithelial cells. After the terminal cell divisions, the centrosomes and nuclei of intestinal cells move near the future apical membranes, and the post-mitotic centrosomes lose all, or most, of their associated microtubules. We show that microtubule-nucleating proteins such as γ -tubulin and CeGrip-1 that are centrosome components in dividing cells become localized to the apical membrane, which becomes highly enriched in microtubules. Our results suggest that centrosomes are critical to specify the apical membrane as the new MTOC. First, γ -tubulin appears to redistribute directly from the migrating centrosome onto the lateral, then apical membrane. Second, γ -tubulin fails to accumulate apically in wild-type cells following laser ablation of the centrosome. These data suggest that the reassignment of MTOC function from centrosomes to the apical membrane is associated with a physical hand-off of nucleators of microtubule assembly. We show that the microtubule severing protein MEI-1/katanin is required for polarization of the epithelial cells; *mei-1* mutants have defects in apical distribution of γ -tubulin and the polarity protein PAR-3. These results suggest that the severing or disassembly of microtubules from the centrosome may be required for the redistribution of microtubule nucleators to the apical surface.

1880

Regulation of microtubule nucleation by GIT-PIX signaling cassette interacting with γ -tubulin.

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γ -Tubulin is an evolutionarily highly conserved protein responsible for microtubule nucleation from organizing centers. While essential γ -tubulin-interacting proteins forming the γ -tubulin ring complex responsible for MT nucleation have been identified, the regulatory mechanisms of microtubule nucleation remain largely unknown. Using co-immunoprecipitation experiments followed by mass spectrometry we have found in human osteosarcoma cells U2Os association of γ -tubulin with proteins forming the so-called GIT-PIX signaling cassette. The interactions were confirmed by co-immunoprecipitation experiments with antibodies to GIT and PIX and by the pull-down experiments with GST-tagged proteins. To elucidate the role of the GIT-PIX signaling cassette in microtubule nucleation, we depleted proteins by siRNA and quantified microtubule re-growth in nocodazole washout experiments. For this purpose we have developed automated image analysis software to measure microtubule densities close to the centrosome at various time intervals. Depletion of both proteins led to inhibition of microtubule nucleation from centrosomes. Collectively, our results suggest that the GIT-PIX signaling cassette could regulate microtubule nucleation.

1881

Centrosome homeostasis is controlled by ubiquitylation and deubiquitylation cyclesJ. Li¹, V. D'Angiolella¹, E. Seeley², T. Kobayashi¹, S. Kim¹, M. Pagano^{1,3}, B. Dynlacht¹;¹Cancer Institute, NYU School of Medicine, New York, NY, ²Department of Pathology, University of California, San Francisco, San Francisco, CA, ³Howard Hughes Medical Institute, New York, NY

Centrosome duplication is a pivotal process required for cell division. In order to avoid genome instability, the duplication of centrosomes must be restricted to once per cell cycle. Different mechanisms that control centrosome duplication impinge on the regulation of CP110, an essential component of the centriole duplication process. Excessive CP110 drives centrosome over-duplication while loss of CP110 inhibits centrosome amplification. CP110 levels are controlled through ubiquitin mediated proteolysis by the SCF(cyclin F) during G2 and M phase of the cell cycle. From published mass spectrometry data, we have identified a de-ubiquitylating enzyme (DUB) as a CP110-interacting protein. We report a new mechanism to regulate centrosome duplication that entails DUB-dependent regulation of CP110 levels. Ubiquitylation and deubiquitylation cycles control CP110 stability and centrosome duplication. We further observe that the levels of this DUB and CP110 are markedly elevated in pancreatic ductal adenocarcinoma (PDAC), suggesting a rationale for inhibiting tumors associated with centrosome amplification. These studies have identified one of the first centriolar de-ubiquitinating enzymes whose expression regulates centrosome homeostasis by countering cyclin F-mediated destruction of a key centrosomal substrate.

1882

Small brain and dwarfism: physiological consequences of centrosome abnormalities.J-H. Sir¹, M. Putz¹, G. Woods², F. Gergely¹; ¹Department of Oncology, University of Cambridge, CRUK Cambridge Research Institute, Cambridge, United Kingdom, ²Cambridge Institute for Medical Research, Cambridge, United Kingdom

Mutations in numerous genes coding for centrosomal proteins cause primary recessive microcephaly (MCPH), a condition that results in reduced brain size and low IQ. The molecular mechanism underlying MCPH remains elusive, but the current consensus is that the disease originates from a failure in stem cell expansion during foetal brain development. Like DNA, the centrosome duplicates once during a normal cell cycle. We have previously described a homozygous MCPH-causing mutation in the human CEP63 gene and demonstrated a requirement for CEP63 in centrosome duplication (1). CEP63 interacts with CEP152, a conserved centrosome duplication factor that is also mutated in MCPH (2,3,4). Both CEP63 knockout vertebrate cells and CEP63 patient-derived cells fail to accumulate CEP152 at centrosomes, thereby impairing centrosome duplication. Thus, the CEP152-CEP63 MCPH protein complex is essential for controlling centrosome numbers, a role that seems especially important for human cerebral cortex growth. Intriguingly, however CEP152 mutations also give rise to Seckel syndrome (5), a disease characterised by short stature and bone malformations in addition to microcephaly. Comparing MCPH- and Seckel-associated missense mutations in CEP152, we find that MCPH mutations weakly whereas Seckel mutations drastically impair the capacity of CEP152 to drive centrosome duplication. The degree of impairment thus seems to reflect the severity of the genetic condition with MCPH being less and Seckel more severe. It is therefore feasible that MCPH and Seckel syndrome are caused by the same cellular deficiency, and instead of being distinct diseases, represent different endpoints on a disease continuum. With the aim of identifying a common underlying mechanism, we will present our most recent data on the roles of MCPH and Seckel proteins, and intact centrosomes, in DNA damage signalling and genome stability.

- (1) Sir et al., *Nat Genet* 2011
- (2) Cizmecioglu et al., *J Cell Biol* 2010
- (3) Hatch et al., *J Cell Biol* 2010
- (4) Guernsey et al., *Am J Hum Genet* 2010
- (5) Kalay et al., *Nat Genet* 2011

1883

Neurological impairment in *centrosomin* mutant flies.

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Autosomal recessive primary microcephaly (MCPH) is a neurodevelopmental disease that results in a small brain size due to loss of neural progenitors. The genetic cause of MCPH is heterogeneous and due to mutations in centrosomal genes, one of which is *CDK5RAP2*. In *Drosophila*, the *CDK5RAP2* ortholog *centrosomin* (*cnn*) does not exhibit a small brain phenotype. However, we show that *cnn* mutants, and mutants for other MCPH genes, have neurological defects including severe locomotor performance deficiencies. Actogram data show that *cnn* mutants are less active than isogenic wild type flies, due in large part to more frequent periods of complete inactivity. Our data indicate that the locomotor deficiency in *cnn* mutants is due to post-mitotic activities of the gene. In order to understand the mitotic and post-mitotic roles of *cnn*, we implemented a combination of genetic and proteomic approaches. Using 2D-Difference Gel Electrophoresis (2D-DIGE), we revealed about 60 protein spots that are differentially expressed or post-translationally modified between wild type and *cnn* mutant tissues. Our genetic screen identified 23 mutations that are viable but synthetic lethal with *cnn*. One of these mutants (#125) exhibits male infertility and a visible locomotor defect independent of *cnn*. We are currently mapping the #125 gene. With these approaches, we will investigate the mitotic and post-mitotic roles of *cnn* and understand the physiological basis of MCPH.

1884

An alternative splice product of CNN localizes to mitochondria and converts mitochondria into MTOCs.

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Mitochondria are energy centers in cells. In *Drosophila*, they also play a role in sperm tail elongation by providing a structural platform for microtubule (MT) organization to support the elongating tail. Little is known, however, about the molecular mechanism behind this special function of mitochondria. Centrosomin (CNN) is a core centrosomal protein whose gene expresses several variants. Its centrosomal forms (CNNC) are essential for functional centrosomes, which are the major Microtubule Organizing Centers (MTOCs) in animal cells. We recently found that the other alternative splicing forms of CNN (CNNT) were exclusively expressed in testes. And unlike CNNC, which localizes at centrosomes, CNNT localizes to spermatid mitochondria (nebenkerns). Ectopic expression of CNNT recruited the essential MT nucleating factor γ -tubulin to mitochondria, indicating an MTOC function for CNNT at mitochondria. In addition, expression of CNNT fragments in cell culture shows that the N terminus (shared by CNNC and CNNT) of CNNT functions in γ -tubulin recruitment, whereas the C terminal region (unique for CNNT) of CNNT is responsible for mitochondrial targeting. Ubiquitous expression of CNNT during development results in flies that lose bristles and have wing development defects that are indicative of cell fate determination defects. Ultrastructural examination by EM and live imaging of MTs will define the structure and dynamics of these

MTOCs organized by CNNT. We propose that CNNT promotes assembly of unique MTOCs on the surface of mitochondria where, in elongating sperm cells, it facilitates sperm tail growth.

Cilia and Flagella III

1885

Primary cilia regulate the function of extraciliary TRPV4 in epithelial cells.

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Primary cilia are eukaryotic cell organelle that function as biosensors to sense diverse chemical and mechanical stimuli from the extracellular environment. However, detailed molecular mechanisms underlying cilia-mediated sensing as well as its diversity among different cell types have not been fully understood. The transient receptor potential channel vanilloid superfamily type 4 (TRPV4) forms a divalent cation channel and localizes to cilia of some mammalian cell types, specifying the ciliary functions. TRPV4 is highly expressed in choroid plexus epithelial cells (CPECs), which we demonstrated have multiple primary cilia, but its ciliary localization and functional significance in this cell type have not been reported. In the present study, we investigated the functional relationship between TRPV4 and primary cilia in CPECs. Immunocytochemistry of CPECs demonstrated that TRPV4 was not localized exclusively to the primary cilia but broadly on the apical membrane in CPECs. Treatment of CPEC monolayer with TRPV4-specific agonist, GSK1016790A, augmented the basolateral-to-apical fluid transcytosis, suggesting its regulatory role in the cerebrospinal fluid production. Interestingly, upon deciliation using chloral hydrate, the TRPV4 protein levels as well as cellular responsiveness to GSK1016790A were significantly decreased. These functional relationships between TRPV4 and cilia were reproducible in IMCD3 cells, which also express extraciliary TRPV4: In IMCD3 cells with stable *Ift88* shRNA expression, impaired ciliogenesis was associated with decreases in both TRPV4 protein levels and cellular responsiveness to GSK1016790A. Immunocytochemistry of this cell line also suggested that the plasma membrane targeting of TRPV4 might be perturbed upon inhibition of ciliogenesis, while the global apical membrane protein composition remained unchanged. Overall, the present study demonstrated that loss of primary cilia could disturb the function of extraciliary TRPV4. These findings may provide insights toward better understanding of ciliopathy. In addition, our data also suggest that ciliary transport is dependent not only on the ciliary targeting signals of cargo proteins but also on the cell types, probably due to variations in the structural components of recipient cilia, that would determine which proteins to be retained in cilia.

1886

Asymmetric inheritance of primary ciliary membrane in dividing neural progenitors.

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The primary cilium is a microtubule-based protrusion of the cell surface that is involved in mediating extracellular signals, such as Sonic Hedgehog (Shh). The primary cilium is nucleated by the centrosome containing the eldest centriole (mother centriole). Prior to mitosis, the primary cilium is assumed to be disassembled in order for the centrosomes to be free to serve as the poles of the mitotic spindle.

Recent findings show that asymmetric inheritance of the centrosome containing the mother centriole is related to cell fate decisions during mammalian neurogenesis. However, it is unknown whether centrosome-associated structures such as the primary cilium are involved in this asymmetrical regulation of cell fate. Therefore, we set out to investigate the fate of the primary cilium upon cell division of developing neural progenitors and the possible involvement of the primary cilium in subsequent cell fate decisions in the developing mouse neocortex.

We find that in mitotic neural progenitors and cultured cell lines, a membrane structure containing the ciliary small GTPase Arl13b is associated with the centrosome that contains the mother centriole. By surface biotinylation of the apical membrane in explanted mouse telencephalic hemispheres, we show that this structure contains ciliary membrane and is derived from the primary cilium that was present at the cell surface prior to mitosis. Using live imaging, we find that upon completion of mitosis, this centrosome-associated ciliary membrane is asymmetrically inherited by one of the daughter cells. Furthermore, we show that inheritance of the ciliary membrane causes earlier reestablishment of the primary cilium on the cell surface after division. Interestingly, the association of the ciliary membrane with one centrosome in mitotic neural progenitors decreases as neurogenesis proceeds.

We hypothesize that the observed asymmetry in primary cilium reassembly after mitosis might differentially expose daughter cells to extracellular signals, such as Shh. Therefore, we speculate that inheritance of the ciliary membrane affects cell fate decisions during mammalian neurogenesis.

1887

Primary cilia assembly and signaling are required for normal development and repair of corneal tissue.

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Damage and abnormal repair of the corneal layers due to trauma, diseases or aging can compromise corneal transparency and lead to blindness. Thus, understanding the complex signaling network orchestrating morphogenesis during development and repair

later in life of the cornea is crucial to identify specific molecular targets that can help to counteract loss of vision. Interestingly, patients affected by ciliopathies such as Bardet Biedl and Meckel syndromes present several conditions of the anterior eye including microcornea, sclerocornea, abnormal iridocorneal angle, opacity of the cornea, keratoconus, glaucoma, and cataract. We have recently shown that primary cilia are required for normal patterning of the corneal endothelium (CE) and re-assemble on corneal endothelial cells (CEC) involved in repair (Blitzer et al., 2011 PNAS).

We have now extended these observations to the other corneal cell types. Our data show that cilia assemble during development of the corneal epithelium and stroma and most of the cilia of these tissues disassemble in adults. We have detected ultrastructural differences of the primary cilia in the three corneal cell types that suggest distinct functions. Moreover, upon corneal epithelial injury we have observed a higher number of ciliated cells in newly migrated corneal epithelium on the injured area than we did in intact corneas. To determine the role of cilia during repair of different corneal tissues we have performed a mechanical injury of the corneal epithelium and endothelium in adult transgenic mice (*CAGG-creERTM; ift88^{flox/-}*) in which the IFT88 gene, required for ciliary assembly, had been conditionally inactivated. As expected in control animals CEC neighboring the wound and involved in repair re-assembled a primary cilium, lost cytoplasmic acetylated microtubules, polarized their basal bodies and elongated toward the wound (Blitzer et al. PNAS). In contrast, CEC of *CAGG-creERTM; ift88^{flox/-}* mice lacking IFT88 failed in re-assembling cilia and in disassembling cytoplasmic acetylated microtubules. These cells also failed to polarize and elongate toward the wound. In these mice

healing of epithelial circular wound is also delayed compared to healing of wild-type epithelium. These results suggest that the presence of cilia is required for proper repair of corneal tissues and identify the cilium as a possible pharmacological target in corneal repair.

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1888

BBS4 Traffics Adrenoreceptors In *Chlamydomonas reinhardtii* Flagella.

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Cilia are found on most mammalian cells and play an important role in cell signaling. Mutations in cilia genes cause genetic disorders such as Bardet-Biedl syndrome which presents itself as polydactyly, kidney cysts, retinal degeneration, obesity, or mental deficiencies. Mutations in 16 genes have been identified as causing Bardet-Biedl syndrome. 7 of those genes compose the biochemically purified BBSome, a protein complex actively trafficked in cilia, but whose function remains mostly unknown. The BBSome component BBS4 is required for trafficking G-protein coupled receptors (GPCRs) into mammalian cilia. The BBSome genes are highly conserved between humans and the algae *Chlamydomonas reinhardtii*. When *Chlamydomonas* cells are treated with GPCR targeting compounds (including dopamine, serotonin, and adrenoreceptor-targeting compounds) known to affect flagellar length, *bbs4* mutants show a response that varies from that of wild type cells. Wild type cells also show flagellar binding of a fluorescently labeled adrenoreceptor-targeting compound, while cells lacking BBS4 do not show any flagellar binding. These findings demonstrate the role of BBS4 in adrenoreceptor trafficking, and likely other GPCRs, in flagella of *Chlamydomonas* and illustrate the conservation of BBS4 function.

1889

Transforming growth factor beta (TGF β) signaling is regulated at the pocket region of primary cilia.

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TGF β signaling extensively cross-talks with Hedgehog, Wnt and receptor tyrosine kinase (RTK) signaling to control cell proliferation, migration and differentiation, and when aberrantly regulated leads to developmental defects and cancer. TGF β signaling is activated through the internalization of TGF β receptors via clathrin-dependent endocytosis at which the receptor activates Smad2/3 transcription factors.

Here we investigated the relationship between TGF β signaling and primary cilia using transcriptomics, imaging and molecular biology tools for expression, localization and activation of TGF β signaling components in fibroblasts and in EC and human embryonic stem cells during their differentiation into cardiomyocytes and neurons. During cardiomyocyte differentiation expression of TGF β receptors and Smad transcription factors were up-regulated and targeted to the pocket region of primary cilia, at which the receptor colocalized with clathrin-coated pits and vesicles as well as early endosomes to activate Smad2/3. This activation was blocked by receptor antagonists. In contrast, neuronal differentiation was associated with a loss of TGF β

signaling at the primary cilium. In mouse embryonic fibroblasts (MEFs) and human foreskin fibroblasts (hFFs), TGF β stimulation increased the targeting of TGF β receptors to the ciliary pocket region followed by activation of Smad2/3 signaling to promote cell cycle entry. In Tg737orpk MEFs there was a major reduction in TGF β -induced Smad2/3 phosphorylation, and this was associated reduced activity of clathrin-dependent endocytosis at stumpy primary cilia. Similarly, inhibition of clathrin-dependent endocytosis blocked activation of Smad2/3 at the ciliary pocket region in fibroblasts. Our results suggest that the ciliary pocket functions as a unique site for regulation of TGF β signaling through clathrin-dependent endocytosis and potentially in cross-talking with other signaling pathways during development and in tissue homeostasis.

1890

Epithelial to Myofibroblast transition is associated with the loss of the primary cilium: Topical susceptibility to TGF β in the injured epithelium

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Epithelial-myofibroblast transition (EMyT), a key process in fibrogenesis, involves the activation of a myogenic program (α -smooth muscle actin (SMA) expression) in the injured epithelium. EMyT is an important pathologic feature of polycystic kidney disease, the prototypic ciliopathy. However, the impact of EMyT on the cilium and the underlying mechanisms are largely unknown. Previously we have shown that an intact epithelium is resistant to the EMyT-provoking effect of TGF β . Instead EMyT requires a two-hit scenario, wherein an injury (or uncoupling) of adherens junctions (AJs) and the presence of the fibrogenic cytokine TGF β are both necessary to induce SMA expression in kidney tubular (LLC-PK1) cells. These inputs activate myocardin-related transcription factor and Smad3 respectively, the interplay of which elicits SMA expression. Here we followed ciliary changes in this two-hit EMyT model. EMyT was associated with a biphasic change in the primary cilium, characterized by initial growth followed by dramatic ciliary loss. AJ uncoupling induced by low calcium medium (LCM) triggered a 2-fold growth in ciliary length, TGF β alone had no effect, while LCM+TGF β (EMyT) led to deciliation at 48 h. Differential topical susceptibility to TGF β was also observed in a wound model: TGF β induced EMyT and the loss of the cilium exclusively in cells lining a wound edge (in the first few rows), while cells in the intact part of the same monolayer failed to transform and kept their cilia. Neither TGF β alone in an intact monolayer, nor wounding in the absence of TGF β induced deciliation. In addition to LLC-PK1 cells, human skin fibroblasts treated with TGF β also lost their primary cilium as they differentiated into myofibroblasts. Next we investigated the signaling events that led to deciliation. We found that the loss of the cilium in EMyT is dependent on Smad3 signaling and cell contractility. Thus, downregulation of Smad3, or treatment of the cells with the Rho kinase inhibitor Y-27632 or the myosin blocker Blebbistatin suppressed the LCM+TGF β -provoked deciliation. Additionally cells transfected with the active TGF β RI receptor mutant (Alk5T204D) underwent deciliation only with LCM treatment, whereas those transfected with kinase dead Alk5 did not. AJ uncoupling facilitated the nuclear translocation of the Hippo transcriptional co-activator TAZ, a known nuclear retention factor for Smad3. Remarkably, overexpression of WT or constitutively nuclear TazS89A (while insufficient alone) provoked deciliation upon LCM treatment. We conclude that the myofibroblast is a deciliated cell. The contact-deprived epithelium becomes selectively sensitive to TGF β -triggered deciliation. We propose that this topical susceptibility is due to contact disruption-enhanced, TAZ-promoted Smad3 signaling and a locally increased Rho-dependent contractility.

1891

Differential Localization of Receptors to Primary Cilia and Activation of Ciliary TGF β Signaling During Early Neurogenesis in NT2 Cells.

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We recently showed that the primary cilium is a unique site for TGF β signaling regulated through clathrin-dependent endocytosis at the ciliary pocket region. Canonical TGF β signaling acts through the activation of transcription factors Smad2/3 to control diverse cellular processes during development and in tissue homeostasis. Further, we have shown that SMURF1, an inhibitor of the TGF β pathway, is required for formation of mature neurons in P19.CL6 embryonal stem cells.

In this study we characterized the human teratocarcinoma tera 2 (NT2), pluripotent stem cell line as a model for ciliary TGF β signaling during neurogenesis. Previous studies indicated that canonical TGF β signaling is participating in the maintenance of pluripotency in embryonic stem cells, and that the inhibition of Smad2/3 results in a decrease in the expression of stem cell marker Oct3/4 (James et al., 2005). Initially, we here show that NT2 cells form primary cilia at all stages of differentiation, and transcriptomic analysis on differentially expressed genes in co-regulated gene networks, revealed that multiple pathways are affected during 21 days of NT2 differentiation, including axon guidance as well as TGF β , mTor, Wnt, MAPK, ErbB, and Hedgehog signaling. Immunofluorescence microscopy (IFM), Western blotting (WB), and PCR analysis confirmed formation of mature neurons along with an up-regulation of neuronal markers, including the Paired box protein PAX6, an early transcription factor essential for neurogenesis. Further, IFM analysis showed that components in the various signaling pathways localized in a temporal and spatial manner to the cilium and/or the ciliary pocket region during neurogenesis. As an example, TGF β receptors were localized to the ciliary tip and accumulated at a high level at the ciliary pocket region in non-differentiated cells positive for stem cell markers, SOX2, NANOG and OCT3/4, and this localization was gradually reduced as the cells became positive for PAX6, i.e., entered the early phases of neurogenesis. Conversely, we observed an accumulation of the TGF β signaling inhibitor, Smad7, at the ciliary pocket region upon cell differentiation concomitantly with a reduction in Smad2/3 activation induced by TGF β . In contrast, receptors in Wnt signaling, Frizzled-3, localized to the primary cilium in both undifferentiated and differentiating cells. The differential localization of TGF β signaling components throughout NT2 differentiation, and the observation that the ciliary pocket area is enriched in clathrin-coated vesicles, indicating increased endocytosis in this region, suggest a role for the primary cilium in coordinating TGF β signaling in NT2 cells, and that the absence of TGF β at the primary cilium may participate in the induction of differentiation in NT2 cells.

1892

Hedgehog signaling is dependent on motile cilia in the sea urchin embryo.

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A recent frontier in Hedgehog (Hh) signaling research is the requirement of the primary cilium and intra-flagellar transport (IFT) proteins for signal transduction. Studies in vertebrates have shown that proper trafficking of Hh pathway components within the primary cilium is essential for Hh signaling. This machinery is not necessary, however, for Hh signal transduction in protostomes such as *Drosophila*. As a basal deuterostome, the sea urchin occupies a unique phylogenetic position and can provide crucial insight into the evolution of hedgehog signaling. Here we provide evidence that, as in vertebrates, Hh signaling in the sea urchin relies on the presence of cilia. It has been previously shown by Robert Morris and John Scholey that Kinesin II is essential for cilia assembly. Indeed, knockdown of Kinesin II using an antibody

phenocopies Hh morphants and leads to downregulation of the conserved Hh target Patched. These findings lead to a model of sea urchin Hh signaling that closely resembles the pathway as it functions in vertebrates. Furthermore, analysis of the cilia on Hh receiving cells reveal that they exhibit a 9+2 microtubule morphology consistent with motile cilia and implies that motile cilia are also capable of carrying out Hh signal transduction. This work indicates that the requirement of cilia for Hh signal transduction is not specific to vertebrates or primary cilia, but extends to other phyla and ciliary morphologies.

1893

Smurf1 is a Ciliary Protein Involved in Cardiac Development and Congenital Heart Defects.

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The transforming growth factor β (TGF- β) superfamily controls multiple events in heart development, including cardiomyogenesis, smooth muscle development, vessel stabilization and formation of chambers and endocardial cushion in a spatial and temporal manner. Recent studies in our lab showed that TGF- β signaling is regulated through clathrin-dependent endocytosis at the pocket region of the primary cilium to control cardiomyogenesis.

In this study, we investigated the role of SMURF1 in human heart development and in cardiomyogenesis and formation of beating clusters of cardiomyocytes with pacemaker cells from cultures of P19.CL6 mouse EC stem cells. SMURF proteins are E3 ubiquitin ligases important for embryonic development and regulation of TGF- β signaling in cell differentiation, migration and proliferation. SMURF1 was selected as a candidate disease gene among three genes located in a 486kbp de novo duplication of 7q22.1 in a patient with a congenital heart disease. Immunohistochemical analysis of human embryonic and fetal hearts revealed that SMURF1 has a spatially and temporally restricted expression pattern during cardiogenesis, which suggests that SMURF1 plays a specific role in chamber formation and in controlling the heart beating. Knockdown of SMURF1 by siRNA in P19.CL6 cells increased the rate of differentiation towards cardiomyogenesis, but clusters of cardiomyocytes showed altered morphology compared to mock-transfected cell cultures and were unable to beat. Immunofluorescence microscopy analysis revealed that no or very few pacemaker cells were formed in the clusters of siRNA treated cells, indicating that SMURF1 is required for neuronal development in P19.CL6 cells. Preliminary data shows that the amount of beating clusters and fully mature pacemaker cells increase when SMURF1 is over-expressed. Our analysis further showed that SMURF1 localizes to the ciliary pocket region and interacts with TGF- β receptors and downstream signaling components, including Smad2/3. We suggest that ciliary TGF- β signaling take part in the stimulation of cardiomyogenesis, and that deregulated signaling as a consequence of reduced SMURF1 expression at the cilium prevents the formation of beating clusters of cardiomyocytes due to defective formation and integration of pacemaker cells in the clusters.

1894

Primary Cilia in Appetite and Satiety.

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Although primary cilia are well established as important sensory and signaling structures, their function in most tissues remains unknown. Hyperphagia associated obesity is a feature of some syndromes of cilia dysfunction, such as Bardet-Biedl syndrome (BBS) and Alström syndrome (ALMS). Recent data indicate that obesity in BBS mutant mouse models is due to defects in leptin receptor trafficking and subsequent leptin resistance. Furthermore, induction of cilia loss in leptin responsive POMC neurons results in obesity, implicating cilia on hypothalamic neurons in regulating feeding behavior. Here, we directly test the importance of the cilium as a mediator of the leptin response. In contrast to the current literature, a longitudinal study of conditional *Ift88* cilia mutant mice under different states of adiposity indicates that leptin resistance is present only when mutants are obese. Our studies show that caloric restriction leads to an altered anticipatory feeding behavior that temporarily abrogates the anorectic actions of leptin despite normalized circulating leptin levels. Interestingly, pre-obese *Bbs4* mutant mice responded to the anorectic effects of leptin and did not display other phenotypes associated with defective leptin signaling. Furthermore, thermoregulation and activity measurements in cilia mutant mice are inconsistent with phenotypes previously observed in leptin deficient *ob/ob* mice. Collectively, these data indicate that cilia are not directly involved in leptin responses and that a defect in the leptin signaling axis is not the initiating event leading to obesity associated with cilia dysfunction. Current studies suggest altered melanin concentrating hormone or hedgehog signaling may be responsible for the obesity phenotype associated with cilia dysfunction. This work will provide novel insights into the onset of obesity and more generally the importance of the cilium in regulating normal neuronal activity and energy balance.

1895

Bld10/Cep135 stabilizes basal bodies to resist cilia generated forces.

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Basal bodies nucleate, anchor and organize cilia. As the anchor for motile cilia, basal bodies must be resistant to the forces directed toward the cell as a consequence of ciliary beating. The molecules and generalized mechanisms that contribute to the maintenance of basal bodies remain to be discovered. Bld10/Cep135 is a basal body outer cartwheel domain protein that has established roles in the assembly of nascent basal bodies. We find that Bld10 protein first incorporates at basal bodies early during new assembly. A second Bld10 protein population incorporates at basal bodies after assembly and we predict that this is required to stabilize basal bodies. Additionally, we identify a novel mechanism for Bld10/Cep135 in basal body maintenance so that basal bodies can withstand the forces produced by motile cilia. Bld10 stabilizes basal bodies by promoting the stability of the A- and C-tubules of the basal body triplet microtubules and by properly positioning the triplet microtubule blades. The forces generated by ciliary beating promote basal body disassembly in *bld10Δ* cells. Thus, Bld10/Cep135 acts to maintain the structural integrity of basal bodies against the forces of ciliary beating in addition to its separable role in basal body assembly.

1896

Configuration changes of *Chlamydomonas* dynein-f tail coupled with IC138 phosphorylation.

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Inner-arm dynein-f of *Chlamydomonas* flagella is a hetero-dimeric dynein which has two heavy chains (α and β) forming the respective heads, three intermediate chains and light chains attached to the tail. It has been shown that an axonemal casein kinase I phosphorylates the 138K intermediate chain (IC138) and that this phosphorylation is involved in regulation of flagellar activity. To elucidate the regulation mechanisms of dynein-f functions through the IC138 phosphorylation, we examined molecular configuration changes of dynein-f coupled with IC138 phosphorylation by negative-staining electron microscopy and following single-particle image analyses. For the analyses, we picked up a dynein-f density map from the tomogram of axoneme, prepared projections of the map from various angles, and used tail parts of the projections as the templates for alignments. We found that (1) the distribution of the two heads' positions spread and distance between the α and β heads became closer coupled with IC138 phosphorylation. (2) In ~10% classes, the tail showed forked structure which consisted of thick stem and the thin branch. It was suggested that this thin branch linked to the β head. The averaged image of the thin branch became smearing in phosphorylation conditions, suggesting that the branch positioning became unstable. From these observations, we suppose that the IC138 phosphorylation could first affect the thin branch positioning, then the β -head position should be shifted. In axonemes, position shift of the β head could affect the interactions of dynein-f and adjacent microtubule to change their operation mode.

1897

Composition and function of the nexin-dynein regulatory complex.

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The nexin dynein regulatory complex (N-DRC) is a group of polypeptides thought to coordinate the activity of dynein arms and interconnect doublet microtubules (Piperno et al., 1994; Gardner et al., 1994; Heuser et al., 2009), but its precise role in flagellar motility is poorly understood. Here we take advantage of *drc4* mutations to identify novel components and evaluate the function of the N-DRC. We found that DRC4 is the *PF2* gene product in *Chlamydomonas* (Rupp & Porter, 2003) and that DRC4 is also modified in *sup-pf3* by transposon insertion and alternative splicing. Truncated DRC4 subunits assemble into *sup-pf3* axonemes, but several N-DRC subunits are missing. Transformation of *pf2* and *sup-pf3* with GFP-tagged DRC4 restores wild-type motility and repairs the mutant defects. Dikaryon rescue experiments reveal that DRC4-GFP does not readily exchange with wild-type DRC4, but assembles into *pf2* axonemes from tip to base within ~1 hour after mating. These observations show that DRC4 plays a key role in the assembly of the N-DRC linker domain. Biochemical strategies have identified at least nine N-DRC subunits that associate with DRC4 to form a discrete complex distinct from the radial spokes, calmodulin spoke associated complex, or ribbon fraction. Several subunits contain calcium signaling motifs or AAA domains. Phylogenetic comparisons indicate that N-DRC subunits are nearly ubiquitous in species with motile axonemes. However, *n-drc* mutants still maintain the normal 9+2 arrangement of outer doublet microtubules and also generate flagellar bending. To further evaluate the role of the N-DRC in flagellar motility, we analyzed microtubule sliding in isolated axonemes. Addition of 0.1 mM ATP to axonemes from wild-type or rescued strains results in intact axonemes or reactivated axonemal bending but no sliding

disintegration. However, addition of 0.1 mM ATP to *n-drc* mutant axonemes results in the splaying of outer doublets in the distal three-fourths of the axoneme, followed by occasional cycles of sliding-bending between splayed pairs of doublet microtubules, similar to that seen in protease treated axonemes (Aoyama and Kamiya, 2005). These results suggest that the N-DRC is required to maintain optimal alignment for productive microtubule sliding and that additional structures at the proximal end of the axoneme provide resistance for flagellar bending. (Supported by grants from the NIH to WSS and MEP).

1898

The microtubule associated protein CLAMP and Par-3/Par-6/aPKC are required for radial intercalation of multiciliated cells.

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Multiciliated epithelia can be found in a variety of tissues such as the upper respiratory tract, the fallopian tube and the ependyma. The directed fluid flow created by multiciliated ciliated cells (MCCs) is essential for many biological processes during development as well as during normal adult tissue homeostasis. We use the skin of *Xenopus* embryos as a model system to study the development of multiciliated epithelia. In developing tadpoles, MCC precursors differentiate in an inner epidermal layer of the ectoderm before radially intercalating into the outer epidermal layer. Radial intercalation has been shown to be restricted to cellular vertices formed by multiple cells of the outer epidermal layer however the molecular mechanisms that regulate this process are largely unknown. MCCs contain roughly one hundred basal bodies that duplicate prior to intercalation. We observe that these basal bodies cluster close to the apical surface during intercalation accompanied by a significant accumulation of microtubules at the leading edge of the intercalating cell. Interestingly, disruption of the microtubule network with the microtubule de-polymerizing drug Nocodazole leads to defects in radial intercalation suggesting that microtubules are required for this process. The Par-3/Par-6/aPKC complex has been shown to regulate cell polarity, centrosome positioning and cell migration. Overexpression of dominant negative variants of Par-3 or kinase dead aPKC specifically in MCCs prevents apical positioning of basal bodies and blocks radial intercalation. Furthermore, we observe that the striated rootlet associated protein CLAMP, that exhibits weak homology to EB1, also associates with cytoplasmic microtubules in *Xenopus* embryos. Interestingly, the human homologue of CLAMP has been shown to stabilize microtubules when overexpressed. We show that CLAMP dynamically associates with the microtubule lattice and that depletion of CLAMP by morpholino oligonucleotides severely disrupts radial intercalation. Interestingly, depletion of CLAMP does not prevent apical positioning of the basal body cluster suggesting that CLAMP acts downstream of the Par-3/Par-6/aPKC complex. We propose that radial intercalation of MCCs requires the Par-3/Par-6/aPKC dependent apical localization of basal bodies followed by CLAMP dependent stabilization of apical microtubules.

1899

Outer-Inner Dynein Linker Regulates the Flagellar Beating.

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Cilia and flagella are elaborate organelles for motility and are conserved in a wide range of eukaryotes. Defects in the motility of cilia and flagella result in hydrocephalus, situs inversus, bronchiectasis and infertility in mammals. Thus, it is of great importance to understand the mechanism of the ciliary/flagellar motility. The beating motion of cilia and flagella is driven by

two species of dynein motors: the outer dynein arm (ODA) and the inner dynein arm (IDA). ODA and IDA play distinct roles in the generation of the beating. ODA is required for generating normal beat frequency while IDA is necessary for propagation of the bending waves with appropriate amplitude. However, functional communications between the two species of the motors have not been investigated. In this study, we found that, in *Chlamydomonas reinhardtii*, a mutation introduced to the amino-terminus of the intermediate chain 2 (IC2) of ODA caused reduction in the bend amplitude as well as defects related to the malfunction of ODA. The phenotype of the mutant suggested that the mutation within ODA disrupt not only ODA's function but also the activity of IDA. Recent studies by cryo-electron tomography of axonemes revealed new structures linking between ODA and IDA. We performed three-dimensional reconstructions of the structures of streptavidin-labeled ODA bound to microtubules and showed that the position of the amino-terminus of IC2 coincided ODA-end of the previously reported ODA-IDA linker. Furthermore, we demonstrated that the IC2 was cross-linked with one of the components of the dynein regulatory complex (DRC) within the axoneme. These observations suggest that IC2 is involved in the regulation of the motor activities of both ODA and IDA via ODA-DRC connection. This is the first report that there are physical and functional interactions between ODA and IDA.

1900

The Mia complex is a conserved dynein regulator required for normal ciliary motility.

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Ciliary motility requires precise regulation and coordination of the activities of a complex array of axonemal dyneins. However, the mechanisms that regulate and coordinate ciliary dyneins are poorly understood. We analyzed two *Chlamydomonas* mutants, *mia1* and *mia2* (modifiers of inner arms): the mutants are defective in phosphorylation of IC138 (the 138 kD regulatory intermediate chain of inner dynein arm "I1", also known as dynein "f"), swim slowly and fail to perform phototaxis (King and Dutcher, 1997). These phenotypes mimic mutants defective in I1 dynein. Analysis of four *mia1* alleles and the *mia2* mutant confirmed the altered IC138 phosphorylation and slow swimming phenotype. We also discovered that the *mia* mutants display reduced ciliary beat frequency, a feature consistent with a defect in outer dynein arm function or regulation. By AFLP mapping and sequencing, we determined that the *MIA1* and *MIA2* genes encode conserved coiled-coil proteins, FAP100 and FAP73, respectively. We further determined that these proteins form a complex in the axoneme, the "Mia complex". Protein-protein interaction analysis revealed that the Mia complex interacts with IC138. Consistent with an interaction between I1 dynein and the Mia complex, cryo-electron tomography of *mia*-mutant axonemes revealed the Mia complex is located immediately distal to the IC138 IC/LC sub-complex (Bower et al., 2009) of I1 dynein. Moreover, density reduced in the *mia* mutants appears to extend from I1 dynein to the Nexin-DRC (Heuser et al., 2009). Notably, in axonemes from mutants that lack both the Mia complex and outer dynein arms, I1 dynein fails to assemble in the axoneme, suggesting a role for the Mia complex in stable assembly of I1 dynein. Together, the results demonstrate that the Mia complex regulates I1 dynein activity and, either directly or indirectly, regulates outer dynein arm activity to control axonemal waveform and beat frequency, the key parameters of normal ciliary motility and physiology.

1901

Amplitude and curvature development at the switch-points of the beat cycle in bull sperm are augmented by ADP and reduced by ATP that is un-complexed with Mg²⁺.

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We investigated the roles of ADP, Mg²⁺, and ATP in the beat cycle of Triton X-100- extracted bull sperm models. We recorded the motility of reactivated sperm models that were firmly attached to a glass surface by their heads with their flagella beating freely. The curvature of the flagellum at ~7 μm from the head tail junction was measured on images that were closest to the point of beat direction reversal. These images corresponded to the principal and reverse bend switch-points. The amplitude of the beat frequency was measured by tracking the excursion of a point on the flagellum 20 μm from the head tail junction relative to the origin on the midline axis of the sperm head when the flagellum is straight. Beat frequencies were hand calculated. The curvature at the switch-point, the amplitude at 20 μm and the beat frequency provided a quantitative basis to evaluate changes of the beat cycle in response to changes in ADP, Mg²⁺, and ATP concentration. We found that bull sperm exhibit well-developed beat cycles at all concentrations of Mg-ATP between 0.1 mM and 10 mM, but only if the concentrations of Mg²⁺ and ATP are equal. ATP in excess of the Mg²⁺ concentration resulted in a reduction of beat amplitude and curvature at the switch-points, and increased beat frequency. A concentration of 4 mM or greater ATP combined with 1 mM Mg²⁺ effectively terminated the beat cycle by reducing amplitude and curvature to extinction. Increasing ADP from 0 to 4 mM increased the amplitude and curvature developed at the switch-points and offset the reduction in amplitude and curvature produced by high ATP with Mg²⁺ held at 1 mM. This suggests that ADP has a strong effect on elevating the curvature and amplitude of the beat that is independent of Mg²⁺ concentration. The rescue of the beat by ADP occurs with a reduction in frequency. Therefore, the beat cycle is restored in spite of a slowing of the dynein cross-bridge cycle. These results can best be understood in the context of the geometric clutch (GC) hypothesis. In the GC hypothesis, switching occurs when the transverse force overcomes the adhesion of the dyneins onto their adjacent doublet. If ADP increases that adhesion by increasing dynein microtubule binding affinity and ATP un-complexed with Mg²⁺ has the opposite effect, then the balance between these factors will regulate the switch-point for dynein release in the beat cycle. Support: NSF MCB-0918294.

1902

Analysis of extraordinary flagellar beat patterns suggesting switching mechanisms of flagellar activity.

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Sea urchin spermatozoa show active swimming by beating flagella toward eggs when spawned in sea water. The flagellar motility is based on the activity of dynein motors that hydrolyze ATP and produce power to generate microtubule sliding. In many cases, spermatozoa quickly lose their motility in 10-20 min after spawning probably due to the depletion of energy sources. However, ATP would not be depleted completely since spermatozoa can continue respiration by mitochondria and we sometimes observed active sperm swimming in a tightly sealed chamber for more than an hour. Sperm is a kind of disposable machine for DNA delivering without any repairing and maintaining system such as IFT. Thus, we assumed that accumulation of mechanical damage(s) inside flagellar axoneme may gradually cause the change of wave generation and propagation, which would partially lead to the cessation of active swimming.

Here, we used sea urchin sperm (*Clypeaster japonicus*, *Hemicentrotus puicherrimus*, and *Anthocardaris crassispina*) with extraordinary beating by aging or mechanical damages to examine the details of modified motility. As for the case of extraordinary motility, we chose spermatozoa that showed relatively symmetric beating at the proximal regions of flagella with waves diminishing when propagating towards the distal ends. Since sea urchin spermatozoa have been shown to have loose head-to-neck junction, we can assume the distal straight parts to be a 'non-sliding site' that can be used as for reference points of microtubule sliding instead of sperm heads. Although the flagellar motion was in irregular forms, we expected more precise analysis of sliding activity around the neck region of spermatozoa that could not be clarified by conventional methods. Our results showed that flagellar beat frequency and phase were kept almost constant when propagating along the axoneme. Amplitude or amount of sliding (shear angle amplitude) was constant in the proximal region, but diminished when propagating into the latter distal regions of axoneme. In most cases, the transient point from constant wave propagations to declined sliding activity was at around a 1/2 of wave length (1/3 of flagella length, $\approx 15\mu\text{m}$) from base of flagella. This point would be corresponding to a region of the functional discontinuity of sperm flagella where turn on and off the activity of dynein arms.

1903

Formation of planar waves on sea urchin sperm flagella.

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The function of Ca^{2+} and cAMP in extruding doublet microtubules from sea urchin sperm axoneme and generating flagellar waves was investigated in order to clarify the regulatory mechanism of microtubule sliding and the formation mechanism of beating patterns of cilia and flagella. Almost all potentially asymmetric spermatozoa, which were demembrated with Triton in the absence of Ca^{2+} , beat with planar waves, whereas potentially symmetric spermatozoa, which were demembrated with Triton and millimolar Ca^{2+} , beat with three-dimensional waves if they were reactivated with low MgATP^{2-} . At a low Ca^{2+} , cAMP enhanced the three-dimensionality of the flagellar waves. These changes in the flagellar waves were caused by different regulations of the microtubule sliding by Ca^{2+} and calmodulin and cAMP. Increase in beat frequency with high MgATP^{2-} converted the three-dimensional waves into the planar waves. These results suggest that the planar flagellar waves are generated at high Ca^{2+} and calmodulin or by rapid beating.

1904

Measurement of human airway ciliary waveforms in the presence of polyethylene glycol and dextran.

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Mammalian airways are covered with cilia which transport mucus out of the airways. They are surrounded by a periciliary layer and covered by a distinct mucus layer. Our goal was to gain insight into the mechanism of the ciliary beat from the responses of cilia to altered environments.

Human airway cultures were grown with an air-liquid interface. For imaging, culture membranes were folded to image cells in profile and then sandwiched between cover-glasses forming a channel. Through this channel, buffer with polyethylene glycol (PEG) or dextran was introduced and then washed out. The average molecular weights tested were 8000 for PEG and several molecular weights up to 2×10^6 for dextran. Both were tested with concentrations up

to 50%. Beating cilia were analyzed by manually altering a model superimposed over videos of beating cilia.

The following waveforms were compared: plain buffer, PEG or dextran, and washout of PEG or dextran with plain buffer. Both PEG and dextran caused a decrease in the ciliary beat frequency and amplitude. The dextran effect increased in proportion to its concentration. At the highest concentrations of dextran, some motion was still observed. PEG had a sudden effect above 15% with an increased coordination of adjacent cilia. At higher concentrations, cilia clumped together and motion completely stopped. Both PEG and dextran washed out and resulted in a normal ciliary beat but the PEG solution required a longer washout time. With some cilia in the presence of dextran, it was also possible to observe curvatures that spanned shorter than normal distances along the cilium. This made it possible to measure propagation of curvature which is not seen in cilia beating in plain buffer.

Overall, PEG and dextran acted differently at similar viscosities and this may be due to specific effects of PEG on the ciliary surface. The PEG effects suggest that the surface properties of cilia are important in waveform generation. The application of dextran unmasked a propagating mechanism that was not observed in plain buffer.

Kinetochores

1905

Kinetochore structure dissection. A Super-Resolution Microscopy issue.

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The kinetochore is a highly complex proteinaceous structure that assembles at the primary constriction of sister chromatids. Kinetochores hold a primary role during mitosis because they direct the process of chromosome segregation. In particular, growing interest has been raised towards the Constitutive Centromere Associated Network (CCAN) components, which seem to take part in the formation of a scaffold involved in kinetochore assembly. Therefore much effort has been put in trying to understand both their distribution and their role in kinetochore functions. In the last decade, novel super-resolution microscopy techniques have been developed that can reach a subdiffraction spatial limit resolution of tens of nanometers. Amongst these, Photo-Activated Localization Microscopy has been recently applied to study the organization of extended fibers of kinetochore chromatin. On linearized chromatin fibers, PALM allows us to localize with a resolution of approximately 37 nm single molecules of the blinking fluorescent protein Dronpa fused to the N-terminal region of CENP-A relative to the position of other antigens labeled with Alexa-tagged antibodies. In our study we are taking advantage of the high homologous recombination efficiency in DT40 B-lymphoma chicken cell line which allowed us to obtain conditional knockouts of several CCAN components. We are using those conditional knockouts to study the localization of several CENPs and post-translational histone modifications relative to Dronpa:CENP-A on unfolded centromere chromatin fibres by PALM Microscopy. This system represents a unique and reliable model to visualize at high resolution how CENPs and epigenetic histone marks change either in composition and distribution along the chromatin fibre following the deletion of different CCAN components.

1906

Understanding the architecture of vertebrate kinetochores using quantitative proteomics.

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When cells divide, a series of events must proceed in a timely and co-ordinated manner to ensure that all DNA is replicated and partitioned equally amongst the two daughter cells. A central component of this process is the kinetochore, a large proteinaceous complex found within the centromere of all mitotic chromosomes. Despite the fact that many kinetochore components are conserved from yeast to human and that these sub-complexes are functionally well studied, the way these components come together to form a fully operational kinetochore is still poorly understood.

Recently, chromosome isolation techniques from chicken DT40 cells, combined with stable isotope labeling by amino acids in cell culture (SILAC), were used to define all chromosome associated proteins. Applying the same method we here compare the proteome of mitotic chromosomes isolated from different kinetochore knockout (KO) cell lines. This includes components of the inner kinetochore; CENP-C, CENP-T and CENP-W, and a sub-unit of the Ndc80 complex important for microtubule attachment. With these large data sets we have focused on the impact these depletions have on the architecture of the holo-kinetochore by measuring the SILAC ratios of individual proteins. We have found that proteins within the same complex typically behave in a similar manner across the different KO conditions. Multivariate data analysis reveals dependency pathways, as well as highlighting potential novel kinetochore proteins worthy of further study.

The KO cell lines listed above all exploit the doxycycline inducible shut-down of gene expression that has proven to be a highly valued system in the study of many cellular processes. However, although gene expression is abolished immediately, full loss of function requires that proteins translated before drug addition are degraded. Depending on protein stability, this can take hours, or even days, and lead to the accumulation of unwanted secondary effects. To address this problem, we are using an auxin-inducible degron (AID) system to rapidly reduce the expression of kinetochore sub-units at the protein level. We find that AID-tagged CENP-T in DT40 cells is rapidly depleted within 1 hour of auxin addition, immediately preventing further proliferation. Importantly, this gives us a unique opportunity to study the effects and phenotypes of CENP-T depletion in a highly regulated way at different phases of the cell cycle.

1907

Systematic analysis of kinetochore assembly and disassembly in human cells.

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Accurate chromosome segregation requires assembly of the multi-protein kinetochore complex. Prior work has identified over 100 kinetochore components in human cells. However, little is known about the regulatory processes that specify their assembly upon mitotic entry, or that control the disassembly of the complex at mitotic exit. Previously, we have identified two parallel pathways for kinetochore assembly involving the constitutive centromere proteins CENP-T and CENP-C. Here we demonstrate that multiple regulatory mechanisms converge on these

pathways to control outer kinetochore assembly. Using a live-cell imaging based assay to map kinetochore assembly dynamics, we systematically assess the role of potential regulatory mechanisms in timely kinetochore assembly. We find that kinetochore assembly and disassembly is primarily controlled by the coordinated mitotic phosphorylation of key kinetochore proteins by Cyclin B1/Cdk1. In addition, we find that the subcellular localization of key kinetochore proteins prior to mitosis is important for timely kinetochore formation. In total, we find that the coordinated temporal regulation of parallel kinetochore assembly pathways is essential for accurate cell division.

1908

Phosphorylation of Zwilch by MPS-1 Is Required for Recruitment of the Fibrous Corona.

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Kinetochores are robust protein scaffolds at centromeres that link chromosomes to the mitotic spindle and mediate chromosome movement. Kinetochores are also responsible for generating diffusible signals that inhibit anaphase onset until the successful completion of chromosome alignment. The Vaughan laboratory has deciphered the basis of checkpoint silencing at metaphase focusing on regulation of dynein and dynein-associated proteins at the kinetochore. Ongoing studies reveal that the Rod-Zw10-Zwilch (RZZ) complex is central to both checkpoint signaling and the recruitment of microtubule (MT) binding proteins required for chromosome movement (Kasuboski et al., 2011). The RZZ complex functions as a platform for assembly of the outer layers of the kinetochore and the fibrous corona, including molecules responsible for microtubule binding, chromosome movement and checkpoint signaling. Monopolar spindle-1 (MPS-1) kinase has been linked to recruitment of the SAC components, Mad2, Bub1, Bub3, Mad1 and BubR1/Mad3 to kinetochores (Hardwick et al., 1996; Abrieu et al., 2001; Vigneron et al., 2004; Wong and Fang, 2005). Although MPS-1 has been identified as a critical mitotic kinase, the complete list of substrates is not known. Inhibition of MPS-1 with reversine perturbed recruitment of the fibrous corona, affecting dynein, dynactin, the RZZ complex, spindly, BubR1 and Mad2 but not Zwint-1 or Hec1. We used in vitro kinase assays to identify candidate substrates and identified Zwilch but not Zw10 as a novel substrate for MPS1. LC/MS/MS analysis of phosphorylated Zwilch revealed 3 novel phosphorylation sites. A triple-A (3A) mutant of Zwilch localized to kinetochores and induced defects similar to MPS1 inhibition. 3A-Zwilch expression led to a loss of dynein, dynactin, spindly and anaphase inhibitors from kinetochores. We also observed defects in mitotic progression including the “frozen” phenotype during prometaphase and premature anaphase onset. These studies suggest that MPS1 phosphorylation of Zwilch is required for assembly of the fibrous corona and proper coordination of checkpoint signaling with chromosome alignment.

1909

The role of CLASP2 phosphorylation at the kinetochore-microtubule interface.

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Accurate chromosome segregation is essential for the faithful distribution of the genetic material during cell division. Microtubules attach to chromosomes through the kinetochore, and facilitate metaphase chromosome alignment and anaphase chromosome segregation. During these processes, chromosomes undergo drastic movements yet remain attached to kinetochore microtubules. Therefore, the plus-ends of kinetochore microtubules must remain dynamic and the kinetochore-microtubule attachment has to be precisely regulated. Although an extensive list

of kinetochore components has been identified, the mechanism by which kinetochores regulate their attachments to dynamic microtubules is incompletely understood. CLASPs are proteins that associate with growing microtubule plus-ends and stabilize microtubules in the periphery of interphase cells. During mitosis, CLASPs localize to kinetochores and are thought to mediate microtubule polymerization at the kinetochore. We have found that CLASP2 is hyperphosphorylated during mitosis by Glycogen Synthase Kinase 3 beta (GSK3 β) on serine residues within its plus-end tracking domain. GSK3 β -mediated phosphorylation is dependent on priming phosphorylation of CLASP2 by cyclin-dependent kinases, and completely disrupts CLASP2-microtubule interaction during mitosis. Expression of non-phosphorylatable CLASP2, in which either all phosphorylation sites or only CDK priming sites are replaced with alanine residues, restores CLASP2 microtubule plus-end tracking during mitosis. Because phosphorylated CLASP2 does not bind microtubules in mitotic cells, we hypothesize that CLASP2 phosphorylation is tightly controlled at the kinetochore to regulate kinetochore-microtubule interactions. To test this hypothesis, we are analyzing how non-phosphorylatable and phospho-mimetic CLASP2 constructs affect mitosis and kinetochore dynamics. HaCaT keratinocytes depleted of CLASP2 by lentivirus-mediated shRNA exhibit characteristic chromosome congression and spindle orientation defects. While expression of EGFP-tagged wildtype CLASP2 can partially rescue mitotic defects of CLASP2 knockdown cells, non-phosphorylatable and phospho-mimetic versions of CLASP2 cannot. This indicates that proper phosphoregulation of CLASP2 is required for normal mitosis. We are currently using 3D imaging of CENPA-mCherry labeled kinetochores in combination with advanced computational analysis of kinetochore dynamics to determine how CLASP2 phosphorylation controls kinetochore and spindle dynamics.

1910

Linker-scanning mutagenesis of the Ndc80 kinetochore protein.

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During chromosome segregation, kinetochores physically link centromeric DNA to spindle microtubules. This linkage requires the Ndc80 complex, an essential and conserved kinetochore component. The complex features globular domains on both ends, and a long coiled coil in between. At the N-terminus, the Ndc80 protein contains a microtubule-binding calponin-homology domain. The remainder of Ndc80, most of which is predicted to form a coiled coil, remains uncharacterized. We performed a saturated linker-scanning mutagenesis screen to identify the essential regions of the budding yeast Ndc80 protein. Using an *in vitro* transposition system, a library of ~11,000 insertion events was generated in a plasmid carrying the *NDC80* gene and screened for function *in vivo*. We found that ~4% of insertions were lethal. Illumina sequencing was used to map these lethal insertions. Combining linker-scanning mutagenesis with high throughput sequencing provided a powerful way to identify new functionally important regions of Ndc80. The insertions clustered in five distinct locations within Ndc80. Two clusters are in the calponin-homology domain, and three clusters are in the coiled coil domain. The only proposed function of the latter is to mediate tetramerization of Ndc80 complex components. However, the lethal insertions in the coiled coil domain do not disrupt assembly of the purified recombinant complex. Therefore, linker scanning identified functionally essential regions of the coiled coil that are not involved in complex assembly.

1911

The Ndc80 kinetochore complex directly modulates microtubule dynamics.

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The conserved Ndc80 complex is an essential microtubule-binding component of the kinetochore. Recent findings suggest that the Ndc80 complex influences microtubule dynamics at kinetochores *in vivo*. However, it was unclear if the Ndc80 complex mediates these effects directly, or by affecting other factors localized at the kinetochore. Using a reconstituted system *in vitro*, we show that the human Ndc80 complex directly stabilizes the tips of disassembling microtubules and promotes rescue (the transition from microtubule shortening to growth). *In vivo*, an N-terminal domain in the Ndc80 complex is phosphorylated by the Aurora B kinase. Mutations that mimic phosphorylation of the Ndc80 complex prevent stable kinetochore-microtubule attachment, while mutations that block phosphorylation damp kinetochore oscillations. We find that Ndc80 complex with Aurora B phosphomimetic mutations is defective at promoting microtubule rescue, even while robustly coupled to disassembling microtubule tips. This impaired ability to affect dynamics is not simply due to weakened microtubule binding, as an N-terminally truncated complex with similar binding affinity is able to promote rescue. Together, these results suggest that in addition to regulating attachment stability, Aurora B controls microtubule dynamics through phosphorylation of the Ndc80 complex.

1912

Microtubule-end bound complexes power chromosome movement during chromosome segregation.

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During mitosis, the microtubule plus-ends play an important role in rapidly capturing subcellular organelles and precisely powering their movements. However, microtubule-end bound protein complexes in mitotic cells are not well characterised, limiting our understanding of molecular mechanisms that control microtubule powered organelle movement. Using a combination of inhibitor treatment, immunoprecipitation and mass spectrometry, we have identified protein complexes that interact with the autonomous plus-end binding protein, EB1 in mitotic cells. This first effort has revealed nearly 15 different protein interactors of EB1 in distinct stages of mitosis, including previously known kinetochore - and cortex- bound proteins. To learn about how plus-end complexes power organelle movement, we used a high-throughput imaging assay to identify those plus-end bound proteins that are essential for attaching chromosomes to microtubule ends but not microtubule walls. Plus-end binding proteins important for end-on attachment interact with several members of the KMN network of outer kinetochore proteins and EB1 directly, as revealed by our yeast two-hybrid interaction studies. We are now investigating the mechanisms that control the interactions between protein domains of EB1 interactors and the KMN network to understand how plus-end complexes are loaded and unloaded at the kinetochore to ensure precise control over chromosome movements.

1913

The KMN network makes major conformational changes with kinetochore microtubule formation not exhibited by other outer domain proteins like RZZ and CENP-F.

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Faithful chromosome segregation is important for maintenance of life in all organisms. Kinetochores, assembled at the periphery of centromeres attach chromosomes to the plus ends of spindle microtubules (MTs) to form kinetochore microtubules (kMTs). At metaphase, human kinetochores become attached on average to ~20 kMTs when their chromosomes are properly aligned at the spindle equator. The KMN network of outer kinetochore proteins (KNL1 (Blinkin) complex, Mis12 complex and Ndc80(hsHEC1)) complex are required for robust kMT attachment and control of the spindle assembly checkpoint that governs entry into anaphase. Previous studies have determined the average protein architecture of the KMN network at metaphase human kinetochores, but information is critically needed for unattached kinetochores to understand how the kMT attachment sites are formed.

We have developed a chromosome spin-down assay for nocodazole treated HeLa cells that frequently orients sister chromosome pairs side-by-side on the surface of coverslips. This orientation enhances enormously the accuracy and speed for analysis of the protein architecture of unattached human kinetochores by our K-SHREC fluorescence microscopy method. Measurements of the average separation of red and green fluorescent labels along the sister-sister kinetochore axis were made at nm scale accuracy as described by Wan et al., 2009, Cell.

We find a major change in the conformation of the KMN network of proteins between unattached kinetochores and kinetochores with a full complement of kinetochore microtubules at metaphase. The end-to end separation for the Ndc80 complex and KNL1 (Blinkin) complex are much shorter within unattached kinetochores compared to their separations along kMTs at normal metaphase. However, the average positions of outer kinetochore components, such as CENP-E, CENP-F, and RZZ complex, do not change between both conditions.

These conformational changes indicate that interactions of unattached prometaphase kinetochores with the plus ends of spindle microtubules is important for the self-assembly of metaphase kMT attachment sites. The conformational changes are also likely important for preventing errors in kMT attachment and for controlling the spindle assembly checkpoint. Our measurements also address issues about how the KMN network is linked to chromosomes by members of the Constitutive Centromere Associated Proteins (CCANs), like CENP-T.

1914

Location of the Mad1/Mad2 Complex, Zwint1, the Rod/Zw10/Zwilch (RZZ) Complex, and the Dynein Motor Recruitment Factor Spindly Within the Substructure of the Kinetochore.

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Zwint1, the Rod/Zwilch/Zw10 (RZZ) complex, the Mad1/Mad2 complex, Spindly, and the Dynactin/Dynein motor all localize to kinetochores where they play a major role in the spindle assembly checkpoint (SAC) and kinetochore microtubule (kMT) attachment. An important unresolved question is how these components integrate into the core kMT attachment site comprised of Knl1 (Blinkin), the Ndc80 (Hec1) complex, and the Mis12 complex. We have used high-resolution two-color fluorescence light microscopy at the nm scale (Delta analysis) to map the average positions of these components within metaphase kinetochores after uncoupling the dynein-dynactin complex from Spindly to prevent their removal during SAC inactivation. Relative to the Hec1 CH domain: the Mad2-binding region of Mad1 and the C-terminus of Zw10 are

located ~35 nm internal (i.e. closer to centromeric chromatin) and very close to the measured loci for Knl1 N-terminus; and the N-terminus of Rod, Zwilch, and the N-terminus of Zw10 are ~10 nm internal. The Zw10 interactor Zwint1 is located ~50 nm internal to the Hec1 CH domain and in close proximity to the C-terminus of Knl1. The C-terminus of Spindly is located near the Hec1 CH domain while its N-terminus is about 70 nm outside the outer end of the Ndc80 complex and within the coronal domain of the kinetochore. Our measurements suggest that the checkpoint protein module is closely juxtaposed with Mis12, the Ndc80 complex and Knl1 in these metaphase-arrested cells. Depletion of Zwint1 by RNAi reduced RZZ and Mad1 kinetochore localization by 60 & 40% respectively, while depletion of Knl1 abolished RZZ, Mad1 and Zwint1 kinetochore localization at kinetochores of nocodazole-treated cells. Zwint1 depletion reduced Knl1 kinetochore localization by 60%, suggesting a co-dependence between these tightly complexed proteins, which may explain the intermediate effect of Zwint1 depletion on RZZ and Mad1 localization. These results establish how checkpoint complexes are positioned relative to components of the core kMT attachment site. Consistent with the observation that Zwint1 forms a tight complex with Knl1, our results also suggest that the Knl1-Zwint1 complex, rather than Zwint1 by itself, recruits RZZ and the Mad1/Mad2 complex to kinetochores.

1915

A structural basis for kinetochore recruitment of the Ndc80 complex via two distinct centromere receptors.

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The Ndc80 complex, composed of Ndc80, Nuf2, Spc24 and Spc25 subunits, is the key microtubule-binding element of the kinetochore. In contrast to the well-characterized interaction of Ndc80/Nuf2 heads with microtubules, little is known about how the Spc24-25 heterodimer connects to centromeric chromatin. Here we present the crystal structure of Spc24-Spc25 in complex with the histone-fold protein Cnn1/CENP-T illustrating how this interface ultimately links microtubules to chromosomes. The conserved Ndc80 receptor motif of Cnn1 is bound as an alpha-helix in a hydrophobic cleft at the interface between Spc24 and Spc25. Interestingly, point mutations that disrupt the Ndc80-Cnn1 interaction are lethal in yeast, though Cnn1 is a non-essential gene. We demonstrate that the same mutations in Spc24-25 abrogate the interaction with the essential Mtw1 complex, and we identify a Cnn1-related binding motif in its Dsn1 subunit. Phosphorylation of the Cnn1 N-terminal domain serves to coordinate the Ndc80 interaction with two competing binding partners. Together our data reveal a modular kinetochore architecture, in which Ndc80 complexes connect to distinct interaction partners through a common interface.

1916

CENP-A at human centromeres and neocentromeres forms octameric nucleosomes with loose superhelical termini.

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The centromere is the chromosomal locus that serves as the site of kinetochore formation and spindle microtubule attachment in mitosis. Centromere protein A (CENP-A) is a histone H3 variant that epigenetically specifies centromere location. Conflicting evidence has emerged regarding the histone composition and stoichiometry of CENP-A nucleosomes, as well as the

handedness of DNA that wraps them. While we have proposed that these disparate models might be reconciled by a cell cycle-coupled program of centromeric nucleosome maturation, the predominant form of the CENP-A nucleosome remains controversial. Indeed, two very recent microscopy-based studies (Bui et al., 2012, *Cell* 150:317-326; Shivaraju et al., 2012, *Cell* 150:306-316) propose that CENP-A exists in half-nucleosomes or 'hemisomes' (i.e. one copy each of CENP-A, H4, H2A, and H2B) for the majority of the cell cycle. We have developed novel genomic analysis tools that we marry to existing massively parallel paired-end, long-read DNA sequencing technology to probe CENP-A nucleosome structure at centromeres. Using this approach in patient-derived cells harboring a neocentromere—a newly arisen functional centromere—we simultaneously identify the length and complete sequence reads of nucleosome positions at base pair resolution on normal repetitive centromere DNA and the unique sequences underlying neocentromeres. Unlike bulk nucleosomes from the same preparations that predominantly protect a single peak size (~150 bp) from nuclease digestion, CENP-A nucleosomes occupy two major peak sizes (~130 and ~110 bp) and a minor peak (~150 bp). Each peak is substantially longer than what could be protected by a hemisome or any other tetramer of histones (i.e. protection of ~60-80 bp from nuclease digestion). Further, the prominent position on centromeric DNA of the 110 bp peak is internal to the most prominent position of the 130 bp peak, indicating a stable, protected nucleosome core with loose termini. Strikingly, pure, reconstituted octameric CENP-A nucleosomes yield similarly sized terminally digested DNA fragments under conditions where their canonical H3-containing counterparts protected ~150 bp of nucleosomal DNA. Our findings indicate that the fundamental unit of functional centromeric chromatin is an octameric nucleosome with loose termini.

1917

The kinetochore protein CENP-Q directs chromosome movement by controlling microtubule dynamics.

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During mitosis the faithful alignment of chromosomes to the metaphase plate and their segregation during anaphase are crucial to ensure accurate partitioning of genetic material into the two daughter cells. These processes are mediated by the kinetochores, protein complexes that assemble at the centromere of each chromatid providing an attachment site for bundles of spindle microtubules (k-fibers) as well as driving chromosome movement. Here we show that the kinetochore protein CENP-Q, a subunit of the CCAN, is required for normal kinetochore movement during mitosis. Depletion of CENP-Q by siRNA results in chromosome congression defects with a polar chromosome population and a pronounced mitotic delay. High temporal and spatial resolution imaging of kinetochores revealed that normal oscillatory dynamics are perturbed in CENP-Q depleted cells, as was the ability of these kinetochores to make possessive movements in one direction. Kinetochore movements are largely powered by microtubule dynamics and our previous work has shown that CENP-Q is able to bind microtubules (Amaro et al., *Nat. Cell. Biol.*, 12: 319-29, 2010). We find that depletion of CENP-Q increases the number of kinetochore-bound microtubules, reduces their rate of turnover but decreases their attachment stability. Using in vitro reconstitution we find that purified CENP-Q directly modulates microtubule dynamics. We propose that CENP-Q directly controls kinetochore-microtubule dynamics to promote chromosome congression during mitosis.

1918

Deformations within Moving Kinetochores Reveal Different Sites of Active and Passive Force Generation.

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Kinetochores mediate chromosome segregation at mitosis. They are thought to contain both active, force-producing and passive, frictional interfaces with microtubules whose relative locations have been unclear. We inferred mechanical deformation within single kinetochores during metaphase oscillations by measuring average separations between fluorescently labeled kinetochore subunits in living cells undergoing mitosis. Inter-subunit distances were shorter in kinetochores moving toward poles than those moving away. Inter-subunit separation decreased abruptly when kinetochores switched to poleward movement, and decreased further when pulling force increased, suggesting that active force generation during poleward movement compresses kinetochores. The data revealed an active force-generating interface within kinetochores, and a separate passive frictional interface located at least 20 nm away poleward. Together, these interfaces allow persistent attachment with intermittent active force generation.

1919

A New Fidelity Mechanism Operating at the Kinetochore During Chromosome Segregation.

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Fidelity of chromosome segregation requires the spindle checkpoint, which senses unattached kinetochores, and Aurora B kinase, which detects and corrects attachment errors. Here, we describe a new fidelity mechanism mediated by direct crosstalk between the dynein motor module and the Ndc80 complex at the kinetochore. We show that the core microtubule-binding activity of the kinetochore, resident in the Ndc80 complex is inhibited by removal of dynein from the kinetochore. Prior work had proposed that Rod/Zwilch/Zw10 (RZZ) complex, which recruits dynein, is responsible for this inhibition and transiently regulates the core microtubule attachment made by the Ndc80 complex in early mitosis. We have identified Ndc80 complex variants that are resistant to RZZ-mediated inhibition *in vivo*, employing a single copy transgene insertion system in the *C. elegans* embryo. Mutation of 4 Aurora B sites in the basic N-terminal tail of Ndc80 did not affect regulation by RZZ *in vivo*. In contrast, deletion of the Ndc80 tail eliminated RZZ-mediated regulation even though it did not affect load-bearing attachment formation or lead to visible missegregation in the one-cell embryo. The Ndc80 tail deletion, but not the Aurora B site mutant, was compromised for viability and displayed a phenotypic spectrum consistent with reduced fidelity of chromosome segregation. Biochemical and yeast two-hybrid analysis revealed a direct interaction between the Rod subunit of RZZ and Ndc80 that is dependent on the basic tail of Ndc80. Taken together, these findings suggest that RZZ directly interacts with Ndc80 at the kinetochore in an Ndc80 tail-dependent manner to hold the microtubule-binding activity of the Ndc80 complex in check until a dynein-dependent reaction turns off this inhibition. We propose that this regulation, which is distinct from the spindle checkpoint and Aurora B kinase-mediated error correction, acts as a mechanical fidelity mechanism, ensuring the proper hand-off from dynein-mediated initial capture to Ndc80-mediated stable end-on attachments during chromosome segregation.

1920

Spatial contribution of chromatin and kinetochores to microtubule nucleation and meiotic spindle assembly *in vitro*.

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We can distinguish two important regions on chromosomes taking part in microtubule (MT) nucleation: the chromosome arms (chromatin) and kinetochores. These two areas send two different signals to trigger MT nucleation. It's been recently shown that kinetochores play a larger role in the chromosome-mediated nucleation pathway of mitotic spindle assembly compared with chromatin in centrosomes ablated cells. Importantly, *Xenopus laevis* eggs lack centrosomes and therefore, egg extract serves as a perfect system for investigating chromatin-mediated spindle formation. The RanGTP gradient created by RCC1 localization on chromosome arms is sufficient for MT nucleation and spindle assembly. However spindles can assemble in *Xenopus* egg extract also in the absence of RanGTP gradient, but in that case they form around sperm nuclei and not around chromatin-coated beads indicating that kinetochores are required. Recent work further showed that kinetochore signaling involves Aurora B kinase, a part of the Chromosomal Passenger Complex (CPC). For precise analysis of signaling pathways involved in chromosome-mediated MT nucleation we physically separate different signals that are normally overlapping in living cells. Using RCC1 coated beads and CPC coated beads in our *in vitro* spindle array we can discriminate the specific contribution of the two signaling pathway for MT nucleation, the temporal order and importance of each one of them.

1921

Investigating the structural and mechanical properties of the kinetochore by combining laser microsurgery and confocal microscopy.

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Accurate chromosome segregation during cell division ensures equal partitioning of the genome, and depends on proper attachment of microtubules (MTs) to kinetochores (KTs). The KT is a multidomain, multi-layer structure that assembles during mitosis to create the MT-binding sites on the centromere, thus playing a key role in ensuring accurate chromosome segregation. Although mounting evidence suggests that the mechanical properties of KT may contribute to faithful chromosome segregation, an in-depth characterization of such properties is still lacking. In the present study, we used merotelic KT as a tool to investigate the biophysical properties of the vertebrate KT, with particular focus on its plastic and elastic properties. Merotelic KT are bound to MTs emanating from opposite spindle poles, and during anaphase, they lag behind at the spindle equator and become stretched due to pulling forces exerted by MTs emanating from the two spindle poles. To study the elasto-plastic properties of the KT, we used PtK1 cells stably expressing GFP-tagged Hec1 (outer KT component), in which we promoted the formation of merotelic KT using a nocodazole-washout protocol. We then microinjected X-rhodamine-labeled tubulin to visualize the KT fibers in cells that displayed merotelically attached anaphase lagging chromosomes. Finally, we performed laser ablation on one of the two MT bundles attached to the merotelic KT, and analyzed the changes in KT size/stretching upon release of the pulling forces from one side. We found that stretched KT progressively shortened after MT severing with a very significant decrease in length immediately following the ablation, after which a majority of KT continued to progressively and slowly shorten over time, whereas for a smaller fraction of KT the shortening seemed to arrest after the change that immediately followed the ablation. Preliminary observations also indicate that

none of the KT's shortened enough to regain the size of un-stretched KT's, suggesting that the outer KT displays both elastic and plastic properties. Future experiments will be performed to investigate the behavior, and thus the biophysical properties, of different KT domains (e.g., inner vs. outer KT), with the aim of unveiling the biophysical properties of the KT and its subdomains.

Mitosis III

1922

The role of KIF4A in microtubule organization and midbody formation

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The spindle is a microtubule-based machine used for the segregation of chromosomes into two daughter cells. During anaphase and through to cytokinesis, anti-parallel microtubules of the spindle midzone undergo radical repositioning, from relatively loosely associated, individual microtubules, into a highly organized and compact midbody. The midbody acts as a docking site for numerous proteins essential for the finale of cell division, abscission. Midbody formation is orchestrated by the recruitment of several key midbody components in a spatio-temporal dependant manner.

KIF4A is a plus-end directed kinesin motor protein with a dynamic localization pattern: It is nuclear (during interphase), and during mitosis it localizes to the chromosome axes, midzone microtubules and midbody. Previous studies have revealed functions for KIF4A in chromosome condensation, chromosome segregation and spindle organization. The purpose of this study was to further characterize the role of KIF4A during late mitosis and cytokinesis.

We have established a KIF4A conditional knockout in chicken DT40 cells, where expression of KIF4A is rapidly suppressed through the addition of doxycycline to culture media. Using both fluorescence and correlative light electron microscopy (CLEM) we have analyzed KIF4A depleted cells, in which frequent abscission failure was observed. It is likely that this phenotype was a result of several other defects identified, including: an abnormal spindle length, mis-localization of other midbody proteins and a loss of midbody microtubule organization. These results begin to further define the role for KIF4A in the regulation of midzone and midbody microtubule organization and dynamics.

1923

Behavior of Sister vs. Non-Sister Pericentric Chromatin Reveals Motor and Chromatin Cross-linking in the Spindle.

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In the mitotic spindle apparatus, spindle microtubules exert an extensional force pulling sister chromatids towards opposite poles while the pericentric chromatin, chromatin proximal to the microtubule attachment site, resists with contractile spring-like properties. Tension generated from these opposing forces silences the checkpoint giving way to segregation. However, it is unknown how the cell senses tension across multiple attachment sites bound to microtubules with stochastic dynamics. In budding yeast, there is one microtubule attachment site per chromosome. Using this model system we label the pericentric chromatin on two different chromosomes using LacO/LacI and TetO/TetR. Non-sister attachment sites on the same side of

the spindle display coordinated motion dynamics relative to the spindle pole whereas sisters are not coordinated. Pericentromere stretching occurs rarely in wild-type cells, visualized as LacO/LacI-GFP transitioning from a focus into a line signal. Pericentromere stretching is also coordinated between different chromosomes (non-sisters) while sisters show no coordinated stretching. The coordinated motion and stretching is dependent on the kinesin 5 motor, Cin8. In addition, coordinated stretching is also dependent on the chromatin-linking complex cohesin. Both the cross-linking of microtubules by motors and chromosomes by cohesin function to coordinate motion in the mitotic spindle.

1924

RanBP2 interacts with Karyopherin beta1 to orchestrate accurate chromosome segregation in mitosis.

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Cancer cells often display perturbed chromosome segregation and disorganized mitotic spindles during cell division. Several nuclear pore proteins, including RanBP2/Nup358, are often abnormally expressed in cancers. Recent studies indicate a link between RanBP2/Nup358 and chromosome segregation during mitosis; however, the molecular details underlying this dynamic process remain unknown. Here we report a novel interaction between RanBP2/Nup358 and karyopherin beta/importin-beta that functions synergistically in the control of dynamic chromosome segregation. We found that RanBP2/Nup358 directly interacts with the N-terminal of karyopherin beta1 and that karyopherin beta1 binds to the Ran-binding domain (RBD) to zinc finger motif region of RanBP2/Nup358. Observed through live cell imaging, RNA interference-mediated knockdown of RanBP2/Nup358 caused a severe chromosome mis-segregation phenotype and disrupted karyopherin beta1 expression and localization. On the other hand, karyopherin beta1 RNAi also disrupted Nup358/RanBP2 localization and RanGAP1 sumoylation. Notably, we performed a series of rescue and dominant negative experiments to further confirm that the RanBP2-RAN GAP1-karyopherin beta1-RAN subcomplexes orchestrate proper chromosome segregation.

1925

Removal of antagonistic spindle forces can rescue metaphase spindle length and reduce chromosome segregation defects.

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Metaphase describes a phase of mitosis where chromosomes are attached and oriented on the bipolar spindle for subsequent segregation at anaphase. In diverse cell types, the metaphase spindle is maintained at a relatively constant length. Metaphase spindle length is proposed to be regulated by a balance of pushing and pulling forces generated by distinct sets of spindle microtubules and their interactions with motors and microtubule-associated proteins (MAPs). Spindle length appears important for chromosome segregation fidelity, as cells with shorter or longer than normal metaphase spindles, generated through deletion or inhibition of individual mitotic motors or MAPs, showed chromosome segregation defects. To test the force balance model of spindle length control and its effect on chromosome segregation, we applied fast microfluidic temperature-control with live-cell imaging to monitor the effect of switching off different combinations of antagonistic forces in the fission yeast metaphase spindle. We show

that spindle midzone proteins kinesin-5 cut7p and microtubule bundler ase1p contribute to outward pushing forces, and spindle kinetochore proteins kinesin-8 klp5/6p and dam1p contribute to inward pulling forces. Removing these proteins individually led to aberrant metaphase spindle length and chromosome segregation defects. Removing these proteins in antagonistic combination rescued the defective spindle length and, in some combinations, also partially rescued chromosome segregation defects. Our results stress the importance of proper chromosome-to-microtubule attachment over spindle length regulation for proper chromosome segregation.

1926

Characterization of mitotic defects in transformed cells.

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Advances in techniques such as whole genome RNAi screening have led to the finding that cancer cells can become dependent on specific proteins that are non-essential to healthy cells. Interestingly, several such 'synthetically lethal' proteins have been identified that are specifically involved in mitotic cell division. This suggests that the cell cycle in cancer cells has been altered to result in fundamental changes in mitotic chromosome segregation when compared to normal cells. To understand these changes we have generated a set of cell lines that model different levels of the transformation process. Genetic changes were introduced stepwise to primary retinal pigment epithelial cells to create a spectrum of four cell lines that increase in tumor forming capacity. Specifically, cells were modified so that they accumulated specific traits associated with transformation including: (i) potential for infinite replication by expression of hTert, (ii) loss of contact inhibition and anchorage dependence by inhibiting tumor suppressor proteins, (iii) ability to stimulate proliferation pathways independent of environment by hyperactivating Ras. Using these cell lines, we demonstrate that as cells undergo the transformation process, errors in chromosome segregation are increased. In addition, cells decrease in their ability to generate normal kinetochore-microtubule attachments as they undergo transformation. Finally, using this system, we have determined that transformation alters the requirement or regulation of specific kinetochore proteins.

1927

Mitotic Misregulation and Aging.

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Cancer is primarily an age-related disease. How aging promotes late-life cancer remains largely unknown. Our working hypothesis is that cellular aging compromises mitotic fidelity leading to chromosomal instability. To address this issue we have ascertained the effect of both natural aging and replicative senescence (in vitro model of aging) in mitotic progression using long-term time-lapse phase contrast microscopy. We performed comparative analysis between dermal fibroblasts derived from neonatal, young-age and old-age humans and mice, as well as between low passage and high passage/senescent fibroblasts. We found a significant mitotic delay in both aged and replicative senescent adult fibroblasts. High resolution time-lapse microscopy of those fibroblasts indicated a prometaphase delay, which was shown to be dependent on the activation of the spindle assembly checkpoint (SAC). Consistently, fixed cell analysis revealed increased number of prometaphase cells with spindle defects and chromosome misalignment. Furthermore, we found a higher frequency of aneuploid and polyploid cell karyotypes. Our

working model is that by inducing chromosomal instability, aging might lead to potential loss of tumor suppressor pathways.

1928

Chromosomes mis-segregated into micronuclei trigger chromosomal instability by further mis-segregating at subsequent mitoses.

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A micronucleus (MN; plural MNi) is a small nucleus that emerges from a chromosome or chromosome fragment that fails to segregate into a daughter nucleus during cell division. The mitotic behavior of a chromosome or chromosome fragment enclosed in a MN is not clear. Here, we specifically focused on whole-chromosome MNi and investigated the behavior of such chromosomes (mnChrs) at the mitotic cell division following MN formation. To this end, we used PtK1 cells and experimentally induced anaphase lagging chromosomes, which are known to form MNi upon mitotic exit. The day following MN formation, many micronucleated cells underwent further mitotic division. High-resolution phase contrast time-lapse microscopy showed that 89% of dividing micronucleated cells exhibited chromosome segregation errors, including anaphase lagging chromosomes, unaligned chromosomes, and MN reformation. To investigate the contribution of mnChrs to such segregation errors, we used PtK1 cells expressing Photoactivatable GFP-histone H2B, and marked MNi in prophase cells, just before nuclear envelope breakdown. We found that the majority of micronucleated cells progressed into anaphase and segregated their chromosomes. However, the sister chromatids from mnChrs often failed to fully separate at anaphase and co-segregated to the same daughter cell. Moreover, most of the mnChrs formed new MNi in the daughter cells, suggesting that MNi can self-sustain. We also observed that many of the mnChrs were not fully condensed at the time of nuclear envelope breakdown. Accordingly, quantitative fluorescence microscopy showed histone H3 (Ser10) phosphorylation on most mnChrs was delayed compared to chromosomes in the main nucleus of the same prophase cells. Finally, we found that such under-condensed mnChrs could become trapped by the cleavage furrow causing furrow regression and cytokinesis failure. In conclusion, our work shows that although anaphase lagging chromosomes frequently segregate to the correct daughter cell, the MN that they form represents a severe threat to chromosomal stability at subsequent cell cycles.

1929

Activation of p38 prevents proliferation in response to aneuploidy

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Most solid tumors are highly aneuploid and this abnormal number of chromosomes is generated by persistent loss and gain of chromosomes in a process called chromosomal instability. Research from the past few years has shown that normal, diploid cells arrest due to p53 stabilization following chromosome mis-segregation, preventing aneuploid cells from accumulating in healthy tissues. It remains unclear by what mechanism p53 is activated to prevent healthy diploid cells that have mis-segregated chromosomes from proliferating and how this is overcome in cancer cells. We sought to determine what occurs upstream of p53 to generate a cell cycle arrest following chromosome mis-segregation. Here we show that when we induce chromosome mis-segregation in otherwise diploid cells, we see activation of the p38 stress kinase pathway. Within 2 hours of inducing chromosome mis-segregation, we see increased levels of phospho-p38 as well as phosphorylated forms of p38 activators MKK3/6 and

downstream targets HSP27 and MAPKAPK2. Activation of p38 is followed by accumulation of p53 and p21, verifying what has previously been published. This response does not appear to be due to elevated levels of reactive oxygen species, contrary to what has been shown previously for cells with a deficient mitotic checkpoint, suggesting alternative mechanisms may exist to prevent proliferation under conditions of high and low levels of chromosome segregation. In conclusion, our data suggest that a p38 dependent pathway generates an early response followed by proliferative arrest in diploid cells that have mis-segregated chromosomes. We are currently investigating upstream regulators of p38 to determine what signal aneuploidy generates to evoke this p38 response.

1930

A genetic screen to identify components involved in correct transmission of broken chromosomes.

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The mechanisms that safeguard cells against aneuploidy are of great interest as aneuploidy contributes to tumorigenesis. To gain insight into these mechanisms, we studied the behaviour of cells entering mitosis with damaged chromosomes. We used the endonuclease I-Crel to generate acentric chromosomes in *Drosophila* larvae. While I-Crel expression produces acentric chromosomes in the majority of cells, remarkably, it has no effect on adult survival. Live studies reveal that acentric chromatids segregate efficiently to opposite poles. The acentric chromatid poleward movement is mediated through DNA tethers that connect the acentric fragment to its centric partner. These tethers are decorated with Polo kinase, a key mitotic regulator, the spindle checkpoint component BubR1 and two chromosomal passenger complex (CPC) proteins, INCENP and Aurora-B. Reduced BubR1 or Polo function results in abnormal segregation of acentric chromatids, a decrease in acentric chromosome tethering and a great reduction in adult survival. This led to the proposal that BubR1 and Polo facilitate the accurate segregation of acentric chromatids by maintaining the integrity of the tethers that connect acentric and centric fragments.

To identify novel *Drosophila* genes involved in this mechanism, we are taking advantage of our observation that when the function of BubR1 or Polo, two components involved in tether function, is reduced, the survival of third instar larvae to adulthood is dramatically decreased upon I-Crel expression. We have performed an unbiased genetic screen to identify mutations that are synthetic lethal with I-Crel expression. The genes involved in tether function are likely to have additional roles in mitosis or other physiological functions. Consequently, the probability that homozygote null mutations of these genes induce lethality prior to adulthood is high, as is the case for key mitotic players already identified, eg BubR1 and Polo. Therefore, we have favoured the use of ethyl methyl sulfonate, which creates point mutations that are likely to be hypomorphic. We will describe the identification of mutations on the X chromosome that are synthetic lethal with I-Crel expression.

1931

***In vivo* measurement of chromosome stretching tension in budding yeast.**

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During cell division, a microtubule-based mitotic spindle is built by the cell which acts to align and stretch duplicated sister chromatids prior to their ultimate segregation into daughter cells. This chromosome stretching tension is thought to provide an important tension-based signal that promotes proper chromosome segregation, and thus contributes to the prevention of

aneuploidy. However, the magnitude of chromosome stretching tension forces during mitosis have not been characterized. By using a novel imaging-based technique to measure thermal fluctuations of chromosome-associated fluorescent markers, we calculated *in vivo* chromosome stiffness via the equipartition theorem. Our measurements of wild type chromosome stiffness in yeast yielded values of ~ 26 pN/ μ m, and this stiffness remains relatively constant over the range of *in vivo* stretching distances. We then used our stiffness measurements to quantitatively evaluate *in vivo* metaphase chromosome stretching forces in budding yeast cells. We found that chromosome stretching forces are ~ 9 pN, which are well above thermal forces, and are surprisingly high given the presence of single kinetochore-microtubule attachments in budding yeast. These high forces are consistent with a model in which kinetochore-microtubule attachments could be stabilized by mechanical chromosome stretching tension.

1932

Sgo1 recruits PP2A to chromosomes to ensure sister chromatid bi-orientation in mitosis.

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To ensure proper chromosome segregation into mother and daughter cells, each sister-chromatid pair must be bi-oriented on the mitotic spindle. Errors in sister-chromatid attachment are detected by the spindle assembly checkpoint, which inhibits the initiation of anaphase until attachment is corrected. The protein Sgo1 is required in budding yeast mitosis for normal error correction, but its precise function has not yet been identified. Sgo1 recruits the PP2A subunit Rts1 to centromeres in meiosis, and deletion of either protein causes sensitivity to spindle poisons in mitosis. Thus, the mitotic function of Sgo1 might be to recruit PP2A-Rts1 to the centromere, where it somehow promotes correct sister-chromatid attachment. Consistent with this hypothesis, we found, using fluorescence microscopy in budding yeast, that Sgo1 is required for Rts1 localization to the centromere in mitosis. Mutations in the predicted PP2A-binding region of Sgo1 both abolish Rts1 localization and prevent proper sister chromatid bi-orientation after treatment with spindle poisons.

In early anaphase, both Sgo1 and Rts1 disappear from the centromere. Cellular Sgo1 levels also decline abruptly in anaphase, suggesting that its removal from the kinetochore, and thus the removal of Rts1, might depend on destruction of Sgo1 via the anaphase promoting complex (APC). We tested this possibility and found that budding yeast Sgo1 is a substrate for ubiquitination by the APC *in vitro*. We identified a motif required for its ubiquitination, and deletion of this motif rendered it resistant to degradation *in vivo*. Stabilized Sgo1 had no clear mitotic phenotype, and stabilized Sgo1 (as well as Rts1) still detached from the centromere at anaphase. We are currently pursuing the other regulatory mechanisms that govern the removal of these proteins from the chromosome.

1933

Cell lineage-specific cell cycle abnormalities in a kleisin- β mutant mouse

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Condensin complexes regulate higher order chromosome structure and are required for chromosome partitioning during mitosis. This essential function has largely precluded their study in the context of mammalian development. We describe highly cell lineage specific phenotypes in a mouse carrying homozygous missense mutations in *Cap-H2*, encoding the kleisin- β subunit of condensin II. Surprisingly, homozygous *Cap-H2*^{nes/nes} mice are viable and fertile, indicating that this lesion does not perturb condensin function in most cell lineages. However, *Cap-H2*^{nes/nes} homozygotes show a severe but incomplete block during thymic T cell development. In

wildtype resting thymocytes, productive VDJ recombination at the *T Cell Receptor (TCR) β* locus triggers an intense burst of cellular proliferation before cell cycle exit in G1/G0 and rearrangement of the *TCR α* locus. *Cap-H2^{nes/nes}* thymocytes express TCR β chains at normal levels and re-enter the cell cycle, but transcriptome differences between stage-matched mutant and wildtype cells are better explained by cell cycle abnormalities than by direct effects on transcription. Consistent with these findings, nesy thymocytes frequently arrest with a 4n, rather than 2n DNA content and exhibit prolonged metaphase during *ex vivo* culture. The 4n arrested cells lack sister chromatid cohesion, suggesting that they originate from abortive cytokinesis. This results in a >100-fold reduction in the CD4+/CD8+ thymocyte subset, and a reduced peripheral T cell pool. These data demonstrate that subtle perturbation of a condensin II component has catastrophic consequences during T cell development, yet causes little or no problem in other cell lineages, including the parallel pathway of B cell development. Thus, T cell development may impose atypical demands on the chromosome segregation machinery.

1934

Mitotic cells have a proximity sensor that utilizes histone H3.3 Ser31 phosphorylation to identify and mark individual misaligned chromosomes.

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During mitosis chromosomes (chrms) become congressed at metaphase, as their kinetochores bind to microtubules (MTs) emanating from spindle poles. The balance of force between k-fibers and chrm cohesion at centromeres silences the checkpoint that down-regulates cell cycle progression into anaphase. Congression and spindle assembly are often coordinate, but any lagging chrms automatically generate this “wait anaphase” signal to ensure that anaphase does not precede congression, thus preventing segregation mistakes that can generate aneuploidy. However, it is not clear whether misaligned chrms create other positional cues, outside of the kinetochore-mediated spindle assembly checkpoint (SAC). Here we show that misaligned chrms generate a “proximity sensor” that is distinct from the SAC and monitors chrm position within the dividing cell, relative to the position of the other chrms. This sensor involves the highly conserved histone variant H3.3, which assembles into nucleosomes via a replication-independent mechanism, and differs from the canonical H3 by 5 AA substitutions (Cell 2004 116:51-61). One of these is Serine 31, which is conserved from yeast to humans, and during mitosis becomes phosphorylated at the centromeres of all congressed chrms (PNAS 2005 102:6344-9). Here we find that misaligned chrms are decorated with anti-S31P along their arms, whereas congressed chrms in the same cell are decorated only at their centromeres. S31P labeling is highest on severely misaligned chrms that are outside the spindle, i.e. located within the astral MT array between a pole and the cortex. These single, mono-oriented chrms do not recruit chromokinesins -4, -10, or -12 to their arms, but do show a dramatic increase in the amount of kinesin-7 (CENP-E) at kinetochores, which drives chrm congression in the absence of k-fiber attachment (NCB 2009 11:832-8). If BSC1 cells are treated with nocodazole (Noc), MTs disassemble, and the SAC is activated, but there is no increase in S31P at centromeres or localization along arms. However, when Noc is added for 1 hr, then washed out, there is a substantial increase in misaligned chrms, and often single poles detach from the spindle – along with a varying numbers of chrms (1-6). We show that there is a 12x increase in the amount of S31P along individual, misaligned chromosomes associated with a detached pole, compared to labeling of congressed chrms. Interestingly, the levels of S31P sharply decrease as >1 chrms accumulate near a detached pole. This suggests that clustered chrms activate PP1, which normally dephosphorylates S31 H3.3 during anaphase (PNAS 2005 102:6344-9). We have also identified the H3.3 S31 kinase as Aurora A. We propose a model, in which H3.3 S31P increases along the arms of misaligned chrms. As they congress they are brought in proximity of other chrms, and S31P is removed by PP1. The marking of misaligned chrms may recruit

factors necessary to maintain cohesion, or facilitate spindle attachment and congression. We are currently investigating potential binding partners for S31P. However, this is the first time a mitotic function has been described for either H3.3 or its S31P modification. H3.3 is often mutated in cancer cells, suggesting it plays a key role in maintaining ploidy (Nature 2012 482:226-31).

1935

Insight into the Coordination of Pole-to-Pole Separation Forces During Spindle Assembly.

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The mitotic spindle, a transient bipolar array of microtubules, segregates duplicated chromosomes during cell division. Establishment of proper spindle length and bipolarity are essential for its function and are achieved by a concert of forces that regulate both microtubule organization and dynamics. A largely unexplored problem is how individual force components are integrated to generate a structure of constant shape and size at steady state. For example, the coordination of pole-to-pole separation forces acting on kinetochore-microtubules (KMTs) and non-KMTs is not understood. We found that Kif15, a kinesin-12 believed to act redundantly to kinesin-5/Eg5, localizes specifically to KMTs and enriches at the kinetochore-microtubule interface. Depletion of Kif15 reduces spindle length in a KMT-dependent manner. Live cell imaging of spindle assembly in Kif15-depleted cells shows that spindles initially become abnormally long, and then undergo contraction to their characteristically short steady state length. Contraction requires KMTs, suggesting that instability of KMTs alters the force balance within the spindle of Kif15-depleted cells. Consistent with this notion, Kif15 protects KMTs from cold-induced depolymerization. Since Kif15 concentrates at the outer kinetochore, we speculate that it stabilizes microtubule plus-ends directly and are currently testing this using *in vitro* approaches. Our results suggest that Kif15 promotes spindle bipolarity by mediating kinetochore-generated pushing forces involved in spindle pole separation. Thus, Eg5 and Kif15 cooperate to establish spindle bipolarity by acting in parallel: Eg5 affects non-KMT organization whereas Kif15 regulates KMT plus-end dynamics. Our results also demonstrate that the coordination of KMT length and non-KMT sliding is critical for spindle length homeostasis.

1936

Molecular mechanisms governing extrinsic forces in mitotic spindle organization.

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The presence of supernumerary centrosomes in cancer cells often creates the potential for catastrophic multipolar divisions. However, many cancer cells successfully divide because of mechanisms that suppress multipolar mitoses by clustering their extra centrosomes. Our functional genomic study has previously identified key pathways contributing to centrosome organization in cancer cells. In addition to spindle intrinsic forces, we found that cell adhesion patterns can determine the fate of mitosis whether they divide into two (bipolar) or more (multipolar). Yet, the mechanism by which actin and adhesion-dependent forces to control the fidelity of mitosis remain unclear at the molecular level. One model proposes that sites of strong cell matrix adhesion are imprinted as an actin rich structure called retraction fibers during mitosis, and the retraction fibers serve as cortical cues to concentrate force generators and regulators to pull centrosomes. By imaging cancer cells plated on fibronectin micropatterns, here we demonstrate that Myo10, an unconventional myosin identified from our genome-wide

screen, is an essential component linking retraction fiber-mediated forces to astral microtubules (MTs) during spindle organization. Myo10 specifically localizes along and to the tips of the retraction fibers. When cells with extra centrosomes were plated on Y-shaped fibronectin micropatterns, control cells divide tripolar toward Y-adhesion axis; however, cells depleted of Myo10 undergo bipolar division independent of retraction fiber positions, suggesting that Myo10 is a force coupler from retraction fibers to centrosomes. We have hypothesized that intrinsic properties of MTs adjacent to retraction fibers are altered to favor stable interaction between astral MTs and cell cortex. To uncover heterogeneity of MT dynamics in relation to retraction fibers, we have performed live cell imaging of GFP-EB3 with high spatio-temporal resolution in cells plated on fibronectin micropatterns where retraction fiber positions are precisely manipulated. We have developed a system that quantifies and visualizes MT dynamics in different subregions of cells by an automated computer program capable of tracking GFP-EB3 comets. This approach uncovered heterogeneity of MT dynamics around retraction fiber regions. Interestingly, our analyses have revealed that Myo10 is in part responsible for promoting the long-lived and long distance travelling MTs. Taken together, these results suggest that Myo10 is a key adhesion-dependent regulator that confers heterogeneity of mitotic cortex by coupling cell geometry/adhesion to mitotic fidelity and genome stability.

1937

The nucleoporin ALADIN is essential for proper mitotic and meiotic spindle function.

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The nuclear pore complex (NPC) is a large proteinaceous structure composed of multiple copies of 30 proteins termed nucleoporins (Nups). During interphase, the NPC is the sole conduit for the bidirectional transport of soluble molecules between the nucleus and cytoplasm. In higher eukaryotes, entry into mitosis is coincident with nuclear envelope breakdown, NPC disassembly, and dispersion of Nups. Many recent studies have demonstrated that some Nups have functions in mitosis. In order to probe the function of all nucleoporins in mitosis we carried out a comprehensive RNAi screen in S2 cells. We performed automated live and fixed analysis using S2 cells expressing mCherry-tubulin and H2B-GFP. Of the 29 dsRNAs used, 5 showed mitotic index alterations, spindle morphology and/or chromosome alignment defects. We have focused our subsequent studies on ALADIN (a nucleoporin that is mutated in the neurological disorder Allgrove/Triple-A Syndrome), which caused chromosome alignment and segregation defects and shortened mitotic spindles when it was depleted. In ALADIN depleted cells, the rate of microtubule flux in the mitotic spindle is significantly increased over control cells, Aurora B and the microtubule depolymerizing kinesin Klp10A enrich on aligned chromosomes. Curiously, ALADIN does not localize to the mitotic spindle or kinetochores, although it does localize around centrosomes and the mitotic spindle in human and *Drosophila* cells. In human cells, ALADIN colocalizes nicely with the spindle matrix proteins NuMA and Tpr. Spindles that form in HeLa cells lacking ALADIN are shorter and not cold-stable, and ALADIN depletion delays chromosome alignment after monastrol washout. We've found that pools of Sgo1, NuMA, and Plk1 are mislocalized after ALADIN depletion and Plk1 inhibition produces a similar, but not additive, decrease in spindle length, suggesting that ALADIN may function to regulate Plk1 activity. Intriguingly, female mice that lack ALADIN are sterile. Oocytes from ALADIN ^{-/-} mice fail to eject a polar body after the first meiotic division and have poorly aligned chromosomes and weak spindles at metaphase of meiosis II. We therefore hypothesize that ALADIN may be more essential in acentrosomal divisions. In support of this hypothesis we find that ALADIN depletion produces no phenotype in *Drosophila* embryos but is synthetically lethal in the early embryo when centrosomes are ablated. Curiously, the ALADIN meiosis phenotype can be rescued by

expressing a version of ALADIN/AAAs that contains a mutation commonly found in human Triple-A syndrome patients, which suggests that failures in mitosis may not underlie many of the phenotypes associated with Triple-A syndrome.

1938

Aneuploidy causes chromosome mis-segregation and karyotype-dependent phenotypes in cancer cells.

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Cancers display distinct aneuploid karyotypes (i.e., abnormal chromosome numbers) and typically mis-segregate chromosomes at high rates, a phenotype referred to as chromosomal instability (CIN). While it is readily apparent how chromosome mis-segregation can cause aneuploidy, the effect aneuploidy has on chromosome segregation is unclear. To test the effects of aneuploidy on chromosome segregation we utilized the colorectal cancer cell line DLD1 (2n=46) and variants of this line containing defined artificial trisomies for chromosomes 7 and 13 (DLD1+7 and DLD1+13, respectively). We found that DLD1+7 and DLD1+13 exhibited higher rates of chromosome mis-segregation compared to the parental cell line, with higher mis-segregation rates for the aneuploid chromosomes compared to other chromosomes. Further, there was an increase in near-tetraploid DLD1+13 cells, due to an increase in cytokinesis failure rates. Our data indicate that this is due to overexpression of the cytokinesis protein spastic paraplegia 20 (Spartin, or SPG20) located on chromosome 13q13.3. Indeed, overexpression of Spartin in DLD1 cells reproduced the cytokinesis failure phenotype observed in DLD1+13 cells. Conversely, siRNA-mediated Spartin knock down resulted in significant reduction of cytokinesis failure in the DLD1+13 cell line. Overall, our study shows that aneuploidy per se induces chromosome mis-segregation in cancer cells. Moreover, our data indicate that different aneuploidies yield distinct cellular phenotypes.

1939

Transient mitotic spindle defects as a cause of chromosomal instability in human cancer cells.

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Accurate chromosome segregation during mitosis is necessary for stability of the genome in developing organisms, and erroneous chromosome segregation is known to cause miscarriage, birth defects, and cancer in humans. Recent reports in both cancer and non-cancer cells have shown a link between transient abnormal mitotic spindle geometry and the establishment of kinetochore mis-attachments, which in turn cause chromosome mis-segregation in the form of anaphase lagging chromosomes. In particular, two mechanisms have been described, one of which occurs in cells that assemble a multipolar spindle but bipolarize before anaphase onset (transient multipolarity mechanism, TM); and one that is characterized by a delay in centrosome separation (incomplete spindle pole separation mechanism, ISPS), by which the centrosomes are not completely separated at the time of nuclear envelope breakdown (NEB), and instead achieve complete separation after NEB. In this study, we investigated the role that these mechanisms play in cancer cell chromosomal instability (CIN). To this end, we screened a panel of cancer cells from various sites to determine the prevalence of mitotic spindle geometry defects in early mitosis and how such defects correlate to chromosome segregation defects. Our data show that human cancer cells from various sites exhibit one or both of the two

transient mitotic spindle defects that can promote kinetochore mis-attachment and chromosome mis-segregation, thus indicating that transiently abnormal spindle geometry plays an important role in CIN.

1940

Stabilization of kinetochore-microtubule attachments is sufficient to induce chromosomal instability in human cells.

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Chromosomal instability (CIN) – a high rate of chromosome loss or gain – is common in aneuploid tumor cells. The most common cause of CIN is the persistence of errors in kinetochore-microtubule (kMT) attachments. Proper chromosome segregation requires sufficiently stable kMT attachments for chromosome movement, yet the attachments must be dynamic enough to permit efficient error correction before anaphase onset. To date more than 10 proteins have been demonstrated to regulate kMT attachment stability including stabilizers and destabilizers. We previously demonstrated that overexpression of kMT stabilizers or loss of destabilizers would induce CIN and that overexpression of destabilizing proteins in cancer cells is sufficient to suppress CIN. These data suggest that regardless of what protein is targeted, stabilizing kMT attachments is sufficient to drive chromosome missegregation and CIN. To test this we treated chromosomally stable, diploid cell lines with low doses of the MT stabilizing drug taxol. We show that the frequency of lagging chromosomes in anaphase increases from 1.0% to 8.4% in RPE-1 cells treated with 2nM taxol. Low dose taxol also increases the frequency of multipolar spindles. Moreover, FISH analyses of clonal populations of p53-deficient HCT116 cells show an increase in aneuploid cells from 5.8% to 14.7% following treatment with 2nM taxol. Finally, measurement of kMT attachment stability using photoactivation of GFP-tagged tubulin shows that low dose taxol treatment stabilizes kMT attachments from 1.8 ± 0.2 min to 2.9 ± 0.4 min in prometaphase and from 3.8 ± 0.4 to 6.0 ± 0.4 min in metaphase. These data demonstrate that stabilizing kMT attachments is sufficient to generate CIN in human cells.

1941

TAO1 maintains genome integrity by mediating the timely maturation of chromosome attachments made to lateral walls of microtubules.

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Chromosomes predominantly attach to lateral walls of microtubules and then transform the lateral attachment to ends of microtubules. Pathways that monitor and mediate this transformation process are critical to maintain genome integrity; however, poorly understood. We report TAO1 kinase as a novel regulator of lateral to end-on transformation as laterally attached kinetochore pairs of TAO1 depleted cells remain unresolved and in some cells, missegregate in a laterally attached fashion. Because the resolution of lateral but not syntelic attachments is perturbed following TAO1 depletion, we propose TAO1 as a specific destabilizer of lateral attachments. We show TAO1's role in mitotic microtubule destabilization and mediating anti-poleward movements of laterally attached chromosomes. Using an error-correction assay, we demonstrate the aneuploid nature of unresolved lateral attachments in TAO1 depleted cells and thereby, uncover a previously unrecognized step in kinetochore-microtubule attachment that prevents aneuploidy

1942

Regulation of microtubule attachment at kinetochores and cell cortex by Cdc42.*E. Vitiello¹, M. Balda¹, K. Matter¹; ¹Cell Biology, UCL Institute of Ophthalmology, London, UK, London, United Kingdom*

Maintenance of epithelial tissue integrity requires correct organization of the polarity plane. This is promoted by assembly of cell-cell junctions, defining distinctive apical and basolateral domains. The establishment of polarity is a key point in tissue organization since it controls the plane in which cells divide. When a cell replicates, it duplicates chromosomes and aligns them before partitioning them evenly between the two daughter cells. Although results from different laboratories indicate a role of cell-cell junctions in the orientation of the mitotic spindle, the coordination between junctions and chromosome capture/alignment is still poorly understood. Previous work suggests that the small Rho GTPase Cdc42 regulates chromosome capture. Cdc42 is also a crucial regulator of cell-cell junction assembly and dynamics. Cell junctions need to remodel during cell division to adapt to the changing cell shape, and allow the condensed chromosomes to be properly aligned. Therefore, we hypothesized that specific regulators of Cdc42 guide the interplay between cell junctions and the mitotic machinery. Using a siRNA approach, we identified a GTPase Activating Protein (GAP) that associates with cell-cell contacts and mitotic spindles, and regulates junctional integrity and chromosome alignment during mitosis. Upon depletion of the GAP in epithelial cells, the normally continuous immunofluorescence staining of junctional markers was disrupted in mitotic cells, and chromosomes were misaligned in metaphase. The orientation of mitotic spindles relative to the substrate was not affected, suggesting that the defect was not due to a loss of polarity. Depletion of the GAP led to increased levels of GTP-bound Cdc42, indicating that deregulation of Cdc42 contributed to the observed phenotypes. Indeed, transfection of dominant negative Cdc42 and partial depletion of Cdc42 by RNA interference rescued the phenotype induced by depletion of the GAP. Live cell imaging revealed that depletion of the GAP led to an extension of prometaphase/metaphase and that the defect in chromosome alignment coincided with a loss in chromosome oscillations. We also found that the GAP associates with the microtubule motor Kif1B, a kinesin-3 family member, and that depletion of Kif1B resulted in a similar phenotype as depletion of the GAP. Based on our observations, we speculate that the GAP regulates Cdc42 to guide association of microtubules with the cell cortex and the kinetochores and to regulate forces they exert at these sites.

1943

Towards Exploring the 3D Supramolecular Architecture of Centrosomes *in situ*.*J. Mahamid¹, A. Hyman², W. Baumeister¹; ¹Department of Molecular Structural Biology, Max-Planck Institute of Biochemistry, Martinsried, Germany, ²Max-Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany*

Centrosomes are the major microtubule organizing centers in most eukaryotic cells. Recent work characterized the structure of centrioles in some detail, however details on the organization of the pericentriolar matrix (PCM) at the molecular level are lacking, leaving the mechanism by which centrosomes nucleate, orient, and anchor microtubules largely obscure. The PCM represents an unusual protein assembly from a structural biology view-point. Its 3D structure is not solely governed by stereospecific interactions between a defined number of protein subunits. It rather assembles as an intracellular matrix formed at the scale of cellular organelles, with local order and substantial amount of disorder. As such, the PCM cannot simply be isolated and studied by the established methods of structural biology, and must therefore be studied within the cellular context.

Cryogenic-electron tomography (CET) in conjunction with pattern recognition methods has the unique potential to visualize the cellular proteome *in situ*, providing 3D images which are interpretable in molecular terms. Here it is applied to a first *in situ* ultrastructural analysis of centrosomes in *Caenorhabditis elegans* embryos and mammalian cell culture.

The application of CET to the investigation of centrosomes within the cell requires the development of novel approaches in sample preparation, primarily due the large dimensions of the specimen and secondly due to the low copy existence of the centrosome within the cell. Correlative Cryo-fluorescence measurements are essential to localize the centrosomes within the large cells and to identify target areas of interest for focused ion-beam (FIB) milling for the production of electron-transparent windows into the cell. CET on these preparations provides 3D view of centrosome organization at molecular resolution. These high resolution structural data are crucial to gain novel insights into the structure and function of centrosomes and of the yet poorly characterized PCM in particular.

1944

Functional genomic screen in human cells reveals novel regulators of centriole number.

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Centrioles are crucial notably for the formation of cilia and flagella, as well as for robust spindle assembly in many cell types. It is critical that cells harbor the correct number of centrioles. The mechanism regulating centriole number are incompletely understood, despite the fact that genetic and genomic approaches have led to the identification of components required for centriole assembly in several organisms. However, no comprehensive functional genomic screen has been reported for mammalian systems, in which centriole assembly may be orchestrated in a more complex manner than in unicellular or invertebrate systems. Therefore, we have developed and executed a genome-wide siRNA-based screen in human cells for genes required for proper centriole number. We used a cell line stably expressing the centriolar component Centrin1 fused to GFP to monitor centrosome number and analyzed the impact of 76138 distinct siRNAs targeting 19593 genes, using a 96-well-based platform. Cells were incubated with siRNAs for 72 hours before fixation, DNA counterstaining and image acquisition. We developed a MATLAB-based algorithm for the automatic counting of centrosomes in each cell, which lead to the preselection of a number of candidate genes. Several subsequent confirmatory steps led to the identification of a final number of 31 new candidates genes required for promoting centriole assembly and of 13 new candidates required for negatively regulating this process. We are in the process of further characterizing the role of a subset of these genes in the centriole duplication cycle.

1945

sas-1 maintains centriole integrity during spermatogenesis in *C. elegans*.

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Centrosomes, the primary microtubule organizing centers of animal cells, usually consist of a pair of centrioles surrounded by pericentriolar material (PCM). Centrosome number must be strictly regulated both in the soma and the germline. The single centrosome present at the onset of the cell cycle in somatic tissues duplicates to give rise to two entities, one of which is ultimately inherited by each daughter cell. Gametogenesis poses a unique challenge to this

general pattern of centrosome inheritance because one of the parental centrosomes must be eliminated so that only one centrosome is inherited by the zygote.

In most metazoan organisms, including *C. elegans* and *H. sapiens*, centrioles are eliminated during oogenesis and maintained during spermatogenesis, such that the correct number of centrioles is restored in the zygote upon fertilization. The mechanisms controlling such differential centriole inheritance in the female and male germline, though fundamental for development, are not understood. We use *C. elegans* as a model system to tackle this question. In a screen of paternal-effect embryonic lethal mutations, we identified two alleles of a locus we named *sas-1*. Embryos derived from homozygous mutant animals assemble a monopolar spindle during the first mitotic division. We found that fertilization of wild type oocytes by *sas-1* mutant sperm also confers this phenotype in one-cell stage embryos, whereas wild type sperm can rescue *sas-1* mutant oocytes during early embryogenesis. Serial-section EM analysis suggests that centrioles are abnormal in *sas-1* mutant sperm. Accordingly, centrioles endowed from *sas-1* mutant sperm are not stable and become structurally unrecognizable in the early embryo. Nevertheless, maternal SAS-4 and SAS-6 are recruited in the vicinity of these seemingly defective *sas-1* paternal centrioles. Furthermore, we find that SAS-1 is also required maternally for proper development. Through SNP mapping, whole genome sequencing and subsequent analysis, we uncovered that SAS-1 is a C2 domain-containing protein that localizes to centrioles. Consistent with a role at centrosomes, mutant SAS-1 proteins do not localize efficiently to centrioles. Finally, we show that SAS-1 does not exchange rapidly at the centriole, but instead seems to be recruited during centriole formation and hardly exchanges with the cytoplasmic pool thereafter.

1947

Measurements of forces produced by the mitotic spindle using optical tweezers.

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We used an optical trapping laser (optical tweezers) to measure the forces needed to stop chromosome movement in *Mesostoma* spermatocytes and crane-fly spermatocytes as well as the inward movement of poles in PtK2 cells after cutting across the spindle. We primarily used *Mesostoma* spermatocytes for these experiments because their kinetochores oscillate to and away from the spindle pole for a period of 1 to 2 hours during prometaphase/metaphase; thus we could trap different kinetochores multiple times in the same spermatocyte. We focussed a Nd:YVO4 continuous wave 1064 nm wavelength laser using a 63x oil immersion objective to a single point [Shi et al Microsc Res Tech 69 (2006) 894] in order to trap kinetochores that moved to or away from their poles. When a trap power of 15 mW to 23 mW was applied to the kinetochore, kinetochore movement stopped or the amplitude of the oscillations decreased, and kinetochore oscillations recovered when the trap was released. In crane-fly spermatocytes, less extensive experiments indicated that a trap power of 28 mW to 65 mW could stop or slow poleward chromosome movement. In PtK2 cells a trap power of about 8mW applied to the spindle pole stopped the spindle pole of the irradiated half-spindle from moving in following laser microbeam irradiations across the metaphase spindle. Using the equation $F=QP/c$, with Q coefficients of 0.09 for PtK spindle poles and 0.06 for chromosomes, P = power, c= speed of light, the force to stop PtK2 pole movements was 2.3 pN and the forces to stop chromosome movements in *Mesostoma* spermatocytes and crane-fly spermatocytes were 3 pN to 13 pN. These forces are two orders of magnitude lower than the force to move grasshopper

spermatocyte chromosomes measured via micromanipulation [Nicklas J Cell Bio 97 (1983) 542].

1948

Drosophila mech is Required for Cilium Formation.

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Cilia are conserved eukaryotic organelles that extend from cell surfaces to mediate motility and sensory functions. During ciliogenesis, centrioles elongate and migrate from the centrosome to the cell membrane to initiate axoneme formation, where they are called basal bodies. The region that lies at the interface of the basal body and the axoneme is termed the transition zone and is essential for proper cilium formation. We have identified mech, a *Drosophila* loss-of-function mutant that abolishes cilium formation. Interestingly, mech localizes to the ciliary transition zone. Using hidden Markov modeling, we have identified a highly conserved domain of mech. Mutation of this domain fails to rescue the mech phenotype, suggesting that this region is essential for mech function. We are currently examining the role of this domain in the context of mech function during ciliogenesis.

1949

Matrix stiffness affects mitotic spindle orientation.

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The extra-cellular matrix (ECM) provides cells with a complex array of biochemical and mechanical stimuli. Mechanical stimuli (stiffness of the ECM) can influence many cellular biochemical pathways that regulate cell proliferation, morphology, and differentiation. The signaling network regulating ECM-cell adhesion and cellular cytoskeleton dynamics help the cells to sense the stiffness of the ECM and transduce these mechanical stimuli into biochemical responses. While the influence of ECM stiffness on cell proliferation and morphology has been widely studied, the influence of micro-environment stiffness on mitosis remains poorly understood. In this study, we investigate how matrix stiffness affects the mitotic spindle apparatus. We found that matrix stiffness influences interphase actin cytoskeleton organization and cell morphology. In addition, matrix stiffness influences cortical actin organization in mitotic cells, which in turn could affect proper mitotic spindle orientation.

1950

Chromosome Biorientation by the Chromosomal Passenger Complex is Separable from Its Localization to the Inner Centromere.

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Accurate segregation of the replicated genome requires biorientation of chromosomes on the metaphase spindle, which is ensured by the kinase Aurora B/Ipl1, a member of the 4-subunit chromosomal passenger complex (CPC). Aurora B localization between sister kinetochores at the inner centromere, which depends on the CPC subunit Survivin, is thought to promote biorientation by eliminating incorrect kinetochore-spindle attachments not under tension. We engineered a truncation of the INCENP/Sli15 subunit of budding yeast CPC that eliminates association with Survivin/Bir1 but nevertheless supports proper chromosome segregation during both mitosis and meiosis. The truncated Sli15 fully rescues inviability of Bir1 and Borealin/Nbl1

deletion mutants, and suppresses deletion phenotypes of Bub1 and Sgo1, components responsible for targeting the CPC to the inner centromere via Bir1. This Sli15 mutant no longer localizes in between the sister kinetochores, indicating that the inner-centromeric pool of the CPC is dispensable for accurate chromosome segregation. Truncated Sli15 localizes to pre-anaphase spindle microtubules, suggesting that Aurora B/Ipl1 activation by clustering on microtubules is sufficient for biorientation. Consistent with this, premature targeting of Sli15 to microtubules by preventing Cdk1 phosphorylation partially suppresses the loss of Bir1. These findings suggest that tension-based discrimination of correct versus incorrect attachments occurs within the kinetochore, as opposed to relying on Survivin-dependent localization of the CPC between sister kinetochores. We propose that clustering-based activation of Aurora B/Ipl1 on either chromatin or on microtubules, coupled to kinetochore-intrinsic sensing of tension, is sufficient for chromosome biorientation.

1951

KNL1 mediates recruitment of Aurora B kinase to the kinetochore to regulate microtubule stability.

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Accurate chromosome segregation during mitosis depends on the correct attachment of kinetochores to spindle microtubules. Aurora B kinase destabilizes incorrect attachments by phosphorylating kinetochore substrates that directly interact with microtubules and it has been proposed that such phosphorylation depends on the distance of the substrates from an inner centromeric pool of the kinase. Here, we show that the kinetochore protein KNL1 mediates recruitment of active Aurora B to the outer kinetochore and this recruitment is required for the phosphorylation of Hec1, an Aurora B substrate involved in kinetochore-microtubule attachment regulation. We identify a region in KNL1 sufficient to rescue both active Aurora B localization and Hec1 phosphorylation in KNL1-depleted cells. A portion of this domain is also sufficient for the kinetochore recruitment of the checkpoint protein Bub1, which has been previously implicated in the regulation of Aurora B kinase activity. We find that Bub1 depletion partially inhibits outer kinetochore Aurora B binding and Hec1 phosphorylation, indicating that both Bub1 and KNL1 are crucial factors for optimal kinetochore Aurora B targeting. Our findings suggest that kinetochore-microtubule attachment stability is regulated by direct recruitment of Aurora B to the outer kinetochore rather than a concentration gradient of a spatially distant centromeric Aurora B population.

1952

Cep192/Aurora A/Plx1 pericentriolar material-recruiting complex is a key mediator of centrosome maturation.

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Centrosomes are the primary microtubule (MT)-organizing centers (MTOCs) and play an important role in cell division, polarity, and spindle assembly. They consist of a pair of centrioles and pericentriolar material (PCM), the amount and composition of which determine centrosome function. The role of centrosomes as MTOCs of the mitotic spindle relies on extensive recruitment, at mitosis onset, of additional PCM. This process, termed centrosome maturation, critically depends on the mitotic serine/threonine kinases Aurora A (AurA) and Plk1 and on several centrosomal proteins, including the key regulator of centrosome biogenesis,

Cep192/Spd-2. How these proteins interact at the molecular level to form a functional MTOC has been elusive.

To dissect the mechanisms underlying centrosome maturation, we have devised a set of assays that recapitulate basic aspects of this process in *Xenopus* egg extract. We found that Cep192 targets Aurora A (AurA) and Plx1 (*Xenopus* ortholog of Plk1) to centrosomes, where it assembles these protein kinases into a two-tiered cascade that drives centrosome maturation. Specifically, the Cep192-bound, T-loop-phosphorylated, AurA, phosphorylates Plx1 at its T-loop-located T201 (equivalent of T210 in human Plk1), thereby facilitating Plx1 self-priming and binding to a conserved threonine in Cep192. The active Plx1 then phosphorylates Cep192, at several serine residues located in regions flanking the AurA-interacting domain, to generate the attachment sites for the γ -tubulin ring complex (γ -TuRC) and, possibly, other PCM components. This set of events is essential for centrosome maturation and for the MT-organizing function of a recombinant N-terminal Cep192 fragment that binds AurA, Plx1, and γ -TuRC. Our study reveals a critical mechanism underlying centrosome maturation and identifies the tripartite Cep192/AurA/Plx1 complex, which we denote Pericentriolar Material-Recruiting Complex (PMRC), as a key mediator of this process.

1953

Evolution of GPR regulation in the control of spindle positioning for two *Caenorhabditis* species embryos.

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Asymmetric cell division relies on the proper positioning of the mitotic spindle. However, it is unclear whether genetic networks that control such a highly conserved cellular processes contain cryptic genetic variability. To address this question, we analyzed such a division within nematodes, which represents an evolutionary conserved phenotype. In *C. elegans*, this process occurs in two steps. First, the nuclei/centrosome complex (NCC) is centered and aligned along the anterior/posterior axis. Second, unbalance pulling forces displace the spindle toward the posterior pole of the cell and cause transverse spindle oscillations. By comparisons with *C. elegans* embryos, the NCC of *C. briggsae* embryos was shifted toward the anterior pole during prophase. In addition, *C. briggsae* spindle oscillations were delayed, shorter in duration, and lower in amplitude. The “tug-of-war” model proposes that oscillations – and thus pulling forces contributing to posterior displacement and spindle elongation – are driven by force generators processivity. We extended this model to account for interspecies phenotypic differences by including the dynamics of astral microtubules and found that centrosome needs to reach a posterior enough position to undergo oscillations. The interspecies differences could be explained by changes in levels and localization of the essential G protein regulator GPR. Importantly, localization of GPR to the posterior cortex was conserved between these species, which established a common positional switch for the onset of spindle oscillation. Oscillation onset and the final spindle position are robust to changes in cell size/shape, the initial position of the spindle, and levels of GPR within the active region. This model fully accounts for the phenotypic differences between both species and suggest that the posterior pulling forces are under the double control of processivity – putatively driven by cell cycle –and position of the spindle. We also uncovered cryptic genetic changes underlying an essential cellular process. Whereas the posterior localization of GPR represented a highly constrained parameter of this asymmetric cell division, variations in other aspects of GPR localization were clear manifestations of these cryptic changes.

1954

A tethered Dam1 ring suffices for a minimal force-bearing unit of a kinetochore.

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During mitosis the sister chromatids must segregate properly into the daughter cells. Two principal components are responsible for this task: the microtubules (MTs) and kinetochores, multi-protein chromosomal specializations. Dynamic MTs are thought to be the main anaphase force-generators, but the mechanism that couples such forces to chromosome movement is still unknown. Recent studies both in budding yeasts and *in vitro* using recombinant kinetochore complexes have identified the ring-shaped Dam1 complex as one of the most energy-efficient couplers which can transduce MT depolymerization into cargo movement. However, microbeads carrying this complex on their surface could capture no more than 5 pN from MT disassembly. On the other hand, a single MT is predicted to generate up to 75 pN (Molodtsov et al., 2005), and the force acting on the chromosomes in live cells is about 45 pN per MT (Nicklas, 1983). A plausible explanation for this poor force-transduction by Dam1 complex is that *in vitro* it is likely to promote lateral attachment of microbeads to MT walls. This geometry of a cargo attachment is significantly different from the the end-on MT attachment of metaphase and anaphase kinetochores *in vivo*. Here we show that tethering Dam1 oligomers to the beads with non-kinetochore linker proteins allows formation of the end-on MT-bead attachment and increases the measured force up to 29 pN. Beads with tethered Dam1 show 4-fold increase in force amplitude and 3-fold increase in force duration compared to the control beads that carry Dam1 complexes at their surface with no linker proteins. We also show that beads with tethered Dam1 move under tension with velocities similar to those reported for beads attached to isolated budding yeast kinetochores (Akiyoshi et al., 2010). Beads with tethered Dam1 can move processively under tension up to 30 pN, implying that Dam1 oligomers are bound strongly to the MT wall. Indeed, our direct measurement of Dam1 complex diffusion along MTs estimates a single Dam1 monomer-MT bond strength at 8-10 $k_B T$. Further, the force amplitudes obtained for tethered Dam1 beads are strikingly similar to the rupture forces measured for wild-type kinetochore particles, while the control beads resemble kinetochores isolated from *dad1-1* mutants (Akiyoshi et al., 2010). These results lead to the conclusion that tethered Dam1 complexes reconstitute the force-bearing unit of a budding yeast kinetochore.

Meiosis

1955

Cracking the *C. elegans* Eggshell.

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Metazoan oocytes have an extracellular coating that governs fertilization. Following fertilization, this covering is altered to prevent polyspermy and protect the developing embryo. In *C. elegans*, a vitelline layer covers oocytes prior to fertilization. Fertilization initiates conversion of

the vitelline layer into a trilaminar eggshell consisting of an outer vitelline layer, a middle chitin-containing layer, and an inner layer proposed to serve as a permeability barrier. Here, we characterize CPG-1 and CPG-2, functionally redundant chondroitin proteoglycans that are the first described protein eggshell components. We show that CPG-1 and CPG-2 are delivered to the extracellular space after formation of the chitin layer by cortical granule exocytosis during meiosis I. Although they contain multiple chitin binding domains, CPG-1 and CPG-2 localize to the inner eggshell layer, whereas chitin is confined to the middle layer. We show that the inner eggshell layer is not the permeability barrier for small molecular weight solutes. Instead, this function resides in a previously undescribed layer that assembles between the eggshell and the plasma membrane following meiosis II. Disruption of the permeability barrier leads to solute permeability and osmotic stress. Disruption of the inner CPG-1/2 eggshell layer causes these phenotypes, as well as adhesion of the embryonic plasma membrane to the eggshell and cytokinesis failure. Interfering with chitin layer assembly results in the inner layer phenotypes, plus polyspermy and catastrophic eggshell rupture. We conclude that the eggshell layers and permeability barrier are laid down in a step-wise and cell cycle-dependent fashion, with later assembly events requiring successful completion of previous ones. To build on this work, we conducted an RNAi screen to identify additional genes that regulate eggshell and permeability barrier assembly. Several screen hits rendered the eggshell permeable, with minimal deleterious effects on early embryonic development. We therefore developed a reliable method to permeabilize and immobilize embryos to allow acute drug/inhibitor treatment to study early embryonic processes with live imaging

1956

Identification and characterization of *mel-43*, a gene required for early embryonic development in *C. elegans*.

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The segregation of chromatin during meiotic and mitotic divisions in the embryo occurs via a bipolar microtubule-based structure called the spindle. The female meiotic spindle forms around chromatin without the contribution of centrosomes, even though the centriole pair is introduced into the oocyte cytoplasm with the sperm upon fertilization. The centrosomes mature and nucleate microtubules for mitotic spindle assembly only after the female meiotic divisions are complete (about 40 minutes after fertilization). Therefore, coordination of all cellular events during the meiosis-to-mitosis transition ensures that these two types of spindles do not form at the same time in the newly fertilized embryo. *mel-43* (*sb41*) is a dominant, temperature-sensitive, maternal-effect mutation that is required for early embryonic viability¹. These mutants exhibit cytoskeleton defects at the end of meiosis II and multiple defects during the first mitotic cell division including cytokinesis, microtubule organization and cell polarity. From our imaging of meiosis *in utero*, the mutant embryos displayed defects in chromatin segregation in anaphase II and failure to extrude the second polar body. Similar to wild type, we found cyclin B1 and katanin levels decreased in the *mel-43*(*sb41*) embryos at the end of meiosis, suggesting that the cell cycle machinery was not grossly affected. These embryos revealed extensive cytoplasmic blebbing as well as improper polarity establishment; however, centrosome maturation occurred normally. Genetic duplication analysis showed that *mel-43*(*sb41*) is a hypermorphic gain-of-function mutation. Sequencing revealed a missense mutation in the coding region of *sb41* worms, and *RNAi* against *mel-43* rescued this mutant phenotype. *mel-43* is predicted to encode a novel protein, with two highly similar paralogues in *C. elegans*. *mel-43*(*RNAi*) did not exhibit any obvious phenotype, however, *RNAi* directed against all paralogues resulted in defects in polar body extrusion at the end of meiosis I. These embryos proceeded directly into mitosis

without forming a meiosis II spindle, suggesting a role for *mel-43* and paralogues in promoting meiosis II-specific events.

¹ Mitenko, N. L., et al., 1997. *Genetics*. 147, 1665-74.

1957

Uncovering the Role of Condensin I during *C. elegans* Meiosis.

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Condensin complexes are key determinants of higher-order chromatin structure during meiosis and mitosis in eukaryotes. However the different roles of condensins I and II in meiosis are poorly understood and have yet to be elucidated in higher eukaryotes. Interestingly, analysis of condensin I in *C. elegans*, demonstrates it localizes to a ring shaped domain between chromosomes at the midbivalent during metaphase and to the meiotic spindle between separating chromosomes during anaphase. Other proteins such as the Chromosomal Passenger Complex, BUB-1, KLP-19, HCP-1/2, and CLS-2 also localize to the midbivalent, suggesting that condensin I may function with proteins at the ring shaped domain to promote chromosome orientation, alignment, and separation.

Depletion of condensin I by RNAi interference leads to gross meiotic spindle defects and abnormal chromosome organization. Our data suggests that condensin I supports orientation and movement of chromosomes throughout meiosis. Additionally, condensin I depletion prevents successful chromosome resolution at anaphase.

Future studies will determine how condensin I functions together with other proteins at the midbivalent to influence chromosomal orientation during meiotic congression and segregation in *C. elegans*.

1958

Non-random segregation of unpaired X chromosomes in *C. elegans* female meiosis.

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Attachment between homologous chromosomes during meiosis I is essential for accurate segregation of chromosomes. Surprisingly, humans with three X chromosomes (triploX) have normal fertility and give birth predominantly to children with a normal chromosome complement (Neri, 1984. *American Journal of Medical Genetics* 18:357). If the unpaired X segregated randomly at anaphase I, triploX mothers would produce 50% eggs with one X and 50% eggs with two X chromosomes. The low frequency of XXX offspring observed suggests that human female meiosis possesses a mechanism that prevents the inheritance of unpaired X chromosomes. In the *C. elegans* *him-8* mutant, which possesses two unpaired univalent X chromosomes at metaphase I, a similar situation occurs. If the two univalent X chromosomes segregated randomly at anaphase I, *him-8* mutants should produce 25% XO male, 50% XX hermaphrodite and 25% XXX progeny. Instead 38% XO male, 56% XX hermaphrodite and 8% XXX progeny are seen, suggesting that female meiosis in *C. elegans* also possesses a mechanism of selective removal of unpaired X chromosomes (Hodgkin et al., 1979. *Genetics* 91(1):67-94).

We are currently testing the hypothesis that univalent X chromosomes are selectively extruded into the first polar body. Using live imaging and fixed immunofluorescence, we found that 95% of *him-8* metaphase I spindles have 7 chromosomes at metaphase I whereas 100% of wild-type metaphase I spindles have 6 chromosomes. This result demonstrates that both univalent X chromosomes in *him-8* worms are still present during metaphase I. 100% of wild-type

metaphase II spindles have 6 chromosomes whereas 40% of him-8 metaphase II spindles have 5 chromosomes, 55% have 6 chromosomes and 5% have 7 chromosomes. These numbers suggest that univalent chromosomes are lost between metaphase I and metaphase II.

Interestingly, 88% of him-8 anaphase I spindles have 1 or 2 lagging chromosomes, something seen in only 2% of wild-type, suggesting that univalents lag during anaphase I. Quantification of the fates of lagging chromosomes revealed that 63% of these are expelled into the polar body and 37% of them are retained in the embryo. Fixed immunofluorescence of him-8 worms with REC-8 antibody revealed univalent chromosomes that biorient at metaphase I. During anaphase I, lagging univalents were also bioriented. H3K9-trimethylation staining of repetitive DNA arrays specifically integrated on the X chromosomes, suggested that both chromosomes lost in embryos with 5 chromosomes at metaphase II are univalent X chromosomes. Our results are consistent with a model in which univalent X chromosomes biorient at metaphase I but lag at anaphase I because cohesin between sister chromatids is not cleaved. The delayed, poleward movement of these bioriented univalents is biased toward the polar body end of the spindle. Preliminary data suggest that contractile ring activity is necessary for the bias seen in expulsion of lagging chromosomes. Our results reveal a cellular process that might allow production of a euploid embryo from a trisomic oocyte.

1959

The role of CDK-1 dependent phosphorylation of Dynactin in meiotic spindle rotation in *C. elegans*.

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Meiosis is a specialized cell division required for sexual reproduction characterized by a single round of DNA replication followed by two rounds of chromosome segregation. The molecular machine responsible for accurately segregating chromosomes is the meiotic spindle. In female meiosis, to properly segregate chromosomes the spindle must correctly orient its pole-to-pole axis perpendicular to the cell cortex. In *C. elegans*, this perpendicular orientation is dependent on activation of the anaphase promoting complex (APC), the molecular motor dynein, and the dynein modulator dynactin (Ellefson and McNally, *Mol. Biol. Cell*, 20: 2722). Furthermore, dynein/dynactin dependent rotation was found to be inhibited by CDK-1 (Ellefson and McNally, *J. Cell Biol.* 193: 1229). These results suggested that phosphorylation of a CDK-1 target inhibits dynein-dependent spindle rotation. Analysis of the *C. elegans* phosphoproteome (phosida.com; phosphopep.org) revealed 7 *in vivo* phosphorylation sites within the basic domain of p150 dynactin. We hypothesize that CDK-1-dependent phosphorylation of the p150 basic domain inhibits dynactin from binding microtubules and thus inhibits recruitment of dynein to the spindle during meiotic metaphase. Furthermore, APC-dependent inactivation of CDK-1 would result in dephosphorylation of the basic domain, which would increase binding of dynactin to spindle microtubules and allow spindle rotation. Mutation of all 7 phosphorylation sites to alanine resulted in increased spindle association by the CAP-Gly + basic domains of *C. elegans* p150 dynactin in transfected *Xenopus* A6 cells. Mutation of all 7 sites to glutamate caused reduced spindle association in transfected cells. Because the *C. elegans* phosphoproteome was generated from mixed stage worms, we then attempted to determine the sites within the p150 basic domain that are phosphorylated in a CDK-1-dependent manner during metaphase of meiosis. We found that metaphase extract isolated from *Spisula solidissima* oocytes is able to phosphorylate *C. elegans* dynactin on 4 residues in the basic domain and 3 of these phosphorylations are prevented by the CDK-1 inhibitor, purvalanol A. In the future we will test these sites for their involvement in regulating dynactin microtubule association in both transfected A6 cells and *C. elegans* meiotic embryos.

1960

Katanin is required continuously during meiotic metaphase to maintain chromosome position and spindle pole structure.

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Katanin is a heterodimeric microtubule-severing ATPase (McNally and Vale, Cell 75:419). In *C. elegans*, the catalytic subunit (MEI-1) and the regulatory subunit (MEI-2) are both required specifically for assembly of female meiotic spindles (Srayko et al. Genes Dev. 14:1072). The P225L mutation in MEI-1 is representative of a class of mutants that assemble meiotic spindles with no ASPM-1 positive poles and produce only dead embryos. The A338S mutation of MEI-1 is representative of a class of mutations that assemble unusually long but fully functional meiotic spindles with two ASPM-1 positive poles. Wild-type MEI-1 but not MEI-1(A338S) or MEI-1(P225L) caused microtubule disassembly when co-expressed with MEI-2 in *Xenopus* A6 cells (McNally and McNally, Mol. Biol. Cell 22:1550). This led to the hypothesis that MEI-1(A338S) lacks microtubule-severing activity but retains a discrete second activity that is required for spindle pole assembly. In this hypothesis, microtubule-severing activity is required only for controlling spindle length and MEI-1(P225L) lacks both severing and pole-forming activities. To further test this hypothesis, we developed methods for purifying active stoichiometric complexes of MEI-1 and MEI-2 from *E. coli*. In vitro microtubule-severing assays revealed that at 0.5 uM MEI-1/MEI-2 completely disassembled microtubules in 4 min whereas MEI-1(A338S)/MEI-2 exhibited no microtubule disassembly. In contrast, at 1.0 uM, MEI-1(A338S)/MEI-2 exhibited microtubule severing that was just kinetically slower than that of wild type. MEI-1(P225L)/MEI-2 showed no microtubule disassembly activity at concentrations up to 2.0 uM. Wild-type MEI-1 in the absence of MEI-2 severed microtubules but only at concentration of 3 to 4 uM. To elucidate whether microtubule severing is likely occurring in meiotic embryos of MEI-1(A338S) or MEI-2(null) mutants, we are currently determining the in vivo concentration of MEI-1 and MEI-2. As a second approach to understanding the relationship between katanin-dependent spindle length control and katanin-dependent spindle pole assembly, we have begun analysis of a fast-acting temperature sensitive mutation in MEI-1 (O'Rourke et al. PlosOne, 6: e16644). We depleted worms of the anaphase promoting complex subunit, MAT-1, by RNAi at permissive temperature to allow accumulation of embryos with pre-formed metaphase I spindles. We then used both live spinning disk confocal microscopy and fixed immunofluorescence to determine the fate of the spindle immediately after shifting to non-permissive temperature. The earliest and most surprising phenotype observed was movement of metaphase chromosomes to the poles. Spindles then elongated and then became disorganized. Fixed immunofluorescence indicated that ASPM-1 moved from the poles onto the spindle microtubules before dissociating. These results show for the first time that katanin activity is required after initial spindle assembly for maintenance of chromosome position, spindle length and spindle pole structure.

1961

Spire-type actin nucleators cooperate with Formin-2 to drive asymmetric oocyte division.

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Oocytes mature into eggs by extruding half of their chromosomes in a small cell termed the polar body. Asymmetric oocyte division is essential for fertility, but despite its importance, little is known about its mechanism. In mammals, the meiotic spindle initially forms close to the centre of the oocyte. Thus, two steps are required for asymmetric meiotic division, first, asymmetric

spindle positioning, and second, polar body extrusion. Here, we identify Spire1 and Spire2 as new key factors in asymmetric division of mouse oocytes. Spire proteins are novel types of actin nucleators that drive nucleation of actin filaments with their four WH2 actin binding domains. We show that Spire1 and Spire2 first mediate asymmetric spindle positioning by assembling an actin network that serves as a substrate for spindle movement. Second, they drive polar body extrusion by promoting assembly of the cleavage furrow. Our data suggest that Spire1 and Spire2 cooperate with Formin-2 to nucleate actin filaments and that both types of nucleators act as a functional unit. This study not only reveals how Spire1 and Spire2 drive two critical steps of asymmetric oocyte division, but it also uncovers the first physiological function of Spire-type actin nucleators in vertebrates.

1962

Meiotic HORMA domain proteins prevent untimely centriole disengagement during male meiosis.

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Successful embryonic development requires that at fertilization each gamete provide complementary components the zygote. One such component is the centrosome, which influence the organization of the microtubule arrays within the cell. Centrosomes are formed by either one or two cylindrical microtubule structures called centriole(s) that recruit pericentriolar proteinaceous material. In species where oocytes lack centrosomes, sperm must contribute both haploid genome and centrioles to the zygote. Correct centriole organization during male meiosis is critical to ensure normal bipolar mitotic spindle in the zygote. We identified a new role for *C. elegans* meiosis HORMA domain proteins, HIM-3, HTP-1 and HTP-2, in regulating centriole dynamics during spermatocyte meiosis. In *C. elegans*, centrioles undergo two rounds of duplication during male meiosis, resulting in haploid sperm each containing a single tightly engaged centriole pair. Since centriole disengagement can license centriole duplication, spermatocytes must tightly regulate centriole disengagement to ensure that sperm inherit a single centriole pair. In the horma mutants, we observe inappropriate separation of centrioles during meiosis II, resulting in separated centrioles in sperm. Further, an extra pair of centrosomes is detected in a subset of zygotes, reflecting a single additional round of centriole duplication that was enabled by aberrant centriole disengagement in the spermatocytes. These and other data implicate meiotic HORMA proteins in preventing centriole disengagement in meiosis II. We showed previously that HTP-1/2 associates with chromosomes and prevents premature loss of sister chromatid cohesion during the meiotic divisions by inhibiting removal of the REC-8 meiosis-specific cohesin subunit. We find that *rec-8* cohesin mutant spermatocytes have similar inappropriate centriole separation phenotypes to those observed in *htp-1/2* mutants. Further, a mutant form of separase that exhibits elevated concentration at meiosis II spindle poles and in budding spermatids likewise promotes inappropriate centriole separation, consistent with HORMA proteins preventing centriole disengagement by antagonizing separase-dependent cohesin removal. Our data suggest that the same specialized meiotic mechanisms that prevent premature release of sister chromatid cohesion during meiosis I also function to inhibit centriole disengagement at meiosis II, thereby ensuring that the zygote inherits the appropriate complement of chromosomes and centrioles.

Here we will also report our current investigation on both the molecular mechanisms preventing untimely separation of centrioles and on the role of other proteins in centrosome inheritance during gametogenesis.

1963

Sub-optimal APC activity following SAC satisfaction reduces aneuploidy in mouse oocytes.*S. I. Lane¹, K. T. Jones¹; ¹University of Newcastle, Newcastle, Australia*

It has long been established that oocytes have a poor ability to divide their chromosomes faithfully during the first meiotic division, resulting in the generation of aneuploid embryos. This is in contrast to the high fidelity of chromosome segregation in somatic cells. Recently we, and others, have demonstrated in oocytes that Spindle Assembly Checkpoint (SAC) satisfaction is coupled to a majority kinetochore-microtubule attachment, but chromosomes neither have to be congressed on the metaphase plate nor bi-oriented, thus creating the potential for aneuploidy. Additionally, exit from meiosis I is characterised by a long period of APC mediated cyclin B1 and securin destruction culminating in anaphase. We hypothesise therefore that in oocytes, this delayed exit from meiosis I following SAC satisfaction helps to prevent aneuploidy.

To examine this idea further we added flavopiridol, a CDK1 inhibitor, to mouse oocytes maturing in culture, at a time following APC activation (observed by the degradation of cyclin B1-GFP during timelapse microscopy). CDK1 inhibition significantly increased APC activity, bringing forward anaphase-onset by >1 hour, and also raising aneuploidy rates. Furthermore, addition of reversine, an Mps1 inhibitor, or ZM447439, an Aurora kinase inhibitor, or microinjection of Mad2 antibody, all increased APC activity. Given these latter interventions are predicted to ameliorate SAC activity, we propose that the APC in oocytes, although active in degrading cyclin B1, continues to be negatively regulated by the SAC and CDK1 activity throughout meiosis I. The delay caused by this restriction of APC activity may allow oocyte aneuploidy to be significantly lowered in a system where attachment errors cannot generate a signal capable of full APC inhibition.

1964

Determining the mechanism of zinc transporters in zinc accrual during meiosis in the mammalian oocyte.*B. Y. Kong¹, F. E. Duncan¹, T. O'Halloran^{2,3}, T. K. Woodruff^{1,3}; ¹Department of Obstetrics and Gynecology, Northwestern University, Chicago, IL, ²Department of Chemistry, Northwestern University, Evanston, IL, ³Department of Molecular Biosciences, Northwestern University, Evanston, IL*

Zinc is a biologically essential transition metal with well-established roles in many molecular and cellular processes. Recently, we demonstrated that zinc regulates meiosis in the mammalian oocyte. Based on synchrotron x-ray fluorescence microscopy, the total intracellular zinc content in mouse oocytes is nearly a magnitude higher than copper or iron, and increases by over 50% during meiotic maturation which occurs over approximately 14 hours. This increase is required for proper meiotic progression as limiting intracellular zinc availability results in premature arrest at telophase I. Although this accumulation of total zinc is necessary, it is not known how labile zinc changes during meiotic maturation or how zinc levels are modulated. We performed in vitro maturation to obtain oocytes at prophase I, metaphase I, and metaphase II. Using a novel zinc-specific fluorophore, we tracked labile zinc using live imaging at these stages of meiosis. Following zinc imaging, cells were fixed and stained for cortical granules, secretory vesicles required for modification of the zona pellucida at fertilization. Zinc localized to discrete vesicle-like structures that were symmetrically distributed in the cortex during prophase I but was highly polarized at metaphase II. Zinc appeared to have a similar localization as cortical granules. To determine how intracellular zinc levels may be regulated during meiosis, we examined the role of zinc transporters. Of the 14 known mammalian zinc transporters that increase intra-

cytoplasmic zinc levels, zip10 had the highest mRNA levels in the oocyte compared to over 60 other mammalian tissues as determined by microarray analysis. We generated a ZIP10-specific antibody; immunostaining of ZIP10 protein in the oocyte revealed a predominantly cortical distribution with a polarized pattern in the prophase I, metaphase I and metaphase II staged oocytes. ZIP10 localization was thus similar to that of both labile zinc and cortical granules. These results suggest that ZIP10 may be the regulatory pathway by which the oocyte acquires zinc from the extracellular environment during meiosis. Current experiments are focused on determining how zinc insufficiency affects ZIP localization and function and how meiotic progression is affected by disruption of ZIP10 expression. Supported by NIH Grant P01 HD021921 and the W. M. Keck Foundation Medical Research Award. B.Y. Kong is a recipient of the American Association of University Women award.

1965

Effects of taxol on chromosome movements in *Mesostoma* spermatocytes.

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Dividing *Mesostoma ehrenbergii* spermatocytes have 3 bivalent chromosomes that oscillate 4-6µm to and from the poles throughout prometaphase/metaphase and 4 univalent chromosomes that sit at the poles. Notably, univalents sometimes move from one pole to another to achieve correct segregation. We added various concentrations of taxol to dividing spermatocytes and found that 10nM taxol was the minimum concentration needed to stop bivalent oscillations. Oscillations resumed 24-42 minutes after the taxol was washed out, with normal velocities but reduced amplitudes. In 10nM taxol, most kinetochores stopped their oscillations toward the poleward side of their normal oscillation cycle, not toward the equator. Cells in taxol with stopped bivalents entered anaphase nonetheless, with chromosome velocities approximately 58% of those in control cells. Univalent movements also occurred in taxol while bivalents were stopped. Assuming that at this low concentration of taxol microtubules (MTs) were stabilized by reducing MT dynamics at the kinetochore (plus) end (Derry et al. 1995), our data suggest that during bivalent oscillations, movements towards the pole require depolymerization of kinetochore MTs at the pole and movements away from the pole require polymerization of kinetochore MTs at the kinetochores. Oscillations are blocked with kinetochores toward the pole because movements away from the pole are blocked when kinetochore MT plus ends are stabilized. We hypothesize that anaphase takes place in the presence of taxol as a result of MT depolymerizing or MT severing enzymes described by Rath and Sharp (2011) increasing their activity at the onset of anaphase.

1966

Cdk1-Cyclin B Activity Regulates the Timing of Formation of Stable Kinetochore-Microtubule Attachments in Meiosis I.

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Chromosome segregation during cell division depends on stable attachment of kinetochores to spindle microtubules. Mitotic spindle formation and kinetochore-microtubule capture typically occur within ~30 min. In contrast, in meiosis I in mouse oocytes, the bipolar spindle forms within 3-4 hours, but formation of stable kinetochore-microtubule attachments is delayed for an additional 3-4 hours. The mechanism underlying this delay, which likely prevents attachment errors during spindle formation, is unknown. Cdk1-Cyclin B activity increases rapidly at mitotic entry and then remains stable, whereas in meiosis I Cyclin B protein levels and Cdk1 activity increase slowly over a period of ~6 hours. This difference suggests that levels of Cdk1 activity may control the timing of formation of stable kinetochore-microtubules. To test this hypothesis

we manipulated Cdk1 activity in mouse oocytes in two ways. First, reducing Cdk1 activity using low levels of an inhibitor led to delayed formation of stable attachments and late anaphase I onset. Second, over-expression of Cyclin B led to a faster increase in Cdk1 activity and early formation of stable attachments. These results indicate that the slow increase in Cdk1 activity in meiosis I acts as a timing mechanism to control kinetochore-microtubule interactions. We also tested whether inter-kinetochore tension and Aurora B kinase, known regulators of attachment stability, also contribute in meiosis I. We show that disrupting tension by premature loss of cohesion prevents full stabilization of attachments, while Aurora kinase inhibition leads to hyper-stability. We conclude that formation of stable kinetochore-microtubule attachments in meiosis I depends on both established mechanisms and high levels of Cdk1-Cyclin B activity that are reached late in metaphase I.

1967

Protein 14-3-3 eta (YWHAH) is essential for normal meiotic spindle assembly during *in vitro* maturation of mouse oocytes.

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The 14-3-3 (YWHA) proteins are known to be central mediators in various cellular signaling pathways regulating development and growth including cell cycle regulation. We previously found that all seven mammalian 14-3-3 isoforms are expressed in mouse oocytes and eggs, and that 14-3-3 eta (YWHAH) accumulates and co-localizes in the region of meiotic spindle in mouse eggs matured *in vivo* [De, S. et al., 2012, BMC Res Notes. 5(1), 57]. Examination of oocytes matured *in vitro* demonstrated that 14-3-3 eta accumulates in both meiosis I and II spindles. To examine the role of 14-3-3 eta in meiotic spindle formation, we microinjected mouse oocytes with a ~0.1mM translation-blocking morpholino oligonucleotide against 14-3-3 eta mRNA. The morpholino-injected oocytes were held in prophase I arrest for 24 hours to allow reduction of existing 14-3-3 eta protein and then allowed to mature *in vitro*. Meiotic spindles in those cells were examined by immunofluorescence staining of 14-3-3 eta and alpha-tubulin along with observation of chromosomes stained with Hoechst dye. Following injection of the morpholino, the spindle was often found to be absent or abnormally formed along with no or reduced accumulation of 14-3-3 eta. In those cells, chromosomes were clumped or disorganized and first polar body formation was absent. Immunofluorescence staining of 14-3-3 eta and alpha-tubulin in control eggs matured *in vitro* from uninjected oocytes and oocytes microinjected with the ineffective, inverted form of a morpholino against 14-3-3 eta, a morpholino against 14-3-3 gamma or deionized water showed normal, bipolar meiosis II spindle assemblies. To further examine the interaction of 14-3-3 eta with alpha-tubulin in meiotic spindles, we performed an *in situ* proximity ligation assay (Duolink In-cell Co-IP; Olink Bioscience) that can detect protein-protein interactions at the single molecule level directly in cells and allows visualization of the actual sites of the interactions. In control eggs, the proximity ligation assay revealed an accumulation of sites of interaction between 14-3-3 eta and alpha-tubulin at the normal meiotic spindles and in the cell cortices adjacent to the spindles, confirming the co-immunofluorescence finding we reported before. However, in cells matured *in vitro* from oocytes microinjected with the morpholino against 14-3-3 eta, there was a marked reduction in the interaction sites for 14-3-3 eta and alpha-tubulin in the regions of the disrupted spindles. These results suggest that 14-3-3 eta is essential for formation of the normal meiotic spindle during *in vitro* maturation of mouse oocytes, in part by interacting with alpha-tubulin.

1968

RNA activates Aurora B kinase to promote meiotic spindle assembly.

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Spindle assembly during cell divisions is a highly controlled process that is regulated by many proteins encompassing a wide range of activities. Our group has shown that, in addition to protein factors, RNA functions in a translation-independent manner to promote spindle assembly. However, the mechanisms by which RNA operates have been unclear. We have now found that one role of RNA is to regulate the kinase Aurora B. Aurora B, the catalytic subunit of the chromosome passenger complex (CPC), plays an important role in aligning chromosomes at metaphase and ensuring their segregation at anaphase and telophase. Its localization and activity are regulated by the other members of the CPC, which stimulate kinase activity and localize it to the inner centromere during metaphase. We have found that RNA is required for maximal Aurora B kinase activity and localization to inner centromeres. One of the main functions of Aurora B during metaphase in *Xenopus* egg extracts is to phosphorylate and inactivate the microtubule depolymerizing kinesin MCAK. Accordingly, inhibiting MCAK largely restores spindle formation in the absence of RNA. Aurora B can be detected in a large RNA-dependent complex, and many of the associated proteins are classified as RNA-binding proteins. Analyzing the transcripts bound to Aurora B revealed that the RNA-binding profile varies between cell-cycle stages, and that some transcripts are specifically bound to Aurora B upon activation by chromatin or microtubules. Finally, we have shown an unprecedented function for one of the associated RNA-binding proteins in localizing Aurora B to meiotic chromosomes in *C. elegans*. These results elucidate a molecular pathway by which RNA influences spindle assembly by serving as a scaffold for protein complex assembly, and suggest a novel mechanism for regulating enzyme functions.

1969

Characteristics of DNA in meiotic recombination hotspots.

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Based on Oxford Dictionary of Biochemistry and Molecular Biology, "genetic information" is defined as "the information carried in a sequence of nucleotides in a molecule of DNA or RNA". Indeed, the genetic code is 'written' in a sequence of nucleotides. Then, is the genetic information carried only in the sequence of nucleotides? The answer is "No". It is also carried in conformation and physical properties of DNA, histones or chromatin. Deciphering the veiled 'code' carried in them is one of the key issues in the current era of epigenetics. We are studying this challenging issue with focusing on the meiotic recombination hotspots.

The current study focused on the genetic background that generates recombination hotspots at meiosis. These sites do not have any consensus sequence. Thus, using the genome databases of *Saccharomyces cerevisiae* and *Mus musculus*, we examined characteristics of the mechanical properties of the hotspots. When averaged, a common distinctive mechanical property was clearly detected at the center region of the hotspots for both organisms. However, very interestingly, the properties themselves were opposite between *S. cerevisiae* and *M. musculus*: i.e., the center region is very rigid in the former while very flexible in the latter. We are now analyzing how these differences are reflected in the conformations of hotspots using atomic force microscopy.

1970

Characterizing Genetic Variation in PRDM9 and its Association with Altered Patterns of Recombination Linked to Chromosome 21 Nondisjunction.

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Nondisjunction (NDJ) of chromosome 21 is the leading cause of trisomy 21, a condition characterized by severe intellectual disability, more commonly known as Down syndrome. Altered patterns of recombination on 21q have long been identified as risk factors for chromosome 21 NDJ. Interestingly, recent studies have shown that variation in the Zinc Finger Binding Domain (ZFBD) of the protein Proline Rich Domain Containing 9 (PRDM9) is associated with altered patterns of recombination. As PRDM9 is a major determinant of the placement of recombination, we hypothesize that variation in the ZFBD of PRDM9 promotes the altered patterns of recombination that predispose chromosome 21 to nondisjoin. We are currently sequencing the ZFBD of DNA collected from over 400 parents who had a chromosome 21 NDJ error leading to an offspring with trisomy 21 (100 paternal cases, 300 maternal cases). If our hypothesis is correct, the distribution of ZFBD alleles identified in the cases should vary from that of the normal population and be correlated with the altered placement of recombination on chromosome 21. These findings may help identify genetic factors that predispose to nondisjunction and refine the risk of having a child with trisomy 21.

1971

Regulation of glucose metabolism and cytoplasmic streaming by *Txnip* during oocyte maturation.

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Previously, we found higher *Txnip* mRNA expression in germinal vesicle (GV) compared to metaphase II (MII) oocytes. *Thioredoxin interacting protein (Txnip)* binds to *Thioredoxin (Trx)* and regulates intracellular redox state. In addition, via *Trx*-independent mechanism, *Txnip* regulates glucose metabolism for maintaining intracellular glucose concentration. However, *Txnip* function in mouse oocytes was not yet known. Therefore, the objective of present study was to determine the function of *Txnip* during mouse oocyte maturation using RNAi.

For *Txnip* RNAi, we performed microinjection of *Txnip* dsRNA into the cytoplasm of GV oocytes. By *Txnip* RNAi, most of oocytes (79.5%) were arrested at metaphase I (MI) stage whereas control (81.25%) or buffer-injected (76.3%) oocytes normally progressed to MII stage. When we used time lapse video microscopy to observe phenotypic changes during oocyte maturation, formation of granules in the oocyte cytoplasm increased after *Txnip* RNAi concurrent with the retarded cytoplasmic streaming. We supposed that these granules were glycogen molecules formed by upregulated lactate production via gluconeogenic pathway following increased glucose uptake by *Txnip* RNAi. For monitoring glucose uptake, oocytes were treated with 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), a fluorescent glucose analog and lactate production was measured using the lactate colorimetric assay kit. After *Txnip* RNAi, not only glucose uptake but also lactate production increased in *Txnip* RNAi-treated oocytes compared to control oocytes. When oocytes were incubated in high lactate-supplemented medium, we observed increased granule formations and retarded cytoplasmic streaming similar to the phenotypic changes observed in *Txnip* RNAi-treated oocytes. Thus, we concluded that *Txnip* RNAi increased lactate production and glucose uptake, and resulted in formation of cytoplasmic glycogen granules. In addition, using immunofluorescence staining, we

observed scattered microtubules and aggregated chromosomes in *Txnip* RNAi-treated oocytes. These results indicate that actin networking was disturbed and thus cytoplasmic streaming was retarded by *Txnip* RNAi.

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1972

Mitochondria are inherited asymmetrically during the first meiotic division.

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Most cell divisions result in the production of two equally sized daughter cells each of which inherit half of the DNA and an equal proportion of cellular organelles. The highly asymmetric cell divisions of female meiosis provide a different challenge because one of the daughters, the polar body, is destined to degenerate, putting at risk essential maternally-inherited organelles such as mitochondria. Whilst the division of DNA during this cytokinesis must be equal, the loss of important cellular organelles to the polar body could be detrimental to oocyte health. This may be particularly relevant in the case of mitochondria which provide the primary source of ATP during oocyte maturation and early embryogenesis due to a block in glycolytic activity that lasts until the blastocyst stage. Furthermore, mitochondrial replication is suppressed in the fully grown oocyte and does not resume until after implantation, such that the mitochondrial complement contained within the oocyte must be capable of sustaining the early stages of mammalian development. However, little is known about how cytoplasmic organelles are organised and distributed during this time.

We find that during oocyte maturation, 42% of mitochondria aggregate around the first meiotic spindle. Disruption of dynein activity using the function-blocking antibody 70.1 decreases the extent of mitochondrial aggregation to 26%, ($p < 0.001$), indicating a role for dynein in this process. Conversely, inhibition of kinesin-1 activity using the function-blocking antibody SUK4 increases mitochondrial aggregation to 54%, ($p < 0.005$) implying a role for kinesin-1 in regulating the extent of mitochondrial association with the spindle.

The arrangement of mitochondria around the spindle places them in an ideal position to be equally segregated between oocyte and polar body, with a resulting loss of ATP generating capacity in the oocyte and early embryo. However, we show that at cell division there is a profound reorganisation of mitochondria into an asymmetrical arrangement. Mitochondria are anchored in the cytoplasm of the oocyte and largely excluded from the polar body, with the result that the percentage area occupied by mitochondria in the oocyte is significantly higher than that in the polar body ($23.7\% \pm 1\%$ vs $4.8\% \pm 1\%$, $p < 0.0001$). Using live cell imaging we show that this rearrangement is initiated around the time of anaphase, and is inversely correlated with the spindle-cortex distance. By inhibiting spindle migration we show that asymmetrical mitochondrial inheritance requires spindle-cortex proximity but find that this alone is not sufficient to initiate asymmetry. Arresting the cell cycle at metaphase with the spindle positioned at the cortex reveals that cell cycle progression is also a requirement for asymmetrical mitochondrial inheritance.

Thus, oocyte-biased inheritance of mitochondria is a variation on rules that normally govern cell division and ensures that essential maternally inherited mitochondria are retained to provide ATP for early mammalian development.

Anterograde and Retrograde Axonal Transport

1973

Molecular mechanisms of axon pruning in the CNS.

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Axon pruning and synapse elimination are critical for both the establishment of neural connectivity and synaptic plasticity. Axon pruning involves removal of ectopic, or misguided, axonal branches that have already formed synaptic connections, thus synapse elimination usually precedes pruning. Stereotyped pruning of axons that originate in the hippocampal dentate gyrus (DG) and extend in the infrapyramidal tract (IPT) occurs during postnatal murine development by neurite retraction and superficially resembles axon repulsion. The chemorepellent *Sema3F* and its holoreceptor complex *neuropilin-2 (Npn-2)/plexinA3 (PlexA3)* are required for IPT axon pruning, in addition to dendritic spine remodeling and repulsion of DG axons. The objective of our study was to determine the signaling events that act downstream of *Sema3F* to regulate IPT axon pruning. Consequently, we uncovered a novel role for *Sema3F*-mediated activation of the *Rac-GAP β2 chimaerin (Chn)* in stereotyped IPT axon pruning and synapse elimination. *β2Chn* is required for axon pruning, but not for *Sema3F*-mediated axon guidance or dendritic spine constraint. Therefore, this selective requirement for *β2Chn* defines a mechanistic distinction among axon pruning, repulsion, and dendritic spine remodeling, all of which are mediated by *Sema3F*. Here, we conducted structure-function analyses *in vitro* and *in vivo* that shed new light onto these signaling events using biochemical and molecular genetic approaches. We first asked whether the lipid binding and the SH2 domains of *βChn* are required for *Npn2* binding *in vitro*, given the known requirement for these domains in a closely related protein, *αChn*, for Ephrin-dependent motor circuit assembly. To test the importance of these domains for IPT pruning *in vivo*, we performed rescue experiments employing stereotactic injection of viral vectors containing WT, Δ SH2 or C246A (lipid-binding deficient) *βChn*. We also analyzed other CNS tracts that are dependent on *neuropilin/plexin* signaling for axon pruning, including the hippocampo-septal (HST) and visual corticospinal (vCST) tracts by performing *in vivo* tracing experiments in WT and *Chn2*^{-/-} mice. In conclusion, these experiments unveil the critical molecular events that mediate axon pruning, and they also lend insight into the differences and similarities between axon guidance and axon pruning during neural development.

1974

Ribosomal transport in Schwann cells: a role for myelination in peripheral nervous system regeneration.

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Local protein synthesis within the axon is known to play a significant role in its regeneration following injury. Schwann cells have also been noted to enhance regeneration but generally are

considered in the capacity of guiding outgrowth and providing trophic factors. Recent evidence has shown ribosomes are transferred *in vivo* from Schwann cells to neurons following axonal injury. Much of this phenomenon has yet to be identified including the underlying mechanisms initiating and driving this process, the development of transferrable ribosomal populations within Schwann cells, and the coupling of this process with that of axonal regeneration. We have developed an *in vitro* myelination system to test several aspects of this process. In this study, we examined the recruitment of ribosomal populations to the SC-neuron boundary through transport during myelination and following injury, and the role that myelination plays in axonal regeneration. Embryonic rat DRG cultures purified for two weeks were supplemented with P2 Schwann cells from sciatic nerves and induced to myelinate through the addition of 50 $\mu\text{g/ml}$ ascorbic acid (AA) to culture media. Schwann cells were transfected with a plasmid encoding the ribosomal subunit L4 tagged with GFP to track ribosomal transport and distribution within Schwann cells. Transport of ribosomes was tracked by time-lapse confocal fluorescence imaging with observations made prior to myelination, during early myelination (3 days post-AA), and late myelination (10+ days post-AA). Corresponding experiments were performed in which either unmyelinated or myelinated axons were severed and allowed to regrow for 3 hours. Images were taken every 10 minutes and axonal lengths measured. Rates of transport within Schwann cells reflected a decrease between initial transfection (.976 $\mu\text{m/s}$) and early myelination (.369 $\mu\text{m/s}$), suggesting a development of residual gradients within projections. Additionally, punctate ribosomal populations were observed on the order of every 30 μm . This value was similar to the known distribution of Schmidt-Lanterman incisures, major candidates for sites of ribosomal transfer. Preliminary data fails to identify a statistically significant difference between axonal regrowth following injury in myelinated (21.27 $\mu\text{m/hr}$) and unmyelinated (18.06 $\mu\text{m/hr}$) cultures ($p > .05$, student's t-test). It is possible that at early time points, myelination physically bars axonal elongation, with positive effects not seen till later time points. In the future it is our desire to investigate the role of axo-glia signaling in this process through the manipulation of calcium and ATP.

1975

A role of the Rac1-TC10 axis in neurite outgrowth.

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Neurite outgrowth is essential for both the initial wiring of neuronal networks during development and regeneration. We have previously shown that Rac1 is locally activated at neurite tips in a variety of neuritogenic processes although the mechanisms of Rac1 activation vary widely. It is well known that the promoting effect of Rac1 on neuritogenesis is mainly through cytoskeletal reorganization. However, neurite outgrowth also requires a local increase in the surface area, which relies on anterograde transport of membrane vesicles. Recently, TC10, which is a Rho family GTPase and located to vesicles and the plasma membrane, has been shown to be required for axonal growth. We are pursuing the possibility that the Rac1-TC10 axis contributes to neurite outgrowth through the membrane addition to neurite tips. In EGF-treated HeLa cells, we previously found that TC10 activity at tethered vesicles dropped immediately before the fusion to the plasma membrane through the Rac1-mediated pathway. In combination with other functional assays, we have proposed that GTP hydrolysis by TC10 is a critical step in vesicle fusion. By using TC10 FRET sensors, here we have shown that TC10 activity decreased on the plasma membrane in parallel with the extension of cell periphery in a range of neuronal cells including hippocampal neurons. In PC12 cells, NGF or dbcAMP treatment rapidly decreased the level of GTP-TC10. This down-regulation was mediated by Rac1 and ROS generation. We found that TC10 activity at the vesicles was markedly higher than that at the plasma membrane

in NGF-treated PC12 cells. We note that TC10 frequently resided on the vesicles containing Rab11, which is a key regulator of recycling endosomal pathways and has been shown to play a role in neurite outgrowth as well as TC10. We think that the role of GTP hydrolysis by TC10 in membrane fusion might be applicable to neurite outgrowth.

1976

Real-time dynamics of autophagosome assembly in primary neurons indicate biogenesis proceeds via a conserved but spatially regulated mechanism.

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Neurons are post-mitotic and thus particularly dependent on efficient degradative pathways such as autophagy to maintain neuronal homeostasis. In fact, neuron-specific depletion of genes required for autophagy is sufficient to cause neuronal cell death. Further, multiple neurodegenerative diseases characterized by excessive protein aggregation exhibit pronounced defects in autophagy. Despite the clear implication of defective autophagy in disease, little is known about the basic mechanisms driving autophagy in neurons. Using live-cell imaging, we investigated autophagosome biogenesis and maturation in primary dorsal root ganglion neurons expressing the autophagosome marker GFP-LC3. We have previously shown that autophagosomes are robustly transported along the axon toward the cell soma in primary neurons. As they move distally to proximally, they undergo maturation into compartments that may more effectively catalyze the degradation of cargo. To determine the source of these retrogradely moving autophagosomes, we imaged the neurite tip and observed constitutive and preferential generation of autophagosomes in the distal axon. Autophagosome formation is initiated with the ordered appearance of Atg13 and Atg5. Atg13 has been previously shown to function in the initial steps of biogenesis by regulating the induction of autophagy, while Atg5 is important for the recruitment of LC3 to the nascent autophagosome structure. We observed de novo appearance of puncta positive for Atg13 and Atg5 that grow progressively in size, followed by recruitment of GFP-LC3. Atg13 and Atg5 then disassemble from the nascent autophagosome leaving the GFP-LC3-positive puncta to grow progressively into ring structures ~800 nm in diameter. These developing autophagosomes are generated in close apposition to the endoplasmic reticulum (ER). Atg13 puncta appear on or near ER structures and often co-migrate with the ER in the distal axon. Further, autophagosomes are enriched in luminal and membraneous components of the ER that persist during transport along the axon toward the cell soma. Taken together, autophagosome formation in primary neurons involves an ordered recruitment of autophagosome assembly factors and a proximal relationship with the ER. Thus, the pathway of autophagosome biogenesis in primary neurons appears to be conserved from yeast and nonpolarized mammalian cells, however, the spatial restriction of autophagosome formation to the distal axon is distinct. Supported by NIH NS060698 to EH.

1977

Slow axonal transport driven by directional actin treadmilling.

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Axonal transport is essential for the growth, maintenance and regeneration of axons, and categorized into the fast component (2-5 $\mu\text{m/s}$), slow component a (0.002-0.01 $\mu\text{m/s}$) and slow component b (0.02-0.09 $\mu\text{m/s}$). Slow component b conveys more than 200 proteins including actin and actin binding proteins. The fast component and slow component a are thought to be transported by kinesins and dyneins along microtubules, however the mechanism for slow

component b has been not well known since it was first reported more than 30 years ago. Previous report showed that an actin rich structure called wave h moves along the axonal shaft at the rate of slow component b, thereby suggesting that the wave transports slow component b (Ruthel & Banker, *Cell Motil. Cytoskeleton*, 1998; Flynn et al., *Dev. Neurobiol.*, 2009).

Here we show that assemblies of actin filaments in wave move anterogradely along the axonal shaft at the rate of slow component b, depending on their directional polymerization/depolymerization, the process called treadmilling, and their anchoring to the substrate.

We found that mass of actin filaments in waves undergo treadmilling in which the polymerizing ends are orientated toward the neurite tip. Inhibition of actin polymerization by cytochalasin B led to a decrease in the velocity of wave movement. Enhancement of the actin polymerization by promoting the cofilin activity led to an increase in the velocity of wave movement. We also found that the treadmilling F-actins are anchored to the substrate, through a clutch protein shootin1 and cell adhesion molecule L1-CAM. Inhibition of the interaction between F-actins and the substrate induced a slippage of treadmilling F-actins. Importantly, this slippage decreased the velocity of wave movement. We also confirmed the actin transport with the wave in vivo.

We propose a novel type of axonal transport system driven by directional F-actin treadmilling anchored to the substrate; this mechanism is consistent with the data that the subaxolemmal region is enriched with slow component b proteins (Heriot et al., *J. Cell. Biol.*, 1985) and appear to explain apparently discrepant previous observations that axonal actin is transported as dynamic and polymeric forms (Tashiro & Komiyta, *J. Neurosci.*, 1989; Okabe & Hirokawa, *Nature*, 1990; Vallee & Bloom, *Ann. Rev. Neurosci.*, 1991).

1978

JIP1 Sustains Long Distance Anterograde Transport via Direct Regulation of Kinesin Autoinhibition and Coordination of Retrograde Motor Association.

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Axonal cargos are transported distances of up to one meter in human neurons. Analysis of the highly processive vesicular cargo APP (amyloid precursor protein) in primary mammalian neurons shows transport characterized by long run lengths with few pauses or directional switches. In order to elucidate the molecular mechanism for sustaining long-distance processive motility, we depleted the adaptor protein JIP1, which directly binds to APP, and observed significant defects in both anterograde and retrograde APP transport. While JIP1 was previously shown to bind kinesin light chain (KLC), we now identify a novel KLC-independent interaction between JIP1 and kinesin heavy chain (KHC) via distinct binding sites in both KHC stalk and tail. Using in vitro TIRF motility assays with single molecule resolution, we found that addition of JIP1 relieves auto-inhibition of KHC and activates motility. In the presence of JIP1, KHC exhibits a ~7-fold increase in run frequency, a ~2-fold increase in run length and ~25% increase in speed. Interestingly, fragments of JIP1 that only bind KHC stalk or KHC tail are sufficient to initiate processive runs, but cannot fully recapitulate the enhancement of KHC speed and sustained processivity seen with full-length JIP1. In addition, JIP1 can coordinate both kinesin- and dynein-driven motility, as we identify a novel interaction between JIP1 and the p150^{Glued} subunit of dynein. Both in vitro and in primary neurons, addition of the JIP1-binding region of p150^{Glued} competitively inhibits JIP1-mediated enhancement of KHC processivity. In contrast to a stochastic tug-of-war model, our data suggest that JIP1 can coordinate the formation of two distinct motile complexes - an anterograde complex that binds directly to KHC and a retrograde complex via its interaction with dynein. Finally, analysis of JIP1 mutants

suggests that JIP1 phosphorylation regulates switching between anterograde and retrograde JIP1 complexes by altering KHC tail binding affinity. Together, our data support a model whereby the vesicle-associated adaptor protein JIP1 mediates bidirectional axonal transport and post-translational modification of JIP1 enhances kinesin activity in order to sustain long-distance anterograde transport.

1979

Recruitment of dynein to endosomes for retrograde survival signal transport requires specific phosphorylation of the dynein intermediate chain by a Trk/ERK pathway.

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The retrograde transport of Trk containing signaling endosomes from the axon to the cell body by cytoplasmic dynein is necessary for axonal and neuronal survival, but little is known about how dynein binding to these organelles is regulated. We investigated the recruitment of dynein to signaling endosomes formed in response to neurotrophin binding to the Trk receptor kinases in rat embryonic neurons and PC12 cells. We identified a novel phospho-serine on the dynein intermediate chains with mass spectrometry. Using an antibody specific for this phosphorylation site, a time-dependent increase in neurotrophin-stimulated phosphorylation of the intermediate chain (IC) was observed in both cell types. Consensus site prediction algorithms, pharmacological studies, over-expression of constitutively active MEK1, and phosphorylation of recombinant protein in vitro demonstrated that the intermediate chains were phosphorylated by the MAP kinase ERK1/2 (extracellular-signal-regulated kinase), a major downstream effector of Trk. In order to investigate the role of the phospho-IC in living cells, we made mRFP-tagged IC-WT, S/A dephospho- and S/D phospho-mimic mutants. Live cell imaging with the fluorescently-tagged IC mutants demonstrated that the dephospho-mimic mutants had significantly reduced co-localization with Trk and Rab7, but not a mitochondrial marker. Analyses of the motility of the mutants in axons indicated that the kinetic properties of the dynein motor were not regulated by phosphorylation. The phosphorylated dynein intermediate chains were enriched on organelles immuno-affinity purified with antibodies to Trk. Inhibition of ERK decreased IC phosphorylation and reduced the total amount of dynein present on the purified organelles. In addition, inhibition of ERK1/2 decreased the amount of Trk and Rab7 co-localization with dynein and reduced the retrograde movement of Rab7 and TrkB containing endosomes in axons. NGF-dependent survival of sympathetic neurons was significantly reduced by the over-expression of the dephospho-mimic mutant IC-1B-S80A, but not WT IC-1B. This provides further evidence that in the absence of IC phosphorylation there was decreased retrograde transport of signaling endosomes. Phosphorylation on this site did not modulate IC binding to the p150 subunit of dynactin, demonstrating that a component other than dynactin was also involved in dynein binding to the endosomes. Since dynein recruitment to mitochondria is not regulated by phosphorylation on this site, our data support a model in which cells utilize different mechanisms to recruit dynein to different organelles. The results demonstrate that neurotrophin binding to Trk initiates the recruitment of cytoplasmic dynein to signaling endosomes through ERK1/2 phosphorylation of intermediate chains and the subsequent retrograde axonal transport of the endosomes that is essential for cell survival. Thus, the Trk containing signaling endosome regulates its own retrograde transport.

1980

Ankyrin-B is required for the regulation of synaptic vesicle transport and for the maintenance of long axonal tracts in neurons.

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It has been established that Ankyrin-B, an abundant membrane adaptor protein in the brain, is essential for linking L1 cell adhesion molecules (L1-CAM) to the spectrin-actin cytoskeleton. These interactions ensure mechanical strength and also play a role in establishing proper axon guidance and connectivity. Consequently, mice lacking expression of the large ankyrin-B isoforms (AnkB KO), and of L1-CAM as a result, exhibit early postnatal lethality, brain malformations and neurodevelopmental defects seen in humans and in mice with L1-CAM mutations.

Interestingly, ankyrin-B binds the dynactin-4 subunit of the dynactin complex, which links the motor protein dynein to membrane cargo and helps regulate bidirectional transport along microtubules. Additionally, β 2-spectrin, which is an ankyrin-B partner, also directly interacts with dynactin through its arp-1 subunit, and has been widely implicated in the regulation of transport dynamics in axons. It is possible that both ankyrin-B and β 2-spectrin collaborate in coordinating motor protein interactions with membrane cargoes and contribute to the regulation of intracellular transport. The efficient and regulated bidirectional transport of organelles, synaptic vesicles, and trophic signals along long distances in axons is critical for axon growth and survival, and for maintaining the functional organization of the adult neuronal circuitry. Not surprisingly, deficiencies in axonal transport are often proposed as common mechanism in the pathogenesis of neurodegenerative diseases.

Here we show that the 220 kDa isoform of ankyrin-B regulates the transport dynamics of synaptic vesicles in mouse hippocampal neuron axons. Loss of this ankyrin-B isoform alters the bidirectional transport of multiple synaptic vesicle proteins, as observed by decreased anterograde and retrograde segmental velocities, shorter run length, and increased percentage of stationary and randomly moving vesicles. Interestingly, transport disruption seems to be specific to synaptic vesicles in the axon, since neither axonal transport of other cargoes nor transport in dendrites is affected. Additionally, cultured hippocampal neurons from AnkB KO animals develop shorter axons and numerous organelle-filled swellings. Lastly, the axonal transport phenotypes of AnkB KO neurons can be rescued by expression of wild-type 220 kDa ankyrin-B, but not ankyrin-B mutant proteins unable to associate with either dynactin or spectrin. In vivo functional characterization using diffusion tensor imaging analysis of neonate brains (PND2) reveals the striking consequences of the deficient axonal transport, as evidenced by severe defects in the development of the corpus callosum, the fimbria and the corticospinal tracts, along with degeneration of the optic nerve.

These results suggest a new role for ankyrin-B in the regulation of transport in axons that may be critical for the development and survival of major brain axonal pathways. Future work will explore additional mechanistic details implicated in these new findings and their potential linkage to neurodevelopmental and neurodegenerative diseases.

1981

Examining the Role for the Survival of Motor Neuron Protein in Axonal mRNA Localization and Transport in Spinal Motor Neurons.

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Spinal muscular atrophy (SMA) is a neuromuscular disease defined by a specific degeneration of motor neurons. SMA results from a reduction in the survival of motor neuron (SMN) protein, which is ubiquitously expressed with a well characterized role in promoting the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs). While some underlying defects in splicing have been observed in an SMA mouse model, these defects are not unique to motor neurons, making it unclear what extent they play in the selective motor neuron degeneration seen in SMA. We and others have demonstrated that SMN undergoes active transport in the axon of primary motor neurons where it also colocalizes with various mRNA binding proteins (mRBPs) known to play vital roles in mRNA transport. Additionally, a defect in axonal localization of β -actin mRNA at the axon terminus in primary motor neurons derived from the SMA mouse model has been observed. These and other data suggest that SMN may play a non-canonical role in the axon, potentially through mediating the assembly of mRNA and mRBPs into actively transported mRNA granules. To explore this hypothesis, we investigated the effects of SMN deficiency on both the localization and transport of messenger ribonucleoproteins (mRNPs) in axons and growth cones of primary motor neurons. Using quantitative fluorescence in situ hybridization (FISH), we observed a significant defect in the localization of total polyadenylated mRNAs in the axons of SMN-depleted motor neurons. Furthermore, we have identified a significant reduction in the axonal levels of *Gap-43* mRNA, a transcript whose stability and transport is regulated by the mRBPs HuD and IMP1. Interestingly, we can show that HuD and IMP1 specifically associate with SMN in motor neuron axons. Quantitative immunofluorescence (IF) revealed a defect in the axonal localization of IMP1 and HuD in motor neurons with depleted SMN protein. Our results support a model where SMN plays a non-canonical role in the specific assembly, transport or both of mRNPs in the axon of motor neurons.

1982

Caspase activity promotes Wallerian degeneration in *Drosophila* motoneurons.

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Axonal degeneration occurs after injury and in many neuropathies and neurodegenerative diseases. Axons that have lost connection with the cell body degenerate through via a self-destruction program whose mechanism is poorly understood. While caspases clearly play a role in developmentally programmed pruning, their role in Wallerian degeneration is less clear. Here we use an *in vivo* injury assay of *Drosophila* peripheral nerves to study Wallerian degeneration defined motoneuron axons and synapses. Our findings indicate that the *Drosophila* initiator caspase Dronc and the effector caspase Dcp-1 are required for the degeneration of injured motoneuron axons. Similarly, overexpression of p35 or the *Drosophila* caspase inhibitor DIAP1 also inhibit Wallerian degeneration. We are currently comparing the role of caspases in different *Drosophila* neuronal types, and testing the role of mitochondria in this caspase-mediated Wallerian degeneration. Consistent with a mitochondrial role in caspase activation, mutations which reduce mitochondria in axons inhibit axonal and synaptic degeneration after injury.

1983

High-Resolution Computational Analysis and Modeling of Tau-Mediated Spatial Organization of Axonal Transport.

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Tau is a microtubule-associated protein that is particularly abundant in axons of neuronal cells. It is thought that, under normal physiological conditions, tau binds and stabilizes axonal microtubules, which provide tracks for axonal cargo transport. It has also been hypothesized that tau *in vivo* may modulate axonal transport spatially by serving as roadblocks and thus facilitates detachment of motor-cargo complexes from microtubules. However, this hypothesis has not been fully tested. Understanding the roles of tau in axonal transport may provide important insights into related spatial-temporal transport regulatory mechanisms. In this study, we combined high-resolution computational techniques with *in vivo* fluorescence imaging to examine the relation between the spatial distribution of tau and the spatial behavior of axonal transport in the segmental nerves of *Drosophila* third instar larvae. Specifically, we analyzed spatial properties of the axonal transport of vesicles containing APP-YFP (amyloid precursor protein tagged with YFP) and Synt-GFP (synaptotagmin tagged with GFP) as well as mitochondria under different genetic backgrounds, including knockdown of endogenous *Drosophila* tau and overexpression of wild type and mutant human tau. We found that transport of these vesicles and organelles follows defined and consistent spatial patterns that are cargo specific. We also quantitatively characterized spatial distribution of tau using immunofluorescence in individual neurons within segmental nerves of the *Drosophila* third instar larvae. Based on these data, we developed quantitative models for analyzing and explaining the spatial behavior of the axonal transport. We tested our models by comparing their predictions of cargo behavior based on computer simulations versus actual experimental measurements. We found that the models we proposed can account for some key aspects of spatial-temporal cargo behavior. Overall, high-resolution computational analysis and modeling reveals important roles of tau in mediating the spatial organization of axonal transport.

1984

Measurements and modeling of axonal transport: Amyloid precursor protein wins over negative charge in the race to the synapse.

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Intracellular transport maintains the architecture and function of neurons. Sorting of cellular components into different functional compartments relies on selective microtubule-based transport yet the details of how cargo-motor interactions produce differences in compartments remains elusive. Several cargo-receptors for kinesin-based transport have been proposed. Two of these--phosphatidylinositol, a negatively charged lipid, and the carboxyl terminus of amyloid precursor protein (APP-C)--mediate motility of cargo in living systems. Here we describe an assay in which the transport dynamics of these defined cargo can be directly observed, measured and modeled. We engineered cargo to display a single type of motor receptor by conjugating polystyrene fluorescent nanospheres (100 nm diameter) to either a charged residue (COOH) or APP-C peptides. As controls we conjugated beads to glycine. After injection into the squid giant axon, bead movements were recorded in time-lapse sequences captured by laser-scanning confocal microscopy. In this report we compare the motility of negatively charged

beads with APP-C beads in the presence of glycine-conjugated control beads. Tracking individual beads in digital sequences revealed instantaneous velocities of 0.279 vs 0.371 $\mu\text{m}/\text{sec}$ yielding calculated attachment/detachment rates of 0.04/0.026 vs 0.03/0.032 sec^{-1} and predicted run lengths (v/k_1) of 10.7 vs 11.5 μm for COOH and APP-C beads respectively. By computationally generating thousands of bead trajectories based on real measurements, we obtained cumulative velocities and dispersion coefficients for each bead type. Using these values in the traffic model of Smith and Simmons (2001) we generated cumulative probabilities of distribution and compared these to the data. The Kolmogorov-Smirnov test for goodness of fit demonstrated similarity between model and bead distributions at long time points ($t=156$, $p=0.96$ and 0.98). Finally we used the model to predict the relative distribution of each bead type and found that APP-C beads achieve greater progress towards the presynaptic terminal with complete separation of distributions after 3 hr of transport from a single injection site. The significance of this data and accompanying model pertains to the role transport plays in neuronal function, connectivity, and survival, and has implications in the pathogenesis of neurological disorders, such as peripheral neuropathies and neurodegeneration as in Alzheimer's. Supported by NS046810, NS062184, U54CA143837 (VC), NIGMS K12GM088021 (JP), P5OGM08273.

1985

N-terminal Fragments of Amyloid- β Precursor Protein are Trafficked in Association with Short Peripherin-containing Neurofilaments.

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Intracellular transport of vesicles over long distances – a process that is particularly active in neurons - is powered by molecular motors, kinesins (plus-end movement) and cytoplasmic dynein (minus-end movement), which are recruited to and move individual vesicles along stationary microtubules. It is thought that cytoskeletal polymers, such as short microtubules and neurofilaments, are transported into neurites in a similar way - by recruiting kinesin-1 and cytoplasmic dynein, which move them along stable microtubule tracks. While using the same motile machinery and transport paradigm, vesicles and short cytoskeletal polymers are thought of moving independent of each other and being under distinct regulatory constraints. By investigating transport of the Amyloid- β Precursor Protein (APP), we identified a novel form of motility where transport vesicles, containing a fragment of APP, move - not by themselves - but by tightly associating with moving cytoskeletal polymers. APP, a type I transmembrane protein, is cleaved in the neuronal soma into N- and C-terminal fragments (NTFs and CTFs); the NTFs and the CTFs are sorted into distinct vesicles, and are separately transported into neurites, to reach fragment-specific destinations. To characterize the transport of the NTFs and CTFs, we used N- and C-terminal, dual-tagged APP constructs: CFP-APP-YFP for live imaging, and FLAG-APP-Myc for immunolocalization. When expressed at low levels in neuronal CAD cells, both constructs revealed a non-overlapping distribution of NTFs and CTFs in neurites, consistent with their independent transport. The distribution of C- and N-terminal epitopes of endogenous APP, revealed with immunolabeling, showed CTFs associated with randomly distributed vesicle-like structures, and NTFs exclusively localized to short, filamentous structures in processes, which we identified as segments of acetylated microtubules and peripherin-positive neurofilaments. A similar, tight distribution along filamentous tracks was detected for the N-terminal FLAG tag, but not for the C-terminal Myc tag of dual-tagged APP, in CAD cells expressing FLAG-APP-Myc. Treatments that selectively destabilized the acetylated microtubules abrogated this distribution of peripherin and NTFs, but did not affect the random distribution of CTFs. In biochemical subfractionation of neuronal extracts, the NTFs copurified with cytoskeletal filaments, to which they bound via ATP insensitive, stable interactions. These

results suggest that vesicles containing NTFs, rather than recruiting themselves molecular motors, attach to short, moving neurofilaments that likely glide over stationary microtubule tracks. Our work expands the current view on vesicle transport in neurons, by offering an alternative model for microtubule-based transport. We propose that, in addition to the established transport paradigm of individual vesicles moving independently on microtubules, molecular motors could transport en bloc short cytoskeletal filaments loaded with vesicles - such as those carrying the NTFs. These findings are highly relevant to the physiology and pathology of the neuron. Supported by NIH award AG039668 (Z.M.) and by an UMDNJ Foundation grant (ZM, VM).

1986

Reduction of Mitochondrial Retrograde Molecular Motors Protein Levels and Mitochondria Total Movement in Hippocampal Cell Culture Before and During Neurodegeneration-Related Protein Aggregation.

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Objective: Decreased mitochondrial transport has been reported after protein aggregation related to several neurodegenerative diseases, however the mitochondrial transport state before and during protein aggregation remains elusive.

The purpose of this study is to analyze protein levels of dynein c1h1 and dynactin, that are mitochondrial retrograde molecular motors and mitochondria total movement in hippocampal cell culture exposed to rotenone a specific inhibitor of mitochondrial NADH dehydrogenase within respiratory chain complex I which can trigger protein aggregation.

Methods: All the procedures were performed in accordance with Institutional and International Guidelines for Animal Experimentation. Hippocampal cell culture of neonate Lewis rats were exposed to 0.1, 0.3 and 0.5 nM of rotenone or DMSO diluted in culture medium for 48 hours. Cells were then lysed and total protein was employed to Western blot analysis of dynein c1h1 and dynactin levels; or stained with Mitotracker™ green for live cell analysis of mitochondria total movement through confocal microscopy. Western blot normalization was done using anti-beta actin labeling. Films were quantified using Image J Software and mitochondria transport was evaluate using Image J plugin Difference Filter. Experiments were run in triplicates and results were analyzed by One-way Anova followed by Tukey post-hoc test.

Results: The analysis demonstrated a significant down-regulation of dynein c1h1 levels of 30% ($p<0.05$), 40% ($p<0.0001$) and 35% ($p<0.001$) respectively after exposure to 0.1, 0.3 and 0.5nM of rotenone respectively, as compared to DMSO. Exposure to 0.1, 0.3 and 0.5nM of rotenone also decreased dynactin levels in 20% ($p<0.05$), 35% ($p<0.0001$) and 50% ($p<0.0001$), respectively. Mitochondria total movement decreased 15% ($p<0.05$) after exposure to 0.5nM of rotenone compared to DMSO, however exposure to either 0.1 or 0.3nM did not alter mitochondria movement.

Conclusion: Decrease of dynein c1h1 and dynactin protein levels is accompanied by decrease in mitochondria total movement decrease after exposure to 0.3 and 0.5nM of rotenone illustrating the impairment of mitochondrial transport during and before protein aggregation, which possibly triggers neurodegenerative processes.

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1987

Damage to Axonal Mitochondria Arrests their Motility prior to Local Mitophagy.

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To minimize oxidative damage to the cell, malfunctioning mitochondria need to be removed by mitophagy. In neuronal axons, two models have been proposed. In one, mitochondria that have lost their membrane potential are retrogradely transported to the soma where they are destroyed. In the other, mitochondria undergo mitophagy locally in the axon.

We have found that mitochondrial depolarization induces accumulation of Parkin on axonal mitochondria, as has previously been shown in non-neuronal cells. In response to damage, PINK1 and Parkin associate with Miro, a mitochondrial adaptor protein that anchors a kinesin motor complex to the mitochondrial surface. This association leads to proteosomal degradation of Miro and arrest of mitochondrial motility in a Parkin-dependent manner. Following their arrest, axonal mitochondria colocalize with the autophagosomal marker LC3 indicating the local onset of mitophagy.

The arrest of mitochondrial motility prior to their local engulfment by autophagosomes may help prevent the spread of oxidative damage throughout the cell.

1988

Image-Based Computational Methods for Analyzing Mitochondrial Dynamics in Axonal Transport.

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Mitochondria are essential organelles of eukaryotic cells, serving a variety of crucial intracellular functions that include energy conversion and distribution as well as signal transduction. In response to changing needs of dynamic cellular processes, mitochondria frequently undergo morphological changes as well as spatial redistribution, a phenomenon referred to as mitochondrial dynamics. Because of their highly polarized configuration, neurons are particularly dependent on proper regulation of mitochondrial dynamics for their survival and function. Quantitative characterization of axonal mitochondrial dynamics is required for studying related functional and regulatory mechanisms. In this study, we developed image-based computational methods for reliable and precise characterization and analysis of mitochondrial dynamics within the axon. To characterize spatial redistribution of axonal mitochondria, we developed automated tracking techniques to recover their complete trajectories. To characterize morphological changes of axonal mitochondria, we developed related image segmentation, shape change detection and quantification techniques. By integrating these techniques, we examined the relation between mitochondrial shape changes and mitochondrial transport in *Drosophila* segmental nerves as well as mouse hippocampal neurons under different perturbations of mitochondrial fusion and fission. We found that sizes of axonal mitochondria follow defined statistical clusters, that mitochondrial morphology affects their motility, and that the maximal velocities of mitochondrial transport are correlated with their sizes. Based on these data, we proposed a quantitative model to account for observed relations between mitochondrial morphology and motility. We present results of testing several prediction of the model using experimental data.

Establishment and Maintenance of Polarity II

1989

Developmental timing determines effects of actin on neuronal process formation

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In the initial development of neuron polarity, localized instability of actin within a growth cone is associated with the formation of a single axon from that neurite, and global destabilization of actin produces multiple axons (e.g. Bradke & Dotti, 1999). The formation and maturation of the dendritic arbor follows a timecourse that is more protracted than that of axons, and so we varied the timing of actin destabilization in cultured hippocampal neurons to test for contributions of actin dynamics in dendritic development. We applied prolonged, global treatment of pharmacological agents to interfere with the state of actin polymerization at 2 key stages of early dendritic growth: after 24 hours in vitro (1-3 days in vitro, DIV), a time point when most cells have begun to initiate axon formation but dendrites remain undifferentiated; and 3-5 DIV, at the onset of robust dendritic outgrowth. Measures of morphological development and molecular polarity were used to classify process phenotype and determine the effects of treatment. Neurons treated from 1-3DIV with actin depolymerizing agents (Cytochalasin D or Latrunculin A) developed supernumerary axons. These cells also formed significantly more dendrites. Thus, at this stage of development a major outcome of actin depolymerization is net production of more processes generally rather than conservation of process number and fate-switching from dendrite to axon. Treatment at 3-5 DIV also lead to a significant increase in axon number, but did not produce additional dendrites, suggesting a critical period for the formation of primary dendrites. Jasplakinolide, a drug that stabilizes actin, lead to dendrite retraction and loss, suggesting that actin dynamics are essential for the structural integrity and maintenance of the dendritic arbor. To test whether molecular polarity of dendrites was also dependent on actin dynamics, cells were immunostained for MAP2, which becomes polarized to dendrites during development, and tau-1, which is localized to axons. A comparison of fluorescence intensity between F-actin-destabilized and control cells revealed a parallel between actin destabilization at 3-5 DIV and reduced polarized distribution for both markers in axons and dendrites. Since treatment at this stage did not alter the number of primary dendrites, these data suggest separate contributions of actin dynamics in the molecular polarity of the dendritic arbor that are distinct from initial outgrowth.

1990

NDR2 affects primary dendrite formation by regulating IQGAP1 and CDC42 in hippocampal neurons.

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NDR kinases (Nuclear Dbf2 Related protein kinase) belong to the AGC group of serine/threonine kinases and include NDR1/2 and Lats1/2 kinases. NDR1/2, conserved from yeast to human, is known to regulate cell morphogenesis. However, the targets and detailed molecular functions of NDR1/2 in hippocampal neurons remain largely unknown. Here we report that NDR2 regulates dendritic morphogenesis through IQGAP1 and CDC42. NDR2 knockdown by shRNA decreased the number of primary dendrite of hippocampal neurons, which was

rescued by NDR2 or CDC42 overexpression. NDR2 overexpression increased the level of activated GTP-bound CDC42, whereas NDR2-knockdown significantly diminished the GTP-CDC42 level. IQGAP1, an effector of CDC42, was identified as an NDR2 interacting molecule, and simultaneous expression of NDR2 and IQGAP1 further increased the GTP-bound CDC42 level. NDR2 prevented the ubiquitin-dependent degradation of IQGAP1. Thus, we propose that NDR2 regulates primary dendrite formation by bringing IQGAP1 and GTP-Cdc42 together and promoting actin cytoskeleton remodeling.

1991

Mechanisms for axon-specific accumulation of an axonal protein JIP1.

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The basic function of neurons is to receive, integrate, and transmit signals. To do so, neurons develop polarity by forming a single axon and multiple dendrites. Rat hippocampal neurons polarize in symmetrical *in vitro* environments (Craig and Banker, *Annu Rev Neurosci*, 1994). The process of neuronal polarity formation was divided into five stages and the neurons first form several immature neurites (minor processes) that are similar in length, and at this stage the neurons appear symmetric (stage 2). Thereafter, one of these neurites outgrows its siblings to break this symmetry and the neurite accumulates axon-specific proteins (stage 3). The longest neurite acquires axonal characteristics, whereas the others later become dendrites, to establish neuronal polarity. Thus, this break in symmetry is the initial step of neuronal polarization. Recent studies reported that both excitatory and inhibitory neurons in the cerebral cortex also undergo symmetry breaking steps (Yamauchi et al, *J Neurosci*, 2010; Hatanaka et al, *Cereb Cortex*, 2012). Previous studies identified a number of axon-specific molecules, which play key roles in the establishment of the polarity (Arimura and Kaibuchi, *Nat Rev Neurosci*, 2007). However, the mechanisms by which these molecules accumulate in the nascent axon remain poorly understood. A cargo adaptor JIP1 is transported along microtubules by kinesin1 and strongly accumulates in the nascent axon during neuronal symmetry breaking. In this study, we analyzed the mechanisms underlying the axon-specific accumulation of JIP1, by focusing on the following three possible processes: 1) selective anterograde transport, 2) retrograde diffusion, and 3) selective anchoring at the neurite tip. Previous studies reported that tubulin acetylation promotes kinesin1 transport (Dajas et al, *Curr Biol*, 2008), while tubulin tyrosination inhibits it (Konishi et al. *Nat Neurosci*, 2009). So using polarizing cultured rat hippocampal neurons during stage 2 and early stage 3, we performed immunostaining for JIP1 and posttranslational microtubule modifications (acetylation and tyrosination) in their neurites. Consistent with a previous report (Nakata et al, *J Cell Biol*, 2011), we found that JIP1 accumulation was not correlated with these posttranslational modifications, during the early stage of neuronal symmetry breaking. On the other hand, JIP1 accumulation showed positive correlation with neurite length. Using photoconvertible KAEDE-JIP1, we quantitatively monitored the retrograde diffusion of JIP1 from neurite tips to the cell body. Inverse correlation between the diffusing velocity and the neurite length was observed, suggesting that the retrograde diffusion would contribute to the neurite length-dependent JIP1 accumulation. In addition, time-lapse live cell imaging of EGFP-JIP1 and mCherry-EB3 showed a possible anchoring of JIP1 in growing neurites. Based on these results, we propose that the retrograde diffusion, and selective anchoring of JIP1 at the neurite tip may play key roles in the axon-specific JIP1 accumulation.

1992

Fluid flows and pattern formation in the one-cell *C. elegans* embryo.

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The interplay between mechanical stresses and cellular signaling systems plays a central role in cellular organization. Our work focuses on such interplay during the asymmetric division of the *C. elegans* one-cell embryo. In the embryo, mechanical stress is generated within a highly cross-linked, contractile actin cortical network underlying the cell membrane. Due to the influence of local cues, asymmetries develop within this network, resulting in long range flow of actin cortex from posterior to anterior. This flow of cortical actin in turn entrains the surrounding cytoplasm giving rise to a complex pattern of fluid flow within the embryo. Our work suggests that the conserved PAR polarity proteins are transported by these bulk cytoplasmic flows due to particular kinetics behaviors of the PAR proteins. In particular, we suggest that it is their ability to undergo a transition from a free, and rapidly diffusing cytoplasmic state into a slowly diffusing cortex-associated state that allows PAR proteins to tap into this bulk transport mechanism, resulting in an induced PAR asymmetry that can drive cell polarization. By accurately quantifying the patterns of fluid flow in the embryo, the diffusive properties of PAR proteins, and incorporating the results within quantitative models, our data provides insight into how cortex-generated flows shape the distributions of PAR proteins throughout the first cell division.

1993

Role of Mob1 in regeneration of a single cell.

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Understanding the processes behind the regeneration of cells and tissues is an important cell biological question. Cells need to identify and react to specific damages in and around them, and in order to understand these processes at a basic level we need a model that exhibits a similar regeneration of specialized structures in the context of a single cell. Perhaps the most classical system for studying regeneration in single cells, pioneered by Thomas Hunt Morgan, F.R. Lillie, Vance Tartar and others, is *Stentor coeruleus*, a large ciliate, ~1mm long, with a highly patterned cell cortex and the ability to regenerate and reorganize after surgical or chemical manipulations. Its large size and complex organization rival those of metazoan organisms and the ease of surgical manipulations gives *Stentor* significant advantages over other ciliate models. Using the traditional surgical techniques unique to *Stentor* as well as modern RNA interference (RNAi) methods, visualization techniques, and genomic sequencing I will revive *Stentor* as a model for studying the regeneration of cell polarity and organization. We have partially sequenced *Stentor*'s macronuclear genome and shown that RNAi machinery is functional in *Stentor*. Using an RNAi approach we have begun a systematic investigation of the molecular basis of single-cell regeneration. Mob1 is a highly conserved kinase regulator and is known to interact with NDR/LATS kinases. It plays a role in a variety of functions including apoptosis, mitosis, morphogenesis, and cell proliferation. Recent work in *Tetrahymena thermophila* suggests that Mob1 also performs important roles in ciliates. Results for RNAi of Alpha-Tubulin and Mob1 in *Stentor* result in dramatic changes in cell polarity and organization of the cortex, showing that we can analyze single-celled regeneration in *Stentor* at a molecular level. Knocking down Alpha-Tubulin, a key structural component in the cortex, results in cortical defects and problems with cell regeneration. This is very different from the Mob1 knockdown, which results in the drastic elongation of cells, cortical aberrations, and an apparent loss of proper cell proportions. Using RNAi in conjunction with the unique microsurgical methods available in *Stentor*, it should be possible to restore this classical system to its previous status

as a central model for understanding the basic principles of cell polarity, biological pattern formation, and cellular regeneration.

1994

Reversing the polarity of migrating Dictyostelium cells.

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Cells migrating in a chemical gradient typically have a polarized morphology, where they display a distinct front and back. Cells can also have different localized sensitivities to chemoattractants depending on their degree and type of polarity. To better understand these phenomena, we used the social amoeba *Dictyostelium discoideum* that expressed GFP-tagged proteins known to localize to distinct regions of the cell during cell migration. *Dictyostelium* cells were lured into a specialized microfluidic device developed in our laboratory which confined the cells in narrow 3-dimensional PDMS channels. The chemoattractant gradient in this experimental platform could be reversed so that the rear of the cell was exposed to a higher concentration of chemoattractant than the front of the cell. Cells in the channels would freeze, become unpolarized, and were then capable of forming a new front at the former rear of the cell. We have observed the temporal and spatial loss and gain of localization of various signaling and cytoskeletal molecules at both the rear and front of the cell as polarity is broken down and re-established. We monitored the localization of PHCrac (a biosensor for PI(3,4)P2 and PI(3,4,5)P3), RBD (a biosensor for Ras activity), PTEN-GFP (a phosphatase which elevates levels of PI(4,5)P2), Tubulin-GFP (a biomarker for microtubules) and redistribution during polarity re-establishment. Further experiments were performed in the absence of an actin cytoskeleton by treating cells with Latrunculin A. These experiments have helped elucidate the molecular mechanisms that contribute to the establishment and maintenance of cell polarity.

1995

How protrusion/retraction switch controls cell polarity, shape, and motion.

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Persistently migrating fish epidermal keratocytes exhibit nearly perfect separation of protrusion and retraction to, respectively, leading and trailing parts of the cell edge, each occupying approximately half of the cell perimeter. During initial cell polarization these protruding and retracting zones consolidate from several smaller regions, sometimes through intermediate stages with multiple leading edges traveling in circles around the cell center. To understand the evolution of cell shape during polarization and motion we analyzed the distribution of the cell edge velocity and the dynamics of transitions between protrusion and retraction. Protrusion velocity was nearly constant along the central part of the leading edge of migrating cells, but exhibited large fluctuations at the cell lateral extremities. Switches between protrusion and retraction occurred preferentially at the cell extremities in both persistently moving and polarizing cells. To investigate the conditions for switch between protrusion and retraction, we designed the experiment where the cell body was mechanically restricted by a flexible glass needle so that it could not move in the direction of the leading edge. In these conditions, the leading edge continued to extend for a while at a nearly constant velocity causing cell elongation until, abruptly, protrusion switched to retraction and the cell reversed its polarity. Reversal was dependent on the myosin II activity and coincided with the maximum of tension

between the leading edge and the cell body. These observations suggest the following empirical rules for the cell edge behavior: the states of protrusion and retraction are both relatively stable, and the switches between the two states occur preferentially at the threshold stress values which arise at the cell extremities. These rules are consistent with the behavior and shape of polarizing, steadily moving and mechanically confined cells. We are developing a numerical top-down model of cell polarization and motion based on these empirical rules. Supported by Swiss National Competence Center for Biomedical Imaging and Swiss National Science Foundation.

1996

Actomyosin Morphodynamics Imprint Spatial Order of the Cytoplasm During Cell Polarization.

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The spatial organization of cells depends on their global geometry and the ability to sense their own shape and size. While the molecular players and interactions involved in the generation and dynamic remodeling of cell shape continue to become identified, a default cell-autonomous mechanism controlling large-scale cell organization remains unknown.

To address this issue, we turn to the use of single epithelial cells, a simplest and evolutionary ancient animal cell type that demonstrates remarkable cell shape plasticity in embryogenesis and postnatal ontogenesis. Investigating this model by multiparametric quantitative live-cell imaging, experimental perturbations, and mathematical modeling, we establish the morphogenetic potential and hierarchy of activities for the universal cytoarchitectural modules, actomyosin and microtubule cytoskeletons.

We show that a balance between myosin-II-dependent cross-linking-contraction and inherent self-organizing activity of polymerizing actin filaments can give rise to structurally and functionally distinct cell morphologies. Decreasing the degree of contractility shifts the balance towards actin-based protrusivity of the plasma membrane. The latter destabilizes cellular shape and can ultimately lead to a spontaneous switch from a planarly symmetric discoid shape of stationary cells to an elongated cell morphology with migratory polarity.

We further establish that a remarkable cooperativity between the actomyosin-driven cell-scale patterns and cytoplasmic microtubule organization mediates the communication process between the global shape of the cell and its interior. The morphologies emerging from the balance between actomyosin contractility and actin protrusivity pose a simple geometric constraint for the self-organization of cytoplasmic microtubules into the patterns that dictate a spatial order of internal cell components. The cooperativity among actin and microtubules in producing the large-scale cell organization is based on the nematic alignment due to collisions of the filaments, and appeared to be independent of previously proposed long-range interactions between the two cytoskeletons.

Thus, we propose that cell-scale morphogenesis is an example of biological self-organization and requires a minimal “tuning” mechanism relying on the pathways directly regulating actomyosin contractility.

1997

Tipping the balance between adhesion and myosin contraction drives spontaneous motility initiation in keratocytes as revealed by modeling and experiment.

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Fish keratocytes spontaneously break symmetry and become motile in the absence of known external cues. It is known that motility initiates at the prospective rear of the cell where centripetal actin flow increases prior to the symmetry break, but what causes this flow increase and whether respective trigger is biochemical or mechanical is not clear. We applied our published model of continuum mechanics of viscous contractile actin network with free boundary to test two simple mechanical pathways of the initial asymmetric flow increase: spatial bias of myosin toward the prospective rear, or weakening of adhesion there. Both scenarios predicted the accelerated actin flow at the rear, but gave opposite predictions about traction force distributions. Experimental measurements showed that the traction forces are attenuated at the rear prior to motility, so the experiment supports the adhesion weakening as the symmetry break trigger. Simulations of the model showed, however, that such transient adhesion change does not support stable motility. We therefore propose a model in which the adhesion strength depends on the actin flow rate or myosin strength such that adhesions slip at high flow or contraction. Simulations of this model with stochastic self-organizing acto-myosin dynamics coupled with mechanosensitive adhesion reproduced the data and made additional predictions on dependence of frequency of the motility initiation on overall adhesiveness of the substrate and localized application of drags that were tested experimentally and confirmed. We propose therefore that the mechanical adhesion-contraction switch underlies the motile polarization in simple keratocyte cells, though additional biochemical mechanisms can complement this switch.

1998

Cooperative regulation of cellular contractility by Rho-kinase/Scrib/Shroom2 complex.

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Rho and Rho-kinase/ROCK regulate cellular morphogenesis and motility through cytoskeletal reorganization. We newly identified Scrib, which is a tumor suppressor and regulator of planar cell polarity, as a substrate of Rho-kinase. Rho- and Rho-kinase-dependent phosphorylation of Scrib was detected in COS7 and MDCK II cells with phospho-Scrib antibodies. We also found that Shroom2 bound to the C-terminal portion of Scrib containing phosphorylation sites by Rho-kinase in a phosphorylation state-dependent manner. Shroom2 is an actin- and Rho-kinase-binding protein, and thought to be involved in tissue morphogenesis via control of topological constriction of cellular sheets. Shroom2 formed a ternary complex with Scrib and Rho-kinase, for which phosphorylation of Scrib by Rho-kinase had a critical role. Both Scrib and Rho-kinase reciprocally stabilized the ternary complex formation. Rho-kinase, Scrib, and Shroom2 were localized at the contractile cortical edge of cell colonies and at the contractile wound edge in MDCK II cells. Ectopically expressed Shroom2 was highly accumulated at the contractile cortical edge and recruited endogenous Rho-kinase and Scrib there, leading to the resultant enhancement of MLC phosphorylation. Whereas, Shroom2 mutant lacking Rho-kinase- and Scrib-binding region was still localized at the edge, but failed to recruit Rho-kinase and Scrib.

Depletion of Scrib in MDCK II cells resulted in the decrease of MLC phosphorylation at the cortical edge. Similar result was obtained when Scrib was depleted in human umbilical vein endothelial cells (HUVECs). These results suggest that Rho-kinase/Scrib/Shroom2 ternary complex regulates cell contractility at the cortical edge, and this mechanism may partly account for the role of Scrib in planar cell polarity signaling cascade.

1999

Analysis of protein interactions involved in rear polarization of rat artery smooth muscle cells in atherosclerosis identify spectrin as a possible interacting partner of RHAMM.

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Using a phosphoproteomic screen followed by mass spectrometry we showed in a previous study (Silverman-Gavrila et al., 2011) that 6 proteins have a higher phosphorylation degree in neointimal smooth muscle cells compared to medial smooth muscle cells: ARPC5_RAT, Actin-related protein 2/3 complex subunit 5; Q9WUF7_RAT, Hyaluronan receptor RHAMM; LMOD2_RAT, Leiomodin-2; TRP13_RAT, Thyroid receptor-interacting protein 13; P2RX3_RAT, P2X purinoceptor 3; Q5U2P5_RAT, Transmembrane protein 24. Further analysis showed that RHAMM and ARPC5 play an important role in rear polarization of microtubule organization centres during migration of smooth muscle cells. Using bio-informatics programs such as BLAST, ClustalW, and PPRSpider, the list of proteins obtained from mass spectrometry was analyzed to reveal additional potential interacting partners. One such target protein that was validated is spectrin, an actin binding protein that was shown to play a role in the production of actin nets. Relative distribution of spectrin to actin, RHAMM, and alpha-tubulin in neointimal smooth muscle cells (SMCs) was further assessed using confocal microscopy in order to identify the localization of spectrin during cell cycle as well as its relative distribution and possible colocalization with members of the identified pathway that play a role in rear polarization of the MTOC. In dividing SMCs spectrin is present at spindle poles from prophase to late anaphase and its presence diminishes as the cell cycle progresses. In telophase and cytokinesis spectrin relocates to the midbody. In neointimal SMCs stimulated to migrate in a wound assay spectrin localizes at the membrane of the advancing edge (lamellipodia) and in the cytoplasm where it colocalizes with RHAMM perinuclearly. Spectrin and actin co-localize in the lamellipodia of interphase migratory neointimal SMCs but there is less colocalization of spectrin and α -tubulin. Our proposed model suggests that spectrin acts as a linker between actin cytoskeleton and RHAMM. The putative interactions will be further validated by biochemistry (co-IP), siRNA, and mutagenesis approaches. We showed previously, that a polygonal actin net localized dorso-lateral of the nuclei in neointimal smooth muscle cells is responsible for rear polarization of microtubule organizing centre during migration from media to neointima. We will further test whether spectrin is involved in organizing actin in the net in neointimal SMCs. Our data show that this multifaceted approach can be a useful tool for identifying interacting partners and decoding pathways involved in polarity regulation during migration of smooth muscle cells in atherosclerosis process that can be used as targets for preventing migration and thus progression of atherosclerosis plaque formation.

2000

Vangl-mediated non-canonical Wnt/planar cell polarity signalling is not required for the planarly polarized cell morphology of endothelium exposed to laminar flow.

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Background: endothelial cells exposed to laminar flow display planarly polarized cell morphology, becoming elongated and orienting along the flow direction. This planarly polarized phenotype is believed to minimize the physical stress that endothelium is exposed to and consequently dampen the possibly mechanical strain-induced inflammatory response, which could contribute to the development of atherosclerosis. Many signaling pathways have been identified to regulate the response of endothelial cells to shear stress, but few has been shown to specifically co-ordinate the response of a confluent sheet of endothelium. In the current study, we investigated the potential role of non-canonical Wnt/planar cell polarity (PCP) signaling pathway, which was first identified in *Drosophila* for their activity in coordinating the planar polarity of confluent epithelial sheets, in coordinating the response of confluent endothelial sheet to laminar flow.

Methods: among several core PCP proteins (Flamingo/Celsr, Frizzled, Dishevelled, and Strabismus/van Gogh), we chose Vang-like protein as our focus as we found that human umbilical vein endothelial cells (HUVEC) only express one of the two mammalian Vang homologs, Vangl1. We modified the expression level of Vangl1 in HUVEC by lentiviral transduction of vectors that encode GFP-Vangl1 or Vangl1-targeting shRNA. Vangl1-modified or control HUVEC were seeded into microfluidic channels and exposed to shear stress of 12 dyens/cm² for 24-48 h. Time-lapse or end-point fluorescent images were taken and the angles of cell nuclei and whole cells were measured using ImageJ.

Results: although there was a slight increase in the endogenous Vangl1 expression level and a change in the sub-cellular distribution of GFP-Vangl1 after cells exposed to shear stress. No difference in the angles of either cell nuclei or whole cell was observed at any time points measured.

Conclusion: Vang-mediated non-canonical Wnt/PCP signaling pathway is not required for the planarly polarized cell phenotype of endothelium under shear stress.

2001

The Arf3p GTPase acts with its novel effector Bud2p to modulate invasive growth in *Saccharomyces cerevisiae*.

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The development of filamentous growth in yeast is composed of various stages, including budding pattern switch, elongated cells, and agar invasion. The regulatory network and cooperative signaling of invasive growth is complicated and has been expansively studied. Bud2p, functioning as a GTPase-activating protein (GAP) for Bud1p/Rsr1p, is known to be required for both the axial and bipolar budding patterns and filamentous growth. Arf3p GTPase, the homologue of mammalian Arf6, was reported to be involved in actin polarization and bud site selection. A large-scale of yeast two-hybrid analysis showed that Arf3p interacts with Bud2p; however, the biological significance of this interaction remains unknown. Here, we provide the evidence that Arf3p promotes Bud2p GAP activity to modulate yeast invasive growth. We observed that Arf3p directly interacts with Bud2p in a GTP-dependent manner and that interaction is necessary for yeast invasive growth. Elevation of active GTP-bound Bud1p or lacking Bud2p GAP activity in yeast prevents invasive growth. Furthermore, we show that Arf3p-Bud2p interaction promotes Bud1/Rsr1p GTP hydrolysis and enhances the Bud2p-Bud1p

association in vivo. Thus, our study identifies Arf3p as a Bud2p's activator for promoting Bud1p-GTP hydrolysis and reveals a novel regulatory signaling of Arf3p-Bud2p-Bud1p for yeast invasive growth.

2002

Role of a cyclin in the establishment of yeast pheromone receptor polarity.

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The formation of shmoo by the budding yeast *S. cerevisiae* during mating is a chemotropic response related to chemotaxis. All chemotropic and chemotactic cells exhibit a remarkable ability to interpret gradients and sense direction. The two haploid yeast mating types, *MATa* and *MAT α* , sense the pheromone secreted by cells of the opposite type, polarize their growth towards the closest mating partner, and fuse to form diploids. How do these cells sense direction? In *MATa* cells, the Ste2 pheromone receptor is the primary gradient sensor. Ste2 is uniformly distributed on the plasma membrane in vegetative cells, but upon ligand binding, it is rapidly internalized. It then reappears as a polarized crescent that coincides with the incipient shmoo site. In mating mixtures, these receptor crescents orient towards the closest mating partner. Although actin-dependent directed secretion stabilizes and amplifies receptor polarity, we have shown that receptor polarization precedes the polarization of actin cables and occurs in the absence of actin-directed secretion. In contrast, internalization of the receptor is essential for its polarization. Our recent data indicate that a receptor polarization site that can be amplified independently of f-actin is established within 15 minutes of pheromone treatment. How does this occur?

To identify key players in the establishment of receptor polarity, we performed a directed genetic screen of haploid-specific genes. *PCL1* deletion conferred a significant defect in receptor polarization. Pcl1 is one of the ten cyclins for the cyclin-dependent kinase (CDK) Pho85. Studies using alexafluor-phalloidin and yDsRed-Sec4 indicated that this phenotype is not due to a defect in the polarization of actin cables or directed secretion. Unlike wild type cells, *pc1 Δ* cells were unable to establish a receptor polarization site that could be amplified independently of actin in the first 15 minutes after pheromone treatment. In contrast, over-expression of Pcl1 caused the receptor to polarize to a very narrow region in response to pheromone. Pcl1 over-expression also greatly increased sensitivity to pheromone as visualized by halo assays and microscopy. Furthermore, slight over-expression of G β in *pc1 Δ* cells caused a moderate increase in pheromone sensitivity. Given that G β is predicted to have a consensus phosphorylation motif for Pho85/Pcl1, we hypothesize that this G β -Pcl1 genetic interaction results from their direct physical interaction. Consistent with its effect on receptor polarization, *pc1 Δ* conferred a directional sensing defect in mating assays. In summary, our data strongly suggest a novel role for the cyclin Pcl1 and the CDK Pho85 in receptor polarization and directional sensing.

2003

Gradient tracking in yeast: role of pheromone receptor phosphorylation and endocytosis.

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Cells are extremely proficient at tracking chemical gradients. Chemical signals are detected by G protein-coupled receptors at the cell surface. In many cells, receptors and their associated G proteins are uniformly distributed on the membrane and are thought to accurately transduce the extracellular spatial profile of ligand into an intracellular profile of active G protein. In contrast, yeast pheromone receptors become heterogeneously distributed over the cell surface following exposure to pheromone. Cells exposed to high concentrations of pheromone arrest in G1 and

undergo polarized growth to form a shmoo projection, and pheromone receptors are concentrated near the tip of the shmoo. Receptor distribution results from (i) polarized secretion of new receptors, (ii) slow diffusion of receptors in the plasma membrane, and (iii) ligand-induced receptor endocytosis and degradation. Why would cells reduce their capacity to sense external gradients by restricting receptor localization in this way? Here we address the potential role of receptor distribution in the context of novel findings indicating that at physiological pheromone concentrations, the patch of polarity proteins “wanders” around the cell cortex, allowing for adjustments in growth orientation. Wandering is biased by pheromone, helping yeast cells grow towards mating partners. We have investigated how the receptor modifications (phosphorylation and ubiquitination) that trigger its endocytosis affect polarity site wandering and gradient tracking. We found that the receptor does not need to be ubiquitinated or endocytosed in order to bias patch wandering. However, receptor phosphorylation appears to be important in regulating patch wandering. These findings suggest novel roles for receptor phosphorylation beyond causing ubiquitination and endocytosis.

2004

Receptor phosphorylation and polarization are regulated by G β interaction with Yck1.

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The mating response of *S. cerevisiae* is directional. Mating cells must interpret the direction of the pheromone gradient and grow toward the closest potential partner. The pheromone receptors are the most upstream component of the mating response. They are distributed uniformly on the plasma membrane during vegetative growth, but in response to pheromone, are rapidly internalized and subsequently polarized to the region where the mating projection will form. We have previously shown that internalization of the receptor is required for its polarization but that actin-dependent directed secretion is not. Pheromone also induces the polarization of the G protein subunits, G α and G β , and this is dependent on receptor internalization. How is receptor polarity established prior to actin-dependent directed secretion? In a genetic screen for proteins that interact with G β , we identified Yck1, one of the two yeast casein kinases required for receptor phosphorylation and internalization. Yck1 interacted with G β at the incipient shmoo site prior to morphogenesis in a bimolecular fluorescence complementation assay, and was identified by mass spectroscopy as a protein that bound to a G $\beta\gamma$ affinity column. A mutant form of G β that cannot be phosphorylated, G $\beta^{\text{P-}}$, showed a decrease in Yck1 interaction in both genetic and biochemical assays. Mathematical modeling suggests that the interaction between G β and Yck1 is important for robust polarization of the receptor in a pheromone gradient. G $\beta^{\text{P-}}$ cells were defective in receptor polarization in both isotropic pheromone and gradient conditions. In mating mixtures, G $\beta^{\text{P-}}$ cells exhibited a defect in asymmetric receptor phosphorylation. Additionally, overexpression of G β inhibited receptor phosphorylation. These data are consistent with a model in which G $\beta\gamma$ interacts with Yck1 to inhibit phosphorylation of the receptor, thereby preventing its internalization. On the up-gradient side of the cell, there is more activated receptor and free G $\beta\gamma$, and therefore, better protection of the receptor. This initiates a positive feedback loop that results in polarization of the receptor and its G protein, thus establishing the growth site.

2005

The yeast guanine nucleotide dissociation inhibitor (GDI) enforces singularity by enhancing competition between polarity sites.

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Polarity establishment employs an evolutionarily conserved machinery centered around the Rho-family GTPase Cdc42p. In response to cell cycle cues, *Saccharomyces cerevisiae* concentrates active GTP-bound Cdc42p at a predictable site defined by landmark proteins. In the absence of landmarks (e.g. *rsr1Δ*), however, yeast cells are able to polarize at a single, random site, a process known as symmetry breaking. The local concentration of GTP-Cdc42p during symmetry breaking grows as a result of a self-amplifying positive feedback mechanism. There is also a negative feedback loop in the polarity circuit that buffers the level of GTP-Cdc42p and stops clusters from growing too large. When cells begin to polarize, they can grow several clusters of polarity factors. However, once key factors become depleted from the cytoplasm, the clusters compete with each other, eventually leaving only one winning cluster, which becomes the bud site. We are trying to understand how competition between clusters works. We find that the yeast guanine-nucleotide dissociation inhibitor (GDI), Rdi1p, is needed for rapid competition between clusters. In the absence of Rdi1p the initial clustering of polarity factors is slowed, and competition is also much slower: in some cases cells still have two clusters at the time of bud emergence, and they form two buds. These phenotypes indicate that Rdi1p accelerates competition between clusters, thereby ensuring that a single winner emerges and only a single bud forms. We are testing possible roles for Rdi1p in this process.

2006

An optogenetic analysis of the minimal requirements for bud-site selection.

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An autocatalytic complex of four proteins (Cdc42, Cdc24, Bem1, PAK) is associated with the selection and maintenance of a single site of polarity in budding yeast, and positive feedback in this complex is required to amplify stochastic fluctuations in Cdc42 activity during symmetry-breaking polarization. However, the relevance of this feedback is not clear for situations where cells polarize towards an established spatial cue, and there has been no direct demonstration that artificially localized cues can be recognized and amplified solely through this mechanism. In order to experimentally manipulate signaling processes such as polarity establishment, we have created tunable, light-controlled interacting protein tags (TULIPs) based on genetic fusion with a photosensory LOV2 domain from plants and an engineered PDZ domain binding partner. These domains can be fused to cellular proteins of interest or localization motifs, thereby making their interaction light-regulated. We have shown that the tags function in yeast and mammalian cells and are readily adaptable to diverse signaling pathways. Here, we use TULIPs tags to confer light sensitivity to the activation of Cdc42 in yeast, thereby eliciting light-directed specification of the site of budding. We show that the multifunctional Cdc42 guanine nucleotide exchange factor (GEF), Cdc24, is sufficient to provide a spatial cue upon localized recruitment to the cortex, and that this artificial cue can override the endogenous cue. Furthermore, the polarity-specifying ability of Cdc24 is lost when either its GEF activity or its ability to interact with the scaffold Bem1 is ablated, even when a wild-type copy of Cdc24 is present. These results suggest that several different functionalities are required to trigger positive feedback and successfully specify the site of budding. Future experiments will explore the timing, cell-cycle dependence, and molecular requirements of light-directed polarity specification.

2007

Electrochemical regulation of budding yeast polarity.

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Electrochemical cues, such as ion fluxes, membrane potential and membrane inner leaflet charges have been proposed to influence polarity establishment in different systems. One striking assay highlighting such effects are electrotactic experiments in which cells ranging from bacteria to neutrophils orient their polarized growth or migration to the direction of small exogenous electric fields (EFs). EFs may signal through the membrane by locally altering electrochemical cues around the cell; however molecular mechanisms remain poorly characterized. Here, we use the budding yeast *S. cerevisiae* as a model for eukaryotic polarity to study these effects. These cells polarize by locally selecting sites of bud emergence or by growing shmoo projections towards a mating partner. We grow budding yeast cells in microfluidic chambers in the presence of EFs and assay for polarity orientation. EFs cause budding cells to bud towards the cathode of the EF, when the bud site selection machinery is weak such as in WT diploids or in haploids *bud1* mutants. In the presence of pheromones (α -factor), the EF directs shmoo growth in the opposite direction, towards the anode. This directional switch depends on downstream activation of Cdc42, as *far1-s* or *cdc24-m* mutants which fail to recruit the Cdc42 GEF, *cdc24* to the mating site, orient shmoo to the cathode, similar to budding cells. A candidate screen for upstream regulators at the plasma membrane, identifies conserved membrane potential regulators such as Nha1 and Trk1, as mediators of the EF response, and put forward the role of negatively charged lipids phosphatidylserine production in these effects. As these charges have been proposed to influence the local binding of Cdc42 for polarity establishment, these experiments begin to define pathways bridging electrochemical signals to the local activation of conserved polarity components. These original studies set the molecular basis of novel layers of polarity regulation that may be relevant to most cell types.

2008

Cdc42 explores the cell periphery for mate selection in fission yeast.

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How cells polarize in response to external cues is a fundamental biological problem. During sexual differentiation, yeast cells polarize growth towards the source of a pheromone gradient produced by cells of the opposite mating type. Polarized growth is controlled by the small GTPase Cdc42, a key eukaryotic polarity regulator that coordinates signaling, cytoskeleton polarization and vesicle trafficking. However, how cells establish polarity and choose a mate in complex cellular environments is poorly understood. We find that, in fission yeast, low-level pheromone signaling promotes a novel polarization state, where active Cdc42, its GEF Scd1 and scaffold Scd2, form co-localizing exploratory dynamic zones that sample the periphery of the cell. Two Cdc42 effectors, the actin cable-directed motor Myosin V Myo52 and the vesicle tethering exocyst complex labeled by Sec6 and Sec8, also dynamically co-localize with active Cdc42. However, prior to mate selection cells do not grow due to a block in the exocytosis of cell wall synthases Bgs1 and Bgs4. At the time of mate selection active Cdc42 zones are stabilized and capable of promoting cell wall synthase exocytosis and polarized growth. However, if cells do not experience prior low-level pheromone signaling, Cdc42 exploration fails and cells polarize growth at cell poles by default. Failure to explore results in altered partner choice, with cells mating preferentially with sister rather than non-sister cells. Thus, Cdc42

exploration serves to orient growth for partner selection and may promote genetic diversification.

2009

Spatial segregation of polarity factors into distinct cortical clusters is required for cell polarity control.

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Cell polarity is key to various cellular processes and much is known about the regulation and general localization of polarity-regulating factors to cortical sub-cellular domains. In contrast how the higher-order organization of polarity factors within sub-cellular domains relates to their function as dynamic positional landmarks is not well understood. We imaged frontally the cortex of live fission yeast (*Schizosaccharomyces pombe*) cells, using advanced imaging techniques including super-resolution microscopy. Interestingly, we found that polarity factors are organized in discrete and dynamic cortical protein clusters, which we can optically resolve to 50nm. Artificial oligomerization of the Kelch-repeat polarity factor Tea1p, in both its wild-type state and with a published deletion of a putative trimerization domain, suggests that the self-oligomerization capacity of polarity factors could be sufficient both to generate clusters and regulate their efficacy as landmarks. We also found from quantitative co-localization analysis that the polarity factors Tea1p and its ERM-family binding partner Tea3p belong to distinct cluster populations, which change in alignment during the cell-cycle becoming maximally co-localized during mitosis. This change in localization can also be associated with changes in the dynamics of both polarity factors. Permanent alignment of the same polarity factors by artificial dimerization created a loss of function, thus spatial segregation of polarity factor clusters provides a means to spatio-temporally control polarity at the cell cortex. We observed similarly clusters in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* cells, indicating this could be a universal regulatory feature.

2010

Tea5p is a Pseudokinase that Functions in Microtubule-Based Cell Polarization.

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Microtubules regulate polarized cell tip growth in the fission yeast *Schizosaccharomyces pombe* by transporting cell polarity factors tea1p and tea4p on their plus ends to the cell tips. These proteins are critical for these rod-shaped cells to initiate growth at the “new” cell end in a process known as new end take off (NETO). How tea proteins regulate cell polarization downstream of microtubules remains unclear. Here we identify a new factor tea5p (also known as ppk2p) required for regulation of cell polarization for NETO. Tea5p is a pseudokinase as it lacks key residues required for kinase activity, and additional mutations in the kinase domain do not affect its function. Overexpression of tea5p leads to a striking loss of polarity, causing cells to become round. Pulldowns show that tea5p associates in a tight complex with tea3p. Genetic studies suggest that tea5p functions with tea3p downstream of tea1p in cdc42p activation via a signaling pathway consisting of the NDR kinase orb6p and a cdc42p GEF gef1p. Thus, tea5p constitutes a novel connection between microtubule +TIP proteins and activation of cdc42p for cell polarization.

2011

Investigating the influence of mechanics on fission yeast cellular shape and growth pattern.

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Cell shape and growth pattern are key to cell division, proliferation and differentiation. Although much is known about the role of the cytoskeletal and cell polarity machineries in defining cell shape and growth pattern, much remains to be understood about the influence of physical constraints on those processes, constraints that must ultimately feedback into the biochemical regulation of the cell.

We seek to clarify how physical constraints influence cell growth and how cell shape is transmitted across generations, using the cylindrically-shaped and genetically tractable fission yeast *Schizosaccharomyces pombe* as experimental model.

We have established a new method to track the fine cortical expansion of fission yeast cells as they grow, by decorating the cell wall with fluorescent nanocrystals (quantum dots, Qdots) and filming their displacement over time. With this method, we have been able to infer a geometrical map of the mechanical strains at play involved in the polar growth of *S. pombe* and to compare how that strain map compares with the cortical localization pattern of different cell growth machineries such as the β -glucan synthase GFP-Bgs4. This approach begins to clarify the respective contributions of mechanical and biochemical regulation in controlling cell morphogenesis in *S. pombe*.

In parallel, we have analysed cell shape transmission in ≈ 30 *S. pombe* gene knockout strains that display a curved phenotype. They include strains lacking not only genes predicted to be involved in cell polarity or the cytoskeleton (*tea1*, *tea2*, *tea4*, *pom1* or *mto1*), but also other genes that lack an obvious direct relation with those processes (*vps71*, *rpl3702* or *swr1*). As not all cells of a given mutant display the curved phenotype, we have investigated the cell shape fate of individual cells and their progeny after cell division, by carrying out pedigree analysis. We find that cell curvature is not predictably transmitted from one cell to its progeny but rather that cells have distinct cell shape inheritance rules related with the penetrance of their mutant phenotype. Importantly, shape inheritance also changes with the type of machinery affected in each mutant and mutants in similar molecular components give rise to similar shape inheritance patterns, indicating multiple layers of biochemical and mechanical cell shape regulation at play, which we are seeking to unravel.

2012

Symmetry breaking in fission yeast germination.

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The cross-talk between cell polarity and mechanics serves as a central paradigm for cellular morphogenesis. The rod-shape fission yeast *S. pombe* is an excellent model to study mechanisms regulating polarity and morphogenesis. In these cells, spatial landmarks at the cortex direct actin assembly and cell wall synthesis for turgor-driven polarized growth at cell tips. In here, we investigate how these cells become rod in the first place. We monitor germinating spores that exit quiescence to become vegetative cells with quantitative time-lapse microscopy. Rounded spores exhibit an initial near-isotropic growth phase and then break symmetry to

establish a single rod-shape growth axis, in a process termed outgrowth. We observe that the polarisome assembles in a single cap long before outgrowth. Strikingly, the polarisome wanders around the rounded spore yielding localized growth sites that fail to progress, disassemble and reform at a new site around the spore surface. Quantitative volume measurements reveal that polarisome stabilization and outgrowth happen when the spore has doubled its volume. This size sensing is independent of G1/S cell cycle transition, initial spore size, and is maintained in cytoskeleton, polarity or secretion mutants. Electron microscopy reveals that outgrowth is concomitant with the local rupture of the outer spore wall, a thin cell wall structure deposited at sporulation and essential for spore resistance. Strain-stress experiments show that this spore wall is 3 times stiffer than the inner wall, suggesting it may hinder or slow down growth. Photoablation of this outer wall hastens symmetry breaking. We propose models that compute the mechanical stress on the outer wall generated by turgor pressure and local growth. These models predict size-sensing mechanisms and morphogenetic features of outgrowth such as tip curvature establishment and growth rate changes.

2013

Role of the conserved NDR kinase Orb6 in the control of a cortical gradient of activated Cdc42.

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Recently our lab has shown that activated Cdc42 GTPase, a key morphology control factor, displays oscillatory dynamics at the two cell tips in fission yeast (Das et al, *Science*, 2012). These oscillations, which are anticorrelated at the two tips, are due to positive and delayed negative feedbacks and competition for Cdc42 activators. In this study, we describe a mechanism by which Cdc42 GEF Gef1 levels are regulated at the cell cortex, thus modulating Cdc42 dynamics at the cell tips.

Conserved NDR kinases regulate cell morphology in eukaryotes. Previous studies in our lab have shown that Orb6 kinase maintains cell polarity by spatially regulating activation of Cdc42 GTPase (Das et al, *Curr Biol*, 2009). *Orb6* mutants exhibit an expansion of the gradient of activated Cdc42 at the cell tips and a loss of polarity. Here we show that Orb6 kinase phosphorylates serine 112 in the N-terminus of Gef1 to facilitate Gef1 binding to the 14-3-3 protein Rad24. Consistent with Rad24 sequestering Gef1 in the cytoplasm, preventing Orb6 kinase phosphorylation of serine 112 leads to increased levels of Gef1 at the cortex. This results in an increased cell diameter, decreased anticorrelation of active Cdc42 oscillations at the cell tips, and precocious bipolar growth activation.

In *S. pombe* cells, activated Cdc42 forms a cortical gradient at the cell tips, which determines cell width (Kelly et al, *Mol Biol Cell*, 2011). We show that NDR kinase phosphorylation of Cdc42 GEF Gef1 modulates cell width, opposing the functional effects of Cdc42 GAP Rga4. Loss of Rga4 in *gef1S112A* mutants leads to complete loss of cell polarity, similar to *orb6* mutants. Thus, our observations indicate that the NDR kinase Orb6 alters Cdc42 dynamics through Gef1 localization to regulate cell diameter and bipolar growth activation.

Mechanotransduction

2014

Differential Contributions of Nonmuscle Myosin II Isoforms to Stress Fiber Mechanics.

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Actomyosin stress fibers (SFs) enable cells to generate traction forces against the extracellular matrix through the contractile activity of nonmuscle myosin II (NMMII). While the NMMII isoforms NMMIIA and NMMIIB are known to play distinct roles in cellular tension generation, their respective contributions to stress fiber mechanics remain unclear. To gain insight into this issue, we used femtosecond laser ablation to sever individual mCherry-LifeAct-labeled SFs in U373 MG human glioma cells in which NMMII isoforms were suppressed by lentiviral shRNAs. We recorded the retraction of the two severed SF ends and fit these data to a Kelvin-Voigt model featuring a viscoelastic time constant and a plateau retraction distance. Our results demonstrate that the two isoforms contribute in distinct ways to the viscoelastic properties of SFs located in the cellular center and periphery. Specifically, we find that NMMIIA suppression slightly speeds SF retraction kinetics for central SFs and reduces the plateau retraction distance for both central and peripheral SFs. By contrast, NMMIIB suppression produces significantly greater reductions in the viscoelastic time constant for peripheral SFs while surprisingly increasing the retraction distance of central SFs. The SF retraction dynamics associated with NMMIIA and NMMIIB suppression qualitatively phenocopy our earlier measurements in the setting of Rho kinase (ROCK) and myosin light-chain kinase (MLCK) inhibition, respectively (Tanner et al., Biophys J 2010). This broadly suggests that one functional relationship exists between central SFs, ROCK, and NMMIIA and that another exists between peripheral SFs, MLCK, and NMMIIB. Our study offers new insight into how NMMII isoforms differentially regulate contractile elements in specific cellular compartments and contribute to stress fiber contractility.

2015

Dynamics of non-muscle myosin II organization into stress fibers and contractile networks.

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The cellular morphology of adhered cells critically depends on the formation of a contractile meshwork of parallel and cross-linked stress fibers along the contacting surface. The motor activity and mini-filament assembly of non-muscle myosin II is an important component of cell-level cytoskeletal remodeling during mechanosensing. To monitor the dynamics of non-muscle myosin II, we used confocal microscopy to image cultured HeLa cells that stably express myosin regulatory light chain tagged with GFP (MRLC-GFP). MRLC-GFP was monitored in time-lapse movies at steady state and during the response of cells to varying concentrations of blebbistatin (which disrupts actomyosin stress fibers). Using image correlation spectroscopy analysis, we quantified the kinetics of disassembly and reassembly of actomyosin networks and compared them to studies by other groups. This analysis suggested that the following processes contribute to the assembly of cortical actomyosin and stress fibers: random myosin mini-filament assembly and disassembly along the cortex; myosin mini-filament aligning and contraction; stabilization of cortical myosin upon increasing contractile tension. We developed simple numerical simulations that include those processes. The results of simulations of cells at steady state and in response to blebbistatin capture some of the main features observed in the

experiments. This study provides a framework to help interpret how different cortical myosin remodeling kinetics may contribute to different cell shape and rigidity depending on substrate stiffness.

2016

Force Fluctuations within Focal Adhesions Mediate ECM Rigidity Sensing to Guide Directed Cell Migration.

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Cell migration towards areas of higher extracellular matrix (ECM) rigidity via a process called “durotaxis” is thought to contribute to neuronal pathfinding, immune response, and cancer metastasis. To understand how cells dynamically sample ECM rigidity to guide durotaxis, we performed the first characterization of cell-generated forces on the nanoscale within single integrin-based focal adhesions (FAs). We found that individual FAs act autonomously within a cell, exhibiting one of two distinct states of force transmission. Traction within FAs is either constant and positionally static, or dynamically fluctuating in magnitude and position. We found that increasing ECM rigidity reduced the proportion of FAs exhibiting traction fluctuations over ~20kPa. Using pharmacological and genetic perturbations we find that a FAK-phosphopaxillin-vinculin pathway is essential for cells to exert high traction and to enable force fluctuations within FA over this 20kPa range of ECM rigidities. We show that traction fluctuations within FA are dispensable for FA maturation, chemotaxis and haptotaxis, but are critical to direct cell migration towards rigid ECM. Together, our findings show that individual FAs dynamically sample rigidity by applying fluctuating pulling forces to the ECM to guide durotaxis, and that a specific pathway downstream of FAK broadens the range of rigidities over which this dynamic sampling process operates, but is dispensable for directing cell migration in response to biochemical gradients.

2017

Sarcomere-Like Units Contract Cell Edges.

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Extracellular matrix rigidity directs cell behavior and key processes in development and tumorigenesis. During spreading and migration, cells produce periodic lamellipodial protrusion retractions cycles that require a rigid substrate and the development of adhesions¹. Moreover, there is evidence that rigidity is detected locally in the region near the cell edge². The motor protein myosin II powers cell retractions, which are simultaneous with rearward flow of the actin filament network¹. However, the pattern and mechanism of forces exerted by cells to test matrix rigidity have not been characterized. Using high-resolution force sensing pillars, we show here that the primary forces powering periodic edge retraction come from local contraction units, which exert inward forces near the cell edge, correlated with outward forces 2-3 micrometers rearwards from the edge. Pillar displacement correlates with the local concentrations of active

myosin II and the actin crosslinking protein alpha-actinin, but not with local levels of other common actin binding proteins. The contractile units therefore display similarity to striated muscle and stress fiber units, which are ~1-3 micrometers long, rely on myosin II for contractility, and contain alpha-actinin as a major component of the complex that anchors actin filaments. Thus, our results indicate that local contractile units, with sarcomere-like organization, are responsible for periodic cell edge retractions and rigidity sensing. References: [1] G. Giannone, B. Dubin-Thaler, M. Sheetz et al. (2007) *Cell* 128, 561-575. [2] S. Ghassemi, G. Meacci, J. Hone et al. (2012) *Proc. Natl. Acad. Sci. USA*, 109, 5328–5333

2018

Cells Maintain a Constant Lamellar Line Tension Responsible for Traction Stress Generation.

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Adherent and migratory cells generate tension and exert stress on their substrate via myosin II motors pulling on the actin cytoskeleton. Recent findings have shown that the bundling of actin into radial stress fibers is unnecessary for force generation. Here we show that the transverse arcs in the lamella are responsible for force generation and that the magnitude of the force exerted scales with the size of the cell. Specifically, the total force is directly proportional to the transverse length of the lamella in the cell. Using deep UV micropatterning of polyacrylamide gels, we created a series of circular patterns of constant area but with increasing aspect ratio. Cells on the circular patterns generated the most force, while cells with the most elongated shapes generated the least force. Traction stresses were only seen under the lamellar region of the cells. We confirmed this finding by creating a series of patterns with a constant arc length at the lamella, but with decreasing pattern area. Cells showed no change in the total amount of force exerted despite up to a 50% reduction in area. These results suggest that cells maintain a constant line tension locally in the lamella. The addition of pharmacological myosin inhibitors enabled us to tune the magnitude but not the behavior of the scaling. Furthermore, we find that this scaling is independent of substrate stiffness. Changes in substrate stiffness impacted a cell's ability to spread and form new stable adhesions, but did not affect the inherent line tension. Finally, this scaling law is independent of cell type, with the magnitude of the line tension representing a characteristic measure of contractility. Together these data suggest that a cell can be modeled as maintaining a constant line tension along its lamella, which is responsible for the generation of traction stresses. This work provides important insights into the mechanisms behind transmission of stress from the cytoskeleton to the substrate during migration and overall cellular behavior on soft substrates.

2019

Spatiotemporal distribution of tensile forces by single stress fibers at the cell-matrix interface.

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Stress fibers (SFs) exert tension on the extracellular matrix (ECM) through focal adhesions (FAs). While it is widely appreciated that these tensile forces play key roles in polarity, motility, fate choice, and other defining cell behaviors, it remains poorly understood how single SFs distribute tension across cellular FAs, where adhesion-dependent signals are triggered. We address this open question by using femtosecond laser ablation to sever single SFs in cells while tracking FA tension changes with a vinculin-based optical tension sensor. Surprisingly, disruption of a single SF reduces tension in FAs located throughout the cell, with enriched

tension reduction in FAs oriented parallel to the targeted SF. Remarkably, some FAs exhibit enhanced tension upon SF irradiation, with individual FAs observed to undergo dramatic, unexpected transitions between tension-enhanced and tension-reduced states. These changes evolve with time following SF disruption and depend strongly on the location of the severed SF, consistent with our earlier finding that different SF pools are regulated by distinct myosin activators (Tanner et al., *Biophys J* 2010). To unify these findings, we present a structural model in which central SFs are more interconnected and mutually reinforced than peripheral SFs due in part to the presence of transverse actomyosin structures that link central SFs into a cohesive network. Tension released upon compromise of a central SF is thus broadly redistributed to other stress fibers and focal adhesions, resulting in stabilization of cell shape. This study represents perhaps the most direct and high-resolution measurements of SF tensional homeostatic contributions to date, as well as a new paradigm for studying molecular-scale transmission of tensile force through the actin cytoskeleton to specific molecules at the cell-matrix interface. In addition, this approach could be extended to investigate the role of these forces in modulating cell-cell adhesion, metalloprotease-mediated matrix digestion, and many other problems.

2020

Myosin II-independent ECM stiffness sensing by nascent adhesions.

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Extracellular matrix (ECM) stiffness is known to impact cell spreading, migration, differentiation and proliferation. Such ECM stiffness-dependent changes in cell physiology are thought to be important for tissue organization and maintenance, while misregulation of stiffness sensing is thought to play a vital role during pathogenesis. Current dogma asserts that ECM stiffness sensing occurs by myosin II-generated forces at mature focal adhesions in the lamella. Here, we show that ECM stiffness sensing modulation of cell spread area for NIH 3T3 fibroblasts occurs at a Young's Modulus of 8 kPa, delineating a "soft" and a "stiff" regime. We find that cells on soft ECMs spread about fifty percent less than those on stiff ECMs. Surprisingly, we find that myosin II activity plays almost no role in this response. Cells that have been treated with blebbistatin, a myosin II ATPase inhibitor, or Y-27632, a Rho Kinase inhibitor, spread to slightly larger areas, but otherwise retain differences between soft and stiff regimes. To identify the mechanisms underlying myosin-II independent stiffness sensing, we analyze protrusive activity of the myosin II-independent lamellipodium and assembly of nascent adhesions. We find that protrusion rates are indistinguishable on soft and stiff, but that the protrusions on soft matrices are unstable and rarely lead to productive cell advance. On soft ECMs, immunofluorescent paxillin images reveal a reduced density of nascent adhesions at the base of the lamellipodium. Live imaging of GFP-paxillin expressing cells confirms that cells on soft ECMs form significantly fewer myosin II-independent adhesions than those on stiff ECMs. We were able to partially recover spread area and myosin II-independent adhesion formation on soft substrates through the addition of PDGF, which increases Rac1 signaling, or Mn^{2+} , which stimulates integrin activation. These data strongly suggest that ECM stiffness regulates myosin II-independent adhesion formation in the lamellipodium and is the origin of the changes in cell spread area on matrices of different stiffnesses.

2021

Modeling actin, myosin and adhesion dynamics based on measured forces and movements in the lamellipodium and lamellum.

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Cell motility relies on the continuous reorganization of a dynamic actin-myosin-adhesion network at the leading edge of the cell, in order to create protrusive force at the leading edge membrane and traction force between the cell and its external environment. We analyze experimentally measured spatial distributions of actin flow, traction force, myosin density, and adhesion density in control and myosin-inhibited epithelial cells and consider a number of mechanical models of this system. A model in which myosin associates and dissociates from the actin network while flowing inward with the network can explain the measured molecular distributions and correctly predict the location of the lamellipodium-lamellum boundary. We use this model to determine the active and passive stresses in the system, and to gain insight into the mechanical properties of the actin network and of the nascent adhesions. The load response characteristics of the nascent adhesions allow the system to maintain a robust inward flow of actin in the presence of greatly varying contractile and traction forces.

2022

Cell intrinsic mechanochemistry of protruding cells.

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While the mechanochemical regulation of the cytoskeleton has been studied in depth, it remains a challenge to study these pathways on the relevant time scale *in vivo*. In addition, the regulation of cell movement depends heavily on substrate architecture and composition. In an effort to address the relationship between mechanochemistry and live cell morphology changes, we examined the cell intrinsic mechanochemistry of rounded, rapidly protruding (~1 min) cells. We used a combination of biochemistry, high resolution microscopy, and quantitative image analysis to elucidate signaling pathways occurring at the actomyosin cortex. We found that myosin II contractility and RhoA activity are spatiotemporally regulated at the cortex and co-localize with actin density. In fact, myosin II contractility positively self-regulates via activation of the RhoA activator GEFH1. Upstream of GEFH1, Pak inhibits both GEFH1 activity and distribution and myosin II light chain Ser19 phosphorylation in rounded cells. We show that the dynamic activation of Pak kinase is required for the structural integrity of the actomyosin cortex, suggesting that Pak dynamics may be responsible for the spatiotemporal regulation of contractility we observe. Pak activity also regulates the cortical response to myosin II activity levels, further indicating that Pak dynamics are essential to the regulation of cell intrinsic mechanotransduction.

2023

Characterizing Tyrosine Phosphorylation in the Vinculin Tail Domain

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Vinculin is an essential cell adhesion protein involved in controlling cell shape, motility and force transduction, in part by coupling the actin cytoskeleton to the extracellular matrix. Vinculin is tightly regulated by intramolecular interactions between the N-terminal head domain (Vh) and the C-terminal (CT) tail domain (Vt) which are linked by a flexible proline-rich region. Vt has been shown to bind and bundle F-actin thereby reinforcing maturing focal adhesions. This actin association is believed to induce Vt dimerization, which in turn promotes F-actin bundling. Given the importance of this interaction in focal adhesion maturation and dynamics, it is likely that vinculin/actin interactions are regulated by additional factors such as phosphorylation. Recent studies from our lab have shown that the CT of vinculin containing the only tyrosine phosphorylation site in Vt is necessary for actin-induced bundling, cellular reinforcement and focal adhesion properties. However, the role of tyrosine phosphorylation in Vt (Y1065) and its full impact on vinculin regulation have not been elucidated. Here, we show that Src-mediated phosphorylation of Vt modulates F-actin bundling. Additionally, we show that a common mutation of Y1065 (Y1065F) exhibits an aberrant F-actin bundling capacity. These results are consistent with our findings that the CT hairpin of Vt, containing Y1065, is critical for actin induced Vt-dimerization and F-actin bundling. We have structurally characterized alternate Vt variants that either mimic tyrosine phosphorylation at this site or cannot be phosphorylated to study the cellular role of Y1065 phosphorylation on focal adhesion dynamics and cellular stiffening in response to force. Results from these studies will be presented.

2024

Membrane tension co-ordinates pseudopod extension during phagocytosis.

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Pseudopod extension in FcγR-mediated phagocytosis occurs in two phases. The first phase is sufficient for the engulfment of small particles (1-3 μm), but a second PI3-Kinase dependent phase is required for ingestion of larger particles. These phases are characterized by differential spatio-temporal regulation of exocytosis and small GTPases. However, the origin of the transition between these two phases is not well understood. We find that this transition is determined by the plasma membrane tension. We show that during frustrated phagocytosis an increase in membrane tension activates exocytosis and dramatically alters the spatial distribution of Rac activation in macrophages. We find the exocytic compartment which responds to membrane tension is linked to an actin-dependent microtubule independent glycosphosphatidylinositol-anchored protein (GPI-AP) recycling pathway. Membrane tension in macrophage plasma membranes increases during phagocytosis of IgG coated beads. By artificially increasing membrane tension with osmotic shock, we dramatically increase phagocytosis efficiency. This effect can partially overcome PI3-Kinase inhibition and microtubule depolymerisation, but fails when secretion is inhibited with NEM. This suggests PI3-Kinase driven protrusion is important for generation of membrane tension during phagocytosis and that tension sensing mechanisms are required to drive exocytosis and the pseudopod transition to complete ingestion of large particles. Given that a GPI-AP rich compartment is recruited to the cell surface during fibroblast spreading and migration, it potentially constitutes a universal membrane area regulation function.

2025

Regulation of cellular tension: Does the cell have a contractile setpoint?

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Adherent cells generate forces through acto-myosin contraction and sense the mechanical properties of their environment. In the context of tissues, these cells have been described as existing in tensional homeostasis with their surroundings, implying an internal regulation of tension at the cellular level. We explicitly test this tension regulation by mechanically perturbing fibroblast cell tension and observing the contractile response. As a cell spreads and contracts sandwiched between two parallel fibronectin-coated surfaces, it exerts a tensile load across the height of the cell. We use a flexible, tipless atomic force microscope (AFM) cantilever as the top surface to measure and to modulate forces, displacements, and stiffness of the contracting cell. Cell spreading is confined on both surfaces to defined regions using micropatterning. We find that within an hour of initial attachment, contraction forces level out to a steady state value as the cell reaches the edges of the fibronectin-coated areas on the cantilever and substrate. To specifically test whether the magnitude of the steady state tension is an internally regulated setpoint, we modulate the tensile load or stiffness once cellular tension has settled at a stable value. Interestingly, fibroblast cells do not maintain the same magnitude of cellular tension after tensile perturbations, while tension remains unchanged for changes in stiffness alone. Our results suggest that the tension cells produce while spreading is not tightly regulated after spreading ceases. These observations have important implications for understanding the timing of cellular control of contraction as a function of environmental loads and stiffness, indicating that the contractile state of cells might be most sensitive to the mechanics of their environment during the initial spreading stage of cellular attachment.

2026

Actomyosin cytoskeletal organization distinguishes lobopodia from lamellipodia.

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Three-dimensional (3D) cell migration through chemically and structurally diverse environments is an important component of embryogenesis, immune surveillance, and wound healing. The regulation of cellular force production (mechanotransduction) by cell-matrix adhesions can control how cells move. For example, the rigidity of 2D extracellular matrix regulates cellular contractility to control the speed of cell migration, while the elastic behavior of 3D matrix governs the mechanical mode of membrane protrusion. Cells in non-linear elastic environments use actin polymerization to extend small, fan-shaped lamellipodia, while cells in linear elastic materials are characterized by blunt, cylindrical lobopodia. To determine how linear elastic 3D extracellular matrix regulates mechanotransduction to control the mode of leading-edge protrusion, we investigated the function of the actomyosin machinery during lamellipodia- and lobopodia-based migration. In non-linear elastic 3D collagen, PIP3 and active Rac1 and Cdc42 were targeted to the leading edge, consistent with 2D lamellipodia-based migration. In contrast, cells migrating inside linear elastic dermal explants and cell-derived matrix (CDM) were characterized by non-polarized Rac1, Cdc42, and PIP3 signaling. Despite these differences in polarized signaling, 3D lamellipodia and lobopodia protruded with similar speed and persistence, and RhoA activity remained relatively uniform within cells using either mode of migration. Significantly, myosin IIA localization was different in lobopodia compared to lamellipodia. Myosin IIA was localized along distinct peripheral F-actin fibers in lobopodia, while

in lamellipodial cells, it was organized in a periodic pattern associated with cortical F-actin beneath the plasma membrane. Switching fibroblasts from lobopodia- to lamellipodia-based migration by inhibiting cellular contractility and mechanotransduction corresponded with the loss of GFP-myosin light chain from the peripheral fibrillar structures. Thus, lobopodia are distinguished by distinct myosin IIA-positive structures that may act to generate the intracellular force necessary for this mechanical mode of lamellipodia-independent 3D migration.

2027

Mechanics of bleb formation and bleb-based migration.

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Protrusion formation at a cell's leading edge is an important step during cell migration. Studies in development and cancer cell motility show that contractility-driven blebs are a common alternative to the well characterized actin polymerization-based lamellipodia or filopodia, particularly in three-dimensional environments. Moreover, a cell's ability to switch between different protrusions is thought to facilitate motility in complex environments and to promote cancer dissemination.

Despite the importance of plasticity in migration, the minimal requirements for switching between protrusions and the physical mechanisms of bleb-based motility remain largely unknown. To address these questions, we use Walker 256 carcinosarcoma cells, which can be selected based on the adhesiveness of the substrate to generate two distinct sublines that form either blebs or lamellipodia. By systematically investigating protrusion switching at the single cell level, we demonstrate that shifting the balance between actin polymerization and actomyosin contractility is sufficient to lead to immediate transitions between blebs and lamellipodia. Furthermore, rapid transitions between blebs and lamellipodia could also be triggered upon changes in substrate adhesion during migration on micropatterned surfaces. Our data indicates that instantaneous switching between protrusions occurs without prior changes in global cell shape or polarity, suggesting that protrusion formation is an independent module in the regulatory network that controls the plasticity of cell migration.

We then asked how cell body translocation was achieved during bleb-based migration. We found that blebbing Walker cells require confined environments for efficient migration. Our data suggest that in these cells, in contrast to lamellipodia-based migration, force transmission during locomotion does not require transmembrane coupling via focal adhesions. Using molecular and biophysical approaches combined with microfluidic engineering we are currently investigating the mechanisms of force transmission during bleb-based motility.

2028

Influence of matrix stiffness on morphology, direction and persistence of motile cells.

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Cell motility is defined as the capacity of cells to move by a self-generated movement. Motility is fundamental to a wide variety of processes such as embryogenesis (development), wound healing (maintenance), immune responses (lifetime) but also in pathological processes as cancer metastasis. According to the disparities of the physico-chemical properties of the extracellular matrix, stationary cells adapt their internal and external organisations, such as the spreading area, the distribution of focal adhesions, the cytoskeletal organisation, etc... However, only few studies have underlined the impact of the physic-chemical properties of the extracellular environment on motile cells.

In this work, we have used fish keratocytes which have a robust morphology, a persistent shape and movement and a very fast motion (up to 18 $\mu\text{m}/\text{minute}$). By using a wide range of ECM stiffnesses covered with a constant cell-ligand density, we have studied the morphology of keratocytes and dynamic parameters (velocity, trajectory, coefficient motility, ...) in response to changes in ECM stiffnesses. We have observed that ECM stiffness controls the cellular shape within the natural phenotypic variability of keratocytes. Then, we have shown that the mechanical rigidity of ECM significantly affects the migratory behavior in terms of directional persistence time, translocation speed and random motility coefficient.

To elucidate the mechanisms by which ECM rigidity affects the internal machinery of motile cells, we have used immunostaining methods and total internal reflection fluorescence (TIRF) microscopy to characterize the organisation of focal adhesions, molecular motors and the cytoskeletal organization in response to ECM stiffnesses.

2029

Silicone gels for cell rigidity sensing and patterned cell adhesion: robust mechanical properties, TIRF/TFM microscopy, and induced cell polarization on soft substrates.

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Substrate rigidity impacts cellular migration, gene expression, development, and tumorigenesis, especially in a range of elastic moduli, E , of 0.1 to 100 kPa. Soft substrates commonly used for cell rigidity sensing studies and cell traction force microscopy (TFM) are hydrogels, such as polyacrylamide gels. Elastic moduli of thin gels are usually measured with atomic force microscopy (AFM), which requires expensive equipment. Elastic moduli of hydrogels can change due to drying, swelling, aging, and hydrolysis of molecular bonds. Here, we formulated and successfully cultured cells in a series of silicone gels with E covering a range from 0.4 to 300 kPa that were all prepared by mixing different proportions of two components of a commercial silicone, Sylgard 184. Silicone pre-polymers were spin-coated onto microscope cover glasses to ~ 35 μm thickness and a specially built microfluidic device was bound to the silicone gel. Elastic moduli of the gels were measured with 5-10% absolute accuracy by tracking displacements of beads on the surfaces of the gels, when known hydrodynamic shear stresses were applied. Mechanical properties of the silicone gel layers were consistent and robust, with $\sim 3\%$ variability within a batch and $\sim 10\%$ change after 6 months of storage.

We also formulated and prepared layers of silicone gels with E from 0.4 to 130 kPa and with refractive index, n , of 1.49. The high value of n made the gels fully compatible with through-the-lens Total Internal Reflection Fluorescence Microscopy (TIRFM) using TIRF objectives with numerical apertures (NA) of up to 1.49. TIRFM enables selective visualization of the dynamics of substrate adhesions, vesicle trafficking, and biochemical signaling at the cell-substrate interface. For TFM, we also chemically functionalized the surface to covalently bind 40nm far-red fluorescent beads and matrix protein to the gel. We plated mouse endothelial cells with fluorescently labeled F-actin on the $n = 1.49$ silicone gels and successfully performed concurrent TIRFM and TFM. We obtained high-quality TIRF images of the cells showing maps of F-actin localization at the substrate and juxtaposed them with high-resolution traction force maps obtained with TFM.

Using moderately soft gels, E of 20 kPa, we also patterned matrix protein on the surface creating arrays with different 50x50 micron shapes (e.i. arrows and circles). Variable surface chemistry enabled blocking of the unpatterned area with PEG and covalent binding of 40nm beads and matrix protein to the patterns. The dimension of the shapes fits only one cell per

shape and constrains formation of focal adhesions to the patterned area, either inducing or preventing cell polarization and orientation.

2030

Lamin-A levels limit 3D-migration but protect against migration-induced apoptosis.

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Cell migration through dense tissue and matrix requires a cell and its nucleus to contort and flow. A strong dependence of 3D-migration processes on the variably expressed nucleoskeletal protein lamin-A is revealed here across widely different cell types relevant to disease and regeneration. Crawling through tissue is modeled by motility through micro-pores, with small decreases in lamin-A producing large increases in net migration. Surprisingly, the largest effects occur when wildtype lamin-A is low relative to constitutive lamin-B's. Nuclear shape changes after micro-pore migration as well as nuclear response times in micropipette aspiration scale strongly with the lamin-A:B ratio across cell types, revealing lamin-A's role in nuclear plasticity and lamin-B's role in nuclear elasticity. Lamin-A also protects against apoptosis induced by micro-pore migration, with deeply deficient cells showing defects in stress-resistance. Xenografts provide in vivo insight and show moderately low lamin-A levels promote growth of the graft. The nuclear lamina thus acts as a physical impediment to motility and also promotes survival in withstanding the mechanical stresses of migration.

2031

Rigidity Sensing in T cells by Actin-Dependent Phosphorylation of Src Family Kinase Substrate Cas-L.

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The rigidity sensing mechanisms involved in T cell activation are unknown. The Crk-associated tyrosine kinase substrate lymphocyte type (Cas-L) is a scaffold protein mainly expressed in lymphocytes and epithelial cells. Previously we showed that phosphorylation of YxxP repeats on the substrate domain of Cas-L-family member p130Cas is involved in sensing extracellular matrix rigidity in fibroblasts in response to integrin engagement. Here, we show that Cas-L acts in T cells like its homologue p130Cas, undergoing increased tyrosine phosphorylation in cells spreading on substrates of higher Young modulus. T cells spreading on stiff poly dimethylsiloxane (PDMS) substrates coated with agonist antibodies against TCR and integrin ligand ICAM-1 show increased Src-family kinase (SFK)-dependent phosphorylation of Cas-L substrate domain in comparison to softer substrates. Importantly, the ability to discern rigid from soft matrices is lost when actin polymerization is disrupted by cytochalasin D. Moreover, actin-disruption as well as myosin-IIA inhibition affects the total levels of phospho-CasL at early stages of cell spreading. This suggests that Cas-L phosphorylation is driven by forces generated by polymerizing f-actin and myosin-IIA contraction. We propose a two-step model to describe how T cells sense substrate rigidity via actomyosin-dependent Cas-L phosphorylation. This work highlights the importance of mechanosensory systems in T cells responding to different rigidity microenvironments.

2032

Effect of substrate mechanical properties on T cell spreading and activation.K. L. Hui¹, A. Upadhyaya¹; ¹Physics, University of Maryland, College Park, MD

T cell activation is a key process in cell-mediated immunity, and is important in directing an effective immune response against infections. Engagement of T cell receptors by peptides on antigen presenting cells leads to activation of signaling cascades as well as cytoskeletal reorganization and large scale membrane deformations. While significant advances have been made in understanding the biochemical signaling pathways, the effects imposed by the physical environment and the role of mechanical forces on cell activation are not well understood. In this study, we have used anti-CD3 coated elastic polyacrylamide gels as stimulatory substrates to enable the spreading of Jurkat T cells and the measurement of cellular traction forces. We have investigated the effect of substrate stiffness on the dynamics of T cell spreading and cellular force generation. We found that T cells display more active and sustained edge dynamics on softer gels and that they exert increased traction stresses with increasing gel stiffness. An intact actomyosin cytoskeleton was required to maintain the forces generated during activation, as inferred from small molecule inhibition experiments. Our results indicate an important role for physical properties of the antigen presenting cell as well as cytoskeleton-driven forces in signaling activation.

2033

Neuronal mechanosensitivity in axonal pathfinding.H. Svoboda¹, L. D. Costa², J. Guck³, C. E. Holt¹, K. Franze¹; ¹Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, United Kingdom, ²Instituto de Fisica de Sao Carlos, University of Sao Paulo, Sao Carlos, Brazil, ³Department of Physics, University of Cambridge, Cambridge, United Kingdom

During the development of the nervous system, neurons migrate and grow over great distances. During these processes, they are exposed to a multitude of signals influencing their speed and direction. Currently, our understanding of nerve tissue development is, in large part, based on studies of biochemical signalling. Despite the fact that forces are involved in any kind of cell motion, mechanical aspects have so far rarely been considered. Here we used custom-built deformable cell culture substrates, traction force microscopy and calcium imaging to investigate how neurons probe and respond to their mechanical environment. The growth rate of retinal ganglion cell axons was increased on stiffer substrates. However, their tendency to grow in bundles (i.e., to fasciculate), which they commonly show in vivo, was significantly enhanced on more compliant substrates. Moreover, if grown on substrates incorporating linear stiffness gradients, neuronal axons were repelled by stiff substrates. Mechanosensing involved the application of forces driven by the interaction of actin and myosin II, and the activation of stretch-activated ion channels leading to calcium influxes into the cells. Applying a modified atomic force microscopy technique in vivo, we found stiffness gradients in developing brain tissue. Importantly, retinal ganglion cell axons followed a soft pathway. The application of chondroitin sulfate, which is a major extracellular matrix component in the developing brain, resulted in stiffening of the tissue and disrupted axonal pathfinding. Hence, our data suggest that neuronal growth in the central nervous system is not only guided by chemical signals – as it is currently assumed – but also by the nerve tissue's mechanical properties.

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Mechanisms of three-dimensional glioma cell motility in non-fibrillar matrices.

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Diffuse infiltration of single cells into brain parenchyma is a hallmark of malignant glioma. This extreme invasiveness makes complete tumor resection difficult and contributes to the high mortality rate associated with this disease. Brain parenchyma has a distinct physical structure characterized by densely packed neural cell processes and sub-micron extracellular space, and is largely devoid of the fibrillar collagen scaffolding typically found in stromal tissue. Consequently, glioma cells migrating in brain slices exhibit a distinct type of motility, with branched protrusions and hourglass-shaped cell-body deformations that help squeeze cells through tight spaces (Beadle *et al.*, *Mol. Biol. Cell* 2008; 19(8):3357-68). However, the mechanistic details of this unique mode of motility remain incompletely understood. To address this question, we synthesized brain-mimetic nanoporous, non-fibrillar extracellular matrices (ECMs) based on cross-linked hyaluronic acid (HA), the major component of brain ECM, and functionalized them with Arg-Gly-Asp (RGD)-containing peptides to facilitate integrin-mediated cell adhesion. In a three-dimensional (3D) spheroid invasion paradigm, glioma cell motility was highly reminiscent of that seen in brain slices, validating the use of these ECMs a model system (Ananthanarayanan *et al.*, *Biomaterials* 2011; 32(31):7913-23). Eliminating RGD peptides from the ECM abolished invasion, suggesting a requirement for integrin-mediated adhesions for this mode of motility. Increasing matrix density, or inhibiting myosin-based cellular contractility by blebbistatin or shRNA-induced knockdown of Myosin IIA also severely impaired motility. We also report preliminary results from two sets of studies: First, we investigate the balance of protrusive and contractile forces in glioma cell motility by pharmacologically and genetically manipulating the Rho GTPases Rac1 and RhoA. Second, we explore the involvement of hyaluronidase-mediated enzymatic remodeling of the HA matrix in this mode of motility. Our studies collectively help define the intracellular and extracellular signals that underlie the distinct mode of glioma cell motility observed in non-fibrillar matrices.

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Regulation of renal cell migration by septin 2.

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Renal cell carcinoma (RCC) is an aggressive metastatic cancer with poor response rates to therapeutic intervention. Recent studies show that the septin 2 GTPase is frequently upregulated in RCCs, which are defective for the von Hippel-Lindau (VHL) tumor suppressor. Septin 2 (SEPT2) is a member of the filamentous septin GTPases, which serve as scaffolds for protein-protein interactions and form diffusion barriers that demarcate distinct membrane compartments. Previous studies have implicated septins in cell motility and migration, but the mechanisms involved remain unknown. Here, we investigate whether and how SEPT2 affects the migration of RCCs. Using the VHL-null RCC cell line 786-O, we show that SEPT2 depletion decreases the rate of wound closure and impairs the ability of 786-O cells to transmigrate along a passive serum gradient. In migrating kidney cells (MDCK) that undergo epithelial-to-mesenchymal transformation after treatment with the hepatocyte growth factor (HGF), SEPT2 filaments appear to localize dorso-distally to paxillin-enriched focal adhesions at the leading edge. Super-resolution microscopy shows that SEPT2 localizes to the tail of focal adhesions and confocal microscopy reveals that SEPT2 colocalizes with the radial actin stress fibers, which are anchored to peripheral focal adhesions. Time-lapse imaging of SEPT2-YFP and

paxillin-mCherry shows that SEPT2 is recruited to the growing focal adhesions of migrating MDCK cells. Interestingly, SEPT2 depletion results in smaller, more numerous and peripheral focal adhesions in both 786-O and MDCK cells. To determine whether SEPT2 affects the maturation of focal adhesions, we stained for phospho-paxillin (pY118), whose levels are known decrease during focal adhesion maturation. We found that focal adhesions in SEPT2-depleted cells contain a two-fold increase in pY118-paxillin relative to control cells. Taken together, these data suggest that SEPT2 regulates the maturation of nascent focal adhesions. On-going studies aim at determining if SEPT2 is essential for the generation and maintenance of mechanical tension at focal adhesions through the assembly and/or contraction of actin stress fibers.

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Filamin is necessary to trigger calcium signaling and cell contraction in vivo.

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Mechanosensation is critical for normal development and tissue function. We are using the *C. elegans* spermatheca as a model system to study how cells sense and respond to mechanical forces in vivo. The spermatheca is a simple myoepithelial tube that naturally experiences cycles of extreme stretching and constriction as oocytes enter, are fertilized, and exit into the uterus. We have discovered that FLN-1/filamin, a stretch-sensitive actin binding and signaling scaffold, is required for this process. PLC-1/phospholipase C- ϵ is also required for the exit of embryos from the spermatheca. Using GCaMP, a genetically encoded calcium indicator, we show that entry of an oocyte into the spermatheca initiates a distinctive series of calcium oscillations which propagate across the tissue via gap junctions and eventually lead to coordinated acto-myosin based contraction and expulsion of the fertilized egg. Genetic analysis indicates FLN-1 acts upstream of PLC-1 to initiate phosphatidylinositol (PI) signaling, calcium release, and contraction of the spermathecal cells. We hypothesize that filamin is required in the spermatheca to respond to increased strain, which initiates calcium oscillations via PLC-1. Current work is focused on understanding the dual roles of filamin as a signaling and structural scaffold, as well as uncovering other components of the pathway.

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Mechanical waves orient cell division during monolayer growth.

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The growth of epithelial tissues is essential to morphogenesis and is recapitulated in postnatal life to enable wound healing and tissue regeneration. Dysregulation of underlying processes contributes to devastating epithelial-derived diseases such as carcinomas. Epithelial growth is increasingly regarded as a mechanical phenomenon in which physical forces not only drive cell motions but also trigger and feedback signaling pathways. The physical forces that drive epithelial growth and the mechanisms by which they regulate cell division and migration remain poorly understood, however. To study these forces we combine traction microscopy, monolayer stress microscopy, and polydimethylsiloxane (PDMS) cell micropatterning. We design PDMS microstencils with a central rectangular pattern and deposit them on top of a collagen-coated polyacrylamide gel. Cells attach and proliferate on the micropatterned area of the gel until they reach confluence and acquire an epithelial phenotype. Membrane removal triggers collective cell migration, generating a mechanical wave of strain rate that propagates slowly throughout the monolayer, traversing intercellular junctions in a cooperative manner. Moreover, as the

monolayer expands, oscillations in cell-cell stress are generated, whose direction and magnitude define the orientation of cell division. This wave suggests a novel mechanism of long range cell guidance and pattern formation during monolayer expansion.

2038

Effects of an inflammatory response and changes in substrate stiffness on human mesenchymal stem cell transmigration through the endothelium.

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Stem cells have been investigated for potential for cell therapy of a wide variety of diseases in the human body. Human mesenchymal stem cells (hMSCs), in specific, have been shown to be present in the bloodstream, and have therefore been believed to hold promise for therapy of diseases such as Parkinson's. However, to date, there have not been many studies aiming to exploit the migration and differentiation of hMSCs for use in novel drug and gene delivery techniques to tackle diseases in all areas of the body. In order to develop such techniques, we must gain a better understanding of the mechanisms of hMSC transmigration through the vascular endothelium, something which has been largely unexplored. Here, we investigate the effects of an inflammatory response and varying substrate stiffnesses on the percentage and speed of hMSC transmigration through the endothelium. The inflammatory cytokine tumor necrosis factor-alpha (TNF- α) was added to human vascular endothelial cell (HUVEC) monolayers to simulate inflammatory conditions. Results show that while TNF- α does not cause a significant change in the percentage of hMSC transmigration, it does cause a decrease in the speed of transmigration. Additionally, experiments were repeated on hydrogels of various biologically-relevant stiffnesses, and results show that substrate stiffness does not cause a change in percentage or speed of hMSC transmigration. Together, our results show that while substrate stiffness may not be an important variable in understanding hMSC transmigration, inflammation of the vascular endothelium affects hMSC transmigration mechanics and therefore needs to be taken into consideration as research continues in the area of stem cell transmigration, so that the application of stem cell therapy may be fully and successfully utilized.

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Fluid shear stress and sphingosine 1-phosphate promote Filamin A membrane translocation and complex formation with VE-cadherin during endothelial cell invasion.

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Cell junctions play a key role in regulating angiogenic responses and the receptors. Vascular endothelial (VE)-cadherin and PECAM-1 have been previously defined as mechanosensors. Filamin A and Filamin B are highly expressed in endothelial cells and have been reported to be regulated by shear stress. We previously demonstrated sphingosine 1-phosphate (S1P) and fluid wall shear stress (WSS) stimulate endothelial cell (EC) invasion in three-dimensional (3D) collagen matrices. In this study we explored the role of junction proteins and filamins in the regulation of EC invasion in response to S1P and WSS. EGTA, a chelator of calcium ions, fully disrupted localization of junction proteins to the plasma membrane and significantly decreased S1P- and WSS-induced invasion. Short hairpin RNA-mediated gene silencing of VE-cadherin, PECAM-1, filamin A, and filamin B reduced S1P- and WSS-induced invasion. Also we observed that S1P and WSS synergistically promoted filamin membrane localization as determined by immunofluorescence. Further, these observations were confirmed by co-immunoprecipitation

studies where S1P and WSS enhanced VE-cadherin interactions with filamin and PECAM-1. Our data show for the first time that filamin A complex with VE-cadherin in response to S1P and WSS to regulate endothelial sprouting in 3D collagen matrices.

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Endothelial Cell Response to Complex Flow Profiles.

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The ability of the endothelial cells that line the interior of blood vessels to sense and respond to fluid flow is an essential component of cardiovascular development, homeostasis, and disease. In particular, the endothelium at arterial bends, branches, and at surface irregularities is prone to chronic inflammation that contributes to the development of atherosclerotic lesions. However, while extensive work has characterized the response of these cells to uniform, laminar flow, the response of endothelial cells to the complex, spatially varying wall shear stresses present in vivo remains poorly understood. We investigated the effects of complex flows on endothelial cells in vitro using a novel impinging flow device that exposes endothelial cells to shear stress gradients and stagnation point flows similar to those found at arterial bifurcations. We investigated multiple shear stress levels over a range found in the aorta (9-200 dynes cm⁻²) and found that in all cases human microvascular endothelial cells migrated toward the vicinity of the stagnation point, and against the direction of fluid flow. We also found that these cells aligned parallel to the flow direction at low levels of shear stress, but perpendicular to the flow direction near the center, stagnation point. These observations suggest that endothelial cell migration and polarization may thus play presently unrecognized roles in the early stages of atherosclerotic lesion formation, particularly in regions of complex flow.

2041

Defining the Mechanism of Enhanced Cellular Invasion Induced by Mechanical Stimulation.

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Formation of secondary tumors by metastasis is a leading cause of death in cancer patients. During metastasis cells separate from the primary tumor, invade the stroma, travel through blood and lymph and colonize tissues distant from the primary mass. Several biochemical factors have been identified which are able to drive the metastatic cascade. However information of the role of mechanical factors in cancer metastasis has primarily been focused on the effect of compliance, overshadowing other mechanical factors that might facilitate metastatic progression. For instance, contractile cells within the stroma can produce tugging and pulling forces in the extracellular matrix as they migrate and remodel the stroma. These activities provide a transient and variable force that would propagate between cells. To simulate this mechanical force, we previously developed an in vitro mechano-invasion assay where cancer cells are subjected to tugging or pulling forces. These forces simulate those produced by contractile cells in the tumor microenvironment, without interference of any cell secreted factors. Surprisingly, we discovered a significant difference in the extent of invasion in mechanically stimulated versus non-stimulated cell culture environments. This mechanically enhanced invasion by cancer cells requires the presence of fibronectin in the extracellular matrix. The objective of our study is to understand the mechanotransduction pathway leading to enhanced invasion. We hypothesized that in response to mechanical forces in the stroma, tumor cells will show an altered expression of genes involved in mechanosensing. We performed expression

profiling of several genes related to cell migration, adhesion and tumor metastasis by real-time PCR analysis. Six genes were confirmed to be differentially expressed between mechanically stimulated and non-stimulated conditions. Surprisingly, one of the genes found to be significantly down-regulated in the mechanically stimulated invasion culture is a fibronectin specific integrin subunit. Over-expression of this gene resulted in a significant decrease in enhanced invasion, supporting its role in sensing the mechanical stimulus. We further propose that mechanical stimulation during invasion, results in integrin crosstalk, leading to the down-regulation of this fibronectin specific integrin while enriching others within the invadopodia.

Chemotaxis and Directed Migration II

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Cell Type Specific Durotactic Response on Polyelectrolyte Multilayer Compliance Gradients.

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To explore the usefulness of polyelectrolyte multilayers (PEMUs) as biocompatible coatings, adhesion and motility of rat aortic smooth muscle A7r5 and human osteosarcoma U2OS cells cultured on PEMUs were investigated. These cells are representative of the vascular smooth muscle and osteoblast cell types that could interact with surfaces of PEMU coated biomedical implants. The PEMUs investigated were built layer-by-layer with a photosensitive negatively-charged polyelectrolyte PAA (poly(acrylic acid)) modified with 4-(2-Hydroxyethoxy) benzophenone (PAABp) and the positively-charged polyelectrolyte PAH (poly(allylamine hydrochloride)). These polyelectrolytes form stable PEMUs through electrostatic interactions and can be covalently crosslinked creating C-C bonds between the layers with ultraviolet irradiation. PEMU surface and bulk properties such as compliance were tuned by varying the degree of ultraviolet light exposure to generate uniform surfaces or gradients of crosslinking using optical filters or edge masking for a steeper gradient. Cell type-specific responses to surface compliance resulted in different adhesion, cytoskeleton organization, and motility behaviors. Although both cell types adhere and spread better on stiffer surfaces, the A7r5 cells were more tolerant than the U2OS cells to a wider range of Young's elastic modulus on which they redistributed vinculin-containing focal adhesions and rearranged stress fibers in response to different surface compliances. In contrast, U2OS were less tolerant of the more compliant surfaces and underwent apoptosis on the softest surfaces. In addition, the A7r5 cells exhibited durotactic responses on both shallow and steep compliance gradients. Along shallow compliance gradients, the A7r5 cells established a polar orientation toward increasing Young's modulus. Windrose plot analysis of cell motility on steeper edge gradients show A7r5 cell durotaxis toward the stiffer portion of the PEMU surface. Analysis of collective cell sheet durotactic response to compliance gradients is ongoing.

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Influence of extracellular matrix proteins and substratum topography on corneal epithelial cell alignment and migration.

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The basement membrane (BM) of the corneal epithelium presents biophysical cues in the form of topography and compliance that can impact the phenotype and behaviors of cells and their nuclei through modulation of cytoskeletal dynamics. It is also well known that the intrinsic biochemical attributes of BMs can modulate cell behaviors. In this study, the influence of an exogenous coating of extracellular matrix proteins (FNC) in addition to substratum topography on cytoskeletal changes, alignment and migration of immortalized corneal epithelial cells (hTCEpi) was investigated. In the absence of FNC coating, a significantly greater percentage of cells aligned parallel with the underlying anisotropically ordered topographic features however their ability to migrate was impaired. Also, changes in surface area, elongation and orientation of cytoskeletal elements were differentially influenced by the presence or absence of FNC. These results suggest that the presence of surface associated ECM proteins modulate the effect of topographic cues. These findings have relevance to the conduction of experiments using culture with biomimetic biophysical attributes as well as the integration of biophysical cues in tissue engineering strategies and the development of improved prosthetics.

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A GEF/GAP modulator defines locomotory and invasive protrusion polarity in migrating tumor cells.

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Locomotory and invasive protrusions are essential first steps that precede tumor cell invasion and migration. Spatial control of actin polymerization is necessary to achieve directional protrusion during cell migration. By using a RhoC biosensor we show that the localization of RhoC activity is crucial to maintain actin polymerization asymmetry in order to sculpt the amplitude and the shape of locomotory and invasive protrusions. This spatial localization of RhoC activity is necessary to place active cofilin right at the leading edge of locomotory protrusions by phosphorylating cofilin behind it. Here we show that RhoC activation at leading edge protrusions is regulated by a p190RhoGEF/p190RhoGAP module that spatially restricts actin barbed end distribution to shape lamellipodia during protrusion. The same mechanism is used to shape invadopodia into needle shaped protrusions. This mechanism reveals a dynamic plasticity in the distribution and amplitude of barbed ends, which can be modulated by fine-tuning RhoC activity by upstream GEFs and GAPs for directed cell motility. p190RhoGEF/p190RhoGAP are able to dynamically tune the location and amplitude of actin barbed ends in protrusions through direct effects on RhoC/Cofilin pathway. In addition, our data show that RhoC is an important component of the cellular steering-mechanism during chemotaxis by defining where actin polymerization takes place in response to external signals.

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Cellular contact guidance through dynamic sensing of nanotopography.*W. Losert¹, M. Driscoll¹, C. Guven¹, X. Sun¹, J. Fourkas¹; ¹University of Maryland, College Park, MD*

We investigate the shape dynamics of the amoeba *Dictyostelium discoideum* on surfaces with nano-topography. Contact guidance (cellular alignment and migration parallel to one-dimensional surface features) has been observed for many cell types migrating on various surfaces. While patterning of focal adhesion complexes (FACs) has been implicated in contact guidance, we observe significant contact guidance of *Dictyostelium* along ridge and groove shaped nano- and microtopographic surfaces, even though *Dictyostelium* lacks FACs. We find that cells that move parallel to the ridges are faster and more elongated than cells that move perpendicular to ridges. Cells that move parallel to ridges are also more protrusive at their fronts than cells that move perpendicular to ridges. Quantification of contact guidance efficiency shows that ridges with a spacing of about 1.5 μm have the greatest contact guidance efficiency. To further analyze contact guidance we measured cell shape dynamics in depth. In a baseline study on flat surfaces, we had demonstrated that cell shapes change in a wave-like manner, with regions of high boundary curvature that propagate from the leading edge toward the back, usually along alternating sides of the cell. Such protrusion waves are easily seen in cells that do not adhere to a surface, such as cells that are electrostatically repelled from surfaces or cells that extend over the edge of micro-fabricated cliffs, but wave-like dynamics of protrusions are also present during migration on surfaces. Therefore we model contact guidance on nanogratings in terms of stochastic cellular harmonic oscillators that couple to the periodicity of the ridges. The wavelength and speed of the oscillations that best couple to the surface are consistent with actin waves, indicating that the dynamics of the actomyosin machinery may be crucial for the contact guidance process. Finally, we explore the possibility to direct cells unidirectionally, utilizing the dynamic nature of the contact guidance process.

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Walking the line: a fibronectin fiber-directed lymphangiogenesis assay.*M. Mitsi¹, M. Schulz², M. Detma², V. Vogel¹; ¹Department of Health Sciences and Technology, ETH, Zurich, Switzerland, ²Institute of Pharmaceutical Sciences, ETH, Zurich, Switzerland*

The terms angiogenesis and lymphangiogenesis refer to complex morphogenetic processes, whereby new blood or lymphatic vessels respectively, grow from the pre-existing vasculature to meet the needs of the tissue, for example during embryonic development, wound healing or cancer. Although several growth factors and signaling pathways have been identified as regulators of angiogenesis, the role of the extracellular microenvironment, including the biomechanical properties of the extracellular matrix, remains poorly understood. In the present work, we aimed to develop a suitable assay, where questions of that nature can be addressed. Our experimental approach involves manually pulled fibronectin fibers interacting with microcarrier beads, on the surface of which a confluent layer of lymphatic endothelial cells is grown. The whole system can be monitored microscopically. Under various experimental conditions, we have observed cell outgrowth from the microcarrier surface to the fibers. Even when the substrate underneath the fibers consisted of fibronectin or collagen, cells preferred to migrate along the fibers, providing evidence for the importance of fibrillar fibronectin as guiding cue for the first steps of sprouting angiogenesis. Consistent with the known ability of VEGF to enhance endothelial cell motility and promote angiogenesis, the number of outgrowth events was increased upon VEGF stimulation. Importantly, we were able in this novel assay to observe and study qualitative differences in the cell movement induced by VEGF. In the absence of

VEGF, the majority of outgrowth events consisted of single cells migrating away from the microcarrier along the fibers. In contrast, when VEGF was added to the medium, the cell activity was dominated by collective cell movement. Although the central role of VEGF in angiogenesis is well established, our assay allows asking new questions regarding the mechanisms concerning the differentiation of tip from stalk cells, as well as factors that coordinate the movement of the cells during sprouting angiogenesis.

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Fluxes of water across AQP9 modulate cell migration and cellular junctions.

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During epithelial wound healing, the cells migrate as an epithelial sheet of interconnected cells often described as collective cell migration. In this process, cell-cell communication is of major importance for guidance of multi-cellular units. In contrast, single cell migration is based on extracellular cues that act at the single cell level, rather than through direct cell-cell communication between front and rear of an entire cell sheet. Fluxes of water across the cell membrane through aquaporins (AQP), have been shown to impact on cell migration. During cell motility, increased expression of AQPs appears to facilitate locomotion. However, the role of AQPs has not been assessed in the context of single cell versus collective cell migration. In this study, we have over-expressed the mammalian AQP9 in epithelial MDCK-1 cells and compared the migration both at single and collective cell levels. We found that in the epithelial wound healing assay, cells over-expressing AQP9 migrated faster than cells expressing an empty vector. In contrast, the latter migrated over larger distances as single cells. Moreover, we found an enhanced transepithelial resistance in cells over-expressing AQP9, indicating a tighter epithelial monolayer. We assume that AQP9 contributes to the strengthening of intercellular attachments in the monolayer, possibly by enabling rapid volume regulation. It is likely that this also enhances the collective migration mode and provides the monolayer with a forward push to migrate into the wound.

2048

EB1-recruited microtubule +TIP complexes coordinate protrusion dynamics during 3D epithelial remodeling

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Epithelial remodeling, in which apical-basal polarized cells switch to a migratory phenotype, plays essential roles in development and disease of multicellular organisms. How microtubule dynamics are controlled or contribute to epithelial remodeling in a more physiological three-dimensional (3D) environment is not understood. We used confocal live-cell imaging to analyze microtubule function and dynamics during 3D epithelial morphogenesis and remodeling of polarized Madin-Darby kidney epithelial cells that undergo a partial epithelial-to-mesenchymal transition (EMT) in response to hepatocyte growth factor (HGF). We found that extensions at the basal surface of HGF-treated cysts are densely packed with microtubules. Computational tracking of EB1-2xEGFP showed large numbers of microtubules growing persistently from the apical domain into these extensions, often deforming the extension tip, and an increase in microtubule growth rate in response to HGF before morphological changes were evident. Next, we tested the role of microtubule plus-end tracking protein (+TIP) complexes in 3D epithelial remodeling by depleting cells of EB1, an adaptor protein that mediates recruitment of other +TIP proteins to growing microtubule plus-ends. In EB1-depleted cells, microtubules displayed rapid

lateral and retrograde movements demonstrating that EB1 is required to anchor and stabilize growing microtubules in HGF-induced extensions. EB1-depleted cysts formed shorter, more branched, extensions further suggesting that EB1 is required for productive HGF-induced extension outgrowth. Analysis of cell-matrix interactions and F-actin dynamics revealed that control extensions progressively pulled on and deformed the extracellular matrix (ECM) typically with one F-actin-rich protrusion near the cell tip. In contrast, EB1-depleted cells produced multiple highly dynamic F-actin rich protrusions that did not productively engage the matrix, resulting in extensions that rapidly protruded, retracted and changed direction. The inability to engage the matrix and stabilize a dominant protrusion was also associated with defects in cell-matrix adhesions. EB1-depleted extensions formed nascent adhesions that did not mature, were mislocalized, and were uncoordinated, in contrast to the highly coordinated adhesions of control cells. Finally we show that trafficking of VAMP3-positive vesicles to the protrusion tip is disrupted in EB1-depleted cells. Together these data suggest that EB1-mediated organization of the MT cytoskeleton and associated vesicle delivery to the tip of HGF-induced extensions are likely required to coordinate cell-matrix adhesion and protrusion dynamics during 3D epithelial remodeling.

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Spontaneous oscillatory rotational behavior during collective cell migration.

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Collective migration is a remarkable behavior observed in nature, from populations as diverse as flocks of birds and schools of fish to the motion of cells during embryonic development and wound healing. Here we show that epithelial cells confined within monolayers spontaneously organize and undergo rotational collective motion. We used microlithography-based techniques to create adhesive islands of fibronectin and time-lapse imaging combined with pseudo-automated cell tracking algorithms to visualize the movements of cells within monolayers cultured on these islands. We found that the coherence of group rotation is affected by the size and shape of the tissue geometry. In addition, while the initial direction of rotation is selected stochastically, disturbances within the monolayers such as those from cytokinesis are able to switch the direction of rotation. Using computational simulations, we found that cells within monolayers behave not as pure random walk particles, but instead as Vicsek-Czirok type self propelled, interacting particles, similar to birds within a flock or fish within a school. These results indicate that basic principles of collective motion that govern animal behavior may be conserved in cellular motion. An improved understanding of these underlying principles will benefit future studies of collective cell migration.

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Rab11 regulates cell-cell communication during collective cell movements.

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Collective cell movements contribute to development and metastasis. The small GTPase Rac is a key regulator of actin dynamics and cell migration. However, the mechanisms regulating Rac in time and space during collective cell migration are poorly understood. Here, we demonstrate that Rab5 and Rab11 regulate Rac activity and polarization during collective cell migration. We

use photoactivatable forms of Rac to demonstrate that Rab11 acts on the entire group to ensure that Rac activity is properly restricted to the leading cell through regulation of cell-cell communication. In addition, we show that Rab11 binds to the actin cytoskeleton regulator Moesin and regulates its activation *in vivo* during migration. Accordingly, reducing Moesin activity also affects cell-cell communication, whereas expressing active Moesin rescues loss of Rab11 function during migration. Our model suggests that Rab11 controls the sensing of the relative levels of Rac activity in a group of cells leading to the organization of individual cells in a coherent multicellular motile structure.

2051

Paxillin Controls Tumor Angiogenesis by Changing Neuropilin-2 Expression.

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Angiogenesis - the growth of new capillary blood vessels - is required for tumor growth; however, tumor vessels commonly exhibit abnormal structure and function, which impairs the targeted delivery of anti-cancer agents. Although a number of growth factors and receptors that control tumor angiogenesis are characterized, relatively little is known about the mechanism by which these factors influence directional endothelial cell migration so as to produce malformed tumor vessels. Recently we reported that the focal adhesion protein, paxillin, is required for directional migration of fibroblasts *in vitro*. Here we show that paxillin knockdown decreases expression of neuropilin 2 (NRP2), which enhances capillary cell migration and invasion, and thereby, interferes with microvessel network formation *in vitro* and *in vivo* in a Matrigel plug implantation model in mice. Soluble factors secreted by tumors also decrease expression of both paxillin and NRP2 in endothelial cells, and over expression of NRP2 restores both oriented cell migration and normal microvessel formation. These results suggest that the paxillin-NRP2 pathway could represent a new therapeutic target for cancer and other angiogenesis-related diseases.

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A Transparent, Implantable Device for the Generation of Chemoattractant Gradients in the Tumor Microenvironment.

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New technologies are needed to provide a better understanding of the role of the tumor microenvironment in promoting metastasis. The Nano Intravital Device (NANIVID) is being developed as a tool to collect cells *in vivo* and study the motility characteristics of invasive cell populations from metastatic mammary tumors. Transparent, biocompatible materials are used to fabricate the device to enable intravital imaging while implanted in the tumor. A reservoir is etched into a glass substrate and loaded with a hydrogel matrix containing epidermal growth factor (EGF) as a chemoattractant for the invasive tumor cell population. The reservoir is sealed using a polymer membrane and a second glass substrate to produce the final device featuring a single outlet for chemoattractant release. When the hydrogel becomes hydrated, EGF diffuses from the outlet and forms a sustained concentration gradient suitable for attracting invasive

cells. The device has overall dimensions of 3 mm x 1.3 mm, and the reservoir outlet is located approximately 100 microns from the back edge of the device. When fully inserted into the tumor *in vivo*, the outlet will be located at the proper depth for intravital imaging of cell migration. Validation of the device has been previously performed *in vitro* in 2D using MTLn3-Mena^{INV} cells, a rat mammary adenocarcinoma cell line. Current work is focused on transitioning the NANIVID to an *in vivo* platform. Experiments are being performed *in vivo* utilizing MDA-MB-231 cell-derived mammary tumors in SCID mice. Cell motility parameters such as velocity, directionality, and displacement will be compared between cell populations in an extended range from the device outlet. Various EGF concentrations will be compared to determine the optimal concentration for attracting tumor cells. Complementary work is also underway focused on *in vivo* cell collection.

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Follower Cells Become Leader Cells during the Closure of Spontaneously Formed Holes in Zebrafish Keratocyte Sheets.

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Collective cell migration plays an important role in epithelial wound healing. *In vitro* studies have used scratch assays and closure of experimentally created holes as models to study the mechanisms of wound healing within epithelial sheets. We have observed a novel form of spontaneously forming holes within zebrafish keratocyte sheets. In 24-hour primary explant culture, keratocytes collectively migrate away from the scale and holes often form within the follower cells of the sheet. The observation that the closure of one hole frequently leads to the opening of one or more holes in adjacent areas suggests that tension may play a role in the formation of these holes. Indeed, the entire sheet is under tension, as illustrated by sheet retraction after inhibition of adhesion formation at the leading edge. In addition, brief serum starvation, likely contributing to disassembly of adhesions, accelerates the rate of hole formation. The time for hole closure varies considerably and appears to depend on hole size and the tension within the sheet. During the initial, spontaneous formation and growth of holes, cells retain the characteristics of follower cells. As holes begin to close, the cells surrounding the hole appear to adopt characteristics of leader cells as evidenced by four observations: 1) An actin cable surrounding the hole forms. 2) Calcium transients, with a frequency and intensity comparable to those seen in leader cells, are observed in the cells surrounding the hole. 3) Keratocytes may break away from the edge of the hole and proceed to migrate individually across the hole in a manner resembling the break-away of cells at the leading edge. 4) The pattern of phosphorylated myosin light chain is similar to the leading edge of keratocyte sheets and individually migrating cells. These results suggest that follower cells may become leader cells during the process of hole closure. Within the keratocyte sheet, a partial second layer of cells that adopt leader cell morphology and apparently migrate independently, may accelerate the rate of hole closure. Ongoing experiments seek to further characterize the mechanism of hole closure and the transition of keratocytes from follower to leader cells within a sheet.

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ADAM control of cranial neural crest cell migration.

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Cranial neural crest cells are embryonic stem cells that can differentiate in many different cell types to contribute to the muscle, bone, cartilage and ganglia of the face. These cells are induced at the border of the neural and non-neural ectoderm and subsequently migrate great distances inside of the embryo both as a collective sheet of cells and then as individual cells.

We have shown that multiple members of the ADAM family of cell surface metalloproteases control key steps of CNC migration. In particular, ADAM9 and ADAM13 cleave the cell adhesion molecule Cadherin-11 producing an extracellular fragment that promotes cell migration. Replacement of Cadherin-11 by a mutant protein that can't be cleaved by ADAM13 does not allow for CNC migration unless a recombinant form of the extracellular fragment is provided. This combination allows us to investigate the relative importance of the homophilic binding site of Cadherin-11 on the integral versus cleaved form of the protein.

In addition to the proteolytic role of ADAM13, the cytoplasmic domain of ADAM13 is cleaved during CNC migration translocates into the nucleus and regulates multiple gene expression. This processes requires an autoproteolytic cleavage of ADAM13 followed by a subsequent cleavage by gamma secretase. In the absence of ADAM13, the cytoplasmic domain of ADAM19 can also perform this function. We further show that the activity of ADAM13 depends on successive activating phosphorylation by GSK3 and Polo Kinase, and that the phosphomimetic mutants of ADAM13 can rescue CNC migration in embryos expressing dominant negative forms of both kinases. Interestingly neither of these phosphorylations is required for the proteolytic activity of ADAM13 *in vitro* and *in vivo* suggesting that they may regulate either the subcellular localization and/or transcriptional activity of the cytoplasmic domain.

While the detail molecular mechanism and regulation of ADAM13 is in itself complex, the protein function is only required *in vivo* in the context of the three dimensional environment of the cell. Using grafts, partial dissections and *in vitro* assays we test when ADAM13 activity is required and when it is dispensable. Our results suggest that ADAM13 controls CNC interaction with the underlying mesoderm and/or extracellular matrix to promote cell migration *in vivo*.

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Long-range Ca²⁺ waves transmit brain-damage signals to microglia.

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One central topic in biology is how the behaviour of motile cells is controlled by positional cues present in the extracellular environment. Unfortunately, in most contexts the spatial and temporal dynamics of these guidance cues is not known due to an inability to visualize these signals in intact tissues. Here, we present a zebrafish larval model system that allows the directional migration of brain phagocytes, microglia, towards neuronal injuries to be studied for the first time *in vivo*. By performing targeted laser neuronal ablations in the brains of transgenic calcium reporter fish, we reveal rapid Ca²⁺ waves whose range defines which microglia will move to the damage site. Preventing their formation by extracellular calcium chelation is sufficient to suppress microglial targeted migration, whereas ectopic induction of Ca²⁺ signals by photo-uncaging mimics the attraction of microglia to neuronal injury. While, we confirm that calcium-transmitted microglial attraction to damage requires ATP, we show that calcium waves formation and propagation are ATP independent and we identify glutamate as a potent inducer of calcium-transmitted microglial attraction. These findings provide a new handle for understanding and controlling microglia. The involvement of glutamate and Ca²⁺ in this process provides great potential for future pharmacological modulation of microglial behaviour.

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Characterization of Leader and Follower Cells in Collectively Migrating Sheets in Zebrafish Keratocyte Explant Cultures.

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Collective cell migration is an important mechanism during tissue repair, embryonic metastasis, and tumor metastasis. Although single cell migration has been well studied, little is known about the mechanisms of collective cell movement. Primary explant cultures of zebrafish keratocytes are an excellent in vitro system to study collective cell migration as keratocytes rapidly migrate out from harvested scales so that real-time video microscopy experiments are possible. Key to the ability to migrate collectively is the complex relationship between leader and follower cells within a migrating cell sheet. To investigate distinctions between leader and follower cells within a zebrafish keratocyte sheet, we have localized adhesion molecules (integrin, vinculin, E-cadherin), cytoskeletal elements (actin, tubulin, actin binding proteins) and signaling pathways (Grb2, Rho GTPases) by immunofluorescence. Our results showed that there are distinct differences between leader cells and follower cells in both cytoskeletal arrangement and adhesion. Initial measurements of migration speed indicate that the rate of advance of the leading edge of the cell sheet is an order of magnitude faster than in other collective cell systems, a difference that is consistent with the relative migration speeds of individual cells. Addition of RGD peptide, to inhibit adhesion formation at the leading edge, produces a rapid retraction of the entire keratocyte sheet. This suggests that the leader cells pull the sheet forward rapidly enough to create tension within the sheet. Possibly as a product of this tension, holes naturally occur within the follower cells of the sheet. Observation of these holes over time suggests that follower cells may rapidly become leader cells to effectuate hole closure. Ongoing experiments are focused on communication between and the interconversion of leader and follower cells within this system.

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Connective Tissue Growth Factor Modulates Macrophage Recruitment During Pancreatic Alcohol Injury in Mice.

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Acute or chronic pancreatitis (AP, CP) are associated with long-term heavy alcohol consumption resulting in pancreatic acinar cell injury and death. Previous data strongly support a pro-fibrotic role for CTGF in CP. Here we report that CTGF is unexpectedly produced by acinar cells at a much earlier time point after injury, corresponding to the time of extensive macrophage infiltration.

6 week old male wild type (WT) C57/BI 6 mice received ethanol once daily six times a week and cerulein injections once a week for 16 days. Control mice received water alone. In a second approach, Swiss Webster mice that were WT or transgenic (TG) for human CTGF and eGreen Fluorescent Protein received ethanol or water for 5 weeks. At the end of the treatment periods, pancreata were analyzed for acinar CTGF, interleukin-1 β (IL-1 β) or macrophage inflammatory protein 1 α (MIP1 α /CCL3), as well F4/80, an activated macrophage marker. To study mechanistic relationships in vitro, a rat acinar cell line (AR42J) was used. Gene expression was evaluated in CTGF-transfected AR42J cells using a PCR Array. CTGF, IL-1 β or MIP1 α /CCL3

mRNA were evaluated by qRT-PCR in ethanol or CTGF stimulated rat AR42J cells. A fluorometric chemotaxis assay was used to test the response of NR8383 rat alveolar macrophages to conditioned medium from CTGF treated AR42J cells with or without BX471, a CCR1 antagonist.

In WT C57/BL6 mice, CTGF was strongly localized in acinar cells by Day 16 of treatment with ethanol + cerulein when no fibrosis was present but CTGF was not detected in the acini of mice receiving water. Ethanol-induced CTGF was spatially and temporally correlated with that of IL-1 β or MIP1 α /CCL3 as well as F4/80-positive macrophage infiltration. In TG Swiss Webster mice receiving ethanol, a marked increase occurred in acinar GFP, CTGF, IL-1 β or CCL3 expression as compared to TG mice receiving water. Macrophage infiltration increased significantly in the pancreata of TG mice receiving ethanol as compared to all other groups. Transfection of AR42J cells with CTGF cDNA resulted in enhanced expression of CCL3. Comparable patterns of CTGF or IL-1 β mRNA expression were stimulated in AR42J cells exposed to ethanol for up to 48 hours. CCL3 and IL-1 β expression also increased in CTGF treated cells. CTGF treated AR42J cell conditioned medium resulted in an increase in NR8383 macrophage chemotaxis which was abrogated in a dose-dependent manner by BX471.

Following alcoholic injury, CTGF is produced by acinar cells and appears to play a novel role in the inflammatory process by increasing infiltration by macrophages and increasing acinar production of inflammatory mediators such as IL-1 β or MIP1 α /CCL3. Inhibition of CCR1, a CCL3,-5 or -7 receptor reduces macrophage chemotaxis to basal levels suggesting a potential role of CTGF in modulating macrophage infiltration via a CCL3-dependent pathway. We conclude that CTGF dynamically regulates inflammatory cell recruitment following pancreatic injury in a way that is novel and distinct from its role in driving fibrogenic pathways in PSC at later stages of injury.

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Mechanisms of the effects of Erythropoietin in wound healing: A morphologic study.

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Recent studies have demonstrated that Erythropoietin (EPO) promotes wound healing. It has been reported that EPO increases angiogenesis and stimulates re-epithelization. EPO administration also shortens the time to final wound closure. Another recent study states that EPO induces bone repair and fracture healing. Further studies are required to clarify the mechanisms of the effects of EPO on wound healing. These studies will determine if preclinical studies can be translated to the clinical settings.

The main aim of the study was to investigate the localization of the following proteins which are shown as important factors during wound healing process:

- (i) Erythropoietin (EPO),
- (ii) Receptor which is specific for Erythropoietin (EPO-R),
- (iii) Basic fibroblastic growth factor (bFGF),
- (iv) Receptor which is specific for basic fibroblastic growth factor (bFGF-R),
- (v) Transforming growth factor-alpha (TGF-alpha)
- (vi) Caspase -3

Study included the comparison of the location of these proteins before and after EPO administration.

Paraffin blocks from our previous experimental study, which is related with EPO administration, were used for the experiments in this study. Sections from these blocks were labeled with primary antibodies which are specific to the proteins listed above. After application of suitable secondary antibodies, staining on the sections were analyzed under light and fluorescence microscopes. The study has revealed the changes on the expression of six proteins which plays important role during wound healing process and angiogenesis. It also provides information about the effect of EPO administration on the expression of these proteins at microscope level.

Dynamics of Focal Adhesions and Invadosomes

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3D Traction force microscopy in fibrin gels.

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The mechanical forces exerted and detected by living cells play integral roles in diverse biological phenomena, including growth and development, wound healing, and cancer metastasis. In the past decade, techniques such as traction force microscopy and micropost arrays have proven to be powerful tools for measuring the forces generated by cells. In particular, traction force microscopy has recently been extended to three-dimensional cell culture environments by embedding tracer beads in either a synthetic polyethylene glycol (PEG) hydrogel (Legant et al., Nat. Meth. 2010) or in collagen gels (Koch et al., PLoS ONE 2012). The embedded beads move in response to cell-generated distortions of the matrix, allowing cell-generated forces to be calculated. We sought to develop an experimental system that would exhibit the excellent mechanical properties of the PEG hydrogel while using a naturally occurring biological matrix. Fibrin gels fulfill both of these requirements: fibrin is elastic up to ~50% strain (Brown et al., Science 2009) and is also widely used for 3D cell culture. Here we describe the use of fluorescently labeled fibrin gels to measure the forces generated by cells in 3D culture. We observe dramatic but elastic deformations of the fibrin matrix surrounding cells as they grow, divide, and migrate. Further, we find that the dynamic forces generated by the cell can be measured using the deformations of the matrix itself, providing a direct observation of how the cell modifies its surroundings. We discuss the use of this new technique in studying matrix remodeling and cell migration.

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VASP highlights endothelial tip cell dynamics *in vivo*.

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Angiogenesis is the process of sprouting and extending new vessels from the sides of pre-existing vessels, and is critical to development and human diseases. Key to angiogenesis is the migration of “tip cells”, which lead the new vessel in its path, using dynamic protrusions to explore the surrounding tissue. While much is known about signals that specify tip cells and induce their migration, little is known about the cytoskeleton and adhesion organization and components that form in tip cell protrusions to guide vessel outgrowth. To analyze the organization and dynamics of the actin cytoskeleton and adhesions during tip cell guidance, we transiently expressed GFP-tagged VASP specifically in zebrafish endothelial cells and analyzed dynamics by time-lapse 3D imaging of the developing vasculature in embryos. In cultured cells, VASP associates with polymerizing actin barbed ends, and localizes to the tips of filopodia and

lamellipodia and focal adhesions. In tip cells of forming intersegmental vessels, we found that GFP-VASP is enriched in the tips of advancing and stable filopodia-like protrusions, but this local enrichment decreases upon filopodia retraction. Filopodia with GFP-VASP show longer excursion times and are more stable in the direction of cell migration than in peripheral directions. GFP-VASP also localizes to focal-adhesion-like structures within the cell body behind the cell leading edge, and to cell-cell junctions both in the dorsal aorta as well as the newly formed adhesions between intersegmental vessels. Transient expression demonstrates that high levels of GFP-VASP expression inhibits normal tip cell migration, while moderate levels of GFP-VASP expression allow for normal vasculature formation through embryo development to adulthood. Since VASP tracks with free polymerizing barbed ends of actin filaments, these data demonstrate that tip cell filopodia cytoskeletal organization is similar to those observed in neuronal growth cones and cells cultured *in vitro*, and that GFP-VASP is a potentially powerful tool for further analyses of actin cytoskeleton organization in normal and pathogenic angiogenesis *in vivo*.

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Adhesions in 3D are guided by myosin activation state and matrix fiber architecture.

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Despite the growing number of studies describing the effects of “dimensionality” on cellular behavior, the mechanisms that cause a cell to have distinct phenotypes in 2D and 3D are not understood. In particular, little is known about the origins of 3D cell morphologies and adhesion phenotype and their relationship to those observed in 2D. For example, substrate stiffness, acting through a myosin contractility feedback loop, is a major determinant of adhesion and cell morphology in 2D; however, while these same basic principles are likely at work in 3D, it is not clear that they fully explain the phenotypes seen in 3D. For example, the architecture of the 3D matrix may alter how stiffness and forces are both experienced and transmitted by cells. We studied how stiffness and myosin II activity contribute to adhesion and morphology in 3D matrices. To delineate the role of myosin in 3D, we manipulated U2OS osteosarcoma cells in collagen matrices with a panel of myosin inhibitors and mutants. We demonstrate that myosin contractility guides adhesion phenotype in 3D matrices similar to that in 2D culture: myosin light chain (MLC) phosphorylation at Ser19 is critical for adhesion maturation, while phosphorylation at both Thr18 and Ser19 promotes larger, more-stable adhesions. Also similar to 2D, MLC di-phosphorylation is responsible for constraining areas of cell protrusion; however in 3D, myosin accumulates along the sides of protrusions resulting in distinctive elongated morphologies. We next tested the effect of changing bulk stiffness in 3D. Unexpectedly, cells in matrices 100-fold softer than our standard matrices still formed similarly sized, although fewer, adhesions, suggesting the cells were not differentiating between the bulk matrix stiffnesses. Time-lapse imaging showed that adhesions forming on fibers that were aligned to the direction of protrusion matured and stabilized while those forming on perpendicular fibers resulted in fiber bending, but no adhesion maturation. Further investigation revealed that local fiber alignment by cells provides both greater resistance to tensile forces and a longer continuous adhesive area, allowing the cells to generate large adhesions, even in soft matrices. Notably, this mechanism is the same in fibrillar substrates regardless of dimensionality or bulk stiffness. Our results indicate that, while myosin-mediated contractility plays similar roles in 2D and 3D, the effect of microenvironment stiffness on adhesion is distinctly local and is modulated by fiber architecture.

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Functional linkages between single-molecule integrin dynamics and edge protrusion in motile cells.

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Integrins are the key receptors in cell-matrix adhesions and play a critical role in cell migration. Although recent advances in high-resolution microscopy have made it possible to localize single molecules such as integrins, following the dynamics of large numbers of molecules in living cells and establishing the relationship between molecular behaviors and cell-level outputs like edge movement has remained a major challenge. To address the difficulties of both the disparity in the spatial and temporal scales of single-molecule and cell-level event dynamics and the inherent complexity and heterogeneity of molecular behaviors, we measured single molecule $\alpha_v\beta_3$ -integrin, talin or actin dynamics at 40 Hz sampling and associated them to the local protrusion and retraction state sampled at 0.1 Hz in analysis windows of 220 x 220 nm. We developed a diffusion mode decomposition algorithm that revealed 4 integrin diffusion states: (1) practically immobile at the 25 ms scale, in which integrins are likely associated with talin and the actomyosin meshwork; (2) slow, in which they are associated with talin only; (3) medium, in which they are most likely associated with a faster-diffusing sub-population of talin and dynamic, metastable actin filaments; and (4) fast, in which they are associated with neither talin nor actin. Using this approach, we discovered local transient changes in molecular dynamics near the leading edge that were strongly linked to the protrusion of the cell. 20-30 s prior to protrusion onset, $\alpha_v\beta_3$ -integrin molecules often slowed down, suggesting formation of nascent adhesions ahead of protrusion. During protrusion, the mobility of integrins increased, reaching a plateau 40-50 s after onset. The increase in integrin mobility was due to a preferential recruitment of the medium and fast integrin sub-populations to the newly-established cell-matrix contact area. These transient changes were limited to $\approx 1 \mu\text{m}$ from the cell edge. Truncation mutants demonstrated that the variations in integrin mobility associated with cell edge protrusion were dependent upon interactions of the tail with cytoplasmic components. In agreement with this notion, talin and actin exhibited parallel changes in mobility and density with cell edge protrusion. Our approach provides a generalizable methodology for extracting localized patterns in molecular dynamics and interactions that underlie higher-order cellular-level behavior.

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A molecular mechanism for pH-dependent autophosphorylation of focal adhesion kinase.

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Activation of focal adhesion kinase (FAK) is one of the early signaling events after integrin-matrix engagement and is necessary for cell spreading and migration. FAK activation is initiated by release of an autoinhibited interaction between N-terminal FERM and C-terminal kinase domains. Release of this autoinhibited conformation unmasks the autophosphorylation site Tyr397 within the linker region between FERM and kinase domains. Phosphorylated Tyr397 is the docking site for Src kinase, which phosphorylates Tyr576/577 in the kinase domain to activate FAK. Our previous data suggested increased intracellular pH > 7.2 is necessary for FAK activation and focal adhesion turnover. Here we show a molecular mechanism of direct pH-dependent FAK activation that involves release of the autoinhibited conformation by deprotonation of His residues at the interface of the FERM and kinase domains. In vitro kinase assays with recombinant FAK show pH-dependent autophosphorylation of full-length FAK

requires the FERM domain and a truncated FAK lacking FERM domain is constitutively active and pH-independent. Also phosphorylation of Tyr397 in trans using separate FERM and kinase domains is pH-dependent and requires pH > 7.2. Three histidines (His41, His58 and His75) are at the FERM-kinase domain interface but our data indicate His58 is the critical residue conferring FAK autophosphorylation at higher pH. A His58Ala mutant has pH-independent Tyr397 autophosphorylation using full-length FAK and phosphorylation in trans using separate FERM and kinase domains. Molecular dynamics simulations with each of the interface histidines charged or neutral also predict deprotonation of only His58 is associated with conformational changes consistent with release of FERM and kinase domains. These data indicate for the first time a mechanism for how the autoinhibited conformation of FAK is released by an increase in pH to allow FAK activation.

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Focal adhesion size uniquely predicts cell migration.

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Focal adhesions are large protein complexes organized at the basal surface of cells, which physically connect the extracellular matrix to the cytoskeleton and have long been speculated to mediate cell migration. However, whether clustering of these molecular components into focal adhesions per se is actually required for these proteins to regulate cell motility is unclear. Here, we use quantitative microscopy to characterize large families of focal adhesion and cell motility descriptors across a wide range of matrix compliance, following genetic manipulations of focal adhesion proteins. This analysis revealed a tight, biphasic relationship between mean size of focal adhesions - not their number, surface density, or shape - and cell speed. The predictive power of this relationship was comprehensively validated by disrupting non-focal adhesion proteins and subcellular organelles (mitochondria, etc.) not known to affect either focal adhesions or cell migration. This study suggests that mean size of focal adhesions robustly and precisely predicts cell speed independently of focal adhesion surface density and molecular composition.

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Proteomic profiling of adhesion complex dynamics.

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Adhesion complexes (ACs) are large macromolecular complexes of integrins and associated proteins that connect the actin cytoskeleton to the extracellular matrix (ECM). In migrating cells ACs are highly dynamic, forming and maturing at the cell front and disassembling at the cell rear. The turnover of ACs enables and localises the necessary traction forces required for cell migration. Evidence exists for spatiotemporal recruitment of specific proteins during AC maturation or disassembly; however, a holistic understanding of the compositional changes to ACs during these states is lacking. We sought to characterise the dynamic changes that occur during AC turnover using a mass spectrometry (MS)-based proteomics approach. A major challenge in studying AC turnover is the desynchronised nature of AC formation, maturation and disassembly within a population of cells. Therefore, a nocodazole-washout assay was used to synchronise mechanical stress-induced AC maturation and microtubule-induced AC disassembly. Immunofluorescence staining for vinculin showed that AC area was increased 3-fold upon nocodazole treatment, and ACs disassembled by 60 minutes of washout. Plasma membrane protein complexes from control and nocodazole treated cells were isolated and both Western blotting and MS analysis demonstrated that AC components were increased upon

nocodazole treatment. Surprisingly, the levels of the fibronectin binding $\alpha_5\beta_1$ -integrin did not change whilst ECM proteins decreased. Immunofluorescence experiments confirmed these findings and suggest the conversion of ACs from fibrillar adhesions to focal adhesions upon nocodazole treatment.

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CLASP-mediated localized exocytosis controls extracellular matrix degradation and focal adhesion turnover.

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Turnover of integrin-based focal adhesions (FAs) with the extracellular matrix is essential for coordinated cell movement during tissue remodeling and wound healing. In collectively migrating human keratinocytes, FAs assemble near the leading edge, grow and mature as a result of actomyosin-mediated contractile forces, and subsequently disassemble underneath the advancing cell body. FA turnover is essential for productive cell migration, and although microtubules have been implicated in FA turnover, the molecular mechanisms by which FA turnover is coordinated are incompletely understood. Here we identify CLASPs that belong to the growing family of microtubule plus-end-tracking proteins, as critical molecules linking microtubules to FA turnover. CLASPs stabilize lamella microtubules in migrating cells, and CLASP-microtubule association is spatiotemporally regulated by GSK3 β phosphorylation. Using high-resolution dual-wavelength confocal microscopy, we found that FA turnover and CLASP dynamics are tightly spatially and temporally correlated: CLASP-decorated microtubules surround FAs at the base of the lamella shortly before FA disassembly. To test the hypothesis that CLASPs function during the disassembly phase of FA turnover, we depleted both mammalian CLASP homologues by lentivirus-mediated shRNA in HaCaT cells stably expressing paxillin-mCherry as a FA marker. FAs in CLASP-depleted cells were larger, turned over more slowly, and were associated with extensive actin stress fibers. In CLASP-depleted cells FAs often disassembled only after extensive sliding across the ventral cell surface suggesting that these FAs were ripped apart by actomyosin-mediated pulling forces. In contrast, FA assembly was not affected in CLASP-depleted cells. Depletion of LL5 β that is required to recruit CLASPs to FAs resulted in similar FA turnover defects. Using Scanning Angle Interference Microscopy, a novel axial super-resolution technique, we further found that close interactions of microtubules with FAs are disrupted in CLASP-depleted cells, indicating that CLASPs anchor microtubules at FAs. In addition, Rab6a-positive vesicle transport to FAs as well as MMP-dependent, FA-associated degradation of the extracellular matrix was disrupted in CLASP-depleted cells. Thus, CLASP-mediated microtubule-tethering at FAs establishes a Rab6a-positive vesicle transport pathway for delivery, docking and fusion of exocytic vesicles near FAs, thereby promoting FA-associated, localized degradation of the extracellular matrix. We propose that CLASPs function as key molecules coupling microtubule organization, vesicle transport and cell interactions with the extracellular matrix, and that local protease secretion may initiate FA turnover by severing cell-matrix connections.

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Regulation of FAK stabilization and cell migration by tyrosine phosphorylated caveolin-1 requires an intact scaffolding domain

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Caveolin-1 (Cav1) is a key component of cell surface caveolae and is also associated with the regulation of multiple cancer-associated processes including tumor growth and cell migration and metastasis. Cav1 is also a major substrate of Src kinase phosphorylated on tyrosine 14 (Y14). Our previous studies revealed that Y14-phosphorylated Cav1 (pCav1) stabilizes focal adhesion kinase (FAK) within focal adhesions, stimulates Rho activation and promotes cancer cell motility. The Cav1 scaffolding domain (CSD), amino acids D82 to R101 in Cav1, was defined by its structural and functional interaction with Src kinase. Using a CSD-disrupting F92A, V94A mutant of Cav1, we have shown that the CSD is required for Cav1 negative regulation of EGFR diffusion and signaling. However, whether the CSD is involved in Cav1 Y14 phosphorylation-dependent activities, such as FAK stabilization in focal adhesions and cell motility has yet to be determined. We have made Cav1 constructs combining mutations on tyrosine 14 (phosphomimetic Y14D, dominant negative Y14F or positively charged Y14R) with the F92A, V94A mutation in the CSD. Stable expression of these constructs in DU145 prostate cancer cells, which express Cav1 but not pCav1, showed that F92A, V94A mutation of the CSD prevented the increased cell migration and focal adhesion stabilization of FAK-GFP due to wild-type Cav1 and Cav1Y14D and Cav1Y14R mutants. CSD mutation of the Cav1Y14F mutant construct did not affect either cell migration or FAK stabilization in focal adhesions. Expression of a membrane-permeable CSD competing peptide (Cav1 amino acids 82-101 fused to an internalization sequence from Antennapedia [AP-CAV]) also prevented the focal adhesion stabilization of FAK-GFP due to Cav1 wild-type, Cav1Y14D and Cav1Y14R. AP-CAV did not affect FAK stabilization in control DU145 cells or Cav1Y14F transfected DU145 cells. This suggests that an intact CSD is specifically required for pY14Cav1-dependent stabilization of FAK within focal adhesions and consequently enhanced cell migration of these prostate cancer cells. pCav1 stabilizes FAK in focal adhesions and stimulates cancer cell motility in a CSD-dependent manner.

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Activation of Rac by Asef2 Promotes Myosin II-Dependent Contractility to Inhibit Cell Migration on Type I Collagen.

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The Rho family of small GTPases, which includes Rho, Rac, and Cdc42, are key regulators of cell migration through their modulation of the actin cytoskeleton. Activation of the small GTPases is regulated by guanine nucleotide exchange factors (GEFs), which catalyze the release of GDP allowing GTP to spontaneously bind and thereby activate the proteins. While the role of the small GTPases in migration has been studied, much less is known about the GEFs that activate them. Here, we show that the Rho family GEF Asef2 inhibits cell migration on type I collagen. The migration speed of HT1080 cells stably expressing GFP-Asef2 was decreased 2-fold compared with control GFP stable cells plated on type I collagen. In a GTPase pulldown assay, the amount of active Rac was increased 1.9-fold in GFP-Asef2 stable cells compared with GFP controls, while no effect was observed on Rho or Cdc42. Moreover, using acceptor photo-bleaching fluorescence resonance energy transfer, a significant enhancement in the level of active Rac was seen in mCherry-Asef2 expressing cells compared

to mCherry controls. Since Rho family GTPases have been linked to actomyosin contractility, we hypothesized that Asef2 regulates cell migration through myosin II (MyoII). Indeed, treatment with the MyoII-specific inhibitor blebbistatin abolished the effects of Asef2 on migration. In addition, a 1.5-fold increase in the amount of active MyoII, as determined by immunostaining for MyoII phosphorylated on serine 19, was observed in GFP-Asef2 cells compared to controls. The Asef2-mediated increase in active MyoII was dependent on Rac, because treatment with the Rac-specific inhibitor NSC23766 abolished this effect. Since integrins have been shown to play an essential role in cell migration, we assessed cell-surface levels of active β 1 integrin using HUTS-4 antibody and flow cytometry. A 2.5-fold increase in the amount of active β 1 integrin was observed in GFP-Asef2 stable cells compared to GFP controls. The higher levels of β 1 integrin affected adhesion dynamics, because adhesions assembled and disassembled 2-fold and 1.5-fold slower, respectively, in GFP-Asef2 stable cells compared with controls. Collectively, these results indicate that Asef2 regulates cell migration on type I collagen by a mechanism that is dependent on Rac, MyoII, and β 1 integrin.

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Essential roles of Crk and CrkL in cell structure and motility in fibroblast cells.

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Cytosolic adaptor proteins containing SH2 and SH3 domains such as Crk and Crk-like (CrkL) are broadly expressed, interact with a variety of proteins, and play key roles in signal transduction pathways triggered by stimulation of receptor tyrosine kinases and non-receptor tyrosine kinases. Crk and CrkL are similar to each other in structure and function, and their distinct and overlapping functions have been studied during development. However, more detailed and systematic analyses of their functions at the cellular level are needed to understand their diverse biological functions. We induced a knockout of Crk, CrkL, or both by infecting fibroblast cells derived from Crk and CrkL floxed mice with a lentivirus expressing GFP and Cre. When both Crk and CrkL were removed from fibroblast cells, the cells became small, round and refractive. This morphological alteration was accompanied by a decrease in spontaneous motility and wound healing. Fibroblast cells lacking Crk and CrkL lost focal adhesions and actin stress fibers, and their microtubule structure collapsed. The results suggest that loss of cellular connections to the extracellular matrix causes altered cell shape, reduced cell motility, and cytoskeletal collapse. A decrease in actin protein followed the loss of Crk and CrkL, and stabilization of polymerized actins with jasplakinolide suppressed the morphological alteration, suggesting that a decrease in cytoskeletal elements including actin might contribute to the cytoskeletal collapse. Partial rescue of the morphological alteration by a reintroduction of CrkI, CrkII, and CrkL to the cells together with small but significant morphological alterations with selective knockout of Crk or CrkL indicates that both Crk and CrkL are important for the maintenance of cell structure. Our results suggest that Crk and CrkL play critical roles in cell structure and motility by maintaining the cytoskeletal connection to the extracellular environments.

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Network analysis of the focal adhesion-invadopodia transition identifies a PI 3-kinase-PKC α invasive signaling axis.

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A central question in cancer is how deregulated signaling leads to acquisition of an invasive cellular phenotype. Here, we modeled the invasive transition as a theoretical switch between focal adhesions and extracellular matrix (ECM)-degrading invadopodia and built molecular interaction network models of each structure. To identify upstream regulatory hubs, we added first degree binding partners and applied graph theoretic centrality analyses. By comparing the results to clustered signaling state data from human head and neck squamous cell carcinomas (HNSCC), phosphatidylinositol 3-kinase (PI3K) and Protein Kinase C alpha (PKCa) were chosen for further combinatorial analysis. Consistent with a previous report, PI3K activity promoted both the formation and activity of invadopodia along with focal adhesion reorganization. Surprisingly, knockdown of PKCa led to divergent effects on invadopodia formation, depending on the activation state of PI3K. Thus, loss of PKCa inhibited invadopodia formation in cells with wild-type PI3K pathway status. Conversely, in cells with either activating mutant PI3K molecules or endogenous loss of the opposing enzyme phosphatase and tensin homolog (PTEN), PKCa knockdown greatly augmented invadopodia formation. This signaling relationship held true for invasive cells from multiple cancer types, including HNSCC, breast and melanoma. Investigation of the molecular mechanism revealed that a negative feedback loop from PKCa dampens PI3K activity and invasive behavior in cells with genetic overactivation of the PI3K pathway. Thus, knockdown of PKCa in PI3K mutant-expressing cells led to a large increase in the ratio of PI(3,4,5)P3 to PI(4,5)P2 in individual cells. Furthermore, similar to the effects of PKCa knockdown, expression of a non-phosphorylatable mutant of the p85 regulatory subunit of PI3K led to greatly increased invadopodia activity. These studies demonstrate the potential of network modeling as a discovery tool and identify a PI3K-high/PKCa-low signaling state as highly invasive.

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Atypical PKC is involved in breast tumor cell invasion through the control of MT1-MMP trafficking.

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The trademark of a metastatic tumor cell is its ability to disseminate from the primary tumor by degrading the extracellular matrix and basement membrane that form a barrier around the tissue. Remodeling of the extracellular matrix by tumor cells requires formation of actin-based protrusions of the plasma membrane called invadopodia, where the trans-membrane matrix metalloproteinase MT1-MMP accumulates. We previously found that in MDA-MB-231 human breast adenocarcinoma cells, MT1-MMP localizes to late endosomes and we identified an exocytic machinery based on the vesicle-docking exocyst complex required for MT1-MMP delivery to invadopodia. Atypical protein kinase C (aPKCs) iota and zeta are upregulated in several cancers including breast carcinomas and exocyst and aPKCs coordinately regulate cell motility. How MT1-MMP transport intermediates are generated from late endosomes to reach invadopodia and whether this mechanism involves aPKCs remain to be established.

We observed a partial association of ectopically expressed aPKC iota with F-actin/cortactin/dynamin-2-enriched domains on MT1-MMP-positive endosomes and found that aPKC phosphorylated cortactin directly. Silencing of aPKC led to cortactin accumulation on MT1-MMP-endosomes and interfered with dynamin-2 recruitment, resulting in endosome tubulation, impaired MT1-MMP trafficking and inhibition of matrix degradation and invasive migration. Analysis of breast tumors revealed a positive correlation of MT1-MMP and aPKC iota mRNA expression, which was associated with poor prognosis. Immunohistochemistry analysis documented an overexpression of aPKC iota in breast tumors and its relocalization from a polarized apical distribution in normal breast epithelial cells to a cytoplasmic vesicular pattern in poorly differentiated carcinomas. A new function for aPKC in controlling MT1-MMP trafficking to the plasma membrane is identified during breast tumor cell invasion.

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Tks5 regulates invadopodium precursor stability through PI(3,4)P₂ in breast cancer cells.

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Cancer cells make invasive protrusions, called invadopodia, whose primary function is to degrade extracellular matrix (ECM) and protrude into degraded space, creating a tunnel-like passage through the extracellular space. Cancer cells are thought to utilize these tunnels to migrate through the primary tumor to enter the bloodstream and eventually metastasize at distant sites to make secondary tumors. A growing body of research implicates invadopodia in cancer cell invasion and metastasis, a leading cause of death among cancer patients. Recently, many invadopodium core molecules (e.g. cortactin, N-WASp, cofilin and Tks5) have been identified and studied. The adapter protein, Tks5 has been shown to regulate invadopodium formation, but the dynamic interaction of Tks5 with other invadopodium molecules is poorly understood. In this study we investigated the role of Tks5 during invadopodium precursor assembly using high spatial and temporal resolution live-cell fluorescence imaging and report here several novel findings: (1) Cortactin, N-WASp and cofilin arrive together to form the invadopodium precursor, followed by Tks5 recruitment. (2) Tks5 is not required for invadopodium precursor formation but is needed for precursor anchoring/stabilization. (3) Invadopodium precursor stabilization is mediated by the interaction of PX domain of Tks5 with PI(3,4)P₂. (4) PI(3,4)P₂ localizes to the invadopodium core, but PI(3)P and PI(4,5)P₂ do not. We also show for the first time that SHIP2, a 5' inositol phosphatase, localizes at invadopodium, modulates PI(3,4)P₂ levels locally at the invadopodium and is required for the maturation of invadopodia. Together, these findings provide novel insights into how invadopodial core proteins and phospholipids cooperate to stabilize the invadopodium precursor, a key step for invadopodium maturation and matrix degradation.

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Ubiquitination of PIPKly by HECTD1 regulates focal adhesion dynamics and cell migration.

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PIPKly binds talin and localizes at focal adhesions (FAs). PIP2 generated by PIPKly is essential for FA formation and cell migration. On the other hand, PIPKly and the β integrin tail

compete for overlapping binding sites on talin. It is unknown how PIPKly binds talin to provide on-site PIP2 without inhibiting the talin- β integrin tail interaction, a key step for integrin activation and FA formation. Here we show that PIPKly is a substrate for HECTD1, an E3 ubiquitin ligase regulating cell migration. HECTD1 ubiquitinated PIPKly at Lys97 and resulted in PIPKly degradation. Expression of PIPKlyK97R enhanced PIP2 and PIP3 production and inhibited FA disassembly and disassembly, cancer cell migration, invasion and metastasis. Interestingly, mutation at Trp647 abolished the inhibition of PIPKlyK97R on FA dynamics and partially rescued cancer cell migration and invasion. Thus, cycling PIPKly ubiquitination by HECTD1 and consequent degradation release the inhibition of PIPKly on the talin- β integrin tail interaction after on-site PIP2 production, providing an essential regulatory mechanism for FA dynamics and cell migration.

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Large Scale Matrix Degradation by Stromal Cells Independent of Invadopodia.

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Metastatic invasion of tumors into peripheral tissues is known to rely upon protease-mediated degradation of the surrounding stroma. This remodeling process is dependent upon complex, actin-based, specializations of the plasma membrane termed "invadopodia" that act to both sequester and release matrix metalloproteases. Two well established components of invadopodia are the large membrane remodeling GTPase Dynamin 2 (Dyn2), and oncogenic Src kinase. Here we report that cells of mesenchymal origin degrade substantial amounts of surrounding matrix by a mechanism independent of conventional invadopodia. Two distinct human, or rat, cultured fibroblast cell lines (HFs/RFs) or a pancreatic tumor stellate cell line (PSC) exhibit little to no matrix degradation when plated on fluorescent gelatin over a 24 hour period. Surprisingly, siRNA mediated knock down of Dyn2 in these cells induces a massive increase (3-4 fold) of substrate degradation. These degradative sites lack the punctate shape of conventional invadopodia but are instead spread along the cell base and are reticular and/or fibrous in character. In strong support of these findings, a tamoxifen-activated knock out of Dyn2 in mouse embryonic fibroblasts also induces a marked increase (7-8 fold) in the degradation of matrix exhibited by these cells. Re-expression of wt Dyn2 or Dyn1, but not a Dyn2-K44A GTPase defective mutant, actually reduces/eliminates matrix degradation to near normal levels. In stark contrast, siRNA knock down of Dyn2, or expression of Dyn2-K44A, in pancreatic adenocarcinoma cell lines (DANG, BXPC3) resulted in a near complete inhibition of canonical invadopodial-based degradation consistent with the past findings of others. As matrix degradation in the mesenchymal cell lines appears to be induced by a reduction in Dyn2 levels and exhibits a pattern distinct from conventional invadopodia, we tested a role for Src kinase in this novel process. While treatment of the pancreatic tumor cells with PP2 or SU6656 inhibitors markedly reduced invadopodia-based matrix degradation these had almost no effect on this process in the fibroblast cell lines. These findings provide evidence for a novel matrix remodeling process conducted by mesenchymal cells that is substantially more effective than conventional invadopodia, distinct in structural organization, and Dyn2 and Src kinase independent. Supported by NCI RO1 CA104125 to MAM.

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N-WASP coordinates the delivery and F-actin mediated reversible capture of MT1-MMP at invasive pseudopods to drive breast cancer cell invasion.

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Metastasizing tumor cells use matrix metalloproteases, such as the transmembrane collagenase MT1-MMP, together with actin based protrusion, to break through extracellular matrix barriers and migrate in dense matrix. Here we show that the actin nucleation factor N-WASP is upregulated in breast cancer and that N-WASP has a pivotal role in mediating the assembly of elongated pseudopodia instrumental in matrix degradation. In actively invading cells N-WASP promotes trafficking of MT1-MMP from late endosomes to “actin-hotspots” in invasive pseudopodia. These actin rich domains on pseudopods in matrix are shown to mature from adhesion sites to degradative foci by actively sequestering MT1-MMP via direct actin binding of the metalloprotease. This clustering of MT1-MMP is shown to be required to achieve matrix degradation and N-WASP mediated actin polymerization is a prerequisite for this process. We demonstrate regulation of the MT1-MMP interactions with actin networks by phosphorylation.

Our results thus suggest a new integrated model of regulated actin mediated receptor targeting in 3D cancer cell invasion.

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Co-ordinated functions of WASH and exocyst complex underlie the biogenesis of invadopodia in metastatic breast tumor cells.

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The trade-mark of a metastatic tumor cell is its ability to disseminate from the primary tumor by degrading the extracellular matrix and basement membranes that form a barrier around the tissue. Remodelling of the extracellular matrix by metastatic cells requires formation of actin-based protrusions of the plasma membrane called invadopodia, where the trans-membrane matrix metalloproteinase MT1-MMP accumulates. How the cell coordinates targeting of MT1-MMP with actin assembly to form a functional invadopodium remains unclear. Here, we describe an interaction between the exocyst complex and the endosomal Arp2/3 activator WASH on MT1-MMP-containing late endosomes in MDA-MB-231 human breast adenocarcinoma cells. Exocyst and WASH regulate actin assembly on MT1-MMP-containing endosomes to control their dynamics. Both protein complexes are required for invadopodia formation and matrix degradation by a mechanism that involves transient tubular connections between the endosomes and the plasma membrane, which ensure focal delivery of MT1-MMP. These connections are accompanied by recruitment of another Arp2/3 activator, N-WASP, to mature invadopodia and loss of WASH. These findings imply a new definition of invadopodia as structures where late endosomes fuse transiently with the plasma membrane to promote actin assembly and deliver the matrix metalloproteinase to focal sites of matrix degradation.

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A specific subset of RabGTPases controls cell surface exposure of MT1-MMP, extracellular matrix degradation and 3D invasion of macrophages.*S. Linder¹, C. Wiesner¹; ¹University Medical Center Eppendorf, Hamburg, Germany*

The matrix metalloproteinase MT1-MMP has a decisive impact on invasive cell migration in both physiological and pathological settings such as immune cell extravasation or metastasis of cancer cells. Surface-associated MT1-MMP is able to cleave components of the extracellular matrix, which is a prerequisite for proteolytic invasive migration. However, current knowledge on the molecular mechanisms that regulate MT1-MMP trafficking to and from the cell surface is limited. Here, we identify three members of the RabGTPase family, Rab5a, Rab8a, and Rab14, as critical regulators of MT1-MMP trafficking and function in primary human macrophages. Both overexpressed and endogenous forms show prominent colocalization with MT1-MMP-positive vesicles, while expression of mutant constructs as well as siRNA-induced knockdown revealed that these RabGTPases critically regulate MT1-MMP surface exposure, contact of MT1-MMP-positive vesicles with podosomes, extracellular matrix degradation in 2D and 3D, as well as 3D proteolytic invasion of macrophages. Collectively, our results identify Rab5a, Rab8a, and Rab14 as major regulators of MT1-MMP trafficking and invasive migration of primary human macrophages

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The proto-oncogene Vav1 promotes Cdc42-dependent invadopodia formation and tumor cell invasion.*G. L. Razidlo¹, B. Schroeder¹, M. A. McNiven¹; ¹Mayo Clinic, Rochester, MN*

Tumor cell metastasis requires an active degradation and remodeling of the extracellular matrix to facilitate migratory invasion into peripheral tissues and vessels. This process is dependent in part on specialized, actin-rich, protrusive structures at the plasma membrane termed "invadopodia" that sequester and dispense active metalloproteases. The oncogenic guanine nucleotide exchange factor Vav1, an activator of Rho family GTPases, is ectopically expressed in pancreatic cancers, where it promotes tumor cell survival and invasive migration. The GOAL of this study was to test if Vav1 contributes to tumor cell invasion by regulating invadopodia function during matrix degradation. Reduction of Vav1 levels in DanG cells by siRNA-mediated knockdown inhibited invadopodia formation and subsequently reduced the degradation of a fluorescent substrate by 80%. Re-expression of WT Vav1 restored degradation in these cells, while expression of a GEF-inactive form of Vav1 did not, demonstrating that Vav1 GEF activity is required for matrix degradation. Because Vav1 can function as a GEF for multiple members of the Rho super-family, we tested which GTPase might mediate the effects of Vav1 on matrix degradation. Vav1-depleted cells were transfected with constitutively active forms of Rac1, RhoA, or Cdc42 in an attempt to bypass a GEF requirement and restore matrix degradation. Importantly, only the expression of active Cdc42, known to be required for invadopodia assembly, but not active Rac1 or active RhoA, restored invadopodia formation and matrix degradation, suggesting that Cdc42 functions downstream of Vav1 to regulate matrix remodeling and invasion by pancreatic tumor cells. These data reveal a novel mechanism by which invadopodia formation is regulated and identify an additional process by which Vav1 contributes to tumor cell invasion. Supported by NCI R01 CA104125 to MAM.

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The role of StarD13 in breast cancer proliferation and motility.*S. Hanna¹, B. Khalil¹, M. El-Sibai¹; ¹Lebanese American University, Beirut, Lebanon*

Breast cancer is one of the most commonly diagnosed cancers in women around the world. In general, the more aggressive the tumor, the more rapidly it grows and the more likely it metastasizes. Cell migration, is a complex process, which requires the dynamic regulation of actin cytoskeleton. Members of the Rho subfamily of small GTP-binding proteins (GTPases) play a central role in breast cancer cell motility. The switch between active GTP-bound and inactive GDP-bound state is regulated by Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and Guanine-nucleotide dissociation inhibitors (GDIs). Here we study the role of StarD13, a newly identified Rho-GAP that specifically inhibits the function of RhoA and Cdc42. We aim to investigate its role in breast cancer proliferation and metastasis. The level of expression of this Rho-GAP in tumor tissues of different grades is assayed using immunohistochemistry. Moreover, the role of StarD13 in breast cancer cell lines is studied using two approaches. StarD13 is overexpressed using a StarD13-GFP construct, in the second approach StarD13 is knocked down using a specific siRNA. The effect on the activity of Rho-GTPases is observed using pull down activation assay, which confirmed StarD13 as a negative regulator for Rho and Cdc42 and not Rac. Our results also showed that StarD13 plays a negative role in cellular proliferation. Moreover to investigate the role of StarD13 in cell motility, StarD13 knock down resulted in an inhibition of cell motility and cells were not able to detach their tail and move forward. Being a Rho-GAP and localizing to focal adhesions, we hypothesize that StarD13 is inhibiting Rho to allow the formation of Rac-dependent focal complexes and the detachment of focal adhesions for the cells to move forward.

Integrins and Cell-ECM Interactions II

2082

Identification of novel Par1b substrates that regulate cell-extracellular matrix signaling.*D. M. Fernandez¹, A. Muesch¹; ¹Albert Einstein College of Medicine, Bronx, NY*

The serine/threonine kinase Par1b is well known for its role in regulating epithelial cell polarity and morphology. Recent evidence suggests that Par1b mediates its function as polarity determinant in part by regulating cell-extracellular matrix (ECM) signaling, which includes the process of cell spreading. How Par1b regulates ECM signaling mechanisms remains to be explored in full detail. We conducted an unbiased screen for novel Par1b substrates in polarized kidney-derived MDCK cells. The identification strategy was based on a chemical genetics approach in which the ATP-binding pocket of a kinase was altered to accommodate bulky ATP-analogues that when offered as phosphate donor in complex mixtures can only be utilized by the engineered kinase to selectively phosphorylate its substrates. The ATP-analogues contain a chemical affinity tag that is transferred onto the substrates and allows their isolation along with the identification of the phosphorylation sites by LC-MS/MS analysis. Using this approach, we have identified a total of 74 novel candidate substrates, as well as 5 known substrates. According to their gene ontology association, more than half belonged to categories, which are known to regulate Cell-ECM interactions. From those novel candidates, we have validated 14 as Par1b substrates. We are currently exploring how regulation of these substrates by Par1b effects cell spreading.

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Role of GNE in cell adhesion.S. Grover¹, R. Arya¹; ¹*School of Biotechnology, Jawaharlal Nehru University, New Delhi, India*

Sialylation of cell surface glycoproteins and glycolipids regulates variety of cellular functions such as adhesion, migration, signaling and immune response. The enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetyl-mannosamine kinase (GNE) is a key regulatory enzyme in the sialic acid biosynthesis. Homozygous missense mutations in this gene lead to Hereditary Inclusion Body Myopathy or Distal Myopathy with Rimmed Vacuoles. The present study depicts the effect of GNE on cell adhesion and migration. We generated stable GNE knockdown HEK cell line using shRNA. Approx. 70% inhibition in the GNE protein expression was observed in the knockdown cell lines compared to wild type HEK using immunoblots with anti-GNE antibody. The colorimetric assays specific for UDP-N-acetylglucosamine-2-epimerase and N-acetylmannosamine kinase activity showed 70-80% reduction in the enzymatic activity of knockdown vs wild type cell lines. Also, the sialic acid content of membrane and cytosolic fractions was reduced to 80-85% in knockdown cell lines. To study the effect of GNE on cell adhesion, knockdown and wild type cell lines were plated on different substrates such as laminin, collagen and fibronectin. GNE knockdown cell lines showed remarkable increase in adhesion property. In addition, GNE knockdown cells showed less migration compared to vector control by live cell imaging. To further elucidate the role of GNE, sialylation levels of key adhesion molecule, $\beta 1$ integrin, was studied. Shift in the electrophoretic mobility of $\beta 1$ integrin from knockdown cell lines confirmed that $\beta 1$ integrin was hyposialylated in the absence of GNE. Our results show for the first time that inhibition of GNE leads to hyposialylation of $\beta 1$ integrin which causes increased cell adhesion and reduced migration of GNE deficient cell lines.

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Regulation of VLA-4 mediated hematopoietic stem/progenitor cell adhesion by CD82.C. M. Termini¹, M. L. Cotter¹, K. D. Marjon¹, T. Buranda¹, K. Lidke², J. M. Gillette¹; ¹*Department of Pathology, University of New Mexico, Albuquerque, NM,* ²*Department of Physics & Astronomy, University of New Mexico, Albuquerque, NM*

The adhesive events between hematopoietic stem and progenitor cells (HSPC) and the bone marrow niche are important for maintenance of HSPC cell number and function. Critical to the HSPC/niche interaction is the spatial arrangement of membrane proteins, such as adhesion and signaling molecules, on the surface of HSPCs. At this time, the regulation of specific adhesion and signaling components remains poorly defined. CD82 or Kai1 is a member of the tetraspanin family of proteins, which function as regulatory scaffold proteins by assisting in the formation of membrane domains. In primary human CD34+ cells and the KG1a human progenitor-like cell line, the site of cell contact with osteoblasts is enriched with CD82. Furthermore, the use of CD82 blocking antibodies inhibited the homing and engraftment of CD34+ cells in vivo. As such, we hypothesize that CD82 is a critical molecule involved in regulating HSPC adhesion within the bone marrow niche. Using the KG1a progenitor-like cell line, we examined the effects of overexpression and knock down of CD82 on HSPC adhesion. We found that the overexpression of CD82 in HSPCs leads to increased adhesion to fibronectin and osteoblasts, while CD82 knockdown led to decreased adhesion. The change in adhesion to fibronectin and osteoblasts suggests the involvement of the $\alpha 4\beta 1$ integrin. Therefore, we treated cells with the $\alpha 4\beta 1$ blocking peptide, LDV, which blocked the CD82-mediated adhesion, and further implicated the $\alpha 4\beta 1$ integrin in HSPC adhesion. With the overexpression of CD82, we detected only a modest increase in $\alpha 4$ and $\beta 1$ surface expression, but no difference in total $\alpha 4\beta 1$ protein levels or $\alpha 4\beta 1$ affinity. Next, we evaluated whether the CD82 overexpression results in greater adhesion due to changes in $\alpha 4\beta 1$ avidity or surface clustering. Using the super resolution imaging technique

dSTORM, we examined the role of CD82 in regulating the clustering of $\alpha 4$ and $\beta 1$ on the surface of HSPCs. We also generated palmitoylation mutants of CD82 to assess the importance of this modification in CD82 function. The CD82 palmitoylation mutants display a reduced adhesion when compared to WT CD82 overexpressing cells suggesting a role for CD82 palmitoylation in mediating HSPC adhesion. Taken together these data suggest that CD82 mediates HSPC adhesion to components of the bone marrow niche by regulating the surface expression of $\alpha 4\beta 1$. Future work will analyze the role of additional CD82 motifs and post-translational modification in mediating $\alpha 4\beta 1$ surface distribution.

2085

Role of Lutheran blood group glycoprotein/Basal Cell Adhesion Molecule (Lu/B-CAM) in cellular interaction with laminin $\alpha 5$

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Lu is a member of the immunoglobulin superfamily and has been studied primarily in the contexts of blood group antigens and sickle cell disease. B-CAM, a splice variant of Lu, has the same N-terminal extracellular domain as Lu, but it lacks the C-terminal 40 amino acids of the cytoplasmic tail which carries an SH3 binding motif and potential phosphorylation sites that could be involved in intracellular signaling pathways. B-CAM was also identified as an up-regulated antigen in carcinoma, suggesting its involvement in tumor progression. Our previous study showed that Lu/B-CAM is a specific receptor for laminin $\alpha 5$, a major component of basement membranes in various tissues. Despite of recent advances, the biological functions of Lu/B-CAM are still unclear. In this study, using transfectants overexpressing Lu/B-CAM, we examined the role of Lu/B-CAM in cellular interaction with laminin $\alpha 5$. The recombinant protein containing laminin $\alpha 5$ was prepared from the conditioned media of HEK293 cells that were transfected with laminin $\alpha 5$, $\beta 1$, and $\gamma 1$ chains. For avoiding clonal variation of transfectants, we used the Flp-In System. Lu or B-CAM gene was cloned into pcDNA5/FRT expression vector and then transfected into HT1080F, human fibrosarcoma, that was Flp-In host cell line. Flow cytometry analysis showed that the transfectants well expressed Lu or B-CAM and did not change the expression of integrin $\alpha 3\beta 1$ and $\alpha 6\beta 1$. Cell attachment assay showed that both transfectants attached to laminin $\alpha 5$ slightly weaker than parent cells. Inhibition assay using anti-integrin and Lu monoclonal antibodies showed that Lu/B-CAM had little, if any, contribution in the cell adhesion to laminin $\alpha 5$. The result showed that the parent cells and transfectants attached to laminin $\alpha 5$ in a similar manner. We also examined the cell migration of transfectants on laminin $\alpha 5$. Cell migration was monitored using time-lapse video microscopy, and the video images were collected at 5 min. The positions of nuclei were tracked to quantify cell motility for 8 hours. Velocities were calculated in micrometers per 1 hour using Image-J. Lu and B-CAM similarly promoted cell migration on laminin $\alpha 5$, suggesting that the cytoplasmic tail of Lu did not involve in cell migration. Furthermore, the cell migration of transfectants on laminin $\alpha 5$ was also suppressed in the present of a function-blocking antibody against Lu/B-CAM. Our results showed that Lu/B-CAM contributed to cell migration rather than cell attachment.

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Selective effects of fungal β -glucans and α -D-mannans on leukocyte integrins Mac-1 and P150,95.*W. Cao¹, E. Pluskota¹, E. F. Plow¹, D. A. Soloviev¹; ¹Molecular Cardiology, Cleveland Clinic Lerner Research Institute, Cleveland, OH*

Poly- and oligosaccharides are important modulators of immune system, particularly as in their role as activators of the leukocyte β 2-integrin subfamily. These integrins are present on the surface of nonstimulated leukocytes in inactive "closed" conformation and activation enhances their recognition of ligands with high affinity. (1,3) β -glucans from *S. cerevisiae* (zymosan) is an activator of integrin Mac-1 (CD11b/CD18, α M β 2, CR3). Upon ligation with the integrin lectin domain, zymosan activates the integrin, stabilize it in an intermediate active conformation, and enhance lectin domain-independent recognition of many Mac-1 ligands such as fibrinogen D-fragment. However, recognition of the ligands interacting with the lectin domain such as *Candida albicans* Pra1 mannoprotein is ablated by β -glucans. Recently, we found that another member of β 2-integrins subfamily, integrin p150,95 (α X β 2, CD11c/CD18, CR4), also contains a lectin-like binding function within its α X subunit. The goal of this study was to compare the effect of fungal (1,3) β -glucans and α -D-mannans on integrins Mac-1 and p150,95. Employing a variety of cell systems including human and mouse neutrophils, monocytes and macrophages, Mac-1 or p150,95-deficient leukocytes, as well as HEK 293 cells overexpressing these integrins, we found that: a) The effect of zymosan on p150,95 was similar to that observed with Mac-1: β -glucans enhanced p150,95-dependent leukocyte adhesion to fibrinogen D-fragment and blocked p150,95 interaction with *C. albicans* Pra1. b) Fungal α -D-mannans promotes both Mac-1- and p150,95-mediated cell adhesion to fibrinogen D-fragment. In contrast, when leukocyte interaction with the lectin domain-dependent ligand *C. albicans* Pra1 was analyzed, α -D-mannans inhibited Mac-1 dependent leukocyte adhesion to this ligand, but had minimal effect ($p=0.068$) on α X β 2-dependent fungal recognition. The observed differences in sugar selectivity of the integrins may play an important role in the regulation of leukocyte activation and host defense against fungal infections.

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The moieties of complement iC3b recognized by I-domain of Integrin α X β 2 and α M β 2*S-U. Nham¹, D. Buyannemekh²; ¹Science Education, Kangwon National University, Chuncheon, Korea, ²Biology, Kangwon National University, Chuncheon, Korea*

The β 2 integrins play a vital role in leukocyte activation including cell adhesion, migration, and phagocytosis. This integrin binds many different types of ligands, including fibrinogen, iC3b, and ICAM-1. Complement fragment iC3b binding to β 2 integrin initiates inflammation and complement-mediated cytolysis. It has been shown that I domains in the α subunits of β 2 integrin (α X and α M I-domains) are the ligand binding moieties. In order to define structural aspect of the binding, we investigated the iC3b binding sites for α M and α X I domains by site directed mutagenesis and solid phase binding analysis. It was found the basic amino acids on the loop of α X I domain are the important residues for iC3b recognition. And the α X and α M I-domains were found to bind two amino acid termini of iC3b: α NTN (D730-E737) and α CUN (S1280-K1319). In α NTN, acidic residues near the N-terminus play an important role on the interaction with the I domains: D730, E731, E746 and E747. The whole chain of α CUN serves as a binding site of the I domains, but some acidic residues are critical for the I domains binding: E1285, E1287 and E1294. In addition, the C3dg domain of iC3b, was identified as an important moiety for the I domains of α X and α M. It was found that three amino acid residues (E1131,

D1134 and K1261) which located at the edge of concave pocket of C3dg were critical for the I domains binding.

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Subtype specific integrin suppression on the integrin binding peptide conjugated polysaccharide matrices.

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Peptide-polysaccharide matrices promote biological activity and are useful as a biomaterial for use in tissue engineering. Previously, we conjugated extracellular matrix derived cell adhesive peptides on polysaccharide, chitosan or alginate, matrices and found that peptide-polysaccharide matrices exhibited biological activities with peptide- and/or polysaccharide-dependent manner. Here, we focus on three integrin binding peptides derived from extracellular matrix proteins, FIB1 (12 aa peptide, from human fibronectin), EF1zz, (20 aa peptide, from mouse laminin α 1 chain), and 531 (14 aa peptide, from human type IV collagen α 1 chain) that bind to α v β 3, α 2 β 1, and α 3 β 1 integrin, respectively. We mixed these peptides and examined their cell attachment and cell spreading activities using human dermal fibroblasts to analyze the cross-talk between integrin subtypes. The FIB1-, EF1zz-, and 531-chitosan matrices promoted cell attachment and spreading with peptide specific cell morphology. When FIB1:EF1zz and FIB1:531 were mixed, mixed-peptide-chitosan matrices showed cell attachment with intermediate cell morphology of two different peptides. In contrast EF1zz:531-peptide-chitosan matrix showed the suppression of cell attachment and spreading. Same results were observed on the mixed-peptide-alginate matrices. These results suggest that β 1 integrin subtypes may compete or compensate each other whereas no effect on different β subunit integrin subtypes. TS2/16 integrin activation anti- β 1 integrin antibody partially recovered the suppression. Further, protein kinase A inhibitor of 14-22 amide promoted cell attachment suppression, whereas protein kinase C inhibitor of Go 6976 did not showed any effect on the suppression. These results suggested that protein kinase A involving integrin activation was down-regulated by the cross-talk of α 2 β 1 and α 3 β 1 integrin. We conclude that the mixed peptide-polysaccharide matrices have a potential to analyze the specific receptor-receptor cross-talk among different receptors.

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Clonal derivation and clonal survival of human embryonic stem cells on human laminin-521-based matrix in xeno-free and chemically defined environment.

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Clinical use of human embryonic stem (hES) cells has been hampered by poorly defined derivation and culturing procedures as well as ethical concerns caused by the destruction of human embryos in the process. Feeder cell layers, currently used as surfaces for derivation of new ES cell lines, are undefined and variable and can be sources of viral and bacterial contaminations. We have cloned, produced and purified human recombinant laminin isoforms that are expressed in hES cells. Laminins, as well as other proteins of extracellular matrix, have been always regarded as a minor, insignificant part of stem cell niches with no specific functions other than cell adhesion. We have shown that laminins have diverse effects on cultured hES and human induced pluripotent stem (iPS) cells. Thus, laminin-521 (LN-521) and LN-511 permit

long-term self-renewal of hES and iPS cells by interaction with $\alpha 6\beta 1$ integrin activating PI-3K signaling pathway. Karyotypically normal stem cells can be cultured for 9 months with stable expression of pluripotency markers and can subsequently be differentiated into cell lineages of all three germ layers. Importantly, a combination of LN-521 with other proteins of the natural hES cell niche allows clonal survival of hES cells. The LN-521-based matrix fully substitutes undefined and potentially hazardous feeder cells for hES cell derivation both from whole inner cell masses of blastocysts or from single blastomere cells without need to destroy the embryo, potentially addressing central ethical issues of hES cell derivation. The derivation from single blastomeres does not imply co-cultures with either the parental blastocyst or established hES cells. The methods provide a fully chemically defined animal component-free culture system for new hES cell line derivation and propagation, which can bridge the gap between academic stem cell research and clinical science.

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Laminin-derived integrin binding peptide-polysaccharide matrices as basement membrane mimetics.

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Basement membrane (BM) is a thin extracellular matrix existing among tissues and plays a critical role on tissue repair. The major components of BM are type IV collagen, laminin, perlecan, and nidogen, and laminin is mainly involved in biological function of BM. Laminins are heterotrimeric BM proteins consisting of α , β , and γ chains and exert multiple biological functions through interactions with other matrix molecules and with cell surface receptors. Matrigel, an extract of BM derived from mouse sarcoma, has been widely used as a three-dimensional cell culture matrix, but it is not possible to apply for human. Previously, we have identified a number of cell adhesive peptides from laminins. Our goal is to develop a synthetic Matrigel and use for tissue engineering. We conjugated the laminin active peptides to polysaccharide, alginate or chitosan, matrices and demonstrated their usefulness for cell transplantation and cell culture [1,2]. Here, we focused on integrin-binding peptides derived from laminins because integrins are known as major cell surface receptors for BM. We synthesized 22 integrin-binding peptides by Fmoc-based solid-phase peptide synthesis and examined their biological functions on both alginate and chitosan matrices. First, we evaluated the cell attachment activity on the peptide-alginate and -chitosan matrices using human dermal fibroblast (HDFs). As a result, ten peptide-alginate and eleven peptide-chitosan matrices showed HDF attachment activity in a dose-dependent manner. Six peptide-alginate and seven peptide-chitosan matrices promoted cell spreading, and the HDF attachment to these matrices was significantly inhibited by EDTA. These results indicate that the six peptide-alginate and seven peptide-chitosan matrices promote integrin-mediated cell adhesion. Next, we evaluated the inhibitory effect of anti-integrin antibodies on the 10 peptide-alginate matrices and 11 peptide-chitosan matrices to identify integrin subtypes. Integrins $\alpha 2$, $\alpha 3$, $\alpha 6$, and/or $\beta 1$ were involved in the HDF adhesion to the peptide-alginate and -chitosan matrices with peptide-dependent manner. We also investigated neurite outgrowth activity of the peptide-alginate and -chitosan matrices using PC12 cells. Six peptides promoted neurite outgrowth on both alginate and chitosan matrices. These results demonstrated that these laminin-derived integrin-binding peptide-polysaccharide matrices are useful to develop a synthetic Matrigel and can be applied for tissue engineering.

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2091

Cell matrix interactions and spheroid formation in 3D alginate cell culture system

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The use of 3-dimensional (3D) matrices for cell culture is gaining popularity as a substitute for traditional 2D cell culture as it can enable formation of multi-cellular structures and a more biologically relevant cell-cell contact such as found in tissues, organs and tumors. NovaMatrix[®]-3D is an alginate-based hydrogel cell culture system comprising an alginate foam matrix and an alginate immobilizing solution. Cells are entrapped uniformly throughout the alginate foam by first suspending them in a solution of sodium alginate that is applied to the foam. As the foam absorbs the suspension, in situ gelation occurs as calcium ions are donated from the foam cross-linking the added alginate. A hydrogel forms in the pores of the foam. Cells then grow in 3D and can form multicellular structures such as spheroids, myotubes, etc.

Some cells, however, require signaling molecules and matrix interaction in order to proliferate. This was evaluated by culturing cells in the presence of alginate to which the peptide sequence RGD (Arg-Gly-Asp) was covalently attached (RGD-alginate) thereby facilitating a signaling cascade via integrin-RGD interaction. Single cells of NHIK 3025 (human cervix carcinoma), OVCAR-3 (human ovarian adenocarcinoma), SKOV-3 (human ovarian carcinoma), NIH:3T3 (murine fibroblasts), C2C12 (murine myoblasts), V79-379A (hamster lung fibroblast) and MDCK (Madin Darby canine kidney) suspended in 0.5 - 1% alginate or RGD-alginate were added to the alginate foams. Cell localization and cell structure within the foam was visualized by confocal microscopy of fluorescently labeled cells. Cell proliferation was measured by counting cells after de-gelling the foam using sodium citrate that chelates calcium. Similar cell proliferation and spheroid formation occurred during three weeks of culture in foams with or without RGD-alginate for NHIK 3025, SKOV-3, 3T3 and V79-379A indicating that these cell lines do not require the presence of RGD within the alginate matrix for cell growth. However, during the same period, cell proliferation of OVCAR-3, MDCK or C2C12 was only seen in the foams containing RGD-alginate. The proliferation rate was affected by the RGD density. Cells that did not proliferate in alginate without RGD were still metabolically viable. Cell-matrix interaction can be controlled by type and concentration of signaling molecules covalently attached to the alginate. Portions of this work were funded by METOXIA project no.222741, EU 7th Framework Programme.

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ECM interaction with signaling events and cellular functions.

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Extracellular matrix is deposited by the cells that are under the control of multiple signaling molecules such as hormones and local factors. Extracellular signals in turn regulates cell behavior. This is called "inside-out" and "outside-in" signaling. Among these extracellular proteins, SPP1 is a unique molecule that possess RGD domain being recognized by integrins as well as stretches of aspartic acid that make the protein to be highly adhesive to calcified tissues. Since this protein is secreted by the cells that produce calcified matrix, the function of this molecule has been considered to be important for the responses of the cells to microenvironment. One of the major stimuli to the individual body is the extra cellular force

stimuli. This is especially important for skeletal tissue as well as cardiovascular tissues where external forces are either due to locomotions or heart beat and blood flow. The force stimuli is in part mediated by neural regulation but also calciotropic hormones. In the case of skeletal tissues, SPP1 is produced by both types of cells including bone forming cells as well as bone resorbing cells. Hormones are regulating such activities. The deletion of this molecule resulted in the loss of tissue response to unloading that is normally enhances bone resorption and suppresses bone formation leading to rapid bone loss in the individual bodies. Quite importantly, this molecule is not only secreted or not only deposited in the extracellular matrix but also being functional inside the cells. Such examples include not only skeletal cells but also immune cells. Such mechanical stimuli against skeletal tissues are also under the control of nervous tissues. In fact, inhibitors for the sympathetic nervous system have been shown to regulate skeletal mass. The receptors for the transmitter molecules expressed on bone cells are considered to convey the regulation by nervous system. Importantly, we have found that this nervous system signaling links to the function of SPP1 inside the cells as well as outside cells. The conversion of the signals from both inside and outside the cells determined the final direction of the cell response to the external force stimuli.

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Tumor Endothelial Marker 8 and its role in vasculogenesis and connective tissue homeostasis.

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The process of angiogenesis is an essential part of vascular development and various human pathologic disease states such as cancer, hemangioma, chronic inflammation, diabetic retinopathy and fibrosis. The Tumor Endothelial Marker 8 (TEM8) gene, which encodes a cell surface integrin-like receptor, also known as Anthrax toxin receptor 1 (ANTXR1), was originally identified as a component of endothelial cells in highly vascularized tumors. The physiological function of TEM8, however, remains elusive. Detailed understanding of TEM8 function will offer valuable mechanistic insights into the etiology of angiogenesis-related diseases.

It has been reported that TEM8 plays a role in adhesion, spreading and migration of endothelial cells through its interaction with extracellular matrix (ECM) components, such as collagen type I and type IV and actin cytoskeleton. In addition, *in vitro* assays show a correlation between increased TEM8 expression and initiation of endothelial tubular structure formation. Altogether, these data suggest that TEM8 can impact the process of vessel formation at any steps of vasculogenesis/angiogenesis. To explore TEM8 function in vascular morphogenesis we have studied a *Tem8* -/- mice, in which eight exons of the gene are removed.

Inspection of mutant animals revealed hemorrhage in digestive, respiratory and reproductive systems that pointed to a defective, leaky vascular system in both *Tem8* -/- and *Tem8* +/- mice. This was confirmed by extravasation of FITC-coupled dextran in skin tissue samples of FITC-dextran-perfused animals. Notably, the overall phenotype was less severe in heterozygous than homozygous animals, suggesting that the level of expression and/or activity of TEM8 is important for vascular morphogenesis. Our data show that proliferation and apoptosis of cells in deep and subcutaneous skin layers are markedly increased in mutants compared to wild type littermates at 7 weeks of age. Moreover, we observed strong correlation between increased CD31 staining and increased vessel density in skin. Furthermore, phenotypic characteristics of dermal and subcutaneous vessels in *Tem8* +/- and *Tem8* -/- animals included (i) thin-walled blood-congested vessels, (ii) defective basement membranes associated with reduced levels of collagen IV and collagen XVIII/endostatin, (iii) abnormal activation of Ang-2/Tie-2 signaling and (iv) mural cell detachment. Intriguingly, in addition to vascular abnormalities *Tem8* null animals exhibited increased deposition of ECM molecules, including collagen I, fibronectin and vitronectin accompanied by fibrosis in various organs.

Matrix deposition and accumulation of fibrous tissue were also found in two recently reported conditional Tem8 mouse models; reminiscent of systemic hyalinosis, a disease caused by mutations in capillary morphogenesis protein-2 (CMG2) sharing 60% identity with TEM8 in the vWF extracellular domain.

Together our data suggest that TEM8 can regulate vascular and connective tissue homeostasis in part through ECM-TEM8 signaling.

2094

Mammary epithelial cells require Integrin Linked Kinase signalling for lactational differentiation and the formation of polarised acini.

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Epithelial cell adhesion to the surrounding extracellular matrix is necessary for their proper behaviour and function. During pregnancy and lactation mammary epithelial cells (MECs) require signals imparted by specific $\beta 1$ integrin-laminin interactions for their functional differentiation in response to prolactin and for the correct formation of polarised secretory acini. Downstream of $\beta 1$ integrin, our lab has identified the scaffold protein Integrin Linked Kinase (ILK) as the key signal transducer that is required for both prolactin driven lactational differentiation and the establishment of apico-basal polarity in MECs. ILK is a multifunctional adaptor protein that links integrins to the actin cytoskeleton and Rho GTPases such as Rac1. ILK forms a ternary IPP complex with PINCH and Parvins, which are central to its adaptor functions. However, it is not known which of ILKs interacting partners are important in controlling tissue-specific gene expression and polarity. We have now established that inducible ILK deletion in MECs from ILK^{fl/fl}CreER mice causes inverted apico-basal polarity and a failure to phosphorylate STAT5 leading to the failure of prolactin-induced milk protein expression. We have also developed a 3-dimensional culture model using the EpH4 mammary epithelial cell line, which respond to prolactin treatment and form polarized acini similar to primary cells. In these cells knocking down beta1-integrin by lentiviral shRNA delivery has a profound effect on beta-casein production. By utilising lentiviral shRNA knockdown of ILK associated proteins, coupled with an ILK mutant rescue approach, we are currently piecing together the role of ILK and its specific binding partners in MEC differentiation and polarity.

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Sarcospan-Dependent Akt Activation is Required for Utrophin Expression, Binding to the Extracellular Matrix, and Muscle Regeneration.

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Utrophin is normally confined to the neuromuscular junction (NMJ) in adult muscle and partially compensates for the loss of dystrophin in mdx mice. We show that Akt signaling and utrophin levels were diminished in sarcospan-deficient muscle. By creating several transgenic and knockout mice, we demonstrate that sarcospan regulates Akt signaling to control utrophin expression. Sarcospan determined α -dystroglycan glycosylation by affecting levels of the NMJ-specific glycosyltransferase, Galgt2. After cardiotoxin injury, regenerating myofibers express utrophin and Galgt2 modified α -dystroglycan around the sarcolemma. Sarcospan-null mice displayed delayed differentiation after cardiotoxin injury due to loss of utrophin and Akt signaling. Treatment of sarcospan-null mice with viral Akt increased utrophin and restored muscle repair after injury, revealing an important role for the sarcospan-Akt-utrophin signaling axis in regeneration. Sarcospan improved cell surface expression of utrophin by increasing

transportation of utrophin and dystroglycan from ER/Golgi membranes. Our studies reveal functions of utrophin in regeneration and new pathways that regulate utrophin expression at the cell surface.

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Amelioration of dystrophin-deficient muscular dystrophy is dependent on $\alpha 7\beta 1$ integrin.

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Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disorder caused by loss of the predominant adhesion complex at the muscle membrane, the dystrophin-glycoprotein complex (DGC), which connects the muscle cell membrane with the extracellular matrix. Over-expression of laminin-binding adhesion complexes, $\alpha 7\beta 1$ integrin or the utrophin-glycoprotein complex (UGC), rescues dystrophic pathology in the mdx mouse model of DMD by compensating for the loss of DGC function. Sarcospan (SSPN) ameliorates mdx muscular dystrophy by increasing abundance of both the UGC and $\alpha 7\beta 1$ integrin at the cell membrane. Here, we show that the rescue effect is dependent on $\alpha 7$ integrin and that SSPN acts synergistically with both $\alpha 7$ integrin and UGC complexes. We biochemically isolated a macromolecular complex containing $\alpha 7\beta 1$ integrin and the UGC. Using several double- and triple-knockout mice, we discovered that $\alpha 7$ integrin and SSPN determine UGC laminin-binding and signaling functions. We show for the first time that $\alpha 7\beta 1$ integrin is required for rescue of mdx dystrophic pathology.

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Tenascin-C aggravates myocardial inflammation in experimental autoimmune myocarditis in mice.

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Tenascin-C (TN-C) is an extracellular matrix glycoprotein with high bioactivity, known to contribute to progression of various autoimmune diseases by mediating innate and adaptive immune responses. In normal adult heart, TN-C is sparsely detected but appears under various pathological conditions closely associated with active inflammation. In the present study, we examined a role of TN-C in regulation of immunoreaction in autoimmune myocarditis. Experimental autoimmune myocarditis (EAM) was induced by immunization with murine cardiac myosin peptide emulsified in complete Freund's adjuvant using knockout (TNKO) mice and comparing them with sibling wild-type (WT) mice. In WT-EAM, TN-C expression started to be upregulated at day 12 and peaked at day 14 after immunization in parallel to histological findings of inflammatory activity. At day 14, heart weight/body, weight myocardial troponin-I levels, severe infiltration of neutrophil, dendritic cell, activated CD4+ T-cells, Th17 cells infiltration, level of cytokines IL-6 and IL-17A in heart markedly elevated in WT-EAM to compare with the WT-control mice. These changes were significantly reduced in TNKO-EAM.

Next, we cultured dendritic cells derived from bone marrow of naive mice. In vitro, TN-C-induced expression of IL-6 which favors the generation of pathogenic Th17 cells and CCL17 that has chemotactic effect on Th17 cells. Silencing of $\alpha 9$ integrin, a receptor for TN-C, by siRNA significantly reduced synthesis of IL-6 and CCL17. However, silencing of toll-like receptor 4, another receptor for TN-C did not show any effect to these cytokine production. These results

indicate that TN-C aggravates inflammatory response in the early stages of EAM by promoting Th17-related cytokine productions and Th17 cell generation via $\alpha 9$ integrin-mediated signaling.

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Increased Collagen Production and Altered Integrin Expression by Fibroblasts in Drug-induced Gingival Overgrowth.

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Drug-induced gingival overgrowth is a side effect associated with 3 types of drugs: anticonvulsants (phenytoin), immunosuppressive agents (cyclosporine A), and various calcium channel blockers for cardiovascular diseases. Gingival overgrowth is characterized by the accumulation of extracellular matrix in gingival connective tissues, particularly collagenous components with various degrees of inflammation. About 30 % of patients treated with cyclosporine A develop gingival overgrowth. Although the mechanisms of this disorder are still unknown, recent studies in rat models suggest that this disorder seems to be induced by inhibition of collagen degradation in gingival connective tissue. However, in human gingival overgrowth collagen synthesis seems to be increased. In order to resolve how collagen is altered in human gingival overgrowth, collagen homeostasis and fibroblast functions were evaluated in cultures of fibroblasts derived from a cyclosporine A-treated patient. Collagen I and metalloproteinase type 1 (MMP-1) and the inhibitor for this metalloproteinase expression were increased. In addition, fibroblasts presented an exaggerated growth rate and a switch in integrin expression. Fibroblasts from a cyclosporine A-treated patient lost expression of $\alpha 4$, $\alpha 5$, and αv integrins, and increased expression of $\alpha 6$ and αL integrins. These data suggest that cyclosporine A-induced gingival overgrowth, in humans is caused in part by overproduction of collagen and at the same time by inhibition of collagen degradation. In addition, fibroblasts alter their phenotype showing an accelerated cell growth and alteration of integrin-dependent functions, such as adhesion and migration. Acknowledgements: This project was financed by grant IACOD IB200811 (to EUQ) and by grant IN205311-2 (to CR) from Dirección general de Asuntos del personal Académico, Universidad Nacional Autónoma de México.

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Binding of $\alpha v \beta 1$ and $\alpha v \beta 6$ integrins to tenascin-C induces epithelial-mesenchymal transition-like change of breast cancer cells.

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Tenascin-C (TNC), a large hexameric extracellular glycoprotein, has multiple domains and a variety of receptors mediating a wide range of cellular functions. We earlier reported its induction of epithelial-mesenchymal transition (EMT)-like change in breast cancer cells. In the present study, we clarified integrin receptors for involvement in this process. Among integrins previously reported as TNC receptors, substantial expression of $\alpha 2$, αv , $\beta 1$, and $\beta 6$ subunits was detected by quantitative polymerase chain reaction and immunoblotting. Integrin $\beta 6$ mRNA was remarkably upregulated by transforming growth factor (TGF)- $\beta 1$ treatment, and protein expression was prominently increased by additional exposure to TNC. Immunofluorescence labeling demonstrated integrin $\alpha v \beta 6$ in focal adhesions after TNC treatment, especially in combination with TGF- $\beta 1$. The Immunoprecipitation showed increase in $\alpha v \beta 1$ heterodimers, but not $\alpha 2 \beta 1$, after TNC treatment. Activated $\beta 1$ subunits detected by an antibody against the Ca^{2+} -dependent epitope were co-localized with αv in focal adhesion complexes, associated with FAK

phosphorylation at tyrosine 925. Binding analysis of integrins to TNC showed binding of $\beta 1$, $\beta 6$ and αv , but not $\alpha 2$, in a divalent cations-dependent manner. Neutralizing antibodies against αv and $\beta 1$ blocked EMT-like change caused by TNC alone. In addition, combined treatment with anti- $\beta 1$ and anti- $\alpha v\beta 6$ inhibited TGF- $\beta 1$ /TNC-induced EMT. TNC could induce EMT-like change by signaling via integrin $\alpha v\beta 1$ and $\alpha v\beta 6$ recruited to focal adhesions.

Bioengineering of Cell-Matrix Interactions

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High Throughput Analysis of Mechanosensitive Stem Cell Differentiation.

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Human mesenchymal stem cells (hMSCs) have the ability to differentiate based solely on the elastic modulus of the two-dimensional substrate upon which they are plated; substrates with a stiffness value mirroring that of a given tissue will cause hMSC differentiation toward that tissue's lineage. In order to accomplish this, hMSCs must be able to sample their environment for physical information and be able to convert that information into a biochemical signal in a process known as mechanotransduction. The role of mechanotransduction in cell fate decisions has been increasingly scrutinized, but a consensus on the necessary and sufficient signaling pathways for mechanosensitive stem cell differentiation has not yet been achieved. Focal adhesions, due to their position in proximity to the cell-matrix interface, have been proposed to play a role in this process. Linker proteins within focal adhesions have been shown to contain force-sensitive cryptic binding sites that are exposed under physiological strain. To analyze the force-resultant unfolding of focal adhesion proteins, a Cysteine Shotgun/Western Blot (CS/WB) technique was used to examine the extent of differential unfolding and exposure of cryptic kinase sites across focal adhesion proteins in response to substrate stiffness to identify novel stem cell mechanosensors. CS/WB revealed that in response to changes in substrate stiffness, hMSCs exhibit changes in talin unfolding. Talin, in turn, is responsible for the activation of vinculin, which activates MAPK1. In hMSCs, perturbing this pathway has been shown to affect myogenic differentiation of hMSCs on myogenically favorable substrates. Analysis of this system in the web-based proteomics tool Scansite reveals that vinculin contains an especially inaccessible binding domain for MAPK1. Scansite analysis of 47 potential focal adhesion-based mechanosensors further revealed a total of 465 cryptic binding domains. Of these, MAPK1 stood out for both its frequency and its inaccessibility, and was found to have inaccessible binding domains in a total of 21 potential mechanosensors. While force-dependent MAPK1 binding to vinculin specifically regulates myogenesis, monotonically increasing talin unfolding with stiffness suggests a broader, force-sensitive cell regulation. In order to address this, we utilized a 96 well plate polyacrylamide platform with varying stiffnesses to assess how a targeted siRNA screen of these other 21 proteins affects hMSC spreading, migration, proliferation, and differentiation. Combined, the data from both the bioinformatics analysis and the siRNA screen lend support to the heretofore unexplored role of MAPK1 in substrate-stiffness based hMSC differentiation, as well as the role of kinases in mechanically induced myogenesis.

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Matrix Stiffness Controls Nuclear Morphology of Human Mesenchymal Stem Cells in 2D and 3D.

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It is now widely acknowledged that cells also mechanically interact and respond with their surrounding in multiple ways and that these physical cues can be as important as biochemical ones. Especially striking was the demonstration of mechanically guided differentiation of human mesenchymal stem cells (hMSCs) towards distinct lineages based on the stiffness of their native environment. While this process needs several days or weeks we focused on the early time interactions between cells and elastic substrates and analyzed cell morphology, cytoskeletal structure and nuclear morphology of cells cultured on novel hyaluronic acid hydrogels of different Young's moduli E in two and three dimensions. We find that the nuclear morphology is controlled by matrix elasticity via elastic coupling through acto-myosin stress fibers. Our experimental findings and the described theory offers an insight towards a direct mechanical pathway from the physical parameters of the extra-cellular matrix towards gene regulation in the nucleus.

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Integrated biochemical and functional characterization of the contribution of cellular architecture to ES- and iPS-derived cardiac tissue phenotype.

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Utilization of stem cell-derived cardiomyocytes for *in vitro* toxicity assessment applications is hampered by difficulties in driving them toward a physiologically-relevant phenotype. These challenges arise partly from a limited appreciation of the role played by the physical microenvironment during development, and a lack of rigorous, standardized metrics for evaluating the phenotypic state of stem cell-derived cardiac constructs *in vitro*. Traditionally, myocardial differentiation is evaluated using methodologies that provide poor predictions of tissue-level cooperative function, such as patch clamp recordings taken on individual cardiomyocytes. We hypothesized that the functional phenotype of stem cell-derived cardiomyocytes could be optimized and more readily assessed by culturing them in a microenvironment that recapitulates the laminar cellular organization of the native myocardium. To test this hypothesis, engineered cardiac tissues were fabricated using micro-contact printed fibronectin substrates seeded with either neonatal mouse ventricular myocytes (NMVM), murine ES-derived cardiomyocytes (mES), or murine iPS-derived cardiomyocytes (miPS). These myocardial tissue constructs were then subjected to quantitative cytoskeletal structure analysis, assessment of contractile performance, electrophysiological recordings, and gene expression measurements to compare and contrast the tissue-level, functional phenotype of the three types of cardiomyocytes tested. Examination of global sarcomere alignment as judged from immunohistochemically-labeled α -actinin micrographs revealed that the mES and miPS-derived cardiac tissues possessed reduced sarcomere alignment due to the presence of less mature myofibrils relative to the NMVM tissues. Measurement of contractile stress output using our muscular thin film assay revealed that both the mES and miPS constructs generated significantly less contractile stress than the NMVM tissues. Optical mapping studies showed

that tissues constructed from all 3 cardiac cell types exhibited similar conduction velocities. However, the action potential duration at 90% of the amplitude (APD90) for mES-derived cardiac tissues was significantly shorter than that observed in NMVM tissues, whereas APD90 in miPS-derived cardiac tissues was not significantly different from either the mES or NMVM tissues. Statistical comparison of phenotype marker genes commonly used to distinguish ventricular from atrial myocytes revealed that the mES and miPS samples exhibit significantly greater expression of some atrial markers (*Myl4*, *Tbx5*, *Hcn4*) and significantly lower expression of some ventricular markers (*Pln*, *Irx4*, *Kcne1*) than the NMVM tissues. These results show that morphology and organization play an important role in the functional phenotype of myocardial tissues and allow physiologically-relevant characterization of contractile performance.

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Monitoring mouse iPS cell-derived cardiomyocytes behaviors using polypeptide multilayer-coated electrodes.

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Heart disease remains a leading cause of mortality in developed countries. Recently, one of the potential therapy approaches is to derive cardiomyocytes from induced pluripotent stem (iPS) cells. When iPS cells are cultured on gelatin-coated plates, however, the efficiency of cardiomyocyte differentiation rate is still too low. Therefore, to enhance the efficiency of iPS cell differentiation into cardiomyocytes remains challenging. In terms of molecular level, many biochemical assays are unable to describe cells without precluding their therapeutic potential at present. Electric cell-substrate impedance sensing (ECIS) is a real-time label-free noninvasive monitoring technology to characterize therapeutic potential of cells. Polyelectrolyte multilayer film is an emerging method for substrate coating to modulate cellular behaviors. It would be very useful for stabilizing the ECIS electrode coatings with these films for monitoring cell behaviors in real-time. In this study, we used the layer-by-layer assembly to build polyelectrolyte multilayer of cationic poly-L-lysine and anionic poly-L-glutamic acid on ECIS electrode arrays. After build-up, multilayer films were cross-linked with EDC/sulfo-NHS to modulate their stiffness, and were coated with gelatin to enhance cell adhesion. iPS cells were then cultured on gelatin-terminated PLL/PLGA multilayer-coated electrodes and cardiomyocyte differentiation was monitored with ECIS. The occurrences of appearing beating cardiomyocytes were observed when iPS cells were cultured on gelatin-terminated PLL/PLGA multilayer-coated electrodes and on gelatin-coated electrodes as well. In addition, the periodic fluctuations of measured impedance caused by cardiomyocyte beating were observed accordingly. Our results demonstrate that cross-linked and gelatin-coated polypeptide multilayer films can promote iPS cells toward cardiomyocyte differentiation and the cardiomyocyte beating can be monitored with impedance sensing techniques such as ECIS.

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Novel 3D Porous Biodegradable Scaffolds for Stem Cell Based Tissue Regeneration.

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Herein we report a new method to make porous 3D scaffolds by enzyme crosslinking and microbead technology. Gelatin (Gel) conjugated with tyramine (Tyr) was synthesized as Gel-Tyr. Gel-Tyr can be crosslinked by oxidation using horseradish peroxidase to form biodegradable hydrogel scaffolds. In order to increase the porosity of the Gel-Tyr hydrogel scaffolds, we applied bead-leaching technology. Alginate microbeads with diameter of 100 μm were mixed in the Gel-Tyr solution. After the hydrogel crosslinking was completed, alginate microbeads were dissolved by treating with the chelating reagent EDTA. The vacancy of

microbeads formed pores in the scaffold. The Gel-Tyr scaffolds were tested with bone mesenchymal stem cells (MSC). Cell viability and cell proliferation assays demonstrated good biocompatibility for MSCs adherence and proliferation. This approach provides a new straightforward way to fabricate 3D porous scaffolds for cell delivery and tissue regeneration.

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Fibrillar collagen is equivalent to stiff matrix in driving marrow stromal cell differentiation into a matrix-deficient, myofibroblastic-like phenotype.

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Scars tend to be stiffer than normal tissue, which has prompted the use of stiff matrices as models of scars, but scars are also rich in fibrillar collagen-I. Here, we introduce a soft matrix embedded with distinctly fibrillar collagen type-I, and show that this is sufficient to drive bone marrow stromal cells (MSCs) into a contractile, myofibroblastic-like phenotype – ‘myo-MSCs’. These cells have been reported to minimize scarring in a unique wound healing response, exemplified by their application to myocardial infarcts. Transcriptome analysis in response to matrix rigidity points to an upregulation of genes that participate in the cellular contractile machinery, notably α -smooth muscle actin (SMA), but a decreased expression of matrix protein genes for collagen types I and VI, and tenascin-C; TGF β 1 and TGF β RII, implicated in progressive fibrosis, are also downregulated. MSCs cultured on the embedded-fiber, soft matrix exhibit many similarities to cells on rigid substrates: higher SMA but lower collagen type I protein expression. Phosphorylation at serine-1943 (S1943) of non-muscle myosin IIA, which deactivates stress fiber assembly, is decreased on both fibrosis-like and rigid substrates but almost twice higher on a soft substrate. TGF β is found to induce S1943 phosphorylation and SMA and collagen type I production. Surprisingly, inhibition of the TGF β pathway perturbs matrix expression but not SMA, suggesting a response that is unique from those of myofibroblasts. Furthermore, these ‘myo-MSCs’ hint that, unlike myofibroblasts, they do not become hyper-contractile. This supports the notion that MSC engraftment into wounded tissues suppresses fibrosis, highlighting the promise of these cells in restoring normal tissue function.

2106

Elongated Stem Cell Morphology and Matrix Stiffness Influences Lineage by Modulating Contractility.

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Human adult mesenchymal stem cells (MSCs) “feel” the properties of their surrounding extracellular matrix (ECM) via myosin and differentiate in response to them, e.g. stiffness, porosity to direct cell shape, etc. Degenerative diseases, such as muscular dystrophies cause abnormal stiffening as a result of fibrosis, which misdirects stem cell lineage. When both stiffness and cell shape are present in a disparate fashion, e.g. highly elongated cells similar to muscle despite the presence of an abnormally stiff microenvironment, we hypothesized that a myosin contraction-dependent balance could induce a subset of MSCs to become myogenic despite residing in a dystrophic-like stiff niche, i.e. elongated MSCs on stiff ECM would maintain myosin activity similar to cells on normal ECM. MSCs on fibronectin patterned in shapes of varying aspect ratio but common area on polyacrylamide substrates expressed muscle specific

myosin heavy chain (MHC) most strongly on muscle like stiffness of 11 kiloPascal (kPa) and elongated patterns, i.e. 10:1 and 15:1 aspect ratio. In contrast on osteogenic-like matrices of 34 kPa, highest MHC expression corresponded to isotropic and circle shaped cells. Expression of vinculin scaled with cell shape for each stiffness tested and also with substrate stiffness but only on elongated cells. Cell strain energy as determined by traction force microscopy scaled with substrate stiffness and decreased as a function of cell elongation with isotropic cell patterns producing the highest contractile energy in contrast to our hypothesis. Interestingly, these data imply that focal adhesion formation and cell-generated traction stresses follow opposing trends as a function of cell shape. In addition these shape- and stiffness-dependent lineage changes with muscle markers and contractility-based observations suggest that muscle induction may be possible in non-permissive stiffer environments and could prove beneficial to treat fibrotic muscle diseases. Efforts to test the myogenic potential of this subpopulation of MSCs in the mdx mouse model of muscular dystrophy are ongoing.

2107

Mechanically patterned matrices improve adipose-derived stem cells alignment and fusion

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Adipose-derived stem cells (ASCs) more completely undergo extracellular matrix (ECM)-directed myogenesis than their bone marrow-derived counterparts (BMSCs) by expressing myogenic markers 40-fold higher and forming fused muscle in vitro; fusion rates, however, still produce too few myotubes to constitute a clinically viable cell source (~2%). To encourage end-on cell fusion to form aligned skeletal muscle, a mechanically patterned substrate with alternating neuronal- and muscle-like stiffness that mimicks innervated muscle may be a more physically appropriate environment. This mechanically patterned substrate or 'Zebraxis' matrix was fabricated with alternating regions of soft (neurogenic), firm (myogenic), and/or stiff matrix (fibrotic or osteogenic). ASCs, C2C12 myoblasts, and PC12 neuronal cells all differentially sorted themselves based on their stiffness preference: cardiomyocytes, myoblasts, and ASCs all durotaxed to the myogenic regions of the pattern whereas neurons had opposing behavior. With additional alignment, ASCs fused into multi-nucleated myotubes at a rate almost twice that on static hydrogels. Moreover, a great fraction of ASCs-derived myotubes underwent multiple rounds of fusion due to alignment of their cadherin-rich ends. Most importantly, the multi-nucleated myotubes that form are resistant to transdifferentiation when plated onto a stiffer matrix mimicking a more fibrotic-like stiffness. Singly nucleated ASCs are not, suggesting that additional strategies are necessary to achieve a pure myotube fraction. However differential sorting, enhanced fusion, and multiple fusion events supports using ECM to create spatially-patterned in vitro muscle for regenerative uses in fibrotic muscle diseases such as muscular dystrophy.

2108

A fibrous model of the tumor stromal microenvironment promotes mesenchymal morphology but not EMT in epithelial cells.

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It has become increasingly apparent that the tumor microenvironment is a key regulator of cancer progression. However, the role of the acellular, physical components of the microenvironment in tumorigenesis remains poorly understood. The stromal tissue surrounding most metastatic epithelial cancers is largely comprised of aligned, fibrous extracellular matrix primarily made up of type-I collagen. Here we developed an in vitro model system to test the effect of this aligned fibrous environment on cell morphology and behavior in the absence of physiological collagen. We grew cells on an acellular surface composed of electrospun poly-L-lactic acid (PLLA) and then examined the effect of the aligned fibrous environment upon growth rate, morphology, cytoskeletal arrangement, expression of epithelial to mesenchymal transition markers, adhesive strength and migratory capacity. We compared the phenotypically normal breast epithelial cell line MCF10A and the invasive breast cancer epithelial cell line MDA-MB-231 on three different types of substrates: common 2D culture (glass coverslips or plastic culture dishes), a thin film of PLLA (glass coverslips coated with PLLA) or PLLA fibers (glass coverslips onto which PLLA had been electrospun). Both cell types adopted a more mesenchymal morphology when grown on PLLA fibers, and this effect was exaggerated with the more metastatic MDA-MB-231. However, neither cell type underwent the changes in gene expression indicative of epithelial-mesenchymal transition (EMT), despite the changes in cell shape. Nor did they exhibit the decreased adhesive strength or increased migratory capacity typical of metastatic tumor cells. These results indicate that cells can be grown on electrospun PLLA fibers, and that they can undergo a morphological reorganization due to the topology of the substrate. However, they also suggest that changes in cell morphology alone do not promote a mesenchymal phenotype, and consequently that the aligned fibrous environment surrounding epithelial cancers does not, via topography alone, promote EMT.

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CD44-based adhesion and mechanotransductive signaling on engineered hyaluronic acid matrices.

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Glioblastoma multiforme is the most clinically aggressive primary brain tumor and is characterized by diffuse infiltration of glioma cells into brain parenchyma, which is rich in the glycosaminoglycan hyaluronic acid (HA). We present new evidence that the HA receptor CD44 may be responsible for supporting integrin-based adhesion and mechanotransduction of glioma cells. Additionally, under certain conditions, glioma cells are sensitive to the stiffness of HA-based matrices not functionalized with adhesive peptides, suggesting that HA receptors themselves are involved in sensing of the biophysical environment. While comparatively much is known about cell-matrix mechanosensing through integrins and the role it may play in tumor progression, our findings reveal a previously under-appreciated role of CD44 and possibly other HA-specific receptors in this process. These findings have broad implications for the field's fundamental understanding of how glioma cells interact with the tumor microenvironment and may suggest new therapeutic strategies.

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In vivo surface reaction and microstructural degradation of hydroxyapatite in the dog.

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Hydroxyapatite (HA) is widely used as a bioactive ceramics since it forms a chemical bonding to bone. The disadvantage of this material is its poor mechanical properties. In this research, the surface dissolution and disintegration of HA in the dog have been studied and the mechanism by which phase pure HA dissolves in vivo was investigated. The microstructural interactions of HA implant in vivo. is similar to the behaviour of in vitro. Biological properties of HA in in vivo are affected grain boundary dissolution followed by microstructural disintegration. It was caused by grain boundary dissolution initiated at the implant surface extended into bulk following these paths. This kind of dissolution process apparently evidenced grain boundary dissolving causes particle generation. This results indicates that long-term bone in-growth and mechanical properties could be dramatically decreased.

2111

Integrin involvement in the cell response to matrix-bound BMP2.

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We have explored the concept that extracellular matrix proteins bind and present growth factors as “matrix-bound” ligands. As extracellular matrix is crucial for confinement by limiting diffusive range, accessibility, and ligand signaling direction, we have designed a biomaterial offering the characteristics of a permissive niche by combining growth factor presentation with appropriate mechanical properties. The BMP2-containing soft films appear as an appropriate substrate to further investigate the specificity of BMP2 signaling and to decipher the associated signaling cascade. Our results support that signaling can be enhanced by the spatial organization of different type of receptors and that receptor cooperativity is necessary for the achievement of a coordinated cell response leading ultimately to cell differentiation. We show that cross-talk between BMP2 receptors and integrins is required to drive Smad signaling through specific reorganization of the actin cytoskeleton.

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Tuning cellular mechanics and motility through genetic manipulation of Rho GTPase and myosin activity.

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The structural and mechanical properties of cells such as cell size, shape, and stiffness have recently been recognized as important regulators of cell behavior. For example, cells cultured on small areas of adhesive extracellular matrix (ECM) grow slowly and apoptose more frequently than cells spread on large areas (Chen et al., 1997 *Science* **276**: 1425-1428), and cells confined to one-dimensional lines of ECM elongate their cell bodies and can migrate faster than cells cultured on standard unconfined two-dimensional substrates (Doyle et al., 2009 *J Cell Biol.* **184**: 481-490). While there are many strategies to indirectly control such cell behaviors through manipulation of the extracellular environment, there is a marked lack of strategies for controlling these behaviors in more direct and precise ways. To address this unmet need, we have developed a genetic strategy for directly tuning the mechanical properties of cells. Specifically, we have placed genetic mutants of intracellular mechanotransductive signaling

proteins, including RhoA GTPase and myosin light chain kinase (MLCK), under a tetracycline-repressible promoter and introduced a single copy of these constructs into human glioblastoma cells using viral gene delivery. By expressing these proteins from a tetracycline-repressible promoter, we can vary their expression by simply changing the concentration of tetracycline in the cell culture medium. RhoA and MLCK are both known to activate myosin II, which is the motor protein responsible for cellular force generation, and with constitutively active (CA) mutants of these proteins, we demonstrate that we can modulate cytoskeletal architecture, traction force generation, and cellular stiffness in a graded fashion. Moreover, by reversibly switching expression of CA RhoA on and off over time (by adding and removing tetracycline), we can dynamically control cell spreading, migration, and ECM remodeling. We believe that this strategy will serve as a valuable tool for developing quantitative relationships between intracellular signaling pathways, cellular physical properties, and complex cell behaviors. Furthermore, such precise control over cell behavior could allow us to directly control how cells physically interact with their microenvironment and could potentially allow one to “re-engineer” how cells respond to ECM properties, which would be particularly useful in tissue engineering applications that interface cells with synthetic materials.

2113

Structured substrates for the investigation of shape-mediated behavior.*K. E. Broaders¹, Z. Gartner¹; ¹Pharmaceutical Chemistry, UCSF, San Francisco, CA*

The communal behavior of cells in tissues is influenced by the physical arrangement of matrix, cells, and diffusible factors in the microenvironment. The phenotypic dependence of cells on their surrounding environment is important in the context of tumorigenesis, where an initially organized tissue is disrupted and transitions to a less structured but nonetheless reorganized cancerous "pseudo-organ". It has long been appreciated that genetic injury is, in many cases, not sufficient to elicit tumor growth. Furthermore, disrupting tissue integrity has been shown to enable tumor growth in cases where no growth is observed within a formed tissue. Thus, an important question is: what kinds of structural perturbations are involved the breakdown of tissue homeostasis? In this work, I begin an investigation of the effects of tissue structure on mechanical integration on the tissue integrity. To do this, I fashion substrates from polydimethylsiloxane (PDMS), a biocompatible silicone rubber, by casting them on photochemically patterned curved ridges. The resulting channels and intervening flat substrate portions range between 25 and 500 μm in width and have a depth of 50 μm . These PDMS substrates can be made amenable to cell culture by plasma treating them and treating with collagen I. When seeded at high concentration with Madin Darby canine kidney (MDCK) cells, a model epithelial cell line, confluence is achieved within 1 d for all geometrical configurations. Interestingly, over the course of 5 days, cells were observed to clear out of the channels or form bridges over the channels. This effect is not observed when actomyosin activity is inhibited with either blebbistatin or ML-7, indicating that tissue contractility is necessary for the observed clearance to occur. Additionally, decreasing the area of non-channel flat portions of the substrate decreases the cell-clearing phenomenon. This may indicate that the observed clearance of cells from areas of negative curvature is the result of pulling by mechanically integrated cells on a continuous flat zone of the substrate. Although the system investigated is artificial in nature, it may have strong implications for the maintenance of homeostasis in healthy tissues and the steps necessary or sufficient to disrupt it. In addition to tumorigenesis, diseases of epithelial tissues in general often have characteristic structural components such as blistering or flattening of structures. Understanding what degrees of structural perturbation are sufficient to disrupt mechanical integration or initiate tissue reorganization may be important to understanding and eventually treating these illnesses.

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Elucidating the mechanism of durotaxis using a novel cell-on-a-chip assay.

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Rigidity sensing is widely recognized as an important element of wound healing, tissue engineering, and cancer treatment. However, it has been challenging to study rigidity sensing experimentally due to difficulties in locating a sufficient number of isolated cells at a sharp rigidity border. We sought to address this problem by developing a highly efficient model experimental system that confines cells to a simulated rigidity border. To this end, we fabricated a micropatterned composite material of photoresist islands grafted on top of non-adhesive polyacrylamide hydrogels. The design consists of one large adhesive island, which provides the rigid domain, juxtaposed with two rows of small islands that provide the soft domain when grafted on soft gels, due to the minimal force required to deform this region. The objective of the present study was to analyze conditions that cause defects in rigidity sensing using this novel approach. As expected, the majority of NIH 3T3 fibroblasts were confined to the large island after culturing overnight on the composite substrate with a soft underlying hydrogel, while cells on composite substrates with a stiff underlying hydrogel were able to occupy most of the small islands. Interestingly, FAK knockout cells (FAK ^{-/-}) and cells treated with nocodazole, both shown to have elevated activities of the small GTPase Rho, were able to spread across most of the islands regardless of the stiffness of the underlying hydrogel. In addition, treatment of FAK ^{+/+} cells with a Rho activator was sufficient to cause these cells to spread across the rigidity border. Cells expressing F397-FAK, a mutation preventing autophosphorylation without increasing Rho activities, exhibited a normal response comparable to control cells. In summary, our novel model experimental system allowed highly efficient analyses of rigidity sensing for both motile and non-motile cells. Our results indicate that the regulation of Rho activity is critical for rigidity sensing, and we propose that the loss of Rho regulation is responsible for the rigidity sensing defect observed in FAK^{-/-} cells and in cells depleted of microtubules.

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Analysis of cellular responses of MC3T3-E1 mouse osteoblast-like cells to rough surfaced substrates.

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Precise understandings of cell-material interactions are essential for designing biomaterials for clinical applications. It has been recognized that the behavior of cell adhesion, proliferation and differentiation on materials mainly depends on their surface physico-chemical properties including hydrophilicity, roughness, texture, and morphology. In this study, in order to obtain better understandings of the regulatory mechanisms of osteoblast responses to the surface roughness, we focused on the cellular responses such as adhesion, morphology, and proliferation of mouse osteoblast-like MC3T3-E1 cells plated and cultured on the rough-surfaced substrates, and investigated how these early responses influence the subsequent cellular development of osteoblastic differentiation and mineralization. The cellular responses of MC3T3-E1 cells to the surface roughness were examined in the sequential events of cell adhesion, proliferation, differentiation, and mineralization. The MC3T3-E1 cells were plated and cultured on sandblasted borosilicate glass slideslips with different surface roughness. DNA synthesis at day 1 after plating and the cell number at day 5 significantly decreased as the surface roughness increased. The suppressed cell proliferation on the rough-surfaced substrates, closely related to the round cell morphology, caused underdeveloped cell-cell contacts via the gap junction due to the low population of neighboring cells. Expressions of the

representative osteoblastic genes at day 14, alkaline phosphatase activity at day 21, and mineralization at day 28 were markedly reduced on the rough-surfaced substrates. These results clearly indicated that the reduced cell differentiation and mineralization resulted from the early cellular responses of the suppressed cell proliferation depending on the surface roughness and the consequent poor intercellular communication. Furthermore, specific early changes in the gene expression profiles dependent on the surface roughness were analyzed using a mouse DNA chip in order to obtain a better understanding of the regulatory mechanism of the early cellular responses stimulated by the surface roughness. The specific changes in the early gene expression profiles at day 1, depending on the surface roughness, were examined by a large-scale analysis of the gene expression using a mouse DNA chip. As the results, the ribosomal protein S6 kinase polypeptide 1 gene, known to be a cell growth-related gene involved in the PI3-kinase/Akt pathway, was found to be the most down-regulated among the 4277 screened genes by the DNA chip analysis.

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Nanometer Scale Surface Protein Patterns for Spatially Controlled Cell Adhesion.

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Cell adhesion is an important process in both health and disease states such as wound healing and cancer as well as tissue engineering and biosensor fabrication. Electron beam lithography (EBL) is widely used in applied physics to fabricate integrated circuits, waveguides and photomasks; due to its nanometer scale resolution, ability to pattern large areas and design flexibility. We have developed an EBL-based approach to fabricate surface patterns of proteins at the nanometer scale in order to study cell adhesion. We used atomic force, scanning electron and immunofluorescence microscopies to characterize the EBL generated protein patterns and cell adhesion on them. We showed that proteins respond to a focused electron beam and can directly be used as EBL resists without the need for conventional polymers. When we exposed a silicon surface with immobilized protein of interest to a highly focused electron beam with doses ranging from 0.01 to 100 pC, the exposed areas were 'activated' and a second protein of interest selectively bound to these areas in a dose dependent manner as detected by antibody staining. Accelerating voltage dependence indicated that nanometer scale protein patterns are formed by both back and forward scattered electrons, and two dose thresholds define the formation and shape of the patterns (dot or ring). We were able to pattern (i) cell adhesion promoting proteins such as fibronectin or laminin on an anti-cell adhesion background of BSA or K-casein, (ii) hybrid surfaces with laminin nanopatterns on a fibronectin background, with smallest feature size of 100 nm. Furthermore, we demonstrated biofunctionality at the cellular level where fibroblasts, endothelial and epithelial cells formed pattern specific focal adhesions and organized their immediate cytoskeleton accordingly. Our results suggested that a minimum of ≈ 40 fibronectin molecules is required for a cell to form a focal adhesion. Finally, we successfully adopted our EBL based approach for patterning proteins on indium tin oxide (ITO) coated glass surfaces, which, unlike silicon, are transparent and thus make various downstream light microscopy applications possible.

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Nanostructured conducting polymer devices for tissue engineering.

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The ability to control the microenvironment around a cell offers tremendous value in understanding life processes. Cell–cell and cell-microenvironment interactions regulate cell behavior, however, the underlying biological mechanisms remain poorly understood. Cells cooperate with their environment by interacting with neighbouring cells, with their surrounding extracellular matrix and by communicating through secreted soluble factors. Typically, these interactions are well-coordinated, but perturbation of the underlying cellular and molecular interplay may affect intracellular signalling pathways, immunological reactions and tissue homeostasis.

The triggering of the eventual fate of the cell is mediated through chemical cues from the adsorbed proteins and physical cues such as surface energy, stiffness and topography. For example, it has recently been shown that a gradient of surface energy, obtained by applying a bias to conducting polymer, can lead to a gradient in cell density [1]. It is also known that nano-topography [2] and stiffness variations [3] affect cell proliferation, death, differentiation or migration. Interestingly, stiffness modifications can be induced by nano-topography, the ability of nano-pillars to bend defining an effective stiffness [4]. We have achieved the fabrication of such nanotopography by fabricating pores or pillars in conducting polymers over large areas, using porous aluminum oxide membranes as a template. This enables the development of electrically active devices combining topography, stiffness and surface energy control to investigate cell-substrate interactions and cell migration. We report on the fabrication of such devices and show the results of their interactions with cells.

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Nanophysical properties of scaffolds induce cerebral cortical astrocyte response.

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Astrocytes cultured on electrospun polyamide nanofibers whose nanophysical properties may reproduce key aspects of native extracellular matrices have demonstrated promising results in both in-vitro and in-vivo situations. In vitro, astrocytes cultured on nanofibrillar scaffolds assumed morphologies that appeared to recapitulate those observed in native tissues and also demonstrated increased neurite outgrowth by co-cultured neurons. In vivo, the same scaffolds introduced into spinal cord wound sites promoted accelerated hindlimb recovery measured by standardized observational scoring with aligned and fasciculated axon development and revascularization throughout wound sites. Furthermore, low levels of astrocytic scarring were observed in comparison to injury-only controls. The in-vitro and in-vivo results suggest that

nanofibrillar scaffolds could induce preferential astrocyte differentiation leading to minimized glial scar formation, which has positive implications for improved treatment options for central nervous system injury repairs.

The present investigation examined the hypothesis that external physical cues of the nanofibrillar scaffolds can trigger specific signaling cascades with morphological consequences. The nanophysical cues of surface polarity, surface charge and surface roughness were investigated in this study. The morphological response of cerebral cortical astrocytes to nanophysical properties of the nanofibrillar scaffolds was investigated at high-resolution using AFM. The three-dimensional capability of AFM was also used to characterize cell spreading. An initial study of the corresponding activation of GTPase upstream regulators was performed using immunocytochemistry, focusing on investigation of the main GTPase regulators for the observed morphological responses: filopodia, lamellipodia, stress fiber formation, and stellation. The results support the hypothesis that the nanophysical extracellular environment can trigger preferential activation of members of the Rho GTPase family with demonstrable morphological consequences for cerebral cortical astrocytes.

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Microenvironmental Stiffness and Cell Shape Co-Regulate Work Output of Cardiac Myocytes.

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Disease progression in the heart is associated with marked extracellular and intracellular structural remodeling, which compromises the functional output of the heart. For example, many cardiomyopathies are characterized by increased tissue stiffness due to extracellular matrix deposition and fibrosis. On the cellular level, changes in myocyte aspect ratio commonly result from different pathological states. For example, concentric hypertrophy, which is caused by increased ventricular afterload, decreases myocyte aspect ratio from approximately 7:1 to 5:1. We hypothesized that remodeling of cell shape during concentric hypertrophy optimizes force generation in stiff pathological microenvironments. To test this, we performed traction force microscopy on isolated neonatal rat ventricular myocytes cultured on polyacrylamide gels with physiological (13 kPa) and pathological (90 kPa) stiffness. Gels were micropatterned with fibronectin rectangles ranging in aspect ratio from 2:1 to 12:1 to mimic myocyte shapes characteristic of health and disease. Myocytes on pathological substrates generated more force, but not more work, than myocytes on physiological substrates. On physiological substrates, peak systolic work generation was optimized at aspect ratio 8:1. Conversely, on pathological substrates, peak systolic work was optimized at aspect ratio 2:1. Time to peak systole was similar on both substrates, but relaxation time was prolonged on pathological substrates, suggesting that increased microenvironmental stiffness could be an important contributing factor to diastolic dysfunction. Actin alignment increased with aspect ratio, but was not a function of substrate stiffness. These results suggest that cardiac myocytes remodel their shape to optimize systolic work output in response to changes in the compliance of the microenvironment and reveal potentially important relationships between extrinsic mechanical factors and intrinsic structure-function relationships.

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Substratum topography alters YAP-mediated mechanotransduction response in corneal epithelial cells.

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Corneal wound healing is influenced by the extracellular environment both through soluble signals and from biophysical cues such as topography and matrix stiffness. These cues profoundly influence phenotypic characteristics of cells including adhesion, migration, shape, size and proliferation. These changes are mediated by activation/inhibition of specific signaling pathways. Human corneal epithelial cells have been demonstrated to remodel their cytoskeleton and orient differentially on anisotropically ordered topographically patterned substrates. However, there still remains a considerable knowledge gap in understanding the role of cell signaling pathways in response to biophysical cueing and mechanotransduction. YAP (Yes-associated protein) and TAZ (WWTR1; transcriptional coactivator with PDZ-binding motif), two important signaling molecules of the Hippo pathway, have recently been implicated as nuclear relays to mechanical cues sensed by cells. We investigated the effects of nano through micron scale topographic features in altering the YAP/TAZ pathway in human corneal epithelial cells. Primary and immortalized (hTERT) corneal epithelial cells (hTCEpi) were cultured on planar or topographically patterned substrates with biologically relevant dimensions (pitch sizes of 400, 1400 and 4000 nm; pitch = ridge width + groove width) for 12 hr. The role of substratum topography in modulating gene expression following (a) transcriptional activation of YAP via inhibition of HSP90, or (b) knockdown of YAP/TAZ in hTCEpi cells was investigated. RNA was isolated and the expression of YAP, TAZ, 14-3-3 σ and Transforming Growth Factor beta-2 (TGF β 2) was determined by qPCR. Alignment of cells to topography was determined after knockdown.

A differential response to topographic cues was observed. Corneal epithelial cells were most responsive to (biomimetic) 400 nm pitch topography with up-regulation in YAP, 14-3-3 σ , and TGF β 2. Inhibition of HSP90 induced translocation of YAP to the nucleus implying transcriptional activation. Translocation of YAP to the nucleus was associated with increases in YAP, CTGF, and TGF β 2; this expression was independent of topographic cues. In contrast, knockdown of YAP resulted in significantly lower expression of CTGF but not TGF β 2. Interestingly, YAP, CTGF and TGF β 2 were significantly upregulated with increasing pitch after knockdown of TAZ. Also, TAZ knockdown led to a significantly greater number of cells aligning parallel with the long axis of underlying ridges and grooves on pitch sizes greater than 1600 nm compared with control or YAP knockdown cells.

The biophysical attributes of the microenvironment of corneal cells cause changes in cell morphology and are capable of altering cell signaling. Our results strongly suggest that YAP, TAZ, 14-3-3 σ and TGF β 2 play fundamental roles in regulating how corneal cells respond to their biophysical microenvironment.

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Substratum Compliance and Latrunculin B Regulate the Gene Expression of YAP/TAZ in Human Trabecular Meshwork Cells.

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Objective: The compliance of the human trabecular meshwork (HTM) has been shown to dramatically stiffen in glaucomatous patients. Latrunculin-B (Lat-B) is an actin cytoskeleton disruptor that directly targets HTM cells and is currently in clinical trials as a treatment for glaucoma. The purpose of this study was to determine the impact of substratum compliance and Lat-B, on the expression of the mechanotransducers, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding domain (TAZ), in HTM cells. In addition, the effects of substratum compliance and Lat-B on the expression of 14-3-3 σ (a protein important in YAP/TAZ degradation), and connective tissue growth factor (CTGF), a gene that is highly regulated by YAP, were also examined.

Methods: Primary HTM cells were cultured on hydrogels possessing compliance values mimicking those found in normal (5 kPa) and glaucomatous (75 kPa) meshworks, or on tissue culture polystyrene (TCP >1 GPa) as a control substrate. Cells were treated with 2.0 μ M Lat-B in DMSO or DMSO alone. RT-PCR was used to determine the impact of substratum stiffness and/or Lat-B treatment on the expression of YAP, TAZ, 14-3-3 σ and CTGF.

Results: Substratum compliance modulated YAP and TAZ mRNA expression such that both genes were upregulated on the 75 kPa hydrogels in comparison to the 5 kPa hydrogels and TCP. Treatment with Lat-B resulted in a marked decrease of YAP and TAZ mRNA on the 75 kPa hydrogels. Lat-B treatment of HTM cells on the 75 kPa hydrogels also increased 14-3-3 σ mRNA. In addition, Lat-B treatment decreased CTGF mRNA on the 75 kPa hydrogels.

Conclusions: Substratum stiffness modulates YAP/TAZ activity which then may alter the expression of ECM proteins important in glaucoma. In addition to disrupting the actin cytoskeleton, Lat-B may decrease intraocular pressure by modulating 14-3-3 σ . Therefore, YAP and TAZ may represent new therapeutic targets in the treatment of glaucoma.

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Mechanotransduction in Trabecular Meshwork Cells and the Progression of Glaucoma.

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Background and Aims: Two Yorkie homologues, YAP1 and TAZ, have been identified as important mechanotransducers of extracellular cues to the cell nucleus. These proteins relay changes from the extracellular matrix to induce changes in gene and protein expression. Both proteins are co-activator of transcription factors. The trabecular meshwork in the outflow pathway of aqueous humor is thought to be the site of regulation of intraocular pressure. YAP has been shown to increase the levels of TGF- β , CTGF, transglutaminase 2, serpine 1 and thrombospondin, proteins shown to increase in glaucoma or when human trabecular meshwork (HTM) cells are treated with dexamethasone (DEX). Since the glaucomatous meshwork is stiffer than the normal meshwork, we wished to determine how changes in matrix stiffness would influence YAP and TAZ and the expression of genes in HTM cells. Both YAP and TAZ can be phosphorylated in the cytoplasm. After phosphorylation at specific sites, the protein 14-3-3 σ can bind to them and mark them for degradation. We also wished to determine how substrate stiffness would influence 14-3-3 σ .

Methods: Immunohistochemical staining for YAP and TAZ was done on donor HTM. HTM cells from normal donors were cultured on hydrogels with stiffnesses of 5 kPa (mimicking normal

HTM) and 75 kPa (mimicking glaucomatous HTM). The changes in expression of YAP and TAZ on the different substrates were determined. Levels of mRNA for CTGF and 14-3-3 σ were measured.

Results: YAP and TAZ were present in the HTM cells in sections from donor corneoscleral rims. Both YAP and TAZ were present in the cytoplasm as well as the nucleus of HTM cells. TAZ mRNA expression was upregulated on stiffer hydrogels; however, YAP was down regulated. The amount of 14-3-3 σ was lower on the stiffer hydrogels. The level of CTGF mRNA was higher on both hydrogels after DEX treatment.

Conclusions: The mechanotransducers YAP and TAZ may play key roles in HTM cells particularly when the extracellular matrix becomes stiffer as it does in glaucoma. These proteins have the ability to cause upregulation of several of the proteins found to be increased in glaucoma and may influence the progression of disease.

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A plate-and-cone bioreactor method to measure cell attachment on artificial graft material.

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Artificial vascular implants with diameters smaller than 6 mm have a high risk of occlusion, caused by early thrombosis or long-term intimal hyperplasia. As the availability of autologous grafts is often limited, there is a high demand for improving artificial prostheses. As a solution, a confluent inner lining of endothelial cells could (i) avoid the blood contact of the material, (ii) prevent hyperplasia and therefore offers a solution for improving vessel grafts. Currently, there is no biomaterial in clinical use that is designed for endothelial cell attachment [Ravi et al., 2010]. Electrospun polymer fiber mats offer a promise to fulfill both the mechanical properties for a surgically usable implant and the topographic surface structure for optimal cell attachment. To test different surface topologies and material compositions for the attachment of endothelial cells, we applied the principle of a plate-and-cone rheometer as a bioreactor. Here, a uniform wall shear stress up to 10 dyn/cm² can be applied on a defined, ring-shaped region of a culture dish bottom that has been coated by the test materials and cells seeded. To obtain statistical relevant data, six cone-and-plate systems can be used in parallel, either inducing the same or varying shear rates, adjusted by the rotational speed of the cones. The effects on cell proliferation, migration and cell morphology are monitored during stimulation using a laser scanning microscope. Flow conditions of the reactor have been shown to be laminar with uniform wall shear stress on a defined area.

In our experiments, endothelial cells (GFP-labeled HPMECs) were seeded on protein-coated PCL/PLGA (polycaprolactone/poly(lactic-co-glycolic acid) fiber mats with 1.4 to 1.7 μ m mean fiber diameter (95% CI) resulting in a porosity of 80% \pm 2%. As coating proteins, collagen, fibronectin or gelatine were used. Cell proliferation could be quantified and migration could be observed using live cell imaging. Collagen-coated fiber mats showed best results for cellular attachment under shear stresses of 10 dyn/cm². Modifications of three-dimensional fiber structure and surface topology are ongoing to achieve a confluent lining of endothelial cells.

Focal Adhesions and Invadosomes

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Specific protein-interactions regulate the three-dimensional nanoscale organization of vinculin within focal adhesions.

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Vinculin is a ubiquitously expressed protein that localizes to focal adhesions (FAs) in a myosin II-dependent manner, and is important for regulating FA functions including adhesion strength, actin dynamics, and signaling. Vinculin has at least 10 putative binding partners at FAs including actin, talin, Arp2/3, and paxillin. Thus, vinculin may regulate specific FA functions via interaction with particular binding partners. Interferometric photoactivated localization microscopy ("iPALM") is a superresolution light microscopy technique that allows for 20nm lateral resolution and 10nm axial resolution. We previously used iPALM to show that FAs have a conserved stratified structure. We are currently using iPALM to test the hypothesis that the vertical position of vinculin within the FA is dictated by specific protein-protein interactions that modulate its function in reinforcing FA strength, signaling, and regulating actin dynamics. We have focused first on vinculin's interactions with paxillin and actin. The vinculin-paxillin interaction requires FAK-mediated phosphorylation of paxillin, and the vinculin-actin interaction requires vinculin activation. We showed previously that paxillin is located ~5nm axially from the plasma membrane within FAs, while actin is located 30-40nm higher, suggesting that two spatially and functionally distinct populations of vinculin may exist in FAs. Here we found that phosphomimic mutations in paxillin promote vinculin accumulation near the plasma membrane, while FAK inhibition or non-phosphorylatable paxillin mutants shift vinculin localization 30-40nm higher within FAs. We conclude that vinculin interacts with phospho-paxillin near the plasma membrane within FAs. Furthermore, we show that iPALM allows high-resolution measurements of changes in vinculin's 3D localization within the FA structure, suggesting that iPALM is a valid technique to understand the contribution of vinculin binding interactions to vinculin's nanoscale organization and function within FAs. We are currently mapping the 3D nanoscale organization of various vinculin mutants and correlating this nanoscale organization with vinculin's FA function.

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Vinculin interaction with activated α -actinin.

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Vinculin and α -actinin are two major focal adhesion players, whose interaction entails several important aspects in cytoskeletal reorganization at the adhesion sites. Vinculin is one the most crucial proteins functioning in both formation and maturation of focal adhesions. It binds actin in its activated conformation. The structure is composed of a head (Vh) and a tail domain (Vt) that are connected by a neck region. The head and tail domains are associated in the inhibited conformation covering the actin binding domains. It has been postulated that interaction with some binding partners such as talin and α -actinin induces critical conformational changes in the vinculin head domain that leads to activating the molecule for actin binding. α -Actinin acts as a cross-linker for both parallel and anti-parallel F-actin. This rod-shaped molecule is an anti-parallel homodimer. Each monomer has an actin binding domain at the N-terminal that is followed by four spectrin repeats (R1-R4) and a CaM domain at the C-terminal. Vinculin binding site (α VBS) is placed in R4 and is shown to be inhibited in the full-length structure of the

molecule available in the Protein Data bank. The hydrophobic interactions stabilize α VBS in the rod domain in that structure.

In a previous study, we have successfully achieved the activated structure of α -actinin for vinculin binding that was used in the present study. The full length vinculin molecule was prepared using homology modeling. The head domain was placed in the vicinity of α VBS in such a way that the closest atoms are farther than 10 Angstroms. We used all-atom molecular dynamics methods to model the interaction between α -actinin and vinculin. Explicit water model was used to enhance the accuracy. Our results showed that α -actinin has a high affinity for vinculin binding. The helices in the vinculin head domain rearranged their conformation to allow for α VBS insertion. The energy and root mean square deviation (RMSD) plots illustrated a stable interaction. Our MD simulations were used to explore the effect of this binding on vinculin activation. The vinculin head domain was monitored and several conformational changes were detected during the trajectory. Specifically, the interface between head and tail domains weakened but not completely dissociated throughout the equilibration simulation. Finally, the key interactions in stabilizing the inhibited conformation of vinculin were mapped to later be used for therapeutic applications.

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Vinculin-actin interaction mediates engagement of actin retrograde flow to focal adhesions, but is dispensable for actin-dependent focal adhesion maturation.

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Migrating cells form integrin-based focal adhesions (FA) near their leading edges in association with rapid filamentous actin (F-actin) assembly and retrograde flow. How F-actin flow is coupled to FA is unclear, and thus it is not known how F-actin engagement to FA regulates actin cytoskeleton and FA dynamics. We analyzed the role of vinculin in integrating FA and actin dynamics by vinculin gene disruption in primary fibroblasts. Vinculin promotes nascent FA formation and turnover in lamellipodia, establishes a lamellipodium-lamellum border and engages F-actin flow in maturing FA to generate high ECM traction forces. Characterization of a vinculin point mutation that specifically disrupts F-actin binding shows that vinculin F-actin interaction is critical for these cellular functions. However, FA maturation rate correlates with F-actin flow rate independently of vinculin. Thus, vinculin functions as molecular clutch organizing leading edge F-actin, generating ECM traction and promoting FA formation and turnover, while FA maturation is regulated by F-actin flow independently of vinculin.

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CAS interacts with vinculin to control mechanosensing and focal adhesion dynamics in cells.

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Focal adhesions (FAs) are cellular structures through which both mechanical forces and regulatory signals are transmitted. FA-associated proteins, CAS and vinculin were both independently shown to be crucial for cells to transmit mechanical forces and generate cytoskeletal tension. We identified a novel direct interaction between CAS and vinculin, independent of focal adhesion kinase (FAK). This interaction is mediated by the CAS SH3 domain and the proline-rich sequence in the hinge region of vinculin. We found that CAS localization in FAs is partially dependent on vinculin and that CAS-vinculin association is required for stretch-induced phosphorylation of the CAS substrate Y410 domain and ERK1/2 phosphorylation. Moreover, CAS-vinculin interaction significantly affects the dynamics of CAS and vinculin within FAs as well as the size of adhesion structures, but not the cellular adhesion strength. Finally, disruption of CAS binding to vinculin negatively impacts cell stiffness and traction force generation, but increases cell migration and cell fluidity. Taken together, these findings strongly implicate a crucial role of the CAS-vinculin interaction in mechanosensing, force transmission, and focal adhesion dynamics.

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Focal adhesion kinase, isometric tension generation, and extracellular matrix organization.

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Focal adhesions (FA) are specialized cell contact sites of distinct molecular composition and structure that provide for efficient bidirectional transmission of biochemical and mechanical signals between the intra- and extracellular compartments. Focal adhesion kinase (FAK) is a soluble cytoplasmic protein kinase that is essential for the organization, assembly, stabilization, and basal physiological activity of FA. FAK has been proposed to be a key component of FA downstream of integrins that regulate the organization of actin for transduction of cellular forces from inside to outside of the cell. From isometric tension experiments using FAK knockout (KO) mouse embryonic fibroblasts (MEFs) embedded in three-dimensional (3-D) collagen constructs, we found that loss of FAK decreased basal isometric tension and virtually abolished agonist induced force development compared to wild type (WT) MEFs. Gel-compression assays revealed FAK KO MEFs did not compress the 3-D collagen matrix to the same extent as WT controls. Loss of FAK had no effect on basal myosin II regulatory light chain phosphorylation. This data suggested the loss of tension development might be due to the inability of KO cells to interact/organize the extracellular matrix. WT and FAK KO MEFs exhibited distinct morphology in 3-D collagen constructs. FAK KO MEFs are round and over time formed large multicellular spheres while WT MEFs spread forming an interconnected sheet of cells. Second harmonic generation and SEM studies showed WT MEFs generate a highly organized, uniform, dense

fibril collagen network whereas FAK KO MEFs form a loose matrix lacking a distinct organizational pattern. Expression of either WT-FAK or constitutively active FAK in FAK KO MEFs rescued force development, cell spreading, and matrix organization in 3-D collagen constructs. Inhibition of FAK kinase activity with the small molecule inhibitor PF-573,288 or expression of a FAK kinase dead mutant in FAK KO cells had little effect on basal or agonist induced force generation suggesting FAK kinase activity may not be necessary for tension development. Taken together, our findings indicate FAK is an important molecular player in FA facilitating transduction of cellular forces from inside to outside of the cell.

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Actin dynamics and organization drive focal adhesion maturation at vanishing tension

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Cell adhesion to the extracellular matrix (ECM) must be dynamically regulated during directed cell migration, tissue morphogenesis and wound healing. Focal adhesions mediate the attachment between the cell and ECM and play an important role in regulating cell morphology, cell-ECM force transmission and ECM-mediated signaling. Adhesions assemble in an actin polymerization-dependent manner within the lamellipodium and stabilize to the ECM near the lamellipodial base. Myosin II motors within the lamella drives changes in focal adhesion morphology and composition in a “maturation process” that is crucial for regulating focal adhesion dynamics. Understanding the mechanisms regulating focal adhesion maturation has been complicated by the intermingled effects of myosin II on lamellar actin architecture, dynamics and force transmission during focal adhesion maturation. Stresses generated by myosin II drive retrograde flow of lamellar actin networks and bundles as well apply tension to focal adhesions. Myosin-generated stress and cross-linking also facilitate the reorganization of the lamellar actin network into a dense radial stress fiber at the adhesion plaque. It is widely presumed that focal adhesion maturation is a tension-dependent process, although very few studies have been done to decipher the distinct roles of myosin II activity in focal adhesion maturation. However, the extent to which tension is a direct regulator of focal adhesion elongation and stabilization is not well understood. Here, we show that focal adhesion elongation rate stays constant even when the tension build up is reduced by 75%. Instead, focal adhesion elongation rate is correlated with actin retrograde flow, and focal adhesion elongation only halts at stress sufficiently low to impair retrograde flow. The stability of elongated focal adhesions is impaired at low tension, and a critical tension of ~100 Pa is necessary for adhesion stability. However, this force requirement can be bypassed by overexpression of α -actinin 1 or constitutively active Dia1. Our work identifies a minimal threshold of myosin activity required to drive actin retrograde flow that is sufficient to permit focal adhesion elongation. This work further identifies the stresses associated with both focal adhesion elongation and stabilization. However, rather than acting directly, we believe these stresses are those required to drive retrograde flow dynamics and actin stress fiber assembly demonstrating the important role of actin dynamics and organization in controlling focal adhesion morphology.

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Cells exert rotational moments about focal adhesions

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Anchorage-dependent cells maneuver the extracellular matrix (ECM) by exerting traction stresses, which are generated by myosin II contracting on actin filaments and transmitting tension to integrin-mediated adhesions. Forces at adhesions regulate key molecular processes, including cytoskeletal reorganization and adhesion-mediated signaling, and fundamental cellular processes, such as migration, proliferation, and differentiation. Traditional traction measurement methods have assumed that cells apply only shear forces, and only recently, studies have reported significant vertical deformations on the ECM. However, the dynamics and the subcellular locations of multi-dimensional tractions remain unclear. In this study, we developed a multi-dimensional traction force microscopy (2.5D TFM) with high spatio-temporal resolutions to measure the full nature of traction stresses and investigate their relationship to focal adhesions and the actin cytoskeleton. Mouse embryonic fibroblasts (MEFs) expressing GFP-tagged focal adhesion protein (paxillin), actin, or a membrane-marker (farnesyl) were plated on RGD-conjugated synthetic hydrogels. Sub-resolution fluorescent beads embedded in the hydrogels were tracked over time to obtain the 3D deformation field within the substrate. Finite element method was used to generate discretized Green's function to compute cellular traction stresses from the substrate displacements. The dynamics of shear and normal traction stresses relative to the cell body, actin stress fibers, and focal adhesions were determined by co-analyzing volumetric cell images. Cells on planar ECM substrates exert maximum shear tractions directly along focal adhesions, whereas maximum upward and downward normal tractions are generated around focal adhesions and the termini of actin stress fibers. The net effect of these forces produces rotational moments around focal adhesions with a characteristic radius of 10 μm . These moments form only in cell peripheries, where focal adhesions elongate. The moments are also highly dynamic, as they propagate outward with protruding leading edges in migrating and spreading cells over time. Finite element modeling incorporating empirical data and key cytoskeletal components shows that actomyosin contraction on ~ 150 nm tall focal adhesions induces 1 μm lateral displacements and 150-300 nm vertical displacements on the pliable ECM substrate. These findings reveal that cellular interactions with the ECM involve more complex traction stresses, consisting of shearing and compressive forces at focal adhesions.

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Do cancer cells have distinct adhesions in 3D collagen matrices and *in vivo*?

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During metastasis, cancer cells breach the basement membrane and migrate through the stroma mostly composed of a network of collagen I fibers. Cell migration on 2D is initiated by the formation of protrusions of the cell membrane followed by adhesions at the cell front that link the actin cytoskeleton to the underlying extracellular matrix (ECM). Cells then move forward by exerting traction forces on the adhesions at its front and by disassembling adhesions at the rear. In 2D, only the ventral surface of a migrating cell is in contact with the ECM, where cell-matrix adhesions are assembled. In 3D matrices, even though the whole surface of a migrating cell is interacting with the ECM, it is unclear whether discrete adhesion structures actually exist. Using high-resolution confocal microscopy, we imaged the endogenous adhesome proteins in three different cancer cell types embedded in non-pepsinized collagen type I, polymerized at a slow

rate to allow formation of a network resembling the organization of ECM observed *in vivo*. In MDA-MB-231 cells, vinculin, zyxin and paxillin aggregates were detected in the cellular protrusions, frequently colocalizing with collagen fibers, implying they correspond to adhesion structures in 3D. In colon cancer cells HCT116 and CT26, as the distance from the substrate bottom increases, adhesion aggregates become smaller and almost undetectable. This suggests that the distance from hard substrates, and thus the elasticity of the ECM, is a key factor on the formation of cell-matrix adhesions. Using intravital imaging we observed adhesion protein aggregates *in vivo*. These aggregates share similarities with the ones found in 3D collagen matrices. It still remains to be determined if adhesions assembled in 3D and *in vivo* share functional similarities to the well-described adhesions in 2D. This would provide a major step forward in understanding cell migration in more physiological environments.

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Physiological type I collagen organization induces the formation of a novel class of linear invadosomes.

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Invadosomes are F-actin structures capable of degrading the extracellular matrix through the activation of matrix metalloproteases. Invadosomes is a global term including podosomes found in normal cells and invadopodia observed in cancer cells. As fibrillar type I collagen promotes pro-matrix metalloproteinase 2 activation by membrane type 1 matrix metalloproteinase, we aimed at investigating the functional relationships between collagen I organization and invadosome induction. We found that fibrillar collagen I induced linear F-actin structures, distributed along the fibrils, on endothelial cells, macrophages, fibroblasts, and tumor cells. These structures share features with conventional invadosomes, as they express cortactin and N-WASP and accumulate the scaffold protein Tks5, which proved essential for their formation. On the basis of their ability to degrade extracellular matrix elements and their original architecture, we named these structures "linear invadosomes." Interestingly, podosomes or invadopodia were replaced by linear invadosomes upon contact of the cells with fibrillar collagen I. However, linear invadosomes clearly differ from classical invadosomes, as they do not contain paxillin, vinculin, and $\beta 1/\beta 3$ integrins. Using knockout mouse embryonic fibroblasts, blocking antibody and RGD peptide, we demonstrate that linear invadosome formation and activity are independent of $\beta 1$ and $\beta 3$ integrins. This study demonstrates the existence a new type of invadosomes specifically induced by physiological type I collagen organization.

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Profilin1 regulates invadopodium maturation in human breast cancer cells.

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Metastasis is the dissemination of tumor cells from the primary tumor to distant organs. Invadopodia are actin-based membrane protrusions with proteolytic activity. The formation of invadopodia is a multistep process. First, invadopodium precursors are assembled and actin

polymerization induces the formation of mature invadopodia which are capable of matrix degradation upon delivery of MMPs. Profilin1 (Pfn1) regulates actin polymerization in various ways: it can sequester G-actin and inhibit actin polymerization, or it can promote actin polymerization by supplying actin monomers to the barbed end of the actin filaments. Pfn1 can also bind to a number of proteins containing proline-rich sequences that are important for cell migration as well as several phosphoinositides (PI(4,5)P₂, PI(3,4)P₂, PI(3,4,5)P₃), at least, *in vitro*. Seemingly contrary to the essential role of Pfn1 in cell migration in the physiological contexts, Pfn1 is downregulated in several adenocarcinomas and furthermore, loss of Pfn1 expression increases invasiveness of breast cancer cells raising the possibility that its downregulation enhances invadopodium formation. To investigate the role of Pfn1 in the invadopodium formation, we studied the effect of Pfn1 depletion on invadopodium in MDA-MB-231, a metastatic human breast cancer cell line. Depletion of Pfn1 leads to an increase in the number of mature invadopodia and matrix degradation. Consistent with a previous report of Pfn1's negative regulation of membrane PI(3,4)P₂ at the leading edge, we found that loss of Pfn1 expression enhances the level of PI(3,4)P₂ and as a result, the recruitment of PI(3,4)P₂ interacting adaptor protein Tks5 (an invadopodial protein) to the invadopodium. Thus, our results suggest a role for Pfn1 in invadopodia by controlling the levels of PI(3,4)P₂ and accessibility of Tks5 to promote invadopodium formation.

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Intracellular Trafficking of Src and Initiation of Invadopodium Formation.

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During invasion of an extracellular matrix (ECM), tumour cells can form small, finger-like membrane protrusions called invadopodia. Invadopodia extend into the ECM, and contain on their surface both integrins and matrix metalloproteinases (MMP), suggesting these structures participate in the degradation of the ECM while still allowing cells to adhere to it. The formation of invadopodia involves cytoskeletal rearrangement, attachment to the ECM, and secretion of MMP to degrade the ECM facilitating further protrusion of the cell membrane. Intracellular trafficking of invadopodium-associated proteins is hypothesized to contribute invadopodium formation and to support cell invasion of ECM. SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) play a major role in intracellular vesicular traffic, wherein they function to localize vesicles to target membranes. We have begun to characterize the function of SNAREs during the formation of invadopodia in the tumour cell lines HT-1080 and MDA-MB-231. Using a gelatin-based *in vitro* invadopodium formation assay, we have found that inhibition of SNAREs impedes cell invasion and impairs the formation of invadopodia at an early stage, prior to the assembly of actin-based structures at the core of the invadopodia. Expression of a membrane targeted form of Src (Src-CAAX) rescued invadopodium formation, to the point that actin punctae could be detected, but did not restore cell invasion. In addition, SNARE function was required for the formation of a β 1 integrin-Src-containing complex that was observed early during cell invasion of ECM. Our observations suggest that SNARE-dependent trafficking of factors, including β 1 integrin and Src, contributes to both initiation of invadopodium formation and later events in cell invasion.

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Analysis of myosin 1e role in invadopodia dynamics.

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Cell migration and adhesion are essential for both physiological and pathological processes. Embryonic development, tissue repair and the inflammatory response rely on cell migration, while abnormal migration and invasion can cause bone defects, autoimmune disorders and tumor formation or metastasis in cancer. The actin cytoskeleton and its dynamics are crucial for proper cytoskeletal remodeling associated with cell migration and adhesion. Regulation of actin containing structures, such as lamellipodia and invadopodia, relies on coordination and turnover of a variety of proteins involved in cell adhesion. Unconventional myosins 1e (myo1e) and myosin 1f (myo1f) have been implicated in cell adhesion through proteomic studies that detected these proteins in the integrin-containing cell adhesion complexes.

While the functional role for myo1f in regulation of cell adhesion in neutrophils has been confirmed (Kim et al., Science, 2006), the role of myo1e in cell adhesion has not been analyzed. Since myo1e is expressed in a wide range of tissue and cell types, we hypothesized that it may play an important role in regulation of cell-substrate contacts in a variety of cells. We found that myo1e was enriched in invadopodia rather than in focal adhesions. Using v-Src transformed BHK cells as a model of invadopodia formation, we observed that myo1e localized to the actin-rich core of invadopodia. Enlargement of invadopodia rosettes in response to inhibition of tyrosine phosphatase activity was associated with an increase in the concentration of myo1e at the periphery of the expanding rosettes. Our observations suggest a role for myo1e in invadopodia dynamics and turnover. Thus, myo1e may serve as an important target in diseases where abnormal cell migration and invasion caused by defects in cell adhesion result in pathologies.

Structure and Function of the Extracellular Matrix

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Stem Cell-Derived Matrix is Necessary for Lineage Induction.

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While differentiation methods often rely exclusively on growth factors to direct mouse embryonic stem cell (mESC) fate, the ESC niche also contains fibrillar extracellular matrix (ECM) proteins, including fibronectin (FN), collagens, and laminins. Many soluble factors used in ESC differentiation are known to increase ECM expression, e.g. Activin A and FN, yet the ECM's ability to direct ESC fate alone is not well understood and likely occurs at points during development. We examined whether ECM proteins were necessary and/or sufficient to direct mESC differentiation. mESCs, grown as embryoid bodies in the absence of serum FN, maintained pluripotency marker expression unlike those in complete serum. Embryoid bodies also showed a spatiotemporal correlation between expression of FN and GATA4, an endodermal marker, and an inverse correlation between FN and Nanog, a pluripotency marker. Maintenance of mESC pluripotency via leukemia inhibitory factor (LIF) prevented fibrillar matrix production, but mESCs created a fibrillar ECM containing FN and laminin depending on the specific inductive conditions. This mESC-derived matrix was unlike conventional fibroblast-derived matrix, which almost exclusively contained fibronectin. Analysis of naïve mESCs plated

onto mESC- and fibroblast-derived matrix indicated lineage specific differences: cells were more receptive to neuro-ectoderm induction on laminin-containing matrix, but in the presence of endodermal growth factors, cells were more receptive to endoderm induction. Fibroblast-derived matrix with exogenously added laminin lacks any residual growth factors that mESC-derived matrix may contain, and appears to indicate a similar requirement. These data appear to indicate that some lineages require a specific combination of matrix and soluble cues, e.g. endoderm, whereas matrix is sufficient to begin lineage commitment for others, e.g. neuro-ectoderm. Regardless of lineage, these data imply that fibrillar FN is necessary for and can enhance and/or direct mESC differentiation.

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Cross-linked collagen nanofilms stiffen the nuclei of mesenchymal stem cells and promote osteogenesis.

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Matrix elasticity has been shown in culture to direct lineage specification of MSCs, with mechanisms based in part on forces applied to matrix by the cell's actin-myosin contractility system (Engler et al., 2006). MSCs were cultured on highly ordered collagen nanofilms, in which the mechanical properties had been tuned by enzymatic crosslinking with Transglutaminase. In the absence of cross-linking, cells pulling on the films visibly deformed the collagen fibrils. However, cells cultured on cross-linked films responded by altering their morphology, actomyosin organization, and nuclear shape. Mechanically anisotropic, pristine collagen films promoted strong polarization and orientation along the highly aligned fibrils, whereas cross-linked films caused cells to spread isotropically and stiffen their nuclei with increased lamin-A/C expression. Cell morphology on cross-linked films appeared osteoblastic and the early osteogenic transcription factor RUNX2 and the later marker osteonectin were indeed found to be upregulated relative to cells on pristine collagen. Lamin-A/C over-expression was found to enhance osteogenesis and we discovered a positive correlation between matrix and nuclear mechanics on cell differentiation potential. Conversely, low levels of lamin-A,C favor a soft tissue fate. We found that soft matrix, mimicked here with a soft gel (0.3kPa), increased adipogenesis compared to stiff gels (40kPa), while partial knockdown of LMNA more than doubled adipogenesis on soft matrix. Our results demonstrate the exquisite sensitivity of stem cells and their nuclei to the mechanical properties of their matrix and microenvironment.

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A Continuously Expanding Silicone Culture Surface Provides High Yields of Non-Fibrotic Primary Human Dermal Fibroblasts for iPSC Reprogramming.

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Background: The expansion of primary autologous dermal fibroblasts in culture is a pivotal step to obtain sufficiently high numbers for applications where large surface area tissue destructions exceed the body's repair capacity and demand grafting of regenerative cells. Stimulated by the stiff surface of conventional culture plastic, a substantial percentage of fibroblasts spontaneously differentiate into fibrotic myofibroblasts by producing excessive amounts of collagen, de novo expressing α -smooth muscle actin (α -SMA), and consequently developing high contractile force. Myofibroblasts cause pathological tissue contractures characteristic of hypertrophic scars and fibrosis. Hence, suppressing this phenotype during cell culture expansion is crucial for the success of subsequent grafting. We hypothesize that culture on highly elastic surfaces will deliver better quality fibroblasts for iPSC reprogramming by inhibiting

myofibroblast development. Method: To rapidly produce high numbers of fibroblasts for cell therapy we implement a novel method combining dynamic enlargement of the culture surface with fibroblast growth on a highly compliant, and extendable, silicone rubber (HESR). Results: 1) Attachment and proliferation of human dermal fibroblasts on functionalized HESR is similar to conventional tissue culture plastic. 2) Repeated passaging on plastic augments the percentage of fibrogenic myofibroblasts. 3) HESR fibroblast cultures show differential gene expression of putative markers for fibrosis. 4) The pro-fibrotic cytokine TGF- β 1 fails to activate myofibroblast differentiation on HESR. 5) HESR polymer rheology and not polymer chemistry is responsible for the mechanically-induced gene expression changes and 6) HESR dynamic expansion culture generates a multi-fold increase in daily cell yield while retaining the anti-fibrotic properties of static HESR. Conclusions: The low elastic modulus of the HESR membrane provides anti-fibrotic mechanical cues to primary dermal fibroblasts while dynamic expansion culture using the iris-like expansion device maintains a proliferative cell density by preventing contact inhibition of growth.

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Mechano-sensing of cells on viscoelastic biomembrane-mimicking substrates.

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Cells have been shown to sense the mechanical properties of their environment with substantial impact on cellular fate and function. To probe the mechano-sensitivity of cells, previous studies mainly focused on polymeric cell substrates of adjustable elasticity with immobilized cell-substrate linkers. Here, we report on an alternative way of cell substrate design based on a polymer-tethered multi-lipid bilayer system with distinct material properties. Complementary single molecule tracking and magnetic tweezer experiments show that polymer-tethered lipids in the fluid lipid bilayer matrix do not hinder the lateral mobility of individual cell linker molecules, nor obstruct or even prevent the free lateral diffusion of clusters of cell linkers (as determined from focal adhesions). As a consequence, the presented biomembrane-mimicking substrate exhibits a viscous response with respect to individual linkers and a rather elasto-plastic response in the presence of linker clusters. Importantly, the material properties of the multi-bilayer cell substrate can be tuned by altering the number of bilayers in the stack. Experiments using mouse embryonic fibroblasts confirm the mechano-sensitivity of plated cells in response to altered material properties in the multi-bilayer substrate. Mouse embryonic fibroblasts cultured on triple bilayer substrates show an about 50 % reduced spreading area compared to cells cultured on glass. Furthermore, the cell stiffness and tractions decrease by approximately 25 % and 80 %, respectively. Similarly, the focal adhesion size is reduced with increasing number of stacked bilayers. These results illustrate that mouse embryonic fibroblasts are susceptible to changes in substrate elasto-plasticity and linker mobility by changing their morphological and mechanical properties.

2140B927

Self-Assembling Peptide Gels Enable Comprehensive Phenotypic and Molecular Characterization of Cellular Responses to Extracellular Stiffness Cues in 3D.

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The stiffness of the extracellular matrix (ECM) stimulates mechanotransduction pathways that regulate tissue development and tumor progression. We previously showed that a stiff ECM potentiates cell growth and survival and enhances cell migration to drive tumor cell invasion (Paszek et al., *Cancer Cell* 2005). We also reported that the ECM progressively stiffens during malignant progression and that ECM tension drives malignant progression of the mammary gland in culture and in vivo (Levant et al. *Cell*. 2009; Lopez et al., *J Integ Biol* 2011). To identify molecular mechanisms whereby ECM stiffness could modulate the malignant behavior of cells, we have been using nonmalignant and oncogenically-modified human mammary epithelial cells (MECs) cultured in either collagen/reconstituted basement membrane gels in three-dimensions (3D) or on top of protein-laminated polyacrylamide gels. While sufficient in many experimental contexts, polyacrylamide gels fail to accurately recapitulate the 3D interstitial ECM, and collagen gels exhibit a limited dynamic range of stiffness and are non-uniform with respect to ligand availability, pore size, and topology, severely complicating data interpretation. To address this issue we established an alternative 3D culture system using self-assembling peptide (SAP) gels that significantly extends the experimental dynamic range of stiffness conditions while limiting topological changes in the ECM, and in which cells broadly maintain biological phenotypes observed in comparable collagen and matrigel systems (Miroshnikova et al. *Phys. Biol.* 2011). We used this technique to identify a critical ECM stiffness that promotes progressive changes in mammary acini growth, morphology and invasion. While mammary acini interacting with a highly compliant ECM (80 Pa) maintained acini integrity, mammary acini embedded within a highly stiff ECM (5,000 Pa) collapsed and invaded into the SAP gel. These changes in mammary tissue phenotype were accompanied by differences in levels of genes implicated in tumorigenesis, including significant induction of fibronectin. To identify additional force-regulated molecular pathways involved in tumor progression we used Affymetrix expression microarrays to characterize the gene expression profiles of mammary acini exposed to increasing ECM tension (80-5000 Pa). In parallel, we identified changes in protein expression using mass spectrometry-based quantitative proteomics. These studies highlight the significant advantage SAP gels offer over existing 3D systems and provide biological insight into the divergent cellular responses to distinct stiffness environments.

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Coordinated regulation of epithelial cell invasion by the ECM and actomyosin contractility.

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The extracellular matrix (ECM) provides the signals and scaffold for tissue development and is also hijacked in cancer. Abnormal changes in collagen type I, the most abundant ECM component in the stroma, are particularly correlated with poor prognosis in breast cancer. To determine the effect of fibrillar collagen on epithelial cell invasion and dissemination, we explanted epithelial fragments from tumor and normal murine mammary epithelium and cultured them in 3D gels of collagen I. We observed that tumor cells persistently invaded with protrusions

and disseminated into collagen I. Despite initiating with a similar response, myoepithelial cells in normal epithelium went through cycles of extension and retraction, but maintained a directional growth along progressively aligned collagen fibers. Eventually, normal epithelium restored simple epithelial organization and underwent branching morphogenesis. Ceasation of protrusions correlated with reestablishment of basement membrane.

We next sought to identify molecular changes that would induce persistent invasion of myoepithelial cells. We chose to target actomyosin contractility, which plays an important role in cell-matrix adhesion and collagen remodeling. Using pharmacological perturbation, we found that inhibition of actomyosin contractility resulted in an excess of long, thin invasive protrusions of myoepithelial cells. Time-lapse microscopy revealed that cellular extension was normal but retraction was blocked. We also observed a complete inhibition of fiber alignment and a failure to reorganize into an epithelial structure. In addition, inhibition of Rho kinase, an upstream regulator of actomyosin contractility, completely disrupted the myoepithelial cell layer, allowing a direct access of luminal epithelial cells into the ECM. We next asked whether actomyosin contractility is required for the maintenance of epithelial architecture. When we inhibited actomyosin contractility after protrusions had ceased, we observed rapid initiation of basal protrusions. These data suggest a functional role of actomyosin contractility in maintenance of epithelial integrity and prevention of local invasion of epithelial cells into a collagen-rich ECM.

Actomyosin filaments generate contractile force via Myosin II motors, whose properties and functions are mainly determined by myosin heavy chain (MHC) domains. Myoepithelial cells express two MHC isoforms, namely MHCIIA and MHCIIIB. We next aim to use Cre-lox based deletion to unravel the specific contribution of each isoform to the epithelial cell contractility and invasion.

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Epithelial-mesenchymal crosstalk mediates tissue fibrosis.

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The formation of excess fibrous connective tissue (fibrosis) adversely affects the function of many organs and can result from a variety of conditions including aging and inflammation. A central player in fibrosis is the activation of fibroblasts that secrete excessive amounts of extracellular matrix proteins. Despite their pivotal role in fibrogenesis, the molecular mechanisms regulating fibroblast activation remains elusive and is a major roadblock in the development of effective therapeutics against this debilitating pathology. Previous work in a variety of organs has demonstrated that the transcription factor Snail is capable of inducing tissue fibrosis. Its developmental role in mediating an epithelial-mesenchymal transition (EMT) during embryogenesis is widely extrapolated to be the mechanism by which activated fibroblasts are produced from neighboring Snail expressing epithelial cells. However, we have found that transgenic expression of Snail in epidermal keratinocytes of the skin is capable of leading to dermal fibroblast activation and fibrosis without utilizing an EMT. Instead, the Snail expressing keratinocytes secrete factors capable of activating dermal fibroblasts to induce overproduction and reorganization of the extracellular matrix. This new paradigm of epithelial-mesenchymal crosstalk as a mediator of fibrosis will illuminate novel targets for clinical intervention to combat fibrosis, which accounts for up to 45% of deaths worldwide.

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The role of tissue elasticity in the formation of cutaneous fibrosis.

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Fibrosis is the formation of excess fibrous connective tissue and is the cause of life-threatening fibrotic diseases in multiple organ systems, including the skin, blood vessels, heart, lungs, and kidneys. However, the mechanism of fibrosis formation remains to be clarified. We established a fibrotic skin model using conditional transgenic mice which exogenously express the transcription factor Snail in the epidermis of the skin, leading to severe inflammation and fibrosis in dermal tissue. We also established a method to evaluate the mechanical properties of the skin tissue including tissue stiffness and elasticity and identified the Snail transgenic skin demonstrated a significantly higher elastic modulus and stiffness compared to the wild-type skin. The Snail transgenic skin contained increased amount of elastic fibers and exhibited up-regulated expression of fibrosis signature genes such as smooth muscle actin and SPARC. We also observed an increased deposition of Fibulin-5 which is a component of elastic fiber and essential for elastic fiber formation in the fibrotic dermis of the Snail transgenic skin. To investigate the contribution of altered elasticity with accumulated elastic fibers in fibrosis formation, we crossed the Snail transgenic mice with fibulin-5 knock-out mice. The obtained Snail transgenic/fibulin-5 KO skin demonstrated not only a reduced elastic modulus but also a reduced expression level of the fibrosis and inflammation signature genes in the affected skin. These findings suggest that tissue elasticity regulates the formation of skin fibrosis.

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Regulation of cell-generated forces during phenotypic reversion by a novel laminin chain in 3D culture of human breast tumor cells.

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Laminins are heterotrimeric extracellular glycoproteins found in, but not confined to, basement membranes (BMs). They are important components in formation of the molecular networks of BMs as well as in cell & tissue polarity, differentiation and morphogenesis. They are secreted by cells and serve to initiate the template required for multicellular assembly. Mass spectroscopy of the isolated membrane fractions revealed a differential expression of an unexpected laminin chain where human malignant breast cells have a higher expression than that of non malignant cells in 3D IrECM. We thus asked the question what is the role of this endogenously produced laminin for 3D acinar morphogenesis. Recently, we reported that non-malignant cells in a 3D laminin-rich gel generate a centripetal force resulting in coherent angular motion (CAMo) to establish acini[1]. On the other hand, malignant cells are randomly motile but regain the ability to rotate during phenotypic reversion [2]. We show that there is a differential deposition of this laminin in the BM in malignant tissues from human patient biopsies. Modulation of expression via shRNA reveals that malignant cells regain the ability to generate centripetal forces and thus re-enter the acinar morphogenetic program. We determine that the cells' ability to perform CAMo is in part due to both the expression and localization of this particular laminin chain. These data show the intimate dynamic reciprocity between the cells and the ECM to establish polarity and form tissue architecture.

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Functional characterization of tissue inhibitor of metalloproteinase-1 (TIMP-1) N- and C-terminal domains during early *Xenopus laevis* development.

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Extracellular matrix (ECM) remodeling facilitates processes such as cell migration and angiogenesis, and is therefore necessary for proper development. ECM remodeling is mediated by the enzymatic activity of matrix metalloproteinases (MMPs), which cleave the various components of the ECM, and by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs). TIMP proteins contain two structurally and functionally distinct domains. The N-terminal domains bind to and inhibit MMPs, while the C-terminal domains may have cell signaling activity. *In vitro* studies have indicated that TIMP-1 may play important roles in regulating apoptosis and cell growth pathways through its C-terminal domain, however, its role *in vivo* remains unclear. The objective of this study is to examine the individual roles of TIMP-1 N- and C-terminal domains *in vivo*, as they pertain to early *Xenopus laevis* development. Semi-quantitative RT-PCR analysis shows that endogenous TIMP-1 is expressed throughout early *X. laevis* development, with the highest level of expression during organogenesis. RNA microinjection was used to overexpress full-length TIMP-1 or its individual N- or C-terminal domains. Overexpression of each TIMP-1 construct produced unique developmental defects; however, full-length and N-terminal TIMP-1 resulted more similar phenotypic abnormalities (axis defects) and increased lethality compared to C-terminal TIMP-1. Additionally, RT-PCR analysis and zymography showed that overexpression of N- or C-terminal TIMP-1 resulted in unique changes in the expression of axial and proteolytic genes, as well as MMP activity, respectively. Together, this indicates that the individual N- and C-terminal domains of TIMP-1 can play distinct roles during development, which are moderated in the full-length TIMP-1 molecule.

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Tissue inhibitor of metalloproteinase-2 (TIMP-2) with a non-functional N-terminal domain decreases the invasiveness of MCF-7 and MDA-MB231 breast cancer cells.

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The tissue inhibitors of metalloproteinases (TIMPs) are the natural inhibitors of matrix metalloproteinases (MMPs), a family of proteins primarily involved in the proteolytic degradation and remodeling of the extracellular matrix (ECM). By virtue of their activity, TIMPs and MMPs play an important role in the migration and invasion of cells both *in vivo* and *in vitro*. TIMPs possess functionally distinct N- and C-terminal domains. While the N-terminal domain of TIMPs is largely involved in inhibiting specific MMPs, the C-terminal domains of the different TIMPs are capable of different functions depending on the TIMP. The N-terminal domain of

TIMP-2 is an excellent inhibitor of MMP-2, -9 and MT-1 (Membrane Type-1) MMP, while the C-terminal domain has been shown to signal inside the cell through MT-1 MMP and alpha3beta1 integrin. The C-terminal domain of TIMP-2 has been shown to modulate migration through the ERK (Extracellular Regulated Kinase) pathway, and induce expression of RECK (REversion inducing Cysteine rich protein with Kazal motifs), a novel MMP regulator, amongst other functions. The objective of my study was to see how TIMP-2 or ALA+TIMP-2 conditioned media affects the invasion and gene expression of MCF-7 and MDA-MB231 breast cancer cells. ALA+TIMP-2 is a TIMP-2 mutant with a non-functional N-terminal domain, which allows for the isolation of the effect the C-terminal domain of TIMP-2 has on invasion and gene expression. MDA-MB231 cells express much higher levels of MT-1 MMP and MMP-2 than MCF-7 cells and as such are significantly more invasive. Treatment of MCF-7 and MDA-MB231 cells with TIMP-2 and ALA+TIMP-2 conditioned media decreased invasiveness, with ALA+TIMP-2 treatment showing the highest decrease in invasiveness for both cell lines. Treatment of both cell lines with TIMP-2 or ALA+TIMP-2 conditioned media did not change expression of RECK, TIMP-2, beta-1 integrin or MT-1 MMP. However, MDA-MB231 cells show a significant decrease in the expression of MMP-2 after treatment with TIMP-2 and ALA+TIMP-2 conditioned media. These data indicates that the C-terminal domain of TIMP-2 can decrease the invasion of MCF-7 and MDA-231 cells, as well as affect MMP-2 expression.

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Analysis of RECK expression in dorsalized and ventralized *Xenopus laevis* embryos.

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Remodeling of proteins within the extracellular matrix (ECM) is crucial during the development of multicellular organisms. Degradation of ECM components occurs through the combined action of matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs). The reversion-inducing cysteine-rich protein with kazal motifs (RECK) gene encodes a membrane-anchored protein and has recently been found to play an important role in mediating ECM remodeling by inhibiting MMPs. To date, however, few *in vivo* studies exist examining the role of RECK during early development. My research focuses on cloning and examining the expression pattern of RECK during *Xenopus laevis* development. A full-length cDNA clone of the RECK gene was generated and sequence analysis confirmed its identity relative to other species. RT-PCR analysis was used to examine the temporal expression of RECK during early development. Results showed that RECK was expressed at lower levels during gastrulation, and then increased during neurulation and organogenesis. Whole mount *in situ* hybridization was used to examine the spatial expression of RECK. Results showed that RECK expression was localized to the neural regions of the developing embryo, including the brain, neural tube, and notochord. RECK expression was also observed in the blood precursors and the otic placode. Treatment of *Xenopus* embryos with lithium chloride (LiCl) and ultraviolet (UV) irradiation revealed that RECK expression decreased in LiCl-treated (dorsalized) embryos and increased in UV-treated (ventralized) embryos. These results suggest that RECK expression is temporally and spatially restricted during early *Xenopus* development.

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The role of polycystins in the maintenance of extracellular matrix integrity.

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Mutations in Polycystin1 and Polycystin2 account for Autosomal Dominant Polycystic Kidney Disease (ADPKD), the most frequent heritable human genetic disease. ADPKD is characterized by the presence of multiple cysts in both kidneys. In addition to cystic pathology, ADPKD is also associated with vascular aneurysm, cardiac valve defects, and abdominal wall hernia; all pathologies linked to extracellular matrix defects. We have modeled ADPKD in zebrafish by polycystin1 and polycystin2 mutation or knockdown and shown that the strong dorsal axis curvature mutant/morphant phenotype was caused by overproduction of cross-linked collagen II alpha1 protein in the notochord sheath since col2a1 knockdown rescued dorsal axis curvature in polycystin-deficient embryos. Surprisingly, overproduction of notochord collagens was not due to over-expression of col2a1, col9a2, or col27a1 mRNA (qRT-PCR). Instead, over-accumulation of collagen protein is more likely linked to enhanced collagen translation, secretion, or reduced degradation.

Polycystin1 is localized to focal adhesions and interacts with focal adhesion kinase (FAK). Over-expression of Polycystin-1 can activate downstream targets of FAK such as PI3K. Interestingly, we find that inhibition of PI3K with a reversible inhibitor (LY294002) phenocopies the polycystin-deficiency dorsal axis curvature in zebrafish, suggesting that inhibition of the PI3K pathway leads to dorsal axis curvature. Further experiments demonstrated that the PI3K targets phospho-Akt and phospho-GSK3 β are downregulated in polycystin2-deficient embryos. Consistent with a role for PI3K in regulating collagen synthesis or turnover, col2a1 knockdown rescued the dorsal axis curvature in embryos treated with the PI3K inhibitor LY294002, strengthening the idea that over-expression of collagen is necessary to induce this phenotype. Taken together, our results support a model where Polycystins 1 and 2 are required for full activity of the PI3K/AKT pathway. In polycystin-deficient or PI3K inhibitor treated embryos, extracellular matrix synthesis or turnover is dysregulated, leading to axial curvature in zebrafish. A similar mechanism may contribute to disease pathogenesis in human ADPKD patients.

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Tenascin C protects aorta from acute dissection in mice.

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Acute aortic dissection (AAD) is caused by an abrupt destruction of the aortic medial layer that is life-threatening and more prevalent than the rupture of abdominal aortic aneurysm (AAA), another common destructive aortic disease. While recent studies including ours have deciphered the molecular pathogenesis of AAA, that of AAD is largely unknown. During the investigation on pathogenesis of AAA focusing on a matricellular protein tenascin C (TNC) that is associated with tissue destruction in various diseases including AAA, we serendipitously discovered that Tnc-null, but not wild type mice developed AAD by the combination of angiotensin II infusion and periaortic CaCl₂ treatment. CaCl₂ treatment caused the stiffening of aorta, a known risk factor of AAD in human, and augmented the AngII-induced hemodynamic stress and TNC expression in aorta. TNC expression, in turn, was essential to tissue reinforcement by expression of extracellular matrix proteins, and to ameliorating an excessive

inflammatory response both in aortic tissue *in vivo* and aortic smooth muscle cells *in vitro*. Our findings indicate that stress-induced TNC expression is a previously unrecognized protective mechanism in aortic walls, and the failure of the protective mechanism underlies the pathogenesis of AAD.

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Roles of the heterotypic CCN2-CCN3 and homotypic CCN2-CCN2 interactions in matrix synthesis in chondrocytes.

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In healthy tissue CCN family member 2 / connective tissue growth factor (CCN2/CTGF) is highly expressed in cartilage and modulates chondrocyte differentiation and proliferation. Since ectopical overexpression of CCN2 is observed in several pathological states such as fibrosis or cancer, regulation of protein level and activity of CCN2 are required for normal development and maintenance of the physiological conditions in the living organisms. To understand the regulating mechanism of CCN2 activity, we, in the present study, identified CCN2-interactive proteins and investigated the effects of interaction on CCN2 activity in chondrocytes.

To identify CCN2-interactive proteins, we carried out GAL4-based yeast two-hybrid screening using a cDNA library derived from a chondrocytic cell line, HCS-2/8. CCN2/CTGF and CCN3/NOV polypeptides were picked up as CCN2-binding proteins, and CCN2-CCN2 and CCN2-CCN3 binding domains were identified. CCN family members have 4 characteristic domains: insulin-like growth factor binding protein-like (IGFBP), von Willebrand factor type C (VWC), thrombospondin type 1 repeats (TSP-1) and C-terminal cystine knot (CT). Among the four domains of CCN2, IGFBP, VWC and CT domains interact directly with full length CCN2, while only VWC and CT domains, but not IGFBP, of CCN2 bound to CCN3. Furthermore direct binding between CCN2 and CCN3 was confirmed by coimmunoprecipitation *in vitro* and *in vivo* and surface plasmon resonance, and the calculated dissociation constant (Kd) was $1.17 \sim 10^{-9}$ M between CCN2 and CCN2 and $1.95 \sim 10^{-9}$ M between CCN2 and CCN3, respectively. Ectopically overexpressed GFP-CCN2 and Halo-CCN3 in COS7 co-localized as determined by direct fluorescence analysis. In addition indirect immunostaining of endogenous CCN2 and CCN3 with specific antibodies showed subcellular co-localization of these proteins. We also present evidence that CCN2-CCN3 interactions modulated CCN2 activity such as enhancement of *aggrecan* and *col2a1* expression. Curiously, CCN2 enhanced, whereas CCN3 inhibited, the expression of *aggrecan* and *col2a1* mRNA in HCS-2/8 cells; and the combined treatment with CCN2 and CCN3 abolished the inhibitory effect by CCN3. These effects were neutralized with an antibody against the VWC domain of CCN2 (11H3). This antibody diminished the binding between CCN2 and CCN2, but enhanced that between CCN3 and CCN2. Our results suggest that CCN2 could form homotypic and heterotypic dimers with CCN2 and CCN3, respectively. Strengthening the binding between CCN2 and CCN3 with the 11H3 antibody had an enhancing effect on *aggrecan* expression in chondrocytes, suggesting that CCN2 had an antagonizing effect by binding to CCN3.

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Identification of Genes Necessary for Neuronal Attachment.

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The extracellular matrix (ECM) is an essential network of proteins that surrounds cells. The interaction between ECM proteins and cells has been of great interest because cellular attachment is lost in several diseases, such as tumor malignancies. Furthermore, the biology of how cells interact with the ECM has been a subject of intense research in the past years, yet the exact mechanism is not fully understood. Previous work from our lab demonstrated that genes including the epidermal growth factor (EGF) and Kunitz domain containing gene *mec-1* and the collagen-like gene *mec-5* encode proteins localized to the ECM and are needed for the attachment in the touch-sensitive neurons to *C. elegans* epidermis; therefore, the attachment of touch neurons to surrounding tissue is a proven approach to identify new genes of the ECM. In the present study, we aim to find genes important for the attachment in the touch neurons by mutagenizing *C. elegans* with ethyl methanesulfonate (EMS) and screening for animals with defective attachment. These screens have led to the identification of 51 mutant strains that show defective attachment. We have also observed great variability in the mutant phenotype, which suggests that multiple genes have nuanced effects in the attachment of neurons to the ECM. The mutant strains that we have found may carry lesions in genes previously associated with attachment defects or in novel genes. We are currently working on characterizing the genes that carry the mutations in these novel strains. The identification of new genes and alleles responsible for neuronal attachment will shed light on the dynamic interaction of cells with their surrounding tissues.

Defining Therapeutic Targets and New Therapeutics II

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Nanoparticles-emitted light attenuates beta amyloid-induced inflammation and oxidative stress.

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Alzheimer's disease (AD) is the sixth leading cause of death in Americans over 65 with no effective intervention yet available to aid in changing this fact. Lower level light therapy (LLLT) has been shown to be an efficacious treatment for many diseases involving inflammation, such as rheumatoid arthritis. Our lab was the first to show the potential of LLLT to serve as a remedy in AD through its ability to prevent the inflammation and oxidative stress induced by amyloid- β (A β) in vitro. However, the low penetration of light into deep tissues is still the major challenge of LLLT. Therefore, new and innovative light delivery methods without relying upon external energy to produce light should be explored. Here we demonstrate the use of Bioluminescence Resonance Energy Transfer-Quantum Dots (BRET-Qdots) as an alternative near infrared (NIR) light source. BRET-Qdots are conjugated on their surface with luc8, a mutant luciferase which is 4 times as efficient. Exposure of luc8 to its substrate, coelenterazine-h (i.e. luciferin), induces the emission of light of peak wavelength 480 nm through an enzymatic reaction. By the principle of fluorescent resonance energy transfer (FRET), the light energy of luc8, in turn, excites the quantum dot to emit NIR light; thus, no external light source is required for light emission. Our results show that BRET-Qdot-emitted NIR suppresses oxidative stress and inflammatory responses induced by A β in primary rat astrocytes. These data provide a proof of concept using quantum dots to carry out light therapy for neurodegenerative diseases, and

should ultimately initiate a new research direction applying nano-medicine approaches as AD therapeutic strategies.

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Construction of different clones of sC3 transferase prior to testing the effectiveness on growth of cells.

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Reconstruction of neural circuits using cell replacement therapies remains high on the lists of potential treatments for neurodegenerative diseases including Parkinson's Disease (PD) and other CNS injuries. New approaches need to be developed to assist implanted cells in regenerating their axons and to guide that growth for proper connectivity. RhoA mediates the effects on growth cones of diverse extracellular cues present after injury. Biochemical blockade of RhoA activity, conversely, leads to axon growth on inhibitory molecules. C3 transferase, a Clostridial Botulinum exotoxin, irreversibly inhibits RhoA and thus holds great promise for regenerative therapeutics. C3 has been shown to increase axon regeneration in the CNS after injury, and is in clinical trials for the treatment of SCI (spinal cord injury).

More recently, a small peptide of 29 amino acids (residues 154-182, sC3) from the C-terminal region of C3 was capable of promoting both axonal and dendritic growth, as well as branching of hippocampal neurons independently from Rho inhibition. The same peptide was shown to enhance axon regeneration in an animal model of spinal cord injury.

The main goal of this project was to generate GFP plasmids containing a short fragment of the C3 gene (sC3). The control contained GFP with no C3. The 0 bridge plasmid contained the sC3 fused directly to GFP and the last plasmid contained a 25 amino acid bridge between sC3 and GFP. These plasmids were transfected into HEK393 cells as a preliminary system. All the plasmids expressed GFP. In addition, both the 0 and 25 bridge constructs enhanced process length of the cells compared to the control plasmid. Future studies will examine these plasmids in neuronal cells grown on inhibitory substrates. These in vitro studies will provide pilot data for subsequent in vivo studies.

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P12, a novel fibronectin peptide, promotes cell survival by augmenting survival signals PDGF-BB.

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Objective: P12 is a small cationic peptide derived from the first type III repeat of human fibronectin with platelet derived growth factor-BB (PDGF-BB) binding activity. P12 limits injury progression in both rat and porcine burn injury models. Previous experiments demonstrated that P12 has synergistic effects with PDGF-BB to prolong adult human dermal fibroblast (AHDF) survival and that P12 alone can enter cells in a temperature and energy dependent manner, like a cell penetrating peptide. Our global hypothesis is that P12 functions by augmenting PDGF-BB survival signals. Results: Here we demonstrate that P12 slows the uptake of PDGF-BB by human dermal fibroblast under starvation. Nevertheless, P12 in the presence of PDGF-BB enhances PDGFR phosphorylation at Y751 (the docking site of PI3K), enhances ERK1/2 phosphorylation, prolongs Akt phosphorylation. Furthermore, we demonstrated by confocal microscopy co-localization between P12 and PDGF-BB. Conclusion/speculation: P12 promotes

cell survival by augmenting survival signals from PDGF-BB through shifting the distribution of PDGF internalization from Clathrin-mediated endocytosis to macropinocytosis.

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Oxytocin is expressed in epidermal keratinocytes and released upon stimulation with ATP in vitro.

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Various similarities have been found between brain and epidermis, as might be expected, because both of them have a common embryonic origin. For example, epidermal keratinocytes synthesize a variety of neuropeptides and neurotransmitters. Oxytocin is a neuropeptide produced primarily in the hypothalamus and is secreted from the posterior pituitary into blood. It contracts smooth muscle during parturition and lactation. It also influences behavior, memory, and mental state. Systemic oxytocin infusion reduced the repetitive behavior of patients with autism and Asperger's syndrome. Moreover, tactile stimuli increased plasma oxytocin level and influenced emotional state. Thus, oxytocin might be a mediator between tactile sensation and emotional state. Here we show that oxytocin is expressed in both human epidermis and cultured epidermal keratinocytes. We also show that oxytocin is released from keratinocytes after application of ATP, in a dose-dependent manner. ATP is an important signaling molecule for keratinocytes and epidermis, and secreted from these cells in response to external stimuli such as heat and pressure. The oxytocin release was inhibited by removal of extracellular calcium, or by the P2 receptor antagonists. These results suggest that oxytocin is produced in human epidermal keratinocytes and is released in response to calcium influx via P2X receptors. Tactile stimuli might induce ATP secretion from epidermis, leading to oxytocin release from epidermal keratinocytes. Further investigation of the roles of epidermis-derived oxytocin may lead to new treatment methodologies for both skin and mental disorders.

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Cannabinoid receptor ligand blocks abnormal hemichannel activity and emerges as a potential treatment for EKV disease.

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Connexins form gap junction channels between adjacent cells to mediate direct exchange of small cytoplasmic molecules and metabolites less than 1KD. They can also form hemichannels on unopposed plasma membrane to allow the passage of small molecules, such as ATP and glutamine. Mutations in connexin 31 (Cx31) are associated with a variety of genetic disorders, including erythrokeratoderma variabilis (EKV), deafness and peripheral neuropathy. Previous studies suggest that EKV associated Cx31 mutants mistraffic and lead to ER stress associated cell death. Our recent study suggests that Cx31R42P forms constitutive-active hemichannels leading to hemichannel activity-dependent necrotic cell death. Using electronic microscopy, we here provide direct evidence of hemichannel formation by Cx31R42P. We have also identified that WIN55212-2 and SR141716, two cannabinoid receptor ligands, suppress Cx31R42P-induced cell death via small molecule screening. Both compounds inhibit hemichannel activity but via distinct mechanisms. Results from this study suggest that abnormal hemichannel activity is the main reason for Cx31R42P associated cell death and identify cannabinoid receptor ligands as potential treatments for EKV disease.

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Stimulant Effects of Guarana Versus Caffeine on *Dugesia dorotocephala*.

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The planarian is an ideal model organism for both developmental and behavioral studies. Planarians prefer darkness and use photoreceptors to detect and avoid light. This behavioral feature provides one means for the associative conditioning or training of these organisms. Further, the locomotor activity (pLMV) and convulsive (c-type) response of planarians with drug exposure has been shown to provide an excellent system to gauge the effects of drug stimulation, addiction and withdrawal. Caffeine is a natural stimulant found in the nuts, leaves and berries of many plants, and prior studies have documented the effects of caffeine on planarian behavior. Guarana is a climbing plant native to the Amazon and Brazil whose seeds contain approximately four times the amount of caffeine found in coffee beans. The mix of other natural chemicals unique to Guarana is suggested to heighten the tonic-type effects of Guarana over caffeine alone. Because of this additive stimulation, the use of Guarana seed extracts is on the rise. Yet, despite the growing use of Guarana as an additive in energy drinks, and a burgeoning market for it as a nutritional supplement, the scientific literature documenting the short-term and long-term effects purportedly attributed to the chemicals they contain is limited. To demonstrate the effects of Guarana, the locomotor activity and reaction time of the planarian species *Dugesia dorotocephala* was tested to gauge the alertness and activity of the flatworms cultured in the presence of the stimulant. Studies examining the physiological effects of Guarana in contrast to, and in combination with, caffeine and glucose were also begun. This work provides an important background for further experimentation using maze learning and memory tests to assess the longer-term effects of Guarana stimulation.

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Cigarette Smoke Toxins on Surfaces are a Major Health Threat for Children and the Elderly.

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Although at least the western nations are making extensive efforts to reduce cigarette smoking, it remains a major health threat for smokers and nonsmokers alike. Moreover, it becomes increasingly clear that the threat is not only during the act of smoking but also to those who share closed spaces with smokers. It is now well known that Second Hand Smoke (SHS) is much more toxic than directly-inhaled smoke and recently, a new and potentially even greater threat has been discovered – Third Hand Smoke (THS) – the accumulation of SHS on environmental surfaces. THS ages with time, becoming progressively more toxic and making it a serious health threat to children, the elderly and those who work in environments where smoking is or has been allowed. We present here the results in vivo showing the effects of THS on multiple organs. We show that significant damage occurs to liver, lung and healing of wounds and that the THS-exposed animals display altered behavior that resembles hyperactivity in humans. In the liver, exposure of mice to THS leads to enhanced lipid concentrations and nonalcoholic fatty liver disease (NAFLD), a precursor to cirrhosis and cancer and potentially a contributor to cardiovascular disease. In lung, THS stimulates excess collagen production and deposition and high levels of inflammatory cytokines, suggesting propensity for fibrosis with implications for Chronic Obstructive Pulmonary Disease (COPD). Mice exposed to

THS show impaired healing of skin wounds that have many characteristics of the poor healing of surgical incisions in human smokers. Lastly, mice exposed to THS become hyperactive and show reduced ability to socialize, as delineated by several behavioral tests. These data are of broad importance because they demonstrate the need for policies governing cleaning of hotel rooms, rental cars, re-rented apartments, etc. and education of parents who smoke.

2159

The role of redox stress in the development of chronic wounds.

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Cutaneous wound healing is a complex event consisting of sequential, overlapping, phases: hemostasis, controlled oxidative stress response, inflammation, re-epithelialization, granulation tissue formation, and remodeling. When wounds do not progress through these stages, the course of healing derails and problematic healing ensues. Chronic wounds, such as diabetic foot, pressure and venous ulcers, have a significant impact on human health, affecting ~5.7M people and costing ~\$20B/year in the US alone. Advancing knowledge of chronic wound development requires animal models. We have shown recently that absence of the gene for Tumor Necrosis Factor Superfamily Member 14 (TNFSF14/LIGHT) in mice results in abnormal healing that can develop into chronic wounds that mimic chronic ulcers in humans. We hypothesized that oxidative stress is increased in the LIGHT^{-/-} wounds and that it is critically involved in the LIGHT^{-/-} impaired healing. In chronic human wounds, the levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are high and lead to deleterious effects because they cause oxidative stress, with consequent significant cell damage and increased inflammation. We show that the level of H₂O₂ is high in adult LIGHT^{-/-} mice and increases with age much like in humans. However, the levels of the anti-oxidant enzymes catalase and glutathione are not elevated, hence these wounds have excessive ROS. Nitrotyrosine is also high, suggesting elevated peroxynitrite anion, a highly reactive nitrogen species and nitrating agent that damages a wide array of molecules. As a result of high oxidative stress, cell death by necrosis is high, further enhancing inflammation and impaired healing. Perturbation of critical parameters in these wounds leads to chronicity. In conclusion: By modifying the wound environment, *we have successfully produced the first model of chronic wounds*. We are using this model to understand mechanisms involved in chronic wound development to identify potential targets for treatment of humans.

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TP508, a novel peptide, rescues loss of stemness in the colonic crypts of mice in response to radiation damage.

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Background: In recent years, the threat of radiation exposure has significantly increased worldwide. In 2011, Japan was hit with a series of earthquakes, damaging the nuclear reactors and exposing thousands of people to high levels of radiation. More recently, nuclear threats against the US and other countries are becoming a concern. Therefore, it is crucial to develop effective medicinal countermeasures to mitigate the effects of radiation exposure and prevent calamitous death rates wherever an accidental or intentional nuclear event may occur. TP508, also known as Chrysalin®, developed by Dr. Darrell Carney, is an investigational drug which has been shown to mitigate the effects of whole body nuclear radiation (8Gy) in mice, and to

reduce mortality and morbidity of the irradiated mice. In addition, TP508 treatment post-exposure delayed mortality of mice exposed to lethal doses of radiation (12Gy), and prevented radiation-induced apoptosis. We now have examined possible protective effects of TP508 on stemness/phenotype of intestinal crypts, which may contribute to the previously observed reduction in mortality of irradiated mice treated with TP508. Methods: CD-1 (IRC) mice (in groups of 5) were either exposed to whole body gamma radiation (9Gy), or not irradiated, followed by i.p treatment of \pm 500 μ g TP508, 24 hrs post-irradiation. Group 1 received no radiation+saline, group 2 received no radiation+TP508, group 3 received a lethal dose (9Gy) of radiation+saline and group 4 received radiation+TP508. Mice were euthanized 12 days post exposure and colonic crypts were isolated and analyzed for their length and architecture. Colons were also processed for immunofluorescence and Western blot analysis for stem cell populations, expressing DCLK1, LGR5 and CD44. Results: Our results demonstrated that lengths of colonic crypts were significantly decreased in irradiated versus non-irradiated mice; however, treatment with TP508 reversed the deleterious effects of irradiation on the morphology and lengths of colonic crypts. Western blot analysis for the indicated stem cell markers, strongly suggested that TP508 effectively protects against the loss of stem cell populations in the colonic crypts of irradiated mice. Conclusions: These findings suggest that TP508 may protect stem cell populations from deleterious effects of irradiation, and thus contribute towards reversing the damaging effects of irradiation on length/morphology of colonic crypts. We therefore postulate that TP508 can be developed as an effective novel drug for mitigating effects of radiation exposure.

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Stem cell expansion, characterization and health monitoring in a scalable bioreactor system.

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Progenitor cells are an attractive target for clinical study as therapeutic agents. However, current multilayer flatbed culture expansion paradigms are cumbersome, time consuming, costly and typically limited in the ability to monitor cell quality attributes during the growth process. We demonstrated a new expansion paradigm that uses a three liter, single use, stirred tank bioreactor with a microcarrier scaffold for suspension expansion of human mesenchymal stromal/stem cells (hMSCs), one example of progenitor cells. The bioreactor is a scalable system that enables sampling throughout the expansion run so cells can be monitored for quality and to ensure they maintain the desired undifferentiated state. We describe implementation of a panel of assays that characterize the cell state and the identity and purity of the cells during expansion and purification. Cell state assays include cell cycle, viability and apoptosis. Identity and purity were assessed using both the standard recommended ISCT panel of positive and negative markers in addition to markers indicative of hMSC properties. We demonstrated that positive markers such as Thy-1 (CD90), Endoglin (CD105) and SH3/4 (CD73) were expressed in >95% of viable cells, markers of hematopoietic origin such as CD34, CD45, CD11b and other markers such as HLA-DR were expressed in <2% of cells per the ICST guidelines. We also demonstrated the use of additional hMSC markers such as H-Cam (CD44), PD-L1 (CD274), V-CAM (CD106) for characterizing the hMSCs with respect to their age and induction state. When we intentionally change the system, an increased fraction of apoptotic cells (34% versus 10% control) correlated to poor cell yield (50 million cells versus 300 million

cells at day 12). In a perturbed system, deviations from the expected percentages for ISCT panel and expanded marker panel clearly identify poor cell/process quality. The combination of cell state and marker panels for identity and purity can be considered a process monitoring tool for expansion of hMSCs in addition to a quality control assessment following expansion. As processes scale, establishing a validation window within which the process should operate is critical; using markers such as these allows one to establish the boundary conditions. Thus, we have demonstrated use of a scalable bioreactor system to expand, monitor and characterize progenitor cells.

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Efficacy Evaluation of #101 pills on Indomethacin Induced Gastric Mucosal Damage in Rats.

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#101 pills were used in clinically for digestive problems including gastritis. But its efficacy does not evaluated and confirmed on scientific methods such as in vivo approach with animal models. In the present study we have investigated the efficacy of #101 pills on its anti-ulcer effect using indomethacin induced gastric mucosal damage in rats.

We divided the animals into 4 groups as follows: (1) vehicle control, (2) 25 mg/kg indomethacin, (3) 25 mg/kg indomethacin + 250 mg/kg #101 pills [repetitive administration], (4) 25 mg/kg indomethacin + 500 mg/kg #101 pills [single administration]. In groups 2, 3 and 4, indomethacin was given at 30 minutes after #101 pills administration. 25 mg/kg indomethacin was given to all rats (n=10) by oral gavage in 3% sodium bicarbonate. Seven hours after, the entire rats were sacrificed and confirmed gastrointestinal erosions and ulcers.

Pretreatment with #101 pills at 30 minutes before administration of indomethacin resulted in a decrease score in gastric damage areas in the entire group treated. And also, gastric acid volume was decreased in as well. Based on H&E staining and its scoring on histopathology, single administration group with #101 pills had more effective on the reducing of gastric mucosal damage. Our data suggests that #101 pills accomplish the protective effect on indomethacin-induced gastric mucosal damage.

Immune System

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Autoantibodies in Senear-Usher syndrome are the result of inter-molecular epitope spreading.

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Senear Usher syndrome is a pathology that overlaps clinical and serological data of pemphigus foliaceus and lupus erythematosus. Skin biopsies of these patients exhibit acantholysis and immunoglobulin's deposition at desmosomes, together with positive lupus band test. Given that Senear-Usher patients have pemphigus and lupus autoantibodies and the disease clinically behaves as benign variant of pemphigus and cutaneous lupus, the major issues that we attempt to address are the following: It is possible that Senear Usher autoantibodies were connected with a single antigen? If so, such poly-reactivity might result of cross-reactivity or instead: Are the antibodies the result of stimulus to independent B cell clones by different antigens? In such case, the poly-reactivity could result of inter-molecular epitope spreading. Sera from patients

with Senear Usher were studied using immunofluorescence and ELISA against recombinant desmogleins 1 and 3, and RNP/Sm, Ro and La ribonucleoproteins and nDNA to determine the fine antigenic specificity. Bound specific autoantibodies to cow nose (used as antigenic substrate and previously digested with nucleases or trypsin) were eluted by 0.2M glycine and recovered, and then tested by double fluorescence assays to tag desmosome in green or nucleus-cytoplasmic antigens in red.

Our present investigation demonstrates that patients with Senear Usher disease share anti-epithelial antibodies specific to desmoglein 1 or 3, together with anti-nuclear antibodies specific to Ro, La, Sm or double-stranded DNA antigens. By double fluorescence assays using specific anti-epithelial or anti-nuclear antibodies recovered after elution, a lack of cross-reactivity between desmosomes and nuclear or cytoplasmic lupus antigens was demonstrated, therefore this result suggests that Senear-Usher auto antibodies are directed against different antigens and they are produced by independent B cell clones. Additionally, the molecular evolution of the immune specificity response was tracked through ten years, the result of this analysis yield the conclusion that desmoglein complex (Dsg 1) was the initial target, because anti-epithelial antibody appeared several months or years before than the anti-nuclear response. Finally, considering the clinical and serology data we suggest that Senear Usher syndrome behaves as a multiple autoimmunity disease. It is accepted that different disease co-exist in a single patient by multiple autoimmunity, the involved mechanisms are largely unknown however some clues have been arising of certain MHC alleles who define the clinical expression of autoimmunity; for instance in blistering autoimmune diseases the DQB1*0301 allele may drive two simultaneous autoimmune responses that under different stereo chemical conditions can bind two T-dependent epitope residues, like desmogleins or BP 180 and under other condition might bind ribonucleoprotein's, such peculiar antigenic driving would result in a dual autoimmune response one organ-specific for pemphigus and another to express another disease phenotype like the cutaneous lupus erythematosus (Clin Exp Immunol 162:224), such kind of dual response might induce inter-molecular epitope spreading this could be the case of Senear Usher disease.

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Hydrogen sulfide inhibits preoptic prostaglandin E2 production during endotoxemia.

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Hydrogen sulfide (H₂S) is a gaseous neuromodulator endogenously produced in the brain by the enzyme cystathionine β-synthase (CBS). We tested the hypothesis that H₂S acts within the anteroventral preoptic region of the hypothalamus (AVPO) modulating the production of prostaglandin (PG) E₂ (the proximal mediator of fever) and cyclic AMP (cAMP). To this end, we recorded deep body temperature (T_b) of rats before and after pharmacological modulation of the CBSH₂S system combined or not with lipopolysaccharide (LPS) exposure, and measured the levels of H₂S, cAMP, and PGE₂ in the AVPO during systemic inflammation. Intracerebroventricular (icv) microinjection of aminoxyacetate (AOA, a CBS inhibitor; 100 pmol) did not affect basal PGE₂ production and T_b, but enhanced LPS-induced PGE₂ production and fever, indicating that endogenous H₂S plays an antipyretic role. In agreement, icv microinjection of a H₂S donor (Na₂S; 260 nmol) reduced the LPS-induced PGE₂ production and fever. Interestingly, we observed that the AVPO levels of H₂S were decreased following the immunoinflammatory challenge. Furthermore, fever was associated with decreased levels of AVPO cAMP and increased levels of AVPO PGE₂. The LPS-induced decreased levels of cAMP were reduced to a lesser extent by the H₂S donor. The LPS-induced PGE₂ production was potentiated by AOA (the CBS inhibitor) and inhibited by the H₂S donor. Our data are consistent

with the notion that the gaseous messenger H₂S synthesis is downregulated during endotoxemia favoring PGE₂ synthesis and lowering cAMP levels in the preoptic hypothalamus.

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Spontaneous Hypertension Is Associated With Inflammatory Response In The GI Tract: Effects of Captopril.

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Hypertension is associated with oxidative stress and inflammatory responses in the target organs however the status of intestine remains to be elucidated. In this study we employed spontaneously hypertensive rats (SHR) to examine the level of myeloperoxidase as a marker of inflammation. Twelve weeks old SHR and their genetic counterparts Wistar Kyoto rats (WKY) as controls were used in this study. Groups of animals were also treated with captopril, an ACE inhibitor starting from week 4 of age until week 12 through water ad libitum (300 mg/L). Hypertension was characterized in terms of BP, kidney and heart hypertrophy and proteinuria. Animals were sacrificed at the end of week 12, ileum and colon were taken out, cleaned with ice cold PBS and stored at -70°C until use. Myeloperoxidase activity was measured using standard spectrophotometric method. SHR animals showed significant increase in blood pressure, weights of heart and kidney and proteinuria in SHR as compared to WKY controls. In both colon and ileum myeloperoxidase activity was increased significantly in SHR as compared to WKY controls. These changes were significantly reversed by captopril treatment of SHR. Captopril under the experimental condition did not affect these parameters in WKY controls. These findings demonstrate development of ileal and colonic inflammation due to hypertension, and suggests an antiinflammatory action of captopril.

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Telomere Dysfunction in Naïve CD4 T cells in Rheumatoid Arthritis.

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Background: Telomeres are the natural ends of chromosomes. Naïve CD4 T cells of patients suffering from rheumatoid arthritis (RA) display a premature aging phenotype and a loss of telomeric length. The aim of our study was to determine, if telomeres from RA patients display more telomere damage and DNA damage protein localization and if this can be seen in healthy aging or if telomere dysfunction is a disease specific phenomenon.

Methods: CD4 naïve T cells were isolated from RA patients, age and sex matched controls, and old (mean age 80) and young individuals (mean age 30) using magnetic beads. Telomere damage was measured in metaphase spreads treated with colcemid and subsequent FISH staining. DNA damage foci at the telomere were analyzed with double staining for 53BP1, ATR or BRCA1 and TRF2.

Results: Four parameters of telomere damage are used, a doubling of a signal, apposition of the long chromosome arm, fusion of telomeres and loss of telomeric signal. Naïve CD4 T cells show an increase of double signals (42% versus 32.4% of events) and apposition (22.2% versus 12.4% of events) compared to healthy controls. In addition, we only observed telomeric fusion and telomere loss in nuclei from RA patients (2.9% and 1% of events respectively). In addition, DNA damage foci are increased at telomeres in CD4 naïve T cells from patients with RA including 53BP1 foci (8% in control versus 17% in RA, p<0.05), ATR foci (15% in control versus 33% in RA, p<0.05) and BRCA1 foci (20% in control versus 39% in RA, p<0.05). An

increase of DNA damage repair protein 53BP1 at the telomere is not observed in old healthy individuals (10% versus 8% for young individuals, $p=0.44$). To determine, whether the ATR pathway is activated in RA T cells, we determined the number of pChk1 positive cells. In resting conditions, we did not observe a difference between the amount of pChk1 positive cells between healthy controls and RA patients. However, after 3 days of stimulation we only observed pChk1 positive cells in the RA group (6%), indicating an increased activation of the ATR pathway.

Conclusion: We provide evidence, that naïve CD4 T cells from patients with RA have dysfunctional telomeres and increased localization of DNA damage signaling molecules to the telomere. We did not find a similar response in cells from aging individuals, indicating a disease specific phenotype rather than an age specific phenotype.

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Galectin-8 knockout mice have sex-dependent altered homeostasis of T and B lymphocytes and autoimmune features.

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Galectin-8 (Gal-8) regulates a variety of cellular activities interacting with beta-galactosides of cell surface glycoproteins. Its effects in the immune system include stimulation of apoptosis of thymocytes and activated T cells, proliferation of "naïve" T cells and differentiation of B lymphocytes into plasma cells. We have also described function-blocking autoantibodies in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis. However, the requirement of Gal-8 in immunological functions has not been studied "in vivo". Here we analyze the expression of Gal-8 in spleen and lymph nodes and lymphocyte subpopulations in a Gal-8-null/LacZ knock-in mice (Gal-8^{-/-}). LacZ histochemistry and immunohistochemistry show Gal-8 expressing at specific sites of the spleen and lymph nodes. Strikingly, only female Gal-8^{-/-} mice, examined at 8-12 weeks, have increased spleen populations of CD4 and CD8 T lymphocytes and decreased population of B-lymphocytes, without differences in dendritic cells. By the age of 14 months, these female Gal-8^{-/-} mice develop alopecia and splenomegaly accompanied by a significant decrease in the population of CD4 and CD8 T lymphocytes and B-lymphocytes. These mice also generate anti-ANA and anti-dsDNA autoantibodies. These features suggest an autoimmune disease such as SLE. These results reveal an important contribution of Gal-8 to the homeostasis of T and B cells, with sexual differences, such that alterations in its function might lead to autoimmunity. (Financed by Fondecyt postdoctoral project N° 3120061, Fondecyt N° 1100921 and CONICYT project Basal #PFB12/2007).

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Examining the Role of mTOR in T Cell Proliferation and Migration Under Immunosuppression by Rapamycin.

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mTOR (mammalian target of rapamycin) is a serine/threonine protein kinase that has a role in cell growth and metabolism. mTOR also is the core of two functional distinct complexes, mTORC1 and mTORC2. mTORC1 has a prominent role in cell growth and proliferation while

mTORC2 has been shown to affect cell proliferation and cytoskeletal organization. However, the role of mTOR signaling in T cell migration is not fully understood. Studies were conducted to determine the effects of rapamycin, an immunosuppressant that targets mTOR, on Jurkat T cell proliferation and migration. Rapamycin treatment of Jurkat cells was expected to inhibit cell proliferation and cell migration due to its inhibition of normal mTOR signaling. Jurkat T cells were stimulated with PMA (50 ng/ml) and PHA (5 μ g/ml) in the presence or absence of rapamycin (5, 10, 20, 50 nM; 12, 24, and 48 hours). Cell counts at 12, 24, and 48 hours showed a concentration-dependent inhibition of cell proliferation by rapamycin; no significant effects were seen beyond 20 nM of rapamycin. Phosphorylated ERK was also decreased in activated T cells treated with rapamycin as determined by Western blot analysis. Rapamycin also inhibited Jurkat T cell migration toward CXCL12. Results show that even in combination with activating agents, rapamycin inhibits T cell proliferation and migration, suggesting that mTOR signaling may also have an effect on ERK signaling and T cell chemotaxis.

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TNC Microenvironment Reduces IL-12 Responsiveness In Triple Positive Thymocytes.

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Interleukin-12 (IL-12) is a heterodimeric, pro-inflammatory cytokine released by antigen presenting cells (APCs) such as macrophages and dendritic cells as well as activated T cells. IL-12 is well documented to favor T-helper differentiation into Th1 effector cells in the periphery. However, IL-12 is also reported to promote apoptosis among the CD4⁺CD8⁺ TCR^{lo} triple positive (TP) thymocyte subset known to undergo restriction to self major histocompatibility (MHC) proteins in the thymus. Thymic nurse cells (TNCs) are cortical epithelial cells of the thymus that express both class I and class II (MHC) proteins on their cell surfaces. Although TNCs appear to be significant participants in the negative selection of TP thymocytes, they have been shown to rescue a small proportion of these thymocytes from apoptosis. We hypothesized that TNC mediated rescue of TP thymocytes from apoptosis is due to the down regulation of IL-12 responsiveness by these thymocytes. To demonstrate this, timed co-cultures were prepared containing thymocytes and TNCs with and without recombinant IL-12. Thymocytes were harvested from co-cultures and stained with fluorescently-labeled anti-CD4 and anti-CD8 antibodies to identify cells that were double positive for these co-receptors. These thymocytes were also labeled with FITC-conjugated Annexin V for the detection of apoptosis. Labeled cells were analyzed by flow cytometry. As expected apoptosis was found to be severely reduced among double positive thymocytes in all co-cultures containing TNCs. Remarkably, thymocyte rescue from apoptosis was also observed in cultures that included recombinant IL-12, and this rescue was found to be greater than that seen in cultures which did not include the cytokine. Additionally, it was noted that thymocyte rescue from apoptosis in cultures containing recombinant IL-12 did not translate into thymocyte maturation as it did in cultures that did not contain IL-12. Together these observations led to the theory that IL-12 responsiveness among double-positive thymocytes may be down-regulated in the TNC microenvironment. Current experiments are in progress to test this theory.

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RB controls size and cellularity of the mouse thymus.

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The establishment of the thymic microenvironment early in life is crucial for the production of functional T cells. Conversely, thymic involution results in a decreased T cell output. Thymic involution has important health implications especially following bone marrow transplant. Our objective is to determine molecular and cellular mechanisms that will allow for regeneration of involuted thymic tissue, restore production of naïve T cells, and improve immune function. In this pursuit, we have focused on the Retinoblastoma family of tumor suppressor proteins. The main function of the RB pathway is to restrict passage through the G1/S transition of the cell cycle. RB and its two family members, p107 and p130, mediate the action of a broad range of cellular signals to control the proliferation, survival, and differentiation status of a large number of mammalian cell types. We found that inactivation of the RB pathway in the thymus by early deletion of RB family genes prevents thymic involution, promotes expansion of functional thymic epithelial cells (TECs), and increases thymic T cell output. Moreover, we have identified a direct regulatory relationship between RB and the Foxn1 transcription factor. Via E2F transcription factors, where RB/E2F complexes directly repress the Foxn1 promoter, thereby promoting involution. Thus, the RB family is a critical mediator of extra- and intra-cellular signals to regulate thymic epithelial cells and thymus function, and decreasing RB pathway function may promote regeneration of the involuted thymus and restoration of naïve T cell production in patients.

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Gene expression profile of monocytes from patients with chronic kidney disease reveals canonical Wnt signaling activation.

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Objectives: Patients with chronic kidney disease (CKD) have an increased susceptibility to infections and one contributing factor is dysfunctional leukocytes. Monocytes play a central role in immunity as a link between the innate and the adaptive immune responses. This study was conducted to investigate the gene expression profile of monocytes to understand the underlying molecular mechanism behind CKD.

Methods: Monocytes from CKD patients with glomerular filtration rate (GFR) < 20 ml/min were obtained from peripheral blood by density centrifugation and purified with anti-CD14 Mini-MACS beads. Samples from healthy subjects were run in parallel. Microarray gene expression profile was performed using Affymetrix human Gene Chips U133A. Data were interpreted by GeneSpring™ software. Genes demonstrated a differential expression of 1.5 Fold (F) or more and were statistically significant (P<0.05), further analyzed. The on line PANTHER classification system website was used for pathway analysis.

Results: The gene expression profile demonstrated up regulation of 487 genes and down regulation of 258 genes in the CKD group compared to healthy controls. Pathway analysis revealed that Wnt signaling has the highest score among the pathways affected in the CKD group. Several genes demonstrated up regulation including Wnt5a (3.6 F), FZD4 (2 F), LEF1 (1.6 F),

TCF3 (2.2 F) and TCF7 (1.6 F) respectively. Typically, Wnt5a through FZD4 leads to canonical Wnt signaling pathway activation with inhibition of β -catenin degradation.

Conclusions: This study demonstrates crucial difference in the monocyte gene expression profile between CKD patients and healthy subjects. The canonical Wnt signaling pathway is activated in monocytes from CKD patients which leads to lesser β -catenin degradation and hence its accumulation in the cytoplasm. Since β -catenin affects monocyte adhesion and transmigration, our finding may contribute to monocyte dysfunction and to the pathogenesis of complications observed in CKD. Therefore developing strategies to interfere with this pathway could improve prognosis regarding general immunity and prevention of complications.

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Individual platelet contraction dynamics stimulated via two distinct signaling pathways.

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Platelet adhesion to sites of vascular injury occurs when extra cellular matrix components become exposed to the bloodstream. After adhesion, activation of platelets can be mediated by two independent signaling pathways. One involves the exposure of subendothelial matrix collagen, while another relies on the release of tissue factor and its downstream effectors for platelet activation. Surprisingly, much of the previous work studying platelet force generation relied on bulk assays that calculated individual platelet contraction from macroscopic measurements. These results give little insight into how single platelets contribute to the overall force generation of an aggregate. Here we analyzed individual platelet activation dynamics using traction force microscopy, mapping the stress fields of isolated platelets adhered to polyacrylamide substrates coated with physiologically relevant ECM proteins (collagen, fibrinogen and fibronectin). We find that for all substrates, platelets are capable of generating forces of ≈ 100 nN upon contraction. Interestingly, interaction with collagen and fibrinogen coated substrates was sufficient to induce immediate platelet activation, while on fibronectin coated surfaces, platelets would adhere and spread without contracting. Contraction could then be stimulated on fibronectin coated substrates through the addition of epinephrine. These data suggest that we can successfully activate platelets through inside-out and outside-in signaling pathways, which is consistent with previous in vivo thrombus formation studies that identified two independent signaling pathways for platelet activation. Furthermore, since platelets produced the same amount of force on each substrate, these data indicate that the force response of individual platelets is independent of the mechanism of activation. Together these findings highlight the promising new research avenues that can be explored by studying isolated platelet adhesion and contraction.

Establishing and Maintaining Organelle Structure

2173

Obscurin is Required for AnkyrinB-dependent Localization of Dystrophin and Sarcolemmal Integrity in Skeletal Muscle Fibers.

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Obscurin is a recently identified sarcomeric giant protein that participates in the organization of the sarcomere at the M band. In addition to interact with sarcomeric proteins, obscurin can also bind some muscle-specific ankyrins. Interaction between obscurin and the muscle-specific

ankyrin sAnk1.5 has been found to regulate the organization of the sarcoplasmic reticulum in striated muscles. Additional muscle-specific ankyrin isoforms, namely ankB and ankG, are localized at the sub-sarcolemma level and have been shown to contribute to the organization of dystrophin and β -dystroglycan at costameres. Accordingly, we investigated whether obscurin might be involved in the sub-sarcolemmal localization of ankB and ankG and eventually in the assembly of dystrophin and β -dystroglycan at costameres in skeletal muscle fibers. In vitro experiments indicated that obscurin can bind to ankB and ankG. Furthermore, we found that in mice deficient for obscurin, ankB was displaced from its localization at the M-band, while localization of ankG at Z-disk was not affected. In obscurin KO mice, localization at costameres of dystrophin, but not of β -dystroglycan, was altered and the sub-sarcolemma microtubule cytoskeleton was disrupted. In addition, these mutant mice displayed sarcolemmal fragility and reduced muscle strength. Altogether, these results support a model where obscurin, by targeting ankB at the M-band, contributes to the organization of sub-sarcolemma microtubules, localization of dystrophin at costameres and to maintenance of sarcolemmal integrity. These results add a new twist to obscurin function due to interaction with proteins of the extra-sarcomeric cytoskeleton. Accordingly, obscurin appears to represent a multifunctional anchoring protein that on one hand establishes interactions with sarcomeric proteins and on the other hand enables complex formation with extra-sarcomeric proteins, like the muscle-specific ankyrin isoforms, that help to connect the sarcomeres with the SR and with the sub-sarcolemmal cytoskeleton.

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ELP1 connects Endoplasmic Reticulum to Mitochondria.

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Intercommunication between the endoplasmic reticulum (ER) and mitochondrion is important for Ca^{2+} signaling and cell viability. However, the molecular mechanisms controlling this interaction are largely unknown. The ER supplies Ca^{2+} directly to mitochondria via inositol 1, 4, 5-trisphosphate receptors (IP3Rs). Here we found new ER protein ELP1 (endoplasmic reticulum localizes protein 1), is enriched at ER-mitochondria connection. Removal of ELP1 in mouse embryonic fibroblasts or silencing of ELP1 in HeLa cells show ER abnormal morphology and disconnect ER-mitochondria interactions, for this reason leading to a reduced Ca^{2+} signaling into mitochondria via IP3Rs. ELP1 form a complex at ER-mitochondria connection with prohibitin and chaperone, BiP (GRP78). The ER chaperones are important in maintaining cellular Ca^{2+} level, majority of ER chaperones, including BiP, calreticulin, and calnexin, serve to store ER Ca^{2+} as high-capacity Ca^{2+} -binding proteins. Therefore, ELP1 connects ER to mitochondria and regulates ER-mitochondrial Ca^{2+} signaling.

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Plasma membrane tethering of the cortical ER necessitates its finely reticulated architecture.

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The cortical endoplasmic reticulum (ER) is an intricate network of tubules and cisternae tightly associated with the plasma membrane (PM) in plants, yeast and the excitable cell types in metazoans. How the ER is attached to the cell cortex and what necessitates its highly reticulated architecture remain unknown. Using a combination of mutants where the cortical ER

is detached from the PM and a set of artificial ER-PM tethers suppressing this phenotype we show that the footprint of the cortical ER is functionally insulated from the cellular interior in the fission yeast *Schizosaccharomyces pombe*. Conversion of the reticulated ER structure to the sheet-like morphology blocks large areas of the cellular cortex from interaction with the peripherally associated protein complexes. Importantly, our work provides a mechanistic explanation for the severe division site positioning defects in fission yeast cells lacking the ER membrane tubulating proteins. We propose that fine reticulation of the ER network may have emerged as a critical adaptation enabling access of protein complexes to the inner surface of the plasma membrane.

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Type 1 inositol 1,4,5 tri phosphate receptor is required for Endoplasmic reticulum clustering and Calcium oscillation in mouse oocyte maturation.

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Type 1 inositol 1,4,5 tri phosphate receptor (IP3R1) and Endoplasmic reticulum (ER) become redistributed during mouse oocyte maturation from the germinal vesicle (GV) stage to the metaphase II (MII) stage. We investigated is the impact of redistribution of IP3R1 on ER clustering during oocyte maturation by first degrading endogenous IP3R1 with injection of GV oocytes with Adenophostin A (AdA), a non-hydrolyzable analogue of IP3, then expressing exogenous IP3R1 with injection of IP3R1 cDNA. To examine IP3R1 redistribution, we used immunofluorescence and for ER clustering, we used DiIC. There is no effect of injection of AdA and IP3R1 cDNA on the in vitro maturation rate. Western blot analysis of the IP3R1 in eggs after in vitro maturation demonstrated a 85% decrease in immunoreactive mass of the IP3R1 in AdA-injected eggs compared to control eggs. In eggs injected with IP3R1 cDNA following Ada injection, immunoreactive mass of IP3R1 was increased 1.5 fold from that of control eggs. Immunofluorescence of IP3R1 and ER staining of In vitro matured MII egg demonstrated that cortical clusters localize near plasma membrane in control eggs, but Ada injection inhibits cortical cluster formation of IP3R1 and ER. On the other hand, eggs injected with IP3R1 cDNA after Ada injection recovered cortical cluster formation of IP3R1, but ER clusters. Knockdown IP3R1 eggs showed low [Ca²⁺]_i responses to mPLCzeta cRNA injection, but IP3R-1 overexpressing eggs demonstrated persistent, albeit delayed, [Ca²⁺]_i responses to the injection of the mPLCzeta cRNA.

We therefore hypothesize that IP3R-1s are required for ER cluster formation during mouse oocyte maturation via homotypic and heterotypic interactions. From these results, we hypothesize that IP3R1s are required for ER cluster formation during mouse oocyte maturation via homotypic and heterotypic interactions. This research was supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Education, Science and Technology(2012R1A1A3010811)

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Contacts between the endoplasmic reticulum and the plasma membrane mediated by Extended-Synaptotagmins are regulated by PI(4,5)P₂.

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The endoplasmic reticulum (ER) forms a complex network of cisternae and tubules that extends throughout the cell and participates in multiple contacts with virtually all other membranes, including the plasma membrane (PM). Direct contacts between the ER and the PM have long been known to participate in depolarization-contraction coupling in muscle cells via their regulation of Ca²⁺ fluxes. More recently, a general role of ER-PM contact sites in the regulation of Ca²⁺ homeostasis and in other functions, such as lipid transfer and phosphorylation-dephosphorylation of proteins and lipids in the PM by ER-anchored proteins, has been appreciated. However, evolutionary conserved molecular mechanisms that mediate such contacts have remained elusive. In a companion Abstract (Giordano, Saheki et al.), we report that a family of multiple C2 domain-containing proteins, the Extended-Synaptotagmins (E-Syt1, E-Syt2 and E-Syt3), are cortical ER-enriched proteins that mediate such bridges (see also Abstract by Manford et al. on the tricalbins, the yeast homologue of the E-Syts). E-Syts had been proposed to be transmembrane proteins with a single transmembrane span. However, E-Syts lack a signal sequence, and analysis of the membrane topology of the E-Syts revealed that both the N- and C-terminus are localized in the cytosol, suggesting a hairpin-insertion. Accordingly, the single hydrophobic stretch predicted to represent the membrane anchor is longer than a typical transmembrane span and is reminiscent of hydrophobic amino acid stretches shown to form hairpin in the ER membrane such as those of the reticulons and of REEP. The property of E-Syt2 and E-Syt3 to bind the PM, and thus to generate ER-PM contact sites, depends on C2 domains and requires presence of PI(4,5)P₂ in the PM. Acute dephosphorylation of plasma membrane PI(4,5)P₂ via the recruitment of an inositol-5 phosphatase using an optogenetic approach recently developed in our laboratory (Idevall-Hagren et al., PNAS 2012) resulted in the dissociation of the E-Syts from the PM and in their dispersion throughout the entire ER.

Regulated binding of C2 domains to the PI(4,5)P₂ containing bilayer of the PM mediates the recruitment of cytosolic signaling proteins and the interaction and fusion of secretory vesicles, including synaptic vesicles, with this membrane. Our results add a new function to a member of the C2 domain-containing protein family, bridging the ER to the PM.

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A calcium-activated RNase regulates ER morphology.

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Developmental differentiation results in changes to intracellular organization and organelle structure as cells change to fulfill more specific roles. One of the most dramatic intracellular reorganizations in higher eukaryotes occurs at fertilization, when changes in intracellular calcium concentration lead to large-scale changes in the egg to promote formation of an embryo. While calcium signaling is well known to result in changes to the cell cycle state of the egg it is unknown if calcium affects the transcriptome. While studying posttranscriptional RNA regulation at fertilization we discovered an endoribonuclease that is activated by the increase in

calcium concentration at fertilization. We purified this nuclease from *Xenopus* egg extracts and identified the nuclease as EndoU, a ribonuclease previously implicated in snoRNA processing. EndoU is a calcium-activated nuclease in vitro and in vivo with weak sequence preference for poly-U. Interestingly, EndoU copurifies with many ER-resident proteins and localizes to the ER in a variety of cell types. Immunodepletion of EndoU from *Xenopus* egg extracts results in defects in nuclear envelope assembly and dramatic failures in ER-vesicle fusion, suggesting that local degradation of ER-localized transcripts plays a role in controlling ER morphology. We have used RNA-sequencing to identify several hundred transcripts that are degraded at fertilization in an EndoU-dependent manner, which includes many ER-localized transcripts. To determine if EndoU-mediated control of ER morphology is conserved in other cell types we have examined the function of EndoU in human tissue culture cells. Human EndoU is also localized to the ER and RNAi knock-down of hEndoU results in a dramatic change in ER morphology from a balance of ER tubules and sheets to almost exclusively ER-sheets, demonstrating that local control of RNA stability is important for ER morphology in many cell types. Our results identify a novel role for localized transcript destruction in control of organelle structure and may provide a molecular basis for the longstanding observation that calcium signaling is required for nuclear envelope formation and proper ER morphology.

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ER-PM contact sites essential for maintaining both ER morphology and cell signaling pathways.

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Junctions between the peripheral endoplasmic reticulum (ER) and the plasma membrane (PM) are conserved structures with important roles in calcium and phosphoinositide lipid signaling. However, little is known about additional functions for these connections or the structural components that form them. Utilizing quantitative proteomics and electron microscopy experiments in yeast, we demonstrate that the tricalbin proteins (orthologs of the extended synaptotagmins, E-Syt1/2/3, see abstract by Giordano and Saheki), Ist2 (a member of the TMEM16 family of ion channels), and the VAP proteins, Scs2/22, function to tether the cortical ER to the PM. Cells lacking all three families of tethering proteins (a yeast strain deleted for all six genes) display a striking retraction of the cortical ER from the PM, relocating most of the non-nuclear ER into collapsed cytosolic accumulations and long expanded ER sheets. Thus, these conserved ER-PM tethering proteins contribute to an elegant system of regulatory factors (including the reticulons and atlastin) that shape and organize the peripheral ER network. Consistent with previous work (Stefan et al., 2011), phosphoinositide metabolism was severely affected in cells lacking ER-PM tethering proteins, due to the loss of function of the ER-localized phosphoinositide phosphatase Sac1. In addition, the generation of a strain lacking PM-ER junctions allowed us the opportunity to investigate novel functions for these structures. Interestingly, we found that the unfolded protein response (UPR) was constitutively activated in cells lacking ER-PM junctions. Our results provide a molecular mechanism for ER-PM contact site formation and we will present new insights into the function of these organelle junctions in the control of phosphoinositide signaling pathways, as well as other essential cellular stress responses.

Stefan, C.J., Manford, A.G., Baird, D., Yamada-Hanff, J., Mao, Y., and Emr, S.D. (2011). Osh proteins regulate phosphoinositide metabolism at ER-plasma membrane contact sites. *Cell* 144, 389-401.

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Formal evidence using enucleation for the non-genomic effects of STAT5 on endoplasmic reticulum structure.

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STAT5a/b are classically known as a transcription factors that mediate estrogen and prolactin signaling through nuclear transcription. We previously showed that siRNA-mediated knockdown of STAT5a/b in a variety of cell types rapidly led to the development of a dramatic cystic change in the endoplasmic reticulum (ER) characterized by deposition of reticulon-4 and atlastin-3 along cyst membranes and tubule to cyst boundaries, as well as Golgi and mitochondrial fragmentation (Lee et al. 2012). In human pulmonary arterial endothelial cells (HPAECs) the cysts were verified to be dilatation of rough ER by EM identification of ribosomes on their cytoplasmic surface, the presence of CLIMP63 in the lumen and live-cell imaging showing luminal accumulation of KDEL-mCherry. The observation that the cystic ER phenotype developed in response to STAT5a/b siRNAs in the presence of the transcriptional inhibitor, DRB, suggested that the underlying mechanism was independent of the transcription factor function of STAT5a/b i.e. was non-genomic. We have now formally tested the requirement for the nucleus in eliciting the STAT5a/b-siRNA-induced cystic ER phenotype in adherent HPAECs in 35 mm plastic dishes using the cytochalasin B-centrifugation method of enucleation devised by Prescott (1972). In HPAECs, this method routinely yielded 60-80% enucleation as judged by DAPI staining. Enucleated HPAECs displayed normal eNOS localization, ER morphology, and a compact Golgi apparatus. Remarkably, STAT5a/b siRNAs elicited the cystic ER phenotype in the recovered HPAEC cytoplasts demonstrably lacking a nucleus. There was a striking membrane and intracystic accumulation of the ER “spacer” protein CLIMP63 in both nucleated and enucleated cells exposed to STAT5a/b siRNA. Indeed, CLIMP63 siRNA alone led to the development of a cystic ER phenotype; and the combination of STAT5a/b and CLIMP63 siRNAs led to an even further increase in the percentage of HPAECs in a culture exhibiting a cystic ER phenotype (up to 80%). These studies provide (a) formal evidence that the effects of STAT5a/b siRNA in eliciting the cystic ER phenotype are independent of nuclear function and thus are “non-genomic”, and (b) suggest structural and functional involvement of CLIMP63 in the mechanisms underlying development of the cystic ER phenotype. Supported by NIH R01 HL-087176.

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Nitric oxide scavenging induces a tubule to sheet change in endoplasmic reticulum morphology.

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Emerging studies have identified nitric oxide (NO) as a regulator of organellar structure and dynamics. We have previously shown that Golgi structure was dependent on NO in that exposure of human endothelial cells to an NO scavenger (c-PTIO) or eNOS siRNA led to Golgi fragmentation. This was blocked by prior over-expression of the DN K44A-Dynamin2 (Dyn2) mutant but not wt Dyn2, or prior treatment with dynasore. Lipton and colleagues (2011) identified increased mitochondrial fission resulting from S-nitrosylation of the dynamin-2 related protein-1 (Drp1). We now report the dependence of endoplasmic reticulum (ER) structure in endothelial cells on NO. Human pulmonary arterial endothelial cells (HPAECs) exposed to the

NO scavenger c-PTIO showed a dramatic tubule to sheet change in ER morphology as assayed using live-cell fluorescence (ER tracker, KDEL-mCherry), immunofluorescence (for RTN4 or ATL3), and electron microscopy methods. This ER change co-developed with an increase in fibrillar mitochondria, and the previously reported Golgi fragmentation. Although Golgi fragmentation was blocked by the expression of K44A-Dyn2, the sheet-dominant ER morphology persisted suggesting that NO regulates these organelles via different mechanisms. Furthermore, NO scavenging led to the accumulation of Sec31A ER exit sites adjacent to the dispersed Golgi fragments. The tubule to sheet change upon NO scavenging is likely due to posttranscriptional changes in ER structural proteins, because (a) it was observed in enucleated HPAEC cytoplasts, (b) Western blotting and immunopanning data showed that RTN4b was increasingly ubiquitinated following c-PTIO exposure, and (c) the proteasomal inhibitor MG132 also produced a tubule to sheet change in ER morphology. Thus, at least in endothelial cells, the morphology of the ER is regulated by nitric oxide. Supported by NIH R01 HL-087176.

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Mitotic ER reorganization is coupled to the cell cycle in the early *Drosophila* embryo.

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Proper alignment and segregation of the genetic material is a fundamental step during cell division. Lesser known is the proper partitioning of the cytoplasm and its organelles during mitosis. Furthermore, regulation of these organelles and their coupling to karyokinesis is poorly understood. The endoplasmic reticulum (ER), an organelle essential for cellular function, exhibits a dramatic reorganization in mitosis and is also necessary for nuclear pore complex and nuclear envelope formation during mitotic exit. The mechanism driving ER reorganization and its coupling to the cell cycle is currently unknown. Here we show that ER reorganization is directly linked to mitotic progression in the early *Drosophila* embryo. Simultaneous double stranded RNA inhibition of all three *Drosophila* mitotic cyclins (A, B, and B3) or injection of cyclohexamide prior to mitotic entry arrests the embryo in interphase and blocks ER reorganization. We also found that, ER organization can be driven into a mitotic morphology by injection of purified cyclin B. Furthermore, inhibition of the anaphase-promoting complex (APC/C) blocked chromosome segregation and arrested ER organization in a mitotic state. Additionally, small molecule disruptions of the cytoskeleton during mitosis show that changes in ER morphology are dependent upon microtubules and independent of actin filaments. Taken together, these results indicate that ER reorganization during mitosis is directly coupled to the cell cycle and is regulated by mitotic cyclins.

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Regulation of de novo phospholipid synthesis and organelle dynamics in the first division of the *C. elegans* embryo.

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A conserved role for the phosphatidic acid phosphatase Lipin in regulation of nuclear membranes has been proposed, however how Lipin's biochemical function is related to its cell biological role is not clear. Here, we used the *C. elegans* embryo to study how the structural reorganization of the endoplasmic reticulum and nuclear envelope in mitosis is linked to Lipin's role in phospholipid synthesis. In metazoans, the conversion of phosphatidic acid (PA) to diacylglycerol (DAG) by Lipin is required for the formation of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which are the major phospholipids in ER membranes. Phosphatidic acid itself is converted to phosphatidylinositol (PI) via the CDP-DAG pathway,

which makes up a smaller portion of the total ER phospholipids. While the majority of *de novo* phospholipid synthesis takes place in interphase, the ER network and NE undergo dramatic structural changes during mitosis. Lipin is highly phosphorylated and less active in mitosis, however the Lipin activating phosphatase CTD-NEP1^{SCPL-2} when complexed with NEP1R1^{SPO-7} dephosphorylates a pool of Lipin. It has been shown that partial inhibition of Lipin inhibits NE breakdown and causes abnormalities in peripheral ER structure. We found that deletion CTD-NEP1^{SCPL-2} has specific effects on ER/NE membranes that phenocopy partial Lipin depletions. CTD-NEP1^{SCPL-2} is enriched on nuclear membranes and its phosphatase activity is responsible for observed ER/NE phenotypes. Using high resolution quantitative time-lapse microscopy we find that inhibition of CTD-NEP1^{SCPL-2} activity causes premature formation of ER clusters in interphase, and delays the scission of membranes between pronuclei and lamin disassembly in mitosis. The partial delay in lamin disassembly in CTD-NEP1^{SCPL-2} deletion embryos is enhanced by perturbation of a nuclear pore complex protein (NPP-12^{gp210}) that inhibits lamina disassembly without affecting peripheral ER organization. This additive affect on lamina disassembly causes failure in cytokinesis and increased embryonic lethality. Partial inhibition of enzymes downstream of Lipin that convert DAG to PC/PE cause abnormalities in ER structure and inhibition of NE breakdown that resemble partial inhibition of lipin or deletion of CTD-NEP1^{SCPL-2}. These data indicate that either a decrease in PC/PE or an increase in the CDP-DAG pathway for PI synthesis contributes to the observed phenotypes. To test this directly, we depleted enzymes in the CDP-DAG pathway for PI synthesis in the CTD-NEP1^{SCPL-2} deletion background and found a complete reversal of observed phenotypes to wild type. Furthermore, we show biochemically that CTD-NEP1^{SCPL-2} deletion worms have increased PI levels, while PC/PE levels are not significantly changed. Taken together, we propose that activation of a pool of Lipin in mitosis by CTD-NEP1^{SCPL-2} is required to promote synthesis of PC/PE and to maintain low levels of phosphatidic acid for incorporation into PI. These data suggest that modulating PI levels during mitosis has significant effects on ER structure and on the timely disassembly of the nuclear lamina.

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Protrudin/ZFYVE27 acts antagonistically to atlastin GTPases in tubular ER network formation.

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Hereditary spastic paraplegias (HSPs) are inherited neurological disorders characterized by progressive lower limb spasticity and weakness. Though over 50 genetic loci are known (SPG1-52), over half of HSP cases are caused by pathogenic mutations in four genes encoding proteins that function directly in tubular endoplasmic reticulum (ER) network formation: atlastin-1 (SPG3A), spastin (SPG4), reticulon 2 (SPG12), and REEP1 (SPG31). Thus, understanding the cellular mechanism of ER morphogenesis has been postulated to be critical for uncovering the pathogenic mechanisms of HSP. Here we show that the SPG33 protein protrudin/ZFYVE27 interacts with tubular ER proteins through hydrophobic, intramembrane hairpin domains and functions in ER morphogenesis. Recently, lunapark proteins have been shown to alter ER morphology by counterbalancing atlastin-dependent polygon ring closure. Similar to lunapark, protrudin harbors a consensus Zn²⁺-binding site, and protrudin-depleted cells show increases in ER three-way junctions and polygon formation. While N-terminal membrane-binding domains of protrudin are responsible for ER localization, the C-terminus is essential for ER network formation. In contrast to lunapark, protrudin harbors a FYVE domain, and thus may also function in ER contacts with organelles such as endosomes.

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Atlastin-1 mutant P342S, a new mutation causing SPG3A, disrupts the ER network.*P.-P. Zhu¹, C. Blackstone¹; ¹CNU, NIH/NINDS, Bethesda, MD*

Hereditary spastic paraplegias (HSPs) comprise a group of inherited neurological disorders characterized by lower extremity weakness and spasticity. SPG3A, whose protein product is atlastin-1, is the most common gene linked to early-onset, autosomal dominant HSP. Atlastins are an evolutionarily-conserved family, with a GTPase motif near the N-terminus and two closely-spaced transmembrane domains near the C-terminus. The transmembrane domains are required for the interactions with ER-shaping proteins such as REEP1 and reticulon-4a. Atlastin GTPase activity is important for maintaining a normal tubular ER network. In addition, a highly-conserved middle domain is important for oligomerization. Here we describe a novel missense mutation (P342S) in a young girl with SPG3A. This residue is highly conserved among species and in all 3 human atlastins. Mutant atlastin-1 P342S has moderately decreased GTPase activity. Upon overexpression of atlastin-1(P342S) in cells, the ER comprises long tubes and loses its polygonal network. This abnormal morphology is the same as upon expression of the atlastin-1 GTPase mutant K80A. Interestingly, normal ER morphology can be restored in atlastin-1 (P342) overexpressing cells by co-overexpression of atlastin-1 or atlastin-2, but not with atlastin-3. In fibroblasts from the patient, atlastin-1 localization is not different from wild-type cells; in both cases it is punctate along the tubules, including at three-way junctions. In conclusion, the middle domain of atlastins is an important region for atlastin function, probably by affecting oligomerization, but also with some effect on GTPase activity. Atlastin family members can at least partially substitute for one another to maintain the ER network. That signs and symptoms of SPG3A are limited to the central nervous system may reflect not only the fact that atlastin-1 is most enriched there, but also that the very low levels of atlastin-2 in brain are inadequate to compensate for atlastin-1 dysfunction.

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ER Sheet Maintenance is coupled to the Actin Cytoskeleton in Mammalian Cells.*M. Joensuu¹, M. Puhka¹, I. Belevich¹, E. Jokitalo¹; ¹Electron Microscopy Unit, Institute of Biotechnology, University of Helsinki, Helsinki, Finland*

The endoplasmic reticulum (ER) is a large, single-copy, membrane-bound organelle consisting of an elaborate 3D network with diverse structural domains. It is composed of highly curved and tubular regions, ones that are flat and cisternal, and others that are adapted to form contacts with nearly every other organelle in the cell. The ER structure is mainly defined by the integral ER membrane proteins and by the interactions with the cytoskeleton. It is thought that the ER network is spread throughout the cell to accommodate the vast range of ER functions, but it is not known why the organelle is constantly rearranging. The comprehensive picture on how the ER positioning and spreading is maintained and what are different mechanisms employed to accomplish it, remains unclear.

The ER does not move en masse. Instead, the dynamics of ER is accomplished through network remodelling. The studies on the ER dynamics have been concentrating on the tubules and virtually nothing is known about the dynamics and the maintenance of the sheets. To understand the interplay between ER dynamics, morphology and functions, we must first understand more about the factors involved.

We have previously shown that different mammalian cell types show high variation in their sheet and tubule proportions as well as in the morphology of the sheets themselves, which can be large and fenestrated or smaller and intact (Puhka et al., 2012, MBoC, 23:2424). In the present study, we have studied the interactions between the actin cytoskeleton and ER and the dynamic behavior of ER sheets in different mammalian cell lines. We have quantified the general

changes in the ER network induced by actin depolymerisation using a morphometric assay (Puhka et al., 2007, JCB, 179:895). Our results indicate that actin does not seem to have a role on the ER tubules, but rather a maintaining role on the long ER profiles, which correspond to the sheet structures. Our live cell imaging analysis results show that the dynamics of sheets diverges from the tubular dynamics. We also show that the sheets in different mammalian cells lines respond differently to actin cytoskeleton manipulation. The results indicate that the maintenance of the peripheral ER sheets is coupled to the actin cytoskeleton, which affects the sheet persistency and lateral movement of the sheets in some of the mammalian cell types.

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Studies on Golgi biogenesis reveal self-organizing principles of the early secretory pathway.

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More than a century after the first description of the Golgi complex (GC), many aspects of the biology of this key organelle in the secretory pathway are still elusive. How it acquires and maintains its complex structure, as well as its influence in the functionality of other organelles and cellular processes are still open questions. Using laser nanosurgery to remove the GC from living cells, we have previously demonstrated that a functional and structurally normal GC can be *de novo* synthesized in mammalian cells in the absence of a centrosome (Taengemo et al., 2011).

Here, we have observed Golgi biogenesis after laser nanosurgery by high temporal resolution timelapse imaging, providing insights in the process at the light and electron microscopy level. We show that a functional Golgi complex can be *de novo* synthesized with a reproducible sequence of events. In the first phase, for several hours after Golgi removal, despite protein synthesis takes place, the export of secretory cargo from the ER is strongly inhibited. In phase 2, ER-derived carriers are occasionally formed. They move on disorganized microtubule tracks and fuse to each other when they meet. The formation of larger Golgi precursors initiates phase 3. It coincides with the arrangement of a centrally organized microtubule network and with the restoration of the full secretory activity of ER exit sites. As a result of these processes, the Golgi precursors develop into ministacks and cluster in a single perinuclear area (phase 4).

This study reveals a self-organizing nature of the early secretory pathway, and suggests that the GC could play a major role in its spatial and functional organization.

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Identification of GRASP65-interacting proteins and characterization of their roles in Golgi organization.

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The Golgi peripheral protein GRASP65 was identified as a Golgi stacking factor that links adjacent Golgi cisternae by forming mitotically regulated trans-oligomers. Further experiments indicate that GRASP65 function is regulated by proteins in the cytosol. In this study, we have applied biochemical approaches to identify GRASP65-interacting proteins. HeLa cell cytosol was subjected to fractionations using standard biochemical methods followed by affinity chromatography. Proteins that bind to GRASP65 were identified by mass spectrometry and further characterized. Among the identified proteins, clathrin heavy chain, MENA, SHIP2 and Dja1 were confirmed for their interaction with GRASP65 and recruitment to the Golgi membrane. Depletion of these GRASP65 interacting proteins by small interference RNA (siRNA) resulted in Golgi fragmentation in HeLa cells. In both GRASP65-coated bead

aggregation assay and an in vitro Golgi reassembly assay, interphase cytosol which was depleted with GRASP65 interacting proteins had reduced function in GRASP65 oligomerization and Golgi reformation comparing to non-treated interphase cytosol. Our study has identified potential candidates that play essential roles in Golgi stack formation.

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Understanding the molecular mechanism of Golgi biogenesis in *Trypanosoma brucei*.

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The Golgi apparatus is the central organelle in the secretory system, which modifies and sorts secretory cargo. Despite its crucial role in secretion, the biogenesis of this organelle is poorly understood. One question that remains unanswered concerns the source of Golgi components during biogenesis; specifically whether the newly forming Golgi uses material provided by the existing Golgi. Initial studies have provided indirect evidence to suggest this; however direct proof is not yet available.

In this project, we aim to provide direct evidence for the involvement of existing Golgi in the formation of new Golgi. The large number of Golgi stacks in mammalian cells makes it difficult to address mechanistic questions about Golgi biogenesis. To circumvent this problem, we have chosen the simple parasite *Trypanosoma brucei* to study Golgi biogenesis in detail. *T. brucei* cells have a single Golgi apparatus which is duplicated and partitioned precisely during cell division. We established stable cell lines in *T. brucei* that express Golgi components fused to optical highlighter fluorescent proteins such as photoactivatable green fluorescent protein (PAGFP). These cell lines will allow us to track Golgi components during Golgi biogenesis by live-cell microscopy. We will then determine the potential contribution of the existing Golgi to new Golgi formation and investigate whether the kinetics of the contribution of this material correlate with the kinetics of new Golgi formation.

Tracking a subpopulation proteins in the cell using photoactivatable fluorescent proteins is an excellent tool to study order of events and kinetics of organelle biogenesis. Using this approach we hope to address questions on the formation of Golgi apparatus.

Mitochondria, and Peroxisomes

2190B1011

Investigation of the Ubiquitin Proteasome System in Mitochondrial Dynamics.

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Mitochondria produce the majority of cellular energy but, paradoxically, are also the principal producers of free radicals, which can damage nucleic acids, proteins, and lipids. Mitochondria form a dynamic, reticular network that responds, through fission and fusion events, to the energy needs, redox status, and levels of oxidative stress in cells. This network has the capacity to redistribute throughout the cell on microtubules to fulfill the metabolic needs of particular intracellular structures. Recent efforts to elaborate the molecular mechanisms underlying the dynamic nature of the mitochondrial network have revealed that several mitochondrial proteins are targeted for degradation by the ubiquitin proteasome system (UPS). To further investigate the role(s) of the UPS in maintaining mitochondrial morphology, distribution, and function, we treated immortalized, human retinal pigment epithelial cells (RPE-1) with the proteasome inhibitors, MG132 or epoxomicin. These treatments induced a rapid

collapse of the mitochondrial network into perinuclear clusters. The mitochondrial redistribution was abolished when cells were pre-treated with the microtubule depolymerizing drug nocodazole, consistent with the clustering requiring microtubule-mediated transport. Immunostaining against the centrosomal marker γ -tubulin revealed that MG132-induced mitochondrial clusters were oriented around centrosomes, suggesting that inhibition of the proteasome alters the equilibrium of directional microtubule transport of mitochondria. We additionally tested the hypothesis that proteasome inhibition disrupts the balance between mitochondrial fission and fusion. To determine if fission was required for mitochondrial perinuclear aggregation in response to proteasome inhibition, we used siRNA to knock down the major fission factor, Dynamin-related protein 1 (Drp1). Drp1 depletion, however, did not abolish the perinuclear clustering, indicating that intact fission machinery is not required for the mitochondrial redistribution. Ongoing studies are focused on probing the involvement of the mitochondrial fusion machinery as well as microtubule adaptor proteins. In addition, we are pursuing the observation that proteasome inhibition does not ablate the mitochondrial membrane potential as indicated by the efficient uptake and fluorescence of MitoTracker dye into MG132-treated cells. Together, these data support the intriguing idea that the perinuclear clustering of mitochondria represents a cellular defense mechanism. This defense is designed to preserve cellular ATP production in the face of proteotoxic stress resulting from the inappropriate accumulation of proteasome substrates. Identifying the manner in which cells protect their mitochondrial integrity and function is of clinical interest as impaired proteasome activity and a compromised mitochondrial network are hallmarks of several progressive neurodegenerative disorders. Determining how mitochondria react to mismanaged protein levels is anticipated to provide fresh insights into the etiologies of these debilitating diseases.

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Mitochondrial Dynamics Regulates Behavioral Plasticity In Response to Oxygen Deprivation in *C. elegans*.

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Mitochondria are especially important for neuronal function and are known to respond dynamically to stress. Here we report that *C. elegans* neuronal mitochondria undergo fission in response to oxygen deprivation, followed by reconstitution following re-oxygenation. We find that the rate and degree of mitochondria reconstitution is modulated by the hypoxia response pathway, as mitochondria in mutants for the proline hydroxylase gene, *egl-9*, show pronounced mitochondrial "hyperfusion" in a HIF-1 dependent manner. This exaggerated mitochondrial reconstitution requires STL-1, a mitochondrial resident and likely homologue of the mammalian stomatin-like protein, SLP-2. Moreover, we find that these changes in neuronal mitochondrial dynamics are accompanied by changes in locomotory behavior of animals following oxygen deprivation and re-oxygenation. Our findings suggest that the behaviors executed by a simple neural circuit can be regulated through changes in mitochondrial dynamics. In addition, we speculate that adaptive mitochondrial dynamic and behavioral responses to oxygen status can be preconditioned for the nematode, perhaps explaining the extraordinary tolerance of *C. elegans* to extreme changes in oxygen supply. The underlying mechanism might provide valuable insights into the resistance or sensitivity of mammalian neurons to oxygen deprivation stress.

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Biochemical characterization of Pcp1p, a mitochondrial rhomboid protease.

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Pcp1p is a mitochondrial rhomboid protease that localizes to the mitochondrial inner membrane in the budding yeast *Saccharomyces cerevisiae*. *PCP1* codes for a 346 amino acid polypeptide having a cleavable N-terminal mitochondrial-targeting signal followed by six transmembrane alpha-helices. The rhomboid-like active site motif containing the catalytic serine and histidine residues are located in transmembrane helices four and six. Haploid yeast cells deleted for *PCP1* are viable, however they have a slow growth phenotype, are unable to grow on non-fermentable carbon, and exhibit abnormal mitochondrial morphology. Two different substrates have been identified for Pcp1p: Ccp1p (cytochrome c peroxidase) and Mgm1p (mitochondrial genome maintenance). Ccp1p localizes to the mitochondrial intermembrane space and functions to remove hydrogen peroxide and other free radicals generated during respiration; Mgm1p, is a dynamin-related GTPase required for mitochondrial fusion. Recent work has shown that in addition to Pcp1p peptidase activity, Mgm1p processing depends on matrix ATP levels and the Tim23-Pam translocation complex. This dependence suggests a functional link between Pcp1p and the inner membrane protein translocation machinery. As an initial attempt to show a physical association between Pcp1p and members of the Tim23 translocon, a co-immunoprecipitation approach was taken. PCR mediated techniques were used to modify genomic copies of *PCP1*, *TIM23*, *TIM21*, and *PAM18* with sequences coding for 13Myc, 3HA and 3FLAG epitope tags. Preliminary western blot analyses suggest that there is a weak interaction between Pcp1p and components of the Tim23 translocon machinery that is sensitive to different detergents. Unfortunately this interaction was not detected by sucrose gradient ultracentrifugation, although we have determined that Pcp1p exists in a higher order protein complex of ~200kD. Future experiments include a cross-linking approach to stabilize what is likely to be a transient interaction between Pcp1p and the translocon machinery as well as identification of the protein components of the ~200kD Pcp1p complex.

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Genetic insight into the function of the *S. cerevisiae* mitochondrial rhomboid protease, Pcp1p.

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As the central source of energy production in eukaryotic cells, mitochondria are essential for cell viability. One feature that has emerged as a factor impacting mitochondrial activity is organelle morphology. A number of proteins have been identified for their role in controlling mitochondrial dynamics including the mitochondrial rhomboid peptidases, a conserved family of intramembrane serine proteases. The *S. cerevisiae* mitochondrial rhomboid, Pcp1p, localizes to the mitochondrial inner membrane and has been shown to be involved in the proteolytic cleavage of two substrates: Ccp1p and Mgm1p. Ccp1p (cytochrome c peroxidase) is an intermembrane space localized protein involved in the breakdown of reactive oxygen species while Mgm1p (mitochondrial genome maintenance) is critical for mitochondrial fusion-fission based events. Haploid cells deleted for *PCP1* have fragmented mitochondria and are unable to grow when provided with a nonfermentable carbon source. The fact that similar phenotypes have been found for *MGM1* mutants coupled with the finding that Pcp1p cleavage of Mgm1p is required for mitochondrial fusion suggests that Mgm1p processing is the key biological function for Pcp1p. In addition to Pcp1p activity, Mgm1p processing is dependent on mitochondrial ATP

levels and components of the Tim23 translocation machinery. As an initial approach to determine the structural basis of Pcp1p activity and to determine whether Pcp1p function requires an association with additional proteins, we have designed a screen to identify temperature sensitive alleles of *PCP1* generated by hydroxylamine mutagenesis. To date we have screened over 30,000 colonies and have identified four mutants that exhibit a) a temperature sensitive growth profile, b) a respiratory growth defect, c) a defect in Mgm1p and Ccp1p processing, and d) abnormal mitochondrial morphology. Interestingly, the steady state levels of Pcp1p for all four mutants were similar to wild type. All four mutations are a result of single amino acid changes within the fourth and sixth transmembrane domains of Pcp1p, the location of the active site serine and histidine, respectively. Future time-course experiments are designed to better understand the defects associated with these mutants.

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A temperature sensitive screening approach to map amino acids required for Pcp1p activity.

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Pcp1p is a mitochondrial serine protease known to be involved in regulating mitochondrial dynamics and apoptosis. In *Saccharomyces cerevisiae*, Pcp1p is required for the proteolytic cleavage of two substrate proteins: Ccp1p and Mgm1p. Ccp1p (cytochrome c peroxidase) localizes to the mitochondrial intermembrane space and is involved in the elimination of reactive oxygen species. One hundred percent of the Ccp1p polypeptide is cleaved by Pcp1p to generate the mature form of this enzyme. Mgm1p (mitochondrial genome maintenance) is responsible for mitochondrial fusion and fission based events that control mitochondrial morphology. Mgm1p is found in two forms, a large and a small isoform. Pcp1p peptidase activity is responsible for converting the long Mgm1p isoform into the short isoform. On average, approximately half of the Mgm1p present in a cell is processed to the short form; the remaining exists as the long isoform. Overall, our aim is to understand the factors that influence this apparent differential processing of substrate proteins by Pcp1p with the goal of identifying and characterizing potential accessory components. As an initial attempt to identify domains outside of the Pcp1p active site required for function, we have designed a screen to isolate both nonfunctional and temperature sensitive alleles of *PCP1*. Plasmid borne *PCP1* was randomly mutagenized with exposure to hydroxylamine and transformed into our *PCP1* shuffle strain. Colonies were then replica printed to plates containing nonfermentable carbon to identify interesting *pcp1* candidates. To date, we have screened ~36,000 colonies and identified five *pcp1* alleles that eliminate Pcp1p activity and four alleles that function as a 'knockdown' of Pcp1p function. The majority of these single amino acid changes map to the fourth and sixth transmembrane helices of Pcp1p, the location of the active site serine and histidine, respectively. Future experiments are designed to determine how these amino acid changes impact Pcp1p activity including changes in Ccp1p and Mgm1p processing and mitochondrial morphology.

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Mito Tracker labeling in HepG2 Cell Cultures: Influence of different staining techniques on the morphology of mitochondria.

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MitoTracker (MT) probes passively diffuse mitochondrial membrane and accumulate in active mitochondria. This dye is useful for the study of morphology and function of mitochondria on live cells. There are different techniques to visualize mitochondria by use of this stain. These techniques are being used at 37 C chambers. Some techniques use carbondioxide incubator, while others use incubators with no carbondioxide. The dye is being diluted in the medium in several techniques, while the others use phosphate buffer saline for dilution. The aim of this study was to analyze if these different techniques effect the morphology of mitochondria.

HepG2 cells, human hepatocellular carcinoma cell line (ATCC), were seeded on glass coverslips. They were grown in Minimum Essential Medium (ATCC) which contain 10% fetal serum bovine. When they became 70% confluent, they were stained with MitoTracker (Invitrogen/Molecular Probes). There were four experimental groups: I. Group: Cells were stained with MT in medium in a carbondioxide incubator. II. Group: Cells were stained with MT in medium in an incubator without carbondioxide. III. Group: Cells were stained with MT in phosphate buffer solution in a carbondioxide incubator. IV. Group: Cells were stained with MT in phosphate buffer solution (PBS) in an incubator without carbondioxide. The cells from each group were analysed by using Zeiss Axioscope fluorescence microscope and Nikon Eclipse 90i confocal microscope.

Our results showed that the cells were containing mitochondria with classical rode shapes when they were incubated in medium in a carbondioxide incubator. When the cells were incubated in an incubator without carbondioxide, the mitochondria became elongated. The mitochondria in the groups which were stained with MT in PBS also showed alterations in the shape.

In conclusion, the different staining techniques with MT on live cells might effect the morphology of the mitochondria. This information is especially important for the studies related with the morphological changes in mitochondria under certain circumstances.

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Genetic interaction between mitochondrial fission and fusion factors.

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Mitochondria dynamics are regulated by constant fission and fusion and play important roles in many biological processes such as apoptosis and autophagy. The essential fission gene Drp1 and fusion gene Opa1 have been extensively studied but understanding the molecular mechanisms underlying their functions have been hampered by the lack of knockout human cell lines. In this study, we used Talen technology to knock out Drp1, Opa1 and other previously reported mitochondrial fission genes in HCT116 cells. As in RNAi studies, both mitochondria and peroxisomes are excessively elongated in Drp1 KO cells. In contrast, mitochondrial morphology and Drp1 recruitment remain normal whereas peroxisomes are elongated in Mff KO, Fis1/Mff DKO and Opa3/Fis1 DKO cells, indicating that: 1) Mff is essential for peroxisome fission but is dispensable for mitochondrial fission; 2) Mff is not the sole receptor for Drp1 recruitment; 3) Fis1 and Opa3 are not redundant to Mff. Opa1 KO cells display fragmented mitochondria but knocking out Drp1 in the Opa1 KO background leads to highly tubular mitochondria, suggesting the existence of Opa1-independent fusion events. Unlike in yeast,

Opa1 deficiency in Drp1 KO cells fails to restore the mitochondrial morphology back to wild type since the Opa1/Drp1 DKO cells exhibit mitochondrial morphology similar to that of Drp1 single KO, indicating that Drp1 is epistatic to Opa1. However, whereas Drp1 KO cells are resistant to apoptosis, Opa1/Drp1 DKO cells are as hypersensitive to ABT-737 treatment as Opa1 KO cells, suggesting that Opa1 is epistatic to Drp1 in controlling cell death and Drp1 inhibits cytochrome c release through Opa1. Whether Drp1 is involved in the disassembly of Opa1 oligomeric complex is under investigation.

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Roles for tissue-specific ATP synthase subunits in mitochondrial shaping.

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During *Drosophila melanogaster* spermatogenesis, mitochondria aggregate and fuse in post-meiotic spermatids to form the Nebenkern, a spherical structure consisting of two interwoven giant mitochondrial derivatives. Males homozygous for *ms(2)1400* are sterile and show defective internal structure of the Nebenkern, which then cannot disentangle and elongate properly. We mapped *ms(2)1400* to a region containing a candidate gene with testis-enriched expression that encodes an unusually large predicted homolog of ATP synthase subunit d. RNAi knockdown of the candidate gene phenocopied *ms(2)1400*. Analysis of the *Drosophila* genome revealed that three other ATP synthase subunits in the peripheral stalk of the complex have predicted paralogs with testis-enriched expression. RNAi knockdown of these genes in the testis gave Nebenkern defects similar to those seen in *ms(2)1400*. In other systems, the peripheral stalk of ATP synthase is known to mediate higher-order dimerization of the complex, in turn influencing inner mitochondrial membrane curvature and cristae structure. Our results demonstrate ATP synthase involvement in Nebenkern shaping that is consistent with what is known about ATP synthase dimerization and cristae formation in these other systems. These novel findings connect tissue-specific mitochondrial shaping with differential expression of ATP synthase subunit paralogs.

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Uncoupling Protein 2 Controls Mitochondrial Network Fragmentation and Premature Senescence via Regulating Superoxide-mediated p53 Activation.

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Background - Mitochondria, although required for cellular ATP production, are also known to have other important functions that may include modulating cellular responses to environmental stimuli. Most mitochondrial actions are linked to the electron transport chain and the resultant mitochondrial membrane potential ($\Delta\psi_m$), which is involved in ATP synthesis and proton leak. Although the latter is largely regulated via uncoupling proteins, the mechanisms whereby uncoupling proteins impact cellular phenotype are not yet clear.

Methods and Results - We report here that stimuli for endothelial cell proliferation evoke strong upregulation of mitochondrial uncoupling protein 2 (UCP2) in culture. Analysis *in silico* indicated increased UCP2 expression is common in highly proliferative cell types, including cancer cells. Upregulation of UCP2 was critical for controlling $\Delta\psi_m$ and superoxide production. In the absence of UCP2, endothelial growth stimulation provoked mitochondrial network fragmentation and premature senescence via a mechanism involving superoxide-mediated p53 activation.

Mitochondrial network fragmentation was both necessary and sufficient for the impact of UCP2 on endothelial cell phenotype. We also examined human endothelial cells from patients with type 2 diabetes—a condition associated with excess mitochondrial superoxide, and found a reduced UCP2 expression and increased mitochondrial fragmentation in the endothelial cells as well as impaired endothelial function of the patients as determined by flow-mediated dilation.

Conclusions - We demonstrated that endothelial cell proliferation and angiogenesis require upregulation of UCP2 in order to reduce $\Delta\psi_m$ and limit mitochondrial superoxide that otherwise promotes p53-dependent mitochondrial fragmentation resulting in premature senescence. Our data also indicate an important role for UCP2 in determining the endothelial cell function of diabetic patients. These findings reveal a new function for UCP2 that has broad implications for processes that involve the function of endothelial cells.

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Asymmetric inheritance of mitochondrial content in aging budding yeast cells.

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Mitochondria must grow with the growing cell to ensure proper physiology and inheritance upon division. But how does mitochondrial content scale with cell size during asymmetric division? Is the same mitochondrial to cell size ratio maintained uniformly in the cell to produce identical mother and daughter cells? Or is there an asymmetry in the inheritance of mitochondrial content? We measured the physical size of mitochondrial networks by computationally skeletonizing the mitochondrial tubules in 3D. We found mitochondrial inheritance to be very asymmetric; newborn daughters inherited a greater mitochondrial to cell volume ratio than their mothers, who lost mitochondrial content during the budding process. By counting bud scars to measure mother generational age, we found that aging mothers progressively lost mitochondrial volume ratio over successive generations. However, newborn daughters continued to inherit the same average volume ratio. This mother-daughter asymmetry could be enhanced or abolished by altering the expression levels of Ypt11p and Mmr1p, representing two independent mitochondrial inheritance pathways. However the final volume ratio inherited by the newborn daughters remained the same as wild-type cells, suggesting that the mitochondrial volume ratio in the bud is actively regulated by the cell. The cell size of newborn daughters and of the population as a whole, however, decreased for mutants with delayed and increased for mutants with enhanced mitochondrial inheritance. Due to the initial delay in redistribution into the bud, $\Delta ypt11$ mothers inherited a greater volume ratio than wild-type mothers. Over successive generations this resulted in a dramatic decrease of the age-dependent loss of mitochondrial volume ratio in $\Delta ypt11$ cells. Replicative lifespan analysis of $\Delta ypt11$ cells revealed two populations, one short lived, absent in wild-type and one with a longer maximal lifespan than wild-type. Because the difference in volume ratio between wild-type and $\Delta ypt11$ mothers increases with age, the long-lived population represents $\Delta ypt11$ cells with an increased mitochondrial volume ratio compared to wild-type. We are now investigating whether the short-lived population has a decreased volume ratio compared to longer-lived cells. Our results suggest that “mitochondrial content asymmetry” may contribute to the health and aging of cells.

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Lithocholic acid delays aging and exhibits a potent anti-tumor effect by altering mitochondrial composition, structure, and function.

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We use the yeast *Saccharomyces cerevisiae* as a model to study the molecular mechanisms by which age-related changes in mitochondrial membrane lipids regulate longevity. We found that an exogenously added bile acid called lithocholic acid (LCA) extends yeast chronological lifespan and accumulates in the inner mitochondrial membrane (IMM). To define the mechanism by which LCA extends yeast longevity, we 1) examined how LCA influences mitochondrial proteome and lipidome; 2) investigated the effect of LCA on the composition and stoichiometry of respiratory complexes and supercomplexes in the IMM; 3) assessed how LCA affects mitochondrial oxygen consumption, membrane potential and reactive oxygen species (ROS); 4) examined how LCA influences mitochondrial morphology and how it affects the chronology of events characteristic of age-related, mitochondria-controlled apoptosis; and 5) investigated the effect of LCA on the lifespans of long- and short-lived mutants lacking individual components of the mitochondrial fission and fusion machines. Our findings imply that LCA delays yeast aging by increasing the level of phosphatidylserine (PS) and decreasing the levels of phosphatidylethanolamine (PE) and cardiolipin in the IMM. By altering the abundance of these lipid species, LCA greatly expands mitochondrial membrane cristae. In addition, LCA enhances the positive effect of PS and weakens the negative effect of PE on membrane protein machines whose activity they modulate – thereby 1) stimulating protein machines driving mitochondrial respiration, the maintenance of mitochondrial membrane potential and ROS homeostasis, and mitochondrial fusion; and 2) inhibiting protein machines promoting mitochondrial fission and mitochondria-controlled apoptosis. We found that LCA also functions as a potent and selective anti-tumor compound in cultured human neuroblastoma, glioma and breast cancer cells by modulating mitochondrial processes playing essential roles in both cancer and aging. The effects of LCA on these processes seen in cancer cell cultures are opposite of those observed in non-cancerous human cells and in chronologically aged, quiescent yeast.

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Amyloid beta-induced structural alterations of subcellular organelles in Alzheimer's disease.

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Alzheimer's disease (AD) is a progressive neurodegenerative disease and the neuropathologic hallmark of AD is deposition of aggregated amyloid- β (A β). The morpho-functional abnormality of subcellular organelles caused by A β deposition may play important roles in AD pathogenesis. However, molecular and ultrastructural basis for degenerative process of subcellular organelles and ultimate neuronal death in AD still remains unclear. We have performed immunocytochemical and ultrastructural analyses using fluorescence microscopy, conventional electron microscopy, high voltage electron microscopy, and three-dimensional electron tomography in animal- and cell-model for AD to understand how alteration of subcellular organelles are associated with the progression of AD. In the APP/PSEN1 transgenic mice that express human amyloid precursor protein, the deposition of A β plaques and anti-LC3 antibody positive autophagosome were detected in the hippocampus and cortex regions. We also identified the loss of peroxiredoxin 5, an endogenous cytoprotective antioxidant enzyme, and the accumulation of A β in the hippocampal mitochondria of transgenic mice with severe

disruption of mitochondrial cristae. In the murine microglial cell line, BV-2, stimulated A β 1-42 fibrils, which polymerized in *in vitro*, were localized in the periphery of the nucleus as a filamentous structure. In addition, A β -positive signals were detected on structurally deteriorated mitochondria and Golgi complex as similar with APP/PSEN1 transgenic mice. Concomitantly, autophagosomes containing fragmented membraneous compartments of mitochondria and Golgi complex were abundant in cytoplasm of BV-2 cell. These results acquired from *in vivo* and *in vitro* studies provide the evidence that the A β -induced morpho-functional alterations of subcellular organelles and relative accumulation of autophagosomes are closely associated with the neurodegenerative process in AD.

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p62/SQSTM1 promotes the expression of mitochondrial genes in response to increased autophagy.

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Maintaining a pool of functional mitochondria in the cell requires coordinate generation and degradation of these organelles. While several factors have been involved in controlling mitochondrial biogenesis and mitophagy in mammals, little is known about how these two processes are linked together to maintain mitochondrial homeostasis. Using primary human fibroblasts and the autophagy-inducing drug rapamycin, we attempted to define a connection between clearance and renewal of the mitochondrial pool. Stimulating autophagy with rapamycin increased mitochondrial mass and promoted the expression of mitochondrial genes. Chronic exposure to rapamycin also reduced the half-life of the integral mitochondrial protein VDAC, increased mitochondrial membrane potential, and reduced the generation of reactive oxygen species. Furthermore, cells exposed to rapamycin had higher resistance to exogenous stress and delayed the onset of cellular senescence concomitant with a decrease in the p38MAPK stress response pathway activation. These effects appeared to be mediated in part by increased interaction between Keap1 and p62/SQSTM1, a protein involved in the clearance of damaged mitochondria: this increased association promoted nuclear accumulation of the Nuclear Factor Erythroid 2 Like 2 (NFE2L2) in rapamycin treated cells, and increased expression of the Nuclear Respiratory Factor 1 (NRF1) and the mitochondrial Transcription Factor A (TFAM), a protein necessary for mitochondrial genome replication and transcription. Interestingly, p62/SQSTM1 turnover was increased upon exposure to rapamycin, and knockdown of p62/SQSTM1 abrogated the effects of rapamycin on mitochondrial membrane potential and stress resistance, as well as the induction of NFE2L2, NRF1, and TFAM. These results suggest that p62/SQSTM1 coordinates clearance and renewal of mitochondria, and play a key role in maintaining cellular homeostasis.

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Hypertonia-associated mutation impairs Trak1 mitochondrial localization.

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Hypertonia is a pathophysiological condition caused by defects in motor pathways in the central nervous system, resulting in postural abnormalities, jerky movements, and tremor. Hypertonia is associated with a number of neurological disorders, including cerebral palsy, dystonia, Parkinson's disease, stroke, and epilepsy. The molecular mechanisms that trigger the pathogenesis of hypertonia remain poorly understood. The finding that a homozygous

truncation mutation in the trafficking protein, kinesin-binding 1 (Trak1) protein causes a recessively transmitted form of hypertonia in mice highlights the importance of understanding the mechanisms of Trak1 action and the effects of hypertonia-associated Trak1 mutation. We have shown that Trak1 is a ubiquitously expressed protein that localized to both mitochondria and early endosomes. Our subcellular fractionation and immunofluorescence confocal microscopic analyses reveal that the mitochondrial localization of Trak1 is impaired by hypertonia-associated Trak1 mutation. Further characterization of the structural and functional consequences of hypertonia-associated Trak1 mutation will provide new insights into the pathogenic mechanisms of hypertonia.

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MITRAC complexes link mitochondrial protein translocation to respiratory chain assembly and translational regulation.

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The bulk of cellular ATP for most eukaryotic cells is generated by the mitochondrial oxidative phosphorylation system, comprising five transmembrane multi-protein complexes. Defects in function or biogenesis of these key metabolic enzymes have been shown to generate deleterious amounts of reactive oxygen species and are among the most frequent causes for mitochondrial disorders.

The mitochondrial respiratory chain complexes assemble from subunits of dual genetic origin, synthesized both on cytosolic as well as mitochondrial ribosomes. This exceptional situation requires well-balanced and sophisticated regulation of protein expression, transport, and integration of subunits into the inner mitochondrial membrane, where they assemble to form oligomeric complexes. These processes are assisted by a plethora of specific assembly factors, defects of which are the main cause of mitochondrial encephalomyopathies. In yeast, intricate mechanisms are in place to regulate the synthesis of core mitochondrial subunits in response to metabolic needs as well as the assembly status of core subunits themselves. However, due to circumstantial evidence the existence of such mechanisms in mammals has been neglected.

Here, we report a comprehensive dissection of the major human cytochrome c oxidase assembly intermediates by biochemical and mass-spectrometric approaches. Combining native gel electrophoresis with quantitative mass-spectrometry enabled us to define novel intermediates containing newly mitochondria-synthesized and imported respiratory chain subunits, which we term MITRAC complexes. Further biochemical characterization shows that these complexes consist of novel as well as known assembly factors that feedback-regulate mitochondrial translation of central structural subunits, revealing the conservation of this regulating mechanism. Since known constituents of the MITRAC complexes have been implicated in mitochondrial encephalomyopathies, the newly-identified assembly factors are exciting candidates for novel disease genes.

A central yet unresolved question in respiratory chain complex assembly is by which mechanism imported subunits are delivered to assembly-intermediates containing mitochondria-encoded subunits. We find that MITRAC20 is an integral component of the inner membrane presequence translocase. MITRAC20 is dispensable for protein import, but required for assembly of early-assembling presequence-containing subunits into MITRAC complexes. These findings establish an unexpected molecular link between the TIM23 transport machinery and assembling respiratory chain complexes that regulate mitochondrial protein synthesis in response to their assembly-state.

2205B1026

Mitochondrial mistargeting causes autosomal dominant renal Fanconi syndrome.

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Patients with renal Fanconi syndromes show a generalized renal proximal tubular functional defect leading to glucosuria, aminoaciduria, phosphaturia, metabolic acidosis, and small molecular weight proteinuria.

For this study the causative genetic factor as well as the underlying molecular pathology was unraveled in an large family with autosomal dominantly inherited renal Fanconi syndrome without kidney failure. To this end whole genome multipoint parametric linkage analysis was performed resulting in a significant LOD score (3.6) for a single locus. All genes in the linked area were sequenced resulting in the identification of a heterozygous mutation (p.E3K) in a gene, which we called Fanconin. This mutation results in a de-novo formation of a mitochondrial targeting motif as it was predicted with Protein Prowler 1.2. This mutation was not found in ethnically matched controls. To assess the functional impact of the mutation stable permanently transfected cell lines were generated expressing either the mutant or wild type Fanconin protein. These cell lines were obtained from the human cell line TSA201 (HEK cell derivative) and the pig cell line LLC-PK1, the latter which is a renal proximal tubular cell model. Immunohistochemical analysis using these cells showed appropriate intracellular localization for the wild type human Fanconin and mistargeting for human mutant Fanconin to the mitochondria. Immunohistochemical analysis was performed for mouse and human kidneys to investigate the expression of Fanconin. Our results indicated that Fanconin is expressed in renal proximal tubules which coincides with the proximal tubular dysfunction in patients with renal Fanconi syndrome.

Knockout mice for Fanconin, as expected, did not show a proximal tubular transport defect as assessed by aminoacid analysis using GC-MS. We therefore hypothesized that the renal Fanconi phenotype in our family is not caused by haploinsufficiency.

Since mutant Fanconin is localized to mitochondria the impact of this mutation on mitochondrial function was investigated. Respirometric analysis using a high-resolution respirometer showed an impaired oxidative phosphorylation in the mutant Fanconin expressing cells. Transport studies documented abrogated transepithelial transport of water.

2206

Atypical mitochondrial fission upon Listeria infection.

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The bacterial pathogen *Listeria monocytogenes* causes fragmentation of the mitochondrial network through the secreted pore-forming toxin listeriolysin O (LLO) early during infection and the efficiency infection is affected in host cells with perturbed mitochondrial dynamics (Stavru, PNAS (2011) 108:3612-7). Here, we examine mechanistic aspects of LLO-induced mitochondrial fragmentation and find that, surprisingly, it does not depend on key mitochondrial dynamics factors, such as Drp1 and Opa1. LLO-induced mitochondrial fragmentation differs drastically from uncoupler-induced mitochondrial fragmentation and therefore seems atypical. Furthermore, Drp1 appears to dissociate from mitochondria upon LLO-induced fragmentation due to degradation of its recently described receptor Mff. Collectively, our data shows a new mode of regulation for the mitochondrial fission protein Drp1.

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Standardized mitochondrial analysis gives new insights into mitochondrial dynamics and OPA1 function.

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Mitochondria are dynamic organelles that adapt to cellular requirements by changing their shape through processes of fission and fusion to form tubular networks. Defective mitochondrial metabolism is associated with a wide range of human diseases covering most medical subspecialties. The increasing importance of defective mitochondrial dynamics in several diseases including common neurodegenerative disorders calls for the development of reference imaging tools for medical investigation and drug screening. Current approaches to study mitochondrial morphology are limited by low data sampling coupled with manual identification and classification of complex morphological phenotypes. We have therefore normalized the quantification of mitochondrial dynamics by means of micropatterned (from CYTOO SA) designed to standardize the size and shape of cultured fibroblasts. In addition, we present a novel method of imaging mitochondrial networks and nucleoid distribution in 3D, in models of live or fixed cells. Our approach was validated by using fibroblasts with mutations in the OPA1 and MFN2 genes, known to be involved in mitochondrial fusion, as well as with drugs inducing mitochondrial uncoupling which elicited structural mitochondrial defects such as changes in total mitochondrial volume, connectivity of the network, number of mitochondrial branch points, and mitochondrial organization in general. Our results showed that mitochondria emerging from the microtubule organizing centre may be crucial to mitochondrial biogenesis since it appeared to be protected against mitochondrial fragmentation induced by OPA1 mutations. This imaging investigation of mitochondrial dynamics opens new perspectives for establishing the relationship between the description of mitochondrial subcellular distribution and specific experimental and pathological conditions.

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Functional characterization of the role of Pex19p in peroxisome biogenesis in *Pichia pastoris*.

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One aspect of the study of peroxisome biogenesis seeks to understand the underlying processes involved in the initial formation, maturation, and proliferation of peroxisomes. Two peroxins, Pex3p and Pex19p, have essential functions in peroxisome biogenesis; cells lacking Pex3p or Pex19p have no detectable peroxisomes. Pex19p has an N-terminal Pex3p-binding domain and a C-terminal domain known to interact with the membrane peroxisomal targeting signal (mPTS) of other peroxisomal membrane proteins (PMPs). Previous studies have implicated Pex19p in the post-translational insertion of PMPs in the peroxisomal membrane. It was hypothesized that Pex19p is involved in binding and stabilizing newly synthesized mPTS-containing PMPs in the cytoplasm, transporting them to the peroxisomal membrane, where Pex19p docks by its interaction with Pex3p. The interaction between Pex3p and Pex19p is therefore crucial for peroxisome formation. However, recent studies have highlighted the importance of the endoplasmic reticulum (ER) in the *de novo* synthesis of peroxisomes, which proposes a focused assembly of PMPs in a specialized domain of the ER, referred to as the pre-peroxisomal ER. It was shown that PMP-containing pre-peroxisomal vesicles bud from this

domain in a Pex19p-dependent manner and mature by importing matrix proteins sequentially from the cytosol, in a process requiring Pex3p. Nevertheless, the mechanism by which Pex19p promotes the budding process is currently unknown. In support of this novel model of Pex19p function, we have characterized several Pex19p N and C-terminal deletions to determine the minimal structural requirement of the peroxin that supports proper PMP and peroxisome assembly from the ER. As expected, deletions in the N-terminal Pex3p binding site disrupted Pex19p's interactions with Pex3p but preserved interactions with Pex12p, a mPTS-containing PMP known to bind the C-terminal end of Pex19p. In contrast, deletions in the C-terminal mPTS-binding domain of Pex19p disrupted the interaction between Pex19p and Pex12p, while leaving Pex19p-Pex3p interactions intact. However, to our surprise, import competent peroxisomes were still formed in both sets of deletions. Finally with a sequential deletion analysis, we determined a stretch of 61 amino acids as the minimum structural requirement for peroxisome biogenesis. Interestingly, these amino acids of Pex19p lack the classical Pex3p and mPTS binding sites. In conclusion, physical segregation of the Pex3p and PMP binding domains of Pex19p has provided novel insights into the role of a new domain of Pex19p in peroxisome biogenesis.

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Comparison of two expression systems using COS7 cells and yeast cells for expression of heart/muscle-type carnitine palmitoyltransferase 1

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Carnitine palmitoyltransferase 1 (CPT1), catalyzing the transfer of the acyl group from acyl-CoA to carnitine to form acylcarnitine, is located at the outer mitochondrial membrane. Because it is easily inactivated by solubilization, expression systems using living cells are essential for its functional characterization. COS7 cells or yeast cells are often utilized for this purpose; however, the advantages/disadvantages of the use of these cells or the question as to how the CPT1 enzyme expressed by these cells differs are still uncertain. In this study, we characterized the heart/muscle-type isozyme of rat CPT1 (CPT1b) expressed by these two cellular expression systems. The mitochondrial fraction prepared from yeast cells expressing CPT1b showed 25% higher CPT1 activity than that obtained from COS7 cells. However, the expression level of CPT1b in the former was 3.8 times lower than that in the latter; and thus, under the present experimental conditions, the specific activity of CPT1b expressed in yeast cells was estimated to be approximately five times higher than that expressed in COS7 cells. Possible reasons for this difference are discussed.

Endocytic Trafficking III

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ADAM17 is the central modulator of A1 adenosine receptor-mediated EGFR transactivation and apical exocytosis in bladder umbrella cells.

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When exposed to stretch, umbrella cells undergo two sequential bouts of apical exocytosis: the first, is rapid and independent of protein synthesis, whereas the second "late phase" is slower to initiate, requires protein synthesis, and is triggered by transactivation of the epidermal growth factor receptor (EGFR). In this process, a proteinase cleaves the EGFR ligand HB-EGF,

stimulating EGFR activation and mitogen-activated protein kinase (MAPK)-dependent protein synthesis. The steps that lead to EGFR transactivation, and evidence for other stimuli that trigger the late phase exocytic response are unknown or unavailable. Here, we report that activation of apically localized A1 adenosine receptors (A1ar) using the agonist CCPA also stimulated exocytosis at the apical surface of umbrella cells, akin to the “late phase” stretch response. The HB-EGF specific inhibitor CRM-197, the EGFR inhibitor AG1478, the MAPK inhibitor U0126, and the protein synthesis inhibitor cycloheximide inhibited this effect. Furthermore, AG1478 blocked CCPA-stimulated phosphorylation of the EGFR tyrosine residue 1173 (Y-1173), indicating that they acted upstream of EGFR activation. Thus we report a bona fide EGFR transactivation pathway triggered by A1ar. Interestingly, we found that activation of PKC by PMA stimulated a similar exocytic response in these cells. A known effector of PKC and a critical mediator of EGFR transactivation in other cells types is A Disintegrin and a Metalloproteinase 17 (ADAM17). We observed this proteinase to be expressed in vesicles at the apical domain of umbrella cells and upon activation by PMA ADAM17 redistributed to the apical surface. A broad-spectrum metalloproteinase inhibitor GM6001 and an ADAM17 selective inhibitor Tapi-2 blocked CCPA-mediated exocytosis. Finally, using an exogenously expressed human growth hormone (hGH) assay system, we found that hGH is released from the apical surface upon treatment with CCPA and remarkably silencing the expression of ADAM17 using adenoviral-based in situ shRNAs completely blocked CCPA-induced hGH release. We propose a model whereby activation of Gi-protein coupled A1ar triggers PLC-mediated generation of IP₃, which increases intracellular calcium and leads to PKC-mediated activation of ADAM17. In turn, activated ADAM17 cleaves HB-EGF and triggers EGFR activation leading to MAPK-mediated protein synthesis and apical exocytosis.

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A directed RNAi screen for *C. elegans* TGF- β -related DBL-1 trafficking components reveals non-synaptic vesicle machineries in neurons.

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Transforming Growth Factor- β (TGF- β) superfamily members are secreted morphogens that require precise regulation for normal development. Here we report results from a directed RNA inhibition (RNAi) screen we performed to identify gene products required for normal localization of green fluorescent protein (GFP)-tagged *Caenorhabditis elegans* TGF- β member DBL-1. We generated a *C. elegans* screening strain expressing functional, GFP-tagged DBL-1 that is sensitized to the effects of RNAi by an *rrf-3* mutation, which removes endogenous RRF-3/RNA-directed RNA polymerase (RdRP) from the animal. Because our screening strategy is designed to eliminate potential embryonic lethality upon RNAi inhibition of gene function, we were able to identify late roles in TGF- β localization for genes for which early lethal phenotypes were previously identified. We then used this strain to screen an RNAi library for genes required for proper DBL-1 localization *in vivo*.

We discovered that DBL-1 localization is punctate within the nerve cord neurons that secrete DBL-1, and these punctae are not synaptic vesicles. We found that mouse BMP4, another TGF- β superfamily member, has the same function and localization pattern as DBL-1 in *C. elegans*. This localization depends on other TGF- β pathway members in the receiving cell and extracellular matrix constituents. Specific cellular machinery components involved in ubiquitination, sumoylation, and progression of endosomes to lysosomes, specifically Endosomal Sorting Complex Required for Transport (ESCRT) machinery, the adaptor protein (AP) 3 complex, and specific Rab GTPases, are also required for tight localization of GFP-tagged DBL-1. Furthermore, Wnt pathway members affect the localization of GFP-tagged DBL-

1. Reduction of these screen isolates' function by RNAi resulted in spreading of GFP-DBL-1-positive punctae away from neurons. These results provide insights into the cellular mechanisms regulating TGF- β localization and regulation in a living organism.

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Formin-driven actin polymerization is involved in regulation of early endosome dynamics.

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Microtubule- and actin-dependent transport are central to many aspects of cellular homeostasis. Recent work has proposed that formins modulate the dynamics of both the actin and microtubule (MT) cytoskeletons, but their effect on MT-dependent organelle movement remains relatively unexplored. Here, we chose endocytic organelles as a model cargo, defined by the presence of the fluorescently tagged GTPase Rab5. To date, there are some studies that have provided insight into how formins (known to be potent activators of actin polymerization) might regulate endosome dynamics, however, these works have obtained information at low temporal resolution or in fixed cells. We have used computer-based automated particle tracking, with high temporal resolution (33 frames/sec) and a systematic analysis to describe the involvement of formin proteins, specifically the Diaphanus-related formin, mDia1 and its activator GTPase RhoA, in GFP-Rab5 labeled endosome dynamics. By using low levels of GFP-Rab5 expression and low levels of light we avoided perturbation of endosome function. The motility analysis of typically 2000 endosome tracks per imaged cell revealed that endosomal transport, specifically average speed, run length and duration, was profoundly reduced upon activation of mDia1 (mDia1 Δ N3) or RhoA (RhoA-V14) and in cells treated with the small molecule inhibitor of formin homology 2 domains (SMIFH2). The SMIFH2 effects could be reversed after washout of the drug. These results indicate the need for intact mDia1 to allow endosomal movement. All the effects caused by activation of the RhoA-mDia1 pathway in endosome transport were reversibly abolished by treating the cells with Latrunculin A, indicating that the effect of mDia1 on MT organelle movement depends on formin-driven actin polymerization. RhoA-V14 and mDia1 Δ N3 induced perturbation of endosomal transport are independent of the downstream RhoA effector ROCK, since treatment with the ROCK inhibitor Y-27632 did not rescue endosome motility. mDia1 RNAi knock-down caused milder inhibitions. A mean-square displacement analysis revealed that endosome motion in formin disrupted cells was subdiffusive compared to control cells that showed a combination of both subdiffusive and superdiffusive motion. The dominance of a subdiffusive regime suggests that endosomes in formin disrupted cells tended to be either effectively trapped or stalled by cytoskeletal barriers. Altogether these data suggest that the RhoA-mDia1 pathway can regulate endocytosis via its effect on MT-based endosome movement.

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Visualizing the dynamic architecture of the endocytic machinery by high resolution tracking of fluorescent proteins in yeast cells.

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Budding of clathrin-coated endocytic vesicles is driven by a highly dynamic and complex machinery consisting of over 50 different proteins. Although individual proteins have been studied extensively, their function as parts of the endocytic machinery is not well understood.

Especially, it is not known how most of the protein components are organized *in situ* during vesicle budding.

We have developed a live-cell particle tracking approach that measures average positions of fluorescently tagged endocytic proteins in relation to each other during the vesicle budding process with high spatial and temporal resolution. To reconstruct the dynamic organization of the endocytic machinery we combine this tracking data with estimates of numbers of molecules, and with available structural and correlative light and electron microscopy data.

We first investigated coat-associated proteins Sla2 and Sla1. The clathrin coat is structurally well studied but the organization of other coat-associated proteins is poorly understood. Our results suggested that the elongated rod-shaped Sla2 protein is located at the central part of the endocytic coat. By using N- and C-terminal GFP tagging we were able to resolve that Sla2 is oriented perpendicular to the plasma membrane so that its lipid binding domain is at the membrane and the actin binding domain projects away into the cytoplasm. Sla1, in contrast to Sla2, is likely located in the peripheral part of the coat. Next we tracked actin and several actin binding proteins to study the polymerization dynamics of the endocytic actin network. Two opposite models have been proposed about the direction of actin polymerization in relation to the forming vesicle. Our tracking of actin cytoskeletal proteins combined with FRAP experiments supported a model where actin filaments polymerize at the base of the invagination, not at the vesicle surface. Finally, we analyzed the dynamics of a BAR domain protein Rvs161/167 that binds to the membrane at the neck of the invagination to regulate vesicle scission. Our results suggested that Rvs161/167 starts assembling just below the coated tip of the invagination and then extends toward the base as the invagination elongates, eventually covering most of the tubular part of the invagination. The Rvs161/167 assembly ends with a catastrophic disassembly that coincides with vesicle scission. In summary, our results reveal several new insights into the mechanisms of endocytosis and demonstrate that live-cell imaging can provide detailed information about the dynamic molecular architecture of the endocytic machinery.

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Retromer's Role in Insulin-Stimulated GLUT4 Trafficking in Adipocytes.

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The Vps35-Vps29-Vps26 trimeric protein complex, termed Retromer, controls the endocytic sorting of various cargo molecules. Although proteomic studies have revealed that Retromer is present on Glut4 storage vesicle (GSVs) together with transmembrane protein Sortilin (Jedrychowski et al (2010), J. Biol. Chem., 285:104-114), the role of Retromer in cell differentiation and insulin-stimulated Glut4 trafficking remains unknown. We have used the 3T3-L1 cell model to investigate changes in Retromer subunits during differentiation. Interestingly, Vps26B protein levels increased by 50 fold after differentiation, while Vps26A protein levels remains unchanged. Using shRNA knockdown approach, we depleted the Retromer protein levels during the differentiation of 3T3-L1 fibroblasts to adipocytes. Within the cytoplasm, of Vps35 and Vps26A depleted adipocytes we observed a change in lipid droplet morphology with a higher population of small lipid droplets observed. Differentiated adipocytes with depleted Retromer levels have impaired Glut4 trafficking, including translocation to the plasma membrane in response to insulin stimulation.

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Regulated Antagonism between Endocytosis and the Cytoskeleton Controls *Drosophila* Blastoderm Development.D. M. Lee¹, T. J. Harris¹; ¹Cell and Systems Biology, University of Toronto, Toronto, ON, Canada

Actin networks push and pull the plasma membrane for the control of cell structure and behavior. Membrane trafficking also regulates the plasma membrane, suggesting it could impact the membrane cytoskeleton and vice versa. We discovered mutual antagonism between endocytosis and actomyosin networks and its impact on plasma membrane furrow ingression during cellularization of the *Drosophila* embryo. Steppke, a cytohesin Arf-GEF, acts at the tips of ingressing furrows to promote localized endocytosis and restrain actomyosin networks during early furrow ingression. Without Steppke, Amphiphysin-positive endocytic tubules (Amph tubules) no longer form, and the actomyosin networks and associated plasma membrane abnormally expand, disrupting furrow structure and expelling nuclei from the forming epithelium. Overexpressing Steppke induced ectopic Amph tubule formation, reduced furrow F-actin, and shortened furrow length, effects dependent on its Arf-GEF activity. Arguing that the Steppke phenotypes were ultimately due to actin mis-regulation, the overall Steppke loss-of-function phenotype was by suppressed by reducing levels of Rho1, a positive actomyosin regulator. The suppression with Rho1 also restored normal Amph tubules, suggesting that actomyosin overactivity inhibits endocytosis. To independently test whether actomyosin networks inhibits endocytosis, we stabilized actin in embryos using Jasplakinolide, which forced expansion of actomyosin networks and reduced Amph tubule formation. The Steppke phenotypes also suggested that endocytosis inhibits actomyosin networks. To independently test this idea, we examined the effect of chemical inhibitors of actin (Cytochalasin D) and clathrin (Chlorpromazine). Embryos treated with a low dose of Cytochalasin D displayed furrow loss, and remarkably, Chlorpromazine nullified the effect of Cytochalasin D, showing that reducing endocytosis can stabilize actomyosin networks. Overall, these results argue that endocytosis normally inhibits the actin cytoskeleton at cellularization furrows, and elevated actomyosin activity can also act reciprocally to inhibit the endocytosis. We propose that during early furrow ingression Steppke-dependent endocytosis keeps actomyosin cytoskeleton in check, but as cellularization proceeds, the actomyosin cytoskeleton gains strength, blocks the endocytic inhibition, and finally closes off the base of each cell to form the blastoderm.

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Lipid composition as a modulator of liposome uptake in a human astrocytoma cell line.E. Suesca¹, N. Bolanos², C. Leidy¹, J. M. Gonzalez²; ¹Department of Physics, Universidad de los Andes, Bogota, Colombia, ²Facultad de Medicina, Universidad de los Andes, Bogota, Colombia

Astrocytes are the most abundant cells in the central nervous system (CNS). In the CNS, astrocytes form part of the blood brain barrier, supply neurons and oligodendrocytes with substrates for energy metabolism, control homeostasis, regulate neurotransmitter release and modulate the immune response. Glioblastoma multiforme is the most common and severe glioma variant in adults, and is originated primarily from astrocytes. Tumor development induces apoptosis of the surrounding glia cells, neurons, and oligodendrocytes. This implies that the GBM may be exposed to lipid debris generated from the myelin sheath. In previous work, we have shown that lipids found in the myelin sheath upregulate the uptake of liposomes by an astrocytoma cell line. This motivated us to study how lipid composition can be used to enhance liposome uptake in this tumor cell line, as a mechanism for targeted drug delivery. Experiments were carried out using a Human astrocytoma cell line, which was cultured in the presence of

small unilamellar vesicles (SUVs) of varying compositions labelled with NBD-PE. The results show that sulfocerebrosides increase significantly liposome uptake compared to phosphocholine enriched liposomes, without reducing cell viability or enhancing the production of inflammatory response molecules such MHC molecules expression, and pro-inflammatory chemokines secretion. Liposomes are observed through time-lapse fluorescence microscopy to accumulate near the nucleus, indicating that uptake takes place through endocytotic routes. Endocytic inhibitors were evaluated to determine the endocytic route used by liposomes. Cytochalasin D was used to inhibit the macropynocytosis route, by blocking the actin polymerization. Chlorpromazine was used to inhibit the clathrin dependant route, and Nystatin was used to inhibit caveolae mediated endocytosis by diminish cholesterol in the plasma membrane. This information is integrated to propose a model for lipid dependent endocytosis in the astrocytoma cell line.

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Selective transport of pinosomal contents based on the molecular sizes.

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Pinocytosis is a major way immune cells use to capture antigens, thus is fundamental in antigen presentation. However, the mechanisms underlying the processing and trafficking of the engulfed antigens are still mysteries. Here we report a size-based selection of pinosomal contents in which small molecules were sorted out, whereas larger ones were still retained inside pinosomes. Further results suggested that the size-based selection was mediated by kiss-and-run fusion events. Interestingly, disturbance of the selection of pinosomal contents significantly impaired antigen presentation, but had no impact on antigen uptake or T cell survival. Our results indicate that kiss-and-run fusion between vesicles leads to selective transport of pinosomal contents based on their molecular sizes, contributing to efficient antigen presentation.

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Recycling of integral membrane proteins to the cell surface is regulated by their deubiquitination.

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The regulation of protein levels at the cell surface is critical for proper function of a variety of cellular processes. Integral membrane proteins, such as nutrient transporters or receptors that recognize extracellular ligands can be maintained at the cell surface where they function, circulate between endosomes and the plasma membrane (PM), or be sent to the lysosome for degradation. Conjugation of ubiquitin by E3 ubiquitin ligases onto cargo membrane proteins serves as a signal for trafficking into the multivesicular body (MVB) pathway and into lysosomes for degradation. Deubiquitinating peptidases (DUBs) can counteract this regulation by removing the ubiquitin sorting signal. We propose that deubiquitination throughout the endocytic pathway is a regulated mechanism to recycle proteins back to the cell surface, and that many yeast proteins have the capacity to recycle membrane proteins back to the cell surface by default. To test this we have taken advantage of the class E *vps* mutants of *Saccharomyces cerevisiae* that block MVB biogenesis and accumulate ubiquitinated cargo membrane proteins in enlarged endosomes known as Class E compartments. We developed a technique to chemically induce hetero-dimerization of a DUB enzyme onto cargo proteins otherwise trapped on class-E compartment endosomes. This technique quickly and acutely deubiquitinates cargo and

prevents further ubiquitination. We find this approach triggered recycling of membrane proteins such as Ste3 (a GPCR) and nutrient transporters back to the plasma membrane thus revealing the capacity to recycle in the absence of a ubiquitin sorting signal. Currently, pathway analysis indicates that the recycling route taken by “un-ubiquitinated” proteins relies on the Skp1/Rcy1 complex and is distinct from the Retromer-dependent pathway. Future experiments will identify relevant DUBs and protein machinery that facilitate recycling through this “default” pathway.

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Antigen-stimulated exocytosis in RBL mast cells.

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Stimulated exocytosis events are a hallmark of the mast cell allergic response. These processes are triggered by multivalent antigen-mediated crosslinking of IgE bound to its high affinity Fc receptor, FcεRI, on the mast cell surface. We previously showed that in RBL-2H3 mast cells, internalized cholera toxin B (CTXB) bound to ganglioside GM₁ traffics to the plasma membrane in response to antigen stimulation (Naal et al., *Traffic* 4, 190-200, 2003). This result indicated that in addition to secretory granule exocytosis (degranulation), CTXB-labeled recycling endosome (RE)-derived membranes also fuse with the plasma membrane in response to antigen. Here we use the v-SNAREs VAMP2, VAMP3, VAMP7, and VAMP8, C-terminally fused to the pH-sensitive variant of GFP known as super-ecliptic pHluorin, to monitor antigen-stimulated exocytosis. VAMP2, VAMP3, and VAMP8 colocalize at REs, whereas VAMP7 is mostly found in secretory granules. Using these tagged v-SNAREs, we monitor stimulated exocytosis by fluorimetry and visualize individual exocytic events with TIRF microscopy. Similar to constitutive RE trafficking, we find that stimulated RE-derived vesicle exocytosis, as monitored by VAMP2, VAMP3, and VAMP8, is attenuated by expression of dominant negative (S25N) Rab11. Surprisingly, stimulated VAMP7 exocytosis is also reduced in the presence of dominant negative Rab11, suggesting that expression of this mutant broadly impacts the endolysosomal system. We find that stimulated VAMP3 exocytosis is almost completely inhibited in the C1 cell line, a mutant variant of the RBL-2H3 cell line known to have defects in ganglioside expression and antigen-stimulated calcium mobilization necessary for granule exocytosis. In contrast, stimulated exocytosis of antibody-labeled transferrin receptors, which localize to endocytic compartments distinct from the RE, is less affected. Together, our results suggest the existence of multiple, distinct endosome-derived vesicle populations that exocytose in response to antigen.

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Functional characterisation of the Fam21-interacting proteins and their role in regulating WASH complex function.

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The WASH complex is a multimeric protein complex that is recruited to endosomal membranes by retromer. This association is mediated by the interaction of VPS35 with the unstructured tail domain of WASH complex member Fam21, which also binds a number of other proteins. Depletion of the WASH complex members strumpellin, SWIP or Wash1 results in an increased prevalence of endosomal tubules positive for the retromer components Snx1 and VPS26, and the WASH complex is therefore implicated in the regulation of endosomal membrane dynamics. We aimed to elucidate further the role of the WASH complex in these processes. Here we identify and characterise a novel interactor of the Fam21 tail domain. We show that depletion of

this protein causes a profound increase in the presence of retromer-positive endosomal tubules. These tubules are abolished by microtubule depolymerization, but not by inhibition of the V-ATPase using bafilomycin. We show that this tubulation phenotype is not the result of destabilisation of the retromer or WASH complexes. Through the transient expression of truncated forms, we have identified regions in both proteins that are necessary for the interaction to be maintained. Finally, we have extended our investigation of this phenotype to other WASH complex members and known retromer cargoes. The identification of a further WASH complex interactor with roles in endosomal tubulation highlights the importance of this complex in the tight regulation of membrane dynamics, and may provide a means of explaining the mechanism by which depletion of WASH complex members upregulates tubule formation.

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Molecular mechanism of cargo capture and vesicle formation by the ESCRT-III polymer.

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The endosomal sorting complex required for transport (ESCRT) proteins constitute a highly conserved set of proteins involved in MVB formation. ESCRT-III is composed of four subunits (Vps20, Snf7, Vps24 and Vps2) and is the minimal machinery necessary to generate vesicles within MVBs¹. However, the structure and mechanism of ESCRT-III-mediated membrane remodeling remain unresolved. Here, we directly visualize ESCRT-III and propose how it mediates vesicle formation. Mutations promoting Snf7 activation generate long ~9nm protofilaments composed of two ~4nm Snf7 sub-filaments running in parallel². These protofilaments form vast 2-D spiral arrays when assembled on lipid monolayers, which are converted to rings that are ~65 nm in diameter upon addition of ESCRT-II and Vps20². It is currently not understood how this polymer interacts with membrane. Understanding how the ESCRT-III subunits, and the assembled ESCRT-III complex, bind and/or deform membrane is critical for understanding the process of membrane scission and intraluminal vesicle formation. Using the structure of hVps24 as a model, we performed a structural-based mutagenesis screen of Snf7 to identify potential membrane-interacting regions. We have identified two regions important for membrane binding: 1) positively-charged interface on the core region and 2) N-terminal hydrophobic residues. Mutating the positively-charged membrane binding interface affects both membrane localization and function. Mutation of the N-terminal hydrophobic residues affects Snf7's ability to bind membrane *in vitro*, but not to localize to the membrane fraction *in vivo*. Accordingly, mutating the N-terminus affects Snf7's ability to create ILV's and sort cargo, but not to sequester cargo, suggesting it has a key role in vesicle formation. We will present data on the role of the domains in membrane deformation and scission during intraluminal vesicle formation.

Henne WM, Buchkovich NB and SD Emr. The ESCRT pathway. *Dev Cell*, 21:77, 2011.

Henne WM*, Buchkovich NB*, Zhao Y and SD Emr. The endosomal sorting complex ESCRT-II mediates the assembly and architecture of ESCRT-III helices. *Cell*, *in press*

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An Ubiquitin-independent Lysosomal Sorting Pathway: AP-3 and ALIX Coordinate Sorting of PAR1 into Multivesicular Endosomes.

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The sorting of signaling receptors to lysosomes is an essential regulatory process. Many receptors are modified with ubiquitin and sorted by ESCRT-0, -I, -II and -III complexes into

intraluminal vesicles (ILVs) of multivesicular bodies (MVBs) prior to degradation. However, it remains unclear whether a single universal mechanism mediates MVB sorting of all receptors. The protease-activated receptor-1 (PAR1), a G protein-coupled receptor (GPCR) for thrombin, is sorted to lysosomes independent of ubiquitination and the ubiquitin-binding ESCRTs, Hrs and Tsg101. Here we report that PAR1 sorts to ILVs of MVBs through an AP-3- and ALIX-dependent pathway that occurs independent of ubiquitination. AP-3 and ALIX function sequentially to sort PAR1 from early endosomes to late endosomes/MVBs, coupling PAR1 to the ESCRT-III complex prior to entry into ILVs. Depletion of AP-3 inhibits PAR1 degradation and disrupts interaction between PAR1 and ALIX. AP-3 binds to a YXX Φ motif within the PAR1 C-tail, which precedes PAR1 interaction with ALIX. Mutation of the PAR1 YXX Φ motif causes PAR1 to localize to microdomains within the limiting membrane of late endosomes, blocking entry into ILVs of MVBs. ALIX then interacts with PAR1, mediating entry into ILVs by coupling PAR1 to the ESCRT-III complex subunit CHMP4. Depletion of ALIX or mutation of the ALIX Bro-1 domain inhibits PAR1 degradation by blocking PAR1 interaction with CHMP4. The ALIX V-domain binds directly to a YPX_nL motif within the intracellular loop 2 of PAR1. In addition, disruption of ALIX binding by mutation of the PAR1 YPX_nL motif inhibits PAR1 entry into the lumen of MVBs. Interestingly, multiple GPCRs contain YPX_nL motifs within intracellular loop 2, suggesting that ALIX may mediate ubiquitin-independent lysosomal sorting of a wide range of signaling receptors. This study reveals a novel MVB/lysosomal sorting pathway for signaling receptors that bypasses the requirement for ubiquitination and ubiquitin-binding ESCRTs that utilizes AP-3, ALIX and the ESCRT-III complex to facilitate degradation of PAR1.

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An ARF6 / Rab35 GTPase cascade for endocytic recycling and successful cytokinesis.

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Cytokinesis bridge instability leads to the formation of binucleated, tetraploid cells that can be a starting point for tumorigenesis in vivo. Membrane trafficking is crucial for animal cell cytokinesis, and several endocytic pathways regulated by distinct GTPases (Rab11, Rab21, Rab35, ARF6, RalA/B) contribute to the post-furrowing steps of cytokinesis. However, little is known about how these pathways are coordinated for successful cytokinesis.

We previously reported that the Rab35 GTPase controls a fast recycling endocytic pathway and must be activated for SEPTIN cytoskeleton localization at the intercellular bridge and thus for completion of cytokinesis [1-2].

We will present evidence showing that the ARF6 GTPase negatively regulates Rab35 activation and hence the Rab35 pathway. Human cells expressing a constitutively activated, GTP-bound ARF6 mutant display identical endocytic recycling and cytokinesis defects as those observed upon overexpression of the inactivated, GDP-bound Rab35 mutant. This includes defects in Transferrin Receptor fast-recycling back to the plasma membrane, SEPTIN2, MHC-I and PI(4,5)P2 accumulation on abnormal intracellular vacuoles, and post-furrowing cytokinesis failure associated with SEPTIN2 delocalization from early bridges. As a molecular mechanism, we identified the Rab35GAP EPI64B as an effector of ARF6 in negatively regulating Rab35 activation. Unexpectedly, this regulation takes place at clathrin coated pits, and activated ARF6 reduces Rab35 loading into the endocytic pathway. Thus an effector of an ARF protein is a GAP for a downstream Rab protein, and we propose that this hierarchical ARF/Rab GTPase cascade controls the proper activation of a common endocytic pathway essential for cytokinesis [3].

[1] Kouranti et al. Current Biology, 2006

[2] Dambournet et al. Nature Cell Biology, 2011

[3] Chesneau et al., Current Biology, 2012

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GRAF1 and MICAL-L1 co-operate in tubular recycling endosome biogenesis.

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The protein GTPase Regulator Associated with Focal Adhesion Kinase-1 (GRAF1) plays a role in the endocytosis of clathrin-independent carriers. It is found enriched at podosome-like adhesions and implicated in cell spreading. GRAF1 is comprised of: N-terminal BAR domain, PH domain, RhoGAP domain and C-terminal SH3 domain. GRAF1 binds to tubular membranes via its BAR and PH domains. Our group has been studying MICAL-L1, a regulator of endocytic recycling and a component of tubular recycling endosomes, and we hypothesized that GRAF1 and MICAL-L1 might physically and/or functionally interact on these tubular membranes. Indeed, we show that GRAF1 interacts with MICAL-L1 by co-immunoprecipitation. Interestingly, high exogenous expression of GRAF1 displaces MICAL-L1 from tubules, while weakly expressed GRAF1 co-localizes with MICAL-L1 on tubular endosomes. In addition, the depletion of GRAF1 by siRNA induces hyper-tubulation of MICAL-L1- and Rab11-containing endosomes, and leading to delayed recycling of α 1 integrin. However, upon MICAL-L1-depletion, the tubular localization of GRAF1 remains unchanged, suggesting that it localizes to recycling endosomes independently of MICAL-L1. Accordingly, we propose that GRAF1 regulates recycling by controlling the localization of MICAL-L1 to tubular recycling endosomes.

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Exchange and activation of the Nedd4-family adaptor Hua1 is controlled by ubiquitination and ubiquitin-peptidases.

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The sorting of cell surface proteins to the lysosome is triggered by their ubiquitination. The major ubiquitin ligase in yeast responsible for protein sorting to the lysosome/vacuole is Rsp5, a Nedd4-family ligase distinguished by a catalytic HECT domain and 3 central WW domains that bind PPxY motifs in target proteins. Many substrates of Rsp5 do not bind directly, but instead rely on adaptor proteins, which have PPxY motifs and bridge the interaction with Rsp5. Recent studies indicate that these adaptors may be more active in their ubiquitinated state, but it is not clear how ubiquitination “activates” them and whether this step is regulated. Here, we describe a mechanism for how Nedd4-like ubiquitin ligase adaptors are activated. Our studies focused on Hua1, an Rsp5 adaptor that brings Rsp5 to ESCRT-0 to promote sorting of membrane proteins into multivesiculated bodies. Hua1 is also ubiquitinated. When overexpressed, Hua1 inhibits Rsp5 by competing away interactions with other Rsp5 adaptors. A ubiquitin-Hua1 fusion protein, which mimics a constitutively ubiquitinated form of Hua1, is a dramatically more potent inhibitor, while Hua1 lacking its Rsp5 binding motifs or ubiquitinatable lysines lacks the ability to inhibit Rsp5. We also found that Ub-Hua1 binds much tighter to Rsp5 than WT Hua1. Rsp5, like Nedd4, has a binding site within the C-lobe of the HECT domain that mediates non-covalent binding to ubiquitin. Eliminating this ubiquitin-binding domain reduced the potency of inhibition displayed by the Ub-Hua1 fusion protein. Rsp5 also associates with the deubiquitinating enzyme Ubp2. Eliminating Ubp2 increased the inhibitory activity of Hua1 overexpression. These data support a model whereby adaptors become ubiquitinated and bind tighter to Rsp5 via both WW domains and the Rsp5 ubiquitin-binding domain. This tight association allows the adaptor to stay bound to Rsp5 thus directing Rsp5 to a subset of targets. Ubp2 deubiquitinates

adaptors, allowing them to disengage Rsp5 and allow for cycling of adaptors. Loss of Ubp2 results in depriving the full complement of adaptors onto Rsp5, resulting in dysfunction of Rsp5.

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Cargo sorting and endosome-to-Golgi retrograde transport pathways.

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Transport pathways from the endosomal system to the trans-Golgi network (TGN) (retrograde transport) are critical for the recycling of membrane proteins which regulate a range of cellular and development functions. Recent advances have identified multiple retrograde transport pathways each regulated by a distinct set of molecular machinery. For example, the cargoes TGN38 and Shiga toxin are transported by distinct routes to the TGN which are defined by specific machinery which regulate these pathways. Furin is an essential proprotein convertase whose trafficking itinerary also involves cycling between the endosomes and the TGN, presumably allowing it to cleave a variety of substrates as it passes through the different cellular compartments.

Objective: The goal of this study was to compare the retrograde trafficking pathway of furin and TGN38, to define the cargo signals for intracellular transport and to develop high throughput flow cytometry analyses to monitor and quantitate protein localisation changes, eg trafficking, in cells.

Results: Using internalisation assays, we show that furin transits the early and late endosomes en route to the TGN. The late-endosome-localized GTPase Rab9 and the TGN golgin tether GCC185 were both required for TGN-retrieval of furin. In contrast TGN38, which circumvents the late endosome, is Rab9 and GCC185-independent. To identify the sorting signals for the early endosome-to-TGN pathway, the trafficking of furin/TGN38 chimeras were investigated. The diversion of furin from the Rab9-dependent late endosome-to-TGN pathway to the retromer-dependent early endosome-to-TGN pathway required both the transmembrane domain and cytoplasmic tail of TGN38. We present evidence to suggest that the length of the transmembrane domain is a contributing factor in endosomal sorting.

Standard approaches for detecting the intracellular location of cargo involve microscopy-based methods that are highly limited in their throughput capacity, speed of acquisition and quantitation. The development of fast, sensitive and quantitative approaches to detect intracellular localisation and trafficking would represent a significant advance in the field. We have developed a pulse shape analysis (PulSA) to monitor protein localization changes in cells by flow cytometry (Ramdzan et al *Nature Methods*, 2012;9:467-70). We demonstrate that PulSA can be used successfully to monitor the PM to Golgi retrograde transport of cargo in cell populations.

Conclusions: These data show that furin uses the Rab9-dependent pathway from late endosomes and that retrograde transport directly from early endosomes is dependent on both the transmembrane domain and the cytoplasmic tail. The possible underlying mechanisms for this sorting event will be discussed. In addition, with flow cytometry flow rates of >10,000 cells per sec, PulSA provides a major advance in quantitative analysis of retrograde cargo trafficking.

Rab GTPases

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A novel endogenous Rab library.

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Rab GTPases and their effectors are essential coordinators of the intracellular membrane and protein transport machinery. In humans, more than 60 Rab proteins have been identified, and the disruption of Rab-mediated transport has been implicated in several inherited human disorders. While most of the data on intracellular trafficking has come from tissue culture experiments, a model system for studying the role of Rab-mediated intracellular transport in the development and function of tissues is still lacking.

The *Drosophila melanogaster* genome encodes 23 conserved Rab proteins with clear vertebrate homologues. We used homologous recombination to generate a novel Rab library in the fly that includes *rab* genes fused to a fluorophore or a proteolytic cleavage site, as well as precise loss-of-function (LOF) alleles. This new resource will allow us to identify the *in vivo* function and localization of each individual Rab protein at endogenous levels. Moreover, we introduced a CATMAID-based online platform that enables collaborative data collection and annotation towards the decryption of the “Rab code” in *Drosophila* tissues.

We used our novel Rab library to systematically profile and annotate the localization and expression of all conserved Rab proteins in the larval salivary gland, wing disc, fat body, CNS and adult ovary/testis. We found that many Rab proteins show cell type-specific expression, subcellular localization and compartment morphology. Additionally, we observed quantitative differences in protein levels between tissues and developmental stages.

Rab profiling in fat body cells revealed a unique cortical localization of Rab35. We reasoned that this localization could be important for the trafficking of Lipid transfer particle (LTP), which also localizes to the cellular cortex in the fat body. LTP controls the lipidation status of Lipophorin (Lpp) that is required for lipid transport within the organism (Palm *et al.*, 2012). Indeed, when we generated a specific knockdown of our fluorescently tagged Rab35 in fat body cells, we observed a loss of LTP from the cortex. Other markers, including Discs large, F-actin, Lpp, Lava lamp and KDEL, were unchanged, suggesting that general cortical transport, actin bundling, protein secretion and intracellular membrane compartments (i.e., Golgi and endoplasmic reticulum) were unaffected. Furthermore, the knockdown of Rab35-EYFP in the fat body caused an accumulation of lipids in the midgut, resembling the characteristic LTP mutant phenotype. This proves that established genetic knockdown tools directed against the introduced Rab protein tag are suitable to induce specific phenotypes.

The combination of our novel genetic resource and online data management platform comprises a valuable new *in vivo* approach for Rab protein profiling and functional analysis that facilitates new insights into the intracellular trafficking machinery.

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The actin nucleation factor WHAMM cooperates with the small GTPase Rab1 to drive membrane tubulation and procollagen transport.

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The actin cytoskeleton provides mechanical support and generates protrusive forces during plasma membrane dynamics, yet comparably little is known about how actin shapes secretory organelles. In mammalian cells, actin filament networks are assembled and organized by the WASP (Wiskott-Aldrich Syndrome Protein) family of actin nucleation factors, and several of these proteins are recruited to the plasma membrane by small GTPases like Cdc42 or Rac1 to drive endocytosis or cell migration. Another one of the nucleation factors, WHAMM (WASP Homologue associated with Actin, Membranes, and Microtubules), promotes membrane tubulation and ER-to-Golgi transport, but its mechanism of membrane recruitment and its endogenous functions in trafficking have not been well characterized. We therefore tested whether ER- or Golgi-associated GTPases are involved in WHAMM-mediated membrane dynamics and anterograde transport. We found that Rab1 stimulates the formation and elongation of WHAMM-associated membrane tubules, as well as the secretion of collagen, the primary protein component of connective tissue. A portion of WHAMM called the WMD (WHAMM Membrane-interaction Domain) binds directly to a prenylated form of Rab1 in vitro, and active Rab1 recruits WHAMM to membranes in cells. Interestingly, WHAMM and Rab1 co-localize along tubular structures that contain procollagen and require both actin and microtubules for their movement. Finally, depletion or inactivation of WHAMM or Rab1 causes large amounts of procollagen to accumulate within fibroblasts. These results highlight a previously unrecognized ability of Rab1 to signal to the cytoskeleton, and demonstrate that this GTPase cooperates with WHAMM to promote membrane remodeling and procollagen transport.

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Phospho-regulation of ezrin-ACAP4 interaction serves as a switch in polarized membrane trafficking.

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The ezrin-radixin-moesin proteins provide a regulated linkage between membrane proteins and the cortical cytoskeleton and also participate in signal transduction pathways. Our recent biochemical characterization demonstrates that ezrin cooperates with ACAP4, ARF6GTPase activating protein, in spatial control of apical membrane trafficking (J. Biol. Chem. 2010. 285, 18769-80). ACAP4 locates in the cytoplasmic membrane in resting parietal cells but translocates and recruits v-SNAREs to the apical plasma membrane upon histamine stimulation in an ezrin phosphorylation-dependent manner. In these parietal cells, secretagogue stimulation produced a striking apical membrane expansion associated with HCl secretion and the secretory phenotype. Given the fact that phosphorylation of ACAP4 at Tyr733 elicits its interaction with Grb2 (J. Biol. Chem. 2011. 286, 43735-46), we probed for the functional relevance of phospho-Tyr733 of ACAP4 in polarized membrane trafficking by introducing the phospho-mimicking ACAP4 into culture parietal cells. Surprisingly, Y733E mutant ACAP4 was more typically localized to the basolateral membrane associated with Grb2-rich projections. Moreover, H,K-ATPase and v-SNAREs were recruited to the Y733E ACAP4-enriched basolateral domains with non-secretory phenotype. The large basolateral expansion is predicted

to recruit membranes from sources not normally targeted to that surface. We reason that phosphorylation of ACAP4 provide a switch in polarized vesicular trafficking in epithelial cells. Currently, we are investigating how phosphorylation of ACAP4 orchestrates epithelia cell plasticity changes in response to extracellular cues.

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Selective activation of Rab5 by distinct endocytic pathways.

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The process of endocytosis is a complex pattern of membrane vesicle trafficking, which permits the continuous flow of membrane between cell surface and various intracellular compartments, via fusion and fission events, with Rab5 mediating the fusion events of early endosomes. Since it has been well established that varying concentrations of Epidermal Growth Factor (EGF) during the endocytosis of EGF-Receptor can elicit uptake via numerous endocytic routes, we were interested in analyzing which of these endocytic pathways is/are responsible for activation of Rab5. We've discovered activation of Rab5 is dependent on temperature, time of uptake as well as the concentration of ligand available at the cell surface. Furthermore, by utilizing specific inhibitors against key regulators of endocytosis (i.e., chlorpromazine, dynasore or filipin) and by varying the concentration of EGF stimulation, we have provided first evidence for the existence of at least three distinct pathways of Rab5-dependent internalization of EGF-receptors. Overexpression of either Rin1 or Rabex-5 is able to rescue Rab5 activity even in absence of EGF and/or in the presence of any of the employed inhibitors. Taken together, these results constitute evidence for the first time showing differential activation of Rab5 with respect to the different routes of uptake of EGF.

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Rab35 is a novel ciliary protein.

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Primary cilia are microtubule-based mechanosensory organelles that protrude from the cell surface and are essential for several signaling pathways. Cilia proteomic and comparative sequence studies have identified more than a thousand of putative ciliary proteins, including small GTPases, such as Ran, Rab and Arf/ Arf-like (Arl) family members. It is well established that small GTPases regulate different steps of vesicle trafficking, including vesicle budding, transport, tethering, docking and finally fusion with acceptor membranes. Several models have been proposed to explain how ciliary cargo reaches the base of cilia, from where proteins can be transported to the ciliary tip by intraflagellar transport. The most accepted model postulates a trafficking route based on polarized exocytosis, in which the ciliary components are carried into vesicles from intracellular compartments towards the base of the cilium and then, by fusion delivered to the ciliary membrane. It has been shown that the trans-Golgi network can be the source of vesicular carriers destined for the cilium. Alternatively, it was hypothesized that other intracellular compartments, such as the endosome recycling compartment (ERC), can also serve as a source of vesicles that carry ciliary cargo.

We have investigated the localization of Rab proteins known to be involved in endocytic recycling trafficking that could also be involved in ciliary trafficking. We found that Rab35 localizes at the base of primary cilia and along the ciliary axoneme of murine inner medullary collecting duct (IMCD3) cells and NIH-3T3 fibroblasts. Rab35 was previously shown to regulate the endocytic recycling pathway and control the docking of vesicles from the ERC to the plasma membrane. Furthermore, we analyzed the ciliogenesis capacity and the trafficking of ciliary

cargo when Rab35 levels were affected. Taken together, our results suggest that Rab35 is a novel ciliary protein and modulates ciliogenesis.

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EHD1 shapes developing primary cilia and influences transport from the ciliary pocket.

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The primary cilium is a sensory structure important in development and disease pathways. Ciliogenesis involves coordinated assembly of a microtubule-based axoneme from the mother centriole and vesicular membrane transport and fusion forming a ciliary membrane around the developing axoneme. Previously, we demonstrated that a Rab11-Rab8 cascade functions in ciliogenesis. The Rab8 GEF, Rabin8, binds to Rab11 and is transported to the centrosome to activate Rab8 and initiate ciliary membrane assembly. Although it is well established that Rab11 and Rab8 define the ciliary-destined vesicles, it is still unclear how the proteins recruited by these Rab proteins influence cilia formation and maintenance. Here we show the preciliary vesicles carry Rab11/Rab8 associated cargo required for ciliary membrane assembly. Specifically, we find that EHD1 is present on both the preciliary and ciliary membranes via binding to Rab11 and Rab8 effector proteins, all of which are required for ciliogenesis. Live imaging studies show EHD1 is localized to the developing primary cilium membrane prior to Rab8 recruitment, which leads to ciliary membrane extension. This result, along with the known membrane deformation ability of EHD proteins, suggests that EHD1 functions in ciliary vesicle to ciliary membrane sheath transition. Interestingly, in mature cilia EHD1 was detected near the proximal end of the primary cilium. Correlative light-electron microscopy (CLEM) demonstrated that EHD1 is absent from the ciliary membrane that has emerged from the cell surface and is restricted to a region containing the ciliary pocket, an invaginated membrane between the plasma membrane and primary cilium membrane. Using structured illumination microscopy (SIM) and total internal reflection fluorescence microscopy (TIRF) EHD1, unlike Rab8, was often restricted to the pocket membrane and/or the adjacent submerged ciliary membrane. EHD1 displayed dynamic trafficking to the ciliary pocket and was found on membrane tubules forming from the pocket. These tubules also contained proteins that appeared to have exited the primary cilium. The role of EHD1 in primary cilium signal transduction from this poorly understood membrane domain is further described as well as its importance in pocket structure formation and maintenance.

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Rab8 Regulates Invagination of the Furrow Canal During *Drosophila* Development.

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Epithelial sheets create a boundary between the exterior and interior environments of organisms. The maintenance of adhesion within these sheets is critical to the function of the tissue and loss of this maintenance drives the metastasis of many epithelial cancers. In *Drosophila*, the epithelium is created via a process known as cellularization. Cellularization requires the coordinated invagination of plasma membrane to create an epithelial sheet 30µm tall. In the presented work, we show that vesicular trafficking is necessary for this invagination process to occur. Using fixed and live-imaging *in vivo*, we have shown that Rab8, a late exocytic vesicle marker, localizes both to future sites of furrow ingression and to the Furrow Canal (FC) during cellularization. Prior to the onset of cellularization, Rab8 forms tube-like projections, which mark future ingression furrows and precede F-actin, a cytoskeletal element known to play

a vital role during this process. During cellularization, these same tubules follow the length of the Furrow Canal (FC) and lead the basal-most portion of the FC. Knockdown of Rab8 via RNAi leads to a failure in formation of the FC and thus failure to initiate cellularization. We propose that polarized vesicular trafficking via late exocytic pathways is required for providing the membrane components necessary to drive the invagination of the FC and thus form the epithelial sheet of the *Drosophila* embryo.

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The interplay between the Rab27A effectors Slp4 a and MyRIP controls hormone-evoked Weibel-Palade body exocytosis.

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Hormone-evoked Von Willebrand factor (VWF) secretion from endothelial cells (ECs) is mediated by exocytosis of specialized secretory granules called Weibel-Palade bodies (WPBs). This allows the endothelium to rapidly respond to vascular trauma or stress through the release of a pre-synthesized cocktail of hemostatic, inflammatory and angiogenic proteins. The identities of the cellular components that regulate hormone-evoked WPB exocytosis remain unclear. Here we identify new endogenous components of the WPB: Rab3B, Rab3D and the Rab27A/Rab3 effector Slp4-a (granuphilin), and determine their role in WPB exocytosis. We show that Rab3B, Rab3D and Rab27A contribute to Slp4-a localization to WPBs. siRNA knockdown of Slp4 a, MyRIP, Rab3B, Rab3D, Rab27A or Rab3B/Rab27A, or overexpression of EGFP-Slp4 a or EGFP-MyRIP showed that Slp4-a is a positive and MyRIP a negative regulator of WPB exocytosis and that Rab27A alone mediates these effects. We found that ECs maintain a constant amount of cellular Rab27A irrespective of the WPB pool size, and that Rab27A (and Rab3s) cycle between WPBs and a cytosolic pool. The dynamic redistribution of Rab proteins markedly decreased the Rab27A concentration on individual WPBs with increasing WPB number per cell. Despite this, the probability of WPB release was independent of WPB pool size showing that WPB exocytosis is not determined simply by the absolute amount of Rab27A and its effectors on WPBs. Instead we propose that the probability of release is determined by the fractional occupancy of WPB-Rab27A by Slp4-a and MyRIP, with the balance favoring exocytosis.

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A Role of Ypt1 in Preautophagosomal Structure Formation.

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In the autophagic pathway, cellular material is shuttled via a double-membrane organelle termed autophagosome to the lysosome/vacuole for degradation. The first step of the pathway is the formation of the preautophagosomal structure, PAS, which requires the assembly of several autophagy-specific proteins (Atgs) on Atg9-containing membranes. While there are multiple Atg9-containing membranes, there is only one PAS in each cell. Ypt/Rab GTPases regulate all intracellular pathways by recruiting their downstream effectors to membranes. One class of Ypt/Rab effectors is coiled-coil tethering factors, which serve as the first specific linkers in membrane fusion. The established role of Ypt1 and its human homolog Rab1 is in the regulation of ER-to-Golgi transport. Recent studies have shown that Ypt1, with its upstream autophagy-specific regulator Trs85, play a role in autophagy. However, the mechanism by

which Ypt1 regulates autophagy is not known. We found that Ypt1 regulates autophagy via an autophagy-specific downstream effector, Atg11. Atg11 contains four coiled-coil (CC) domains and therefore might act as a tethering factor. We have shown that the CC2 and CC3 domains of Atg11 are necessary for the interaction with Ypt1. The Ypt1-1 mutant protein, which contains a mutation in the effector-binding domain, does not interact with Atg11, as shown by yeast-2-hybrid and recombinant-protein pull-down. In *ypt1-1* mutant cells, PAS components do not localize properly and PAS is not formed. The Trs85 upstream regulator, Ypt1, and the downstream effector Atg11 function together as a module to regulate autophagy. Whereas Trs85 and Ypt1 alone interact on multiple Atg9-containing membranes, all three proteins, Trs85, Ypt1 and Atg11, interact only on PAS. Based on our results, we propose that Trs85 first activates Ypt1 on the Atg9-containing membranes and then Ypt1 interaction with Atg11 initiates fusion of these membranes to form PAS.

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Trs20/Sedlin is Required for TRAPP Conversion.

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Trs20 is one of ten subunits of the modular TRAPP complexes in yeast, which act as nucleotide exchangers on Ypt1 and Ypt31/32, the Rab-family GTPases governing protein transport at the cis and trans Golgi, respectively. Mutations in the mammalian ortholog of Trs20, Sedlin, cause the cartilage-specific disorder SEDT. However, the molecular basis of this disease, as well as the role of Trs20 in the TRAPP complexes, remain unclear. Although Trs20 is currently classified as a subunit of TRAPP I, a complex activating Ypt1, we show that Trs20 is required for the assembly and function of TRAPP II, a larger complex activating Ypt31/32. Trs20 interacts directly with TRAPP II-specific subunit Trs120, and is required for the conversion of TRAPP I to TRAPP II. Furthermore, a mutation analogous to one causing SEDT in humans, D46Y, abolishes interaction between Trs20 and Trs120, but not between Trs20 and the TRAPP I complex. These results indicate that Trs20 is crucial for TRAPP II assembly, and implicate a loss of Trs20-Trs120 interaction as the molecular basis for SEDT.

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Regulation of the Golgi-to-cell surface transport of α 2-adrenergic receptors by Rab26.

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The molecular mechanisms underlying the transport from the Golgi to the cell surface of G protein-coupled receptors (GPCRs) remain poorly elucidated. Here we determined the role of Rab26, a Ras-like small GTPase involved in vesicle-mediated secretion, in the cell-surface export of α 2-adrenergic receptors (α 2-ARs). We found that transient expression of Rab26 mutants and siRNA-mediated depletion of Rab26 significantly attenuated the cell-surface numbers of α 2A-AR and α 2B-AR as well as ERK1/2 activation by the receptors. Furthermore, the receptors were extensively arrested in the Golgi by Rab26 mutants and siRNA. Moreover, Rab26 directly and activation-dependently interacted with α 2B-AR, specifically the third intracellular loop. These data demonstrate that the small GTPase Rab26 regulates the Golgi-to-cell surface traffic of α 2-ARs, likely through a physical interaction. These data also provide the first evidence implicating an important function of Rab26 in coordinating plasma membrane protein transport.

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Unique Regulatory Mechanism of Tbc1d1 RabGAP in GLUT4 Trafficking Revealed by Single Molecule Imaging of GLUT4 Behavior.

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The glucose transporter GLUT4 mediates both insulin- and exercise-responsive facilitation of glucose uptake via its translocation from intracellular storage compartments to the plasma membrane. TBC1 domain family RabGAPs AS160/Tbc1d4 and Tbc1d1 are considered to be key regulators of GLUT4 trafficking in responses to the two stimuli, but precise functional roles remain uncertain due to technical limitations. We previously developed a novel method for quantifying intracellular GLUT4 behavior based on single molecule imaging and revealed that “static GLUT4 retention” and its “insulin-responsive liberation” are critical properties of insulin-responsive GLUT4 trafficking systems. We also found that AS160/Tbc1d4 mediates the latter process. Here, we analyzed functional roles of another key RabGAP Tbc1d1 with this approach. We first found that, in contrast to AS160/Tbc1d4, insulin failed to liberate static GLUT4 in the presence of Tbc1d1. Instead, an AMPK activator AICAR successfully liberated static GLUT4, which occurred concomitantly with increased phosphorylation of Ser237, an AMPK consensus site, but not with that of Thr596, an Akt consensus site, indicating that Tbc1d1 can facilitate GLUT4 liberation similarly to AS160/Tbc1d4, though their proximal regulatory signals differ. Intriguingly, we found that insulin did trigger GLUT4 liberation when the cells had previously been treated with AICAR. Insulin-induced Thr596 phosphorylation was essential for the insulin-responsive liberation since T596A mutant showed no such liberation, but the mutant was fully functional in terms of introductory AICAR-induced liberation. In contrast, S237A mutant completely lost GLUT4 liberation activity with any stimuli, indicating phosphorylation of Ser237 to be crucial for both AICAR-induced GLUT4 liberation and temporal acquisition of insulin responsiveness. Finally, we revealed that the functional PTB domain of Tbc1d1 is essential for the temporal acquisition of insulin responsiveness, since a point mutation in the putative PTB sequence showed no acquired ability to liberate GLUT4 in response to sequential insulin stimulation. A natural mutation of Tbc1d1 which relates to severe obesity and locates in the same PTB domain also showed no acquired insulin responsiveness. In conclusion, we identified a unique regulatory mechanism responsible for temporal acquisition of Tbc1d1 insulin responsiveness that relies on its PTB domain. This regulatory mode shift might be a key to the beneficial effects of exercise on muscle insulin potency.

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Motor Protein Effectors Are Crucial to Rab6 Regulation of Golgi Apparatus Homeostasis and Trafficking.

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Rab6 is the most abundant Golgi associated Rab GTPase and is implicated in regulation of Golgi trafficking and homeostasis. Genetically, Rab6 acts upstream of the retrograde Golgi tether proteins Zeste white 10 (ZW10)/ RINT1 or conserved oligomeric Golgi complex protein 3 (COG3). Knockdown of either tether protein results in Golgi fragmentation. However in epistatic, double knockdown experiments, Rab6 depletion inhibited Golgi ribbon disruption and accumulation of Golgi-derived vesicles induced by ZW10 and COG3 knockdown (Sun et al., 2007, MBoC). In single protein depletion experiments, Rab6 knockdown resulted in the accumulation of Golgi proximal coated vesicles and a concomitant proliferation of Golgi cisternae (Storrie et al., 2012, Traffic). Considering the importance of motor proteins as Rab6 effectors, these results suggest a working hypothesis: proteins affecting vesicle transport could be the immediate Rab6 effector(s) whose depletion produces the observed epistatic

suppression and whose activity is a key regulator of normal Golgi organization and homeostasis.

The goal here was to determine which of the biological cocktail of Rab6 effectors was most crucial to Golgi organization and vesicle transport. Hence, we used our epistatic, double knockdown approach as a screen for candidate effector protein(s) that could mimic the upstream Golgi phenotype effects produced by Rab6 depletion. The Rab6 effectors tested included: Kif1C, Kif5B, Kif20A motors, BicD1, BicD2 dynein regulators, MyosinIIA, Golgin-97 and OCRL. Epistatic knockdown of BicD1, BicD2 and Myosin IIA selectively suppressed ZW10- and COG3-depletion induced Golgi ribbon fragmentation. Depletion of other motor protein effectors, either singly or in the case of Kif1C and Kif5B together, had no suppressive effect. Similarly, neither Golgin-97 nor OCRL depletion was epistatically suppressive. Among the positive candidates, depletion of Myosin IIA alone did not mimic the Rab6 depletion phenotype of Golgi proximal accumulation of coated vesicles and Golgi cisternae proliferation (Storrie et al. 2012, Traffic). Experiments are in progress to test whether knockdown of BicD1/2 mimics the Golgi proximal accumulation of coated vesicles and concomitant proliferation of Golgi cisternae observed with Rab6 knockdown. Based upon the experimental outcomes to date, we speculate that dynein, a motor protein regulated by BicD1/2, is crucial to Golgi cisternal homeostasis and Golgi-derived coated vesicle transport.

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Novel functions of Myosin Vc in melanosome biogenesis through interactions with Rab proteins.

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Rab GTPases are well established as coordinators and markers of intracellular protein trafficking pathways. Rab GTPases function as molecular switches and regulate trafficking through interactions with various effector proteins. In melanocyte cells, the biogenesis and transport of melanosomes is highly dependent on the function of several Rabs. Previous work has established the role of Rab32 and Rab38 in the trafficking of protein cargoes from early endosomes to maturing melanosomes. However, relatively little is known about how Rab32 and Rab38 function with other proteins to facilitate this trafficking.

Here, we report the novel interactions of Rab32 and Rab38 with a class V myosin, Myosin Vc, which was not previously known to function in this pathway. Using the yeast two-hybrid system, the interaction of Myosin Vc with Rab32 and Rab38 has been mapped to partially overlapping regions in the tail of Myosin Vc. This binding is dependent on the GTP-bound state of Rab32 and Rab38 as they bind to Myosin Vc only with constitutively-active, GTP-locked mutations, and is specific to Myosin Vc as Rab32 and Rab38 do not bind to Myosin Vb. Additionally, the binding of Rab32 and Rab38 to Myosin Vc has been mapped to specific amino acid residues on the surface of Rab32 and Rab38 that can be disrupted by mutation of individual residues. Live-cell microscopy experiments demonstrate that Myosin Vc has a polarized distribution within melanocytes and localizes to both small and large structures, likely corresponding to vesicles and melanosomes respectively. Depletion of Myosin Vc from melanocytes causes a phenotype in overall pigmentation and in the trafficking of melanosome protein cargoes. Interestingly, it appears that this Myosin may have dual roles in melanosome biogenesis and transport that future experiments will serve to clarify.

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Rab11-FIP2 Interaction with MYO5B Regulates Movement of Rab11a-Containing Recycling Vesicles.

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Previous investigations have demonstrated that a tripartite association of Rab11a with both Rab11-FIP2 and MYO5B regulates recycling endosome trafficking. We have now sought to define the intermolecular interactions that are required for interaction of Rab11-FIP2 with MYO5B. Using a random mutagenesis strategy to generate mutants of the minimal MYO5B binding region of Rab11-FIP2 (amino acids 129 to 356), we performed yeast 2-hybrid screening to identify point mutants with loss of interaction with MYO5B. Point mutations at S229P or G233E caused a loss of interaction with MYO5B in yeast 2-hybrid assays as well as a loss of interaction of Rab11-FIP2(129-356) with MYO5B tail when expressed in HeLa cells. Single mutations or the double S229P/G233E mutation failed to alter the association of full-length Rab11-FIP2 with MYO5B tail in HeLa cells. However, when the Rab11-binding domain was inactivated with an I481N mutation, the S229P and G233E mutations in Rab11-FIP2 again caused a loss of localization with MYO5B. While EGFP-Rab11-FIP2 wild type co-localized with endogenous MYO5B staining, EGFP-Rab11-FIP2(S229P/G233E) showed a significant decrease in localization with endogenous MYO5B. Analysis of Rab11a-containing vesicle movement in live HeLa cells demonstrated that, compared with cells expressing wild type Venus-Rab11-FIP2, cells expressing Venus-Rab11-FIP2(S229P/G233E) showed patterns of faster vesicle movement that traversed significantly longer paths, all consistent with a decrease in tethering of vesicles to the cytoskeleton. Furthermore, knockdown of Rab11-FIP2 in HeLa cells led to significant increases in vesicle speed, track length, and track displacement which were rescued with full-length Venus-Rab11-FIP2, but not with Venus-Rab11-FIP(S229P/G233E). These results support a critical role for the interaction of MYO5B with Rab11-FIP2 in stabilizing the functional complex with Rab11a, which regulates dynamic movements of membrane recycling vesicles.

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Molecular characterization of melanin transfer from donor melanocytes to recipient keratinocytes.

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The “Epidermal-Melanin Unit” comprises the functional complex in which melanocytes and keratinocytes co-operate in a synergistic fashion to maintain skin pigmentation. The transfer of specialized lysosome-related organelles, called melanosomes, from donor melanocytes to recipient keratinocytes and subsequent redistribution to the supra-nuclear area is a critical process in skin pigmentation and photo-protection against ultra-violet damage. However, the molecular mechanism underlying inter-cellular transfer of melanin remains controversial and the molecular machinery involved in the ultimate fate of melanin within keratinocytes is still poorly characterized.

Using co-cultures of melanocytes with keratinocytes we found that keratinocytes stimulate melanin release and transfer, and that depletion of the small GTPase Rab11b leads to a

decrease in melanin exocytosis. Rab11 is involved in the endocytic recycling pathway and in regulated exocytosis. Interestingly, Rab11 has been shown to be essential for the remodeling of the lytic granules in cytotoxic T-cells, prior to the exocytosis of these lysosome-related organelles. To further investigate the melanin transfer process, we used keratinocytes fed with melanosomes or melanocores, which lack the limiting membrane, isolated from melanocytes. We found that melanin is taken up by keratinocytes and accumulates in lysosome-like structures that resemble the physiological supra-nuclear cap observed in human skin.

Taken together, our observations suggest that the predominant mechanism of melanin transfer is keratinocyte-induced exocytosis, through a Rab11b-dependent remodeling of the melanosome, followed by subsequent endocytosis by keratinocytes. We are currently developing a high-throughput image-based siRNA screen to find novel players involved in keratinocyte-induced melanin uptake and the microtubule-dependent movement towards the perinuclear region of the cell.

2243

Lipid Droplet Breakdown is Dependent on a Novel Dyn2-Rab10 Interaction.

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Lipid droplets (LDs) are dynamic lipid-storage organelles that respond to the metabolic requirements of the cell by alterations in both size and number. During periods of nutritional deficit, LDs are metabolized by mechanisms that are currently poorly defined. However, a substantial number of cytoskeletal and membrane trafficking proteins have been shown to associate with LDs and may participate in the breakdown of these organelles. We postulated that the large GTPase Dynamin-2 (Dyn2), well known to support membrane deformation and cellular protein trafficking events throughout the cell, might also participate in the breakdown of LDs in hepatocytes and provide a central “scaffold” for this process. Importantly, we have found that inhibition of Dyn2 function leads to an accumulation of LDs in hepatocytes. HuH-7 hepatocellular carcinoma cells incubated with 150 μ M oleic acid for 16 hours and starved for 65 hours in low-serum media normally undergo lipolysis, leading to a near-complete loss of LDs. A targeted knockdown of Dyn2 by siRNA resulted in 10 times the number of LDs retained compared to cells treated with a non-targeting siRNA. A substantial attenuation of LD breakdown was also observed in HuH-7 and Hep3B cells treated throughout the starvation period with 80 μ M Dynasore, a widely-used pharmacological dynamin inhibitor. As a third approach toward disrupting Dyn2 function, Dyn2 conditional knockout mouse fibroblasts (courtesy of Ferguson and De Camilli, Yale), treated with tamoxifen to induce KO of Dyn2 and loaded with oleic acid, exhibited a 2.4-fold increase in the number of LDs. Importantly, the transient expression of a Dyn2 mutant lacking residues 300-521 of the middle domain (Dyn2 Δ 300-521) significantly attenuates LD breakdown, suggesting that this domain may be critical to its role in lipolysis. Subsequently, we performed a screen using GST-tagged Dyn2-mid to identify binding partners from rat brain homogenates. A prominent association was observed with Rab10, a small GTPase that mediates vesicle traffic and has been identified previously by proteomic screens to reside on lipid droplets. Specific interaction between Dyn2 and Rab10 was confirmed by co-immunoprecipitation from hepatocyte homogenates. GST-pulldowns using purified proteins in vitro identified a specific 10aa Dyn2-interactive region within the Rab10 effector domain. Subsequent super-resolution immunofluorescence imaging of lipid-loaded HuH-7 cells reveals that both Dyn2 and Rab10 co-localize to the LD surface in prominent “crescent” shaped structures. This localization, and the biochemical interaction, is markedly altered in cells exposed to starvation conditions in which LDs are metabolized. Finally, siRNA knock down of either Dyn2 or Rab10 results in a loss of LD-associated crescents and a corresponding effect on LD metabolism. Together, these findings demonstrate an

important role for a novel Dyn2-Rab10 interaction in LD disassembly. Supported by NIH R01 AA020735 to CAC and MAM.

ER and Golgi Transport

2244

Modulation of the sizes of COPII/KLHL12-coated vesicles.

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COPII proteins (SAR1, SEC23/24, and SEC13/31) carve the ER membrane into transport vesicles that are typically about 60-80 nm in diameter and package cargos into newly forming vesicles. Procollagen forms about a 300-nm-long rigid fibril in the ER and requires COPII proteins for export from the ER. Thus, COPII proteins should generate larger carriers to accommodate procollagen. It has been recently shown that monoubiquitylation of SEC31 by CUL3-KLHL12 is essential for ER export of procollagen. Overexpression of KLHL12 drives the assembly of COPII/KLHL12-coated megavesicles and expedites secretion of procollagen. However, how COPII proteins generate such vesicles remains unanswered. Cranio-lenticulo-sutural-dysplasia (CLSD), an autosomal recessive syndrome, with skeletal features thought to be due to defective deposition of collagen. We identified homozygous *F382L SEC23A* mutation in the original consanguineous family by positional cloning. Recently, we described a new CLSD case with a heterozygous *M702V SEC23A* mutation that was inherited from clinically normal father. Expression of *M702V SEC23A* partially attenuates ER export of procollagen. Purified recombinant *M702V SEC23A*, however, interacts normally with other COPII components and efficiently packages the smaller cargo molecules that we have tested. Interestingly, *M702V SEC23A* accelerates SAR1B GTP hydrolysis when SEC31 is present. Because accelerated SAR1 GTP hydrolysis leads to premature release of COPII proteins from the membrane, premature release of COPII proteins from the membrane may lead to formation of smaller COPII vesicles and exclusion of procollagen from nascent vesicles. Remarkably, when we overexpressed KLHL12, the sizes of KLHL12-coated vesicles became smaller in the *M702V* patient's fibroblasts than in control fibroblasts. We, therefore, hypothesize that the rate of SAR1 GTP hydrolysis or the availability of COPII controls the sizes of COPII/KLHL12-coated megavesicles.

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Human Sar1 paralogs differ biochemically in the assembly of the COPII coat.

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COPII coated vesicles are the primary mediators of ER-to-Golgi trafficking. Sar1, one of the five core COPII components, is a highly conserved small GTPase, which, upon GTP binding, recruits the other COPII proteins to the ER membrane. Mammals have two paralogs of Sar1, SAR1A and SAR1B, which share 90% sequence identity and can both promote vesicle budding *in vitro*. It has been found that mutations in SAR1B, but not SAR1A, are associated with Chylomicron retention disease (CMRD) in humans, suggesting that, despite their strong sequence similarity, they have distinct physiological roles. We have previously identified a functional difference between the paralogs, namely that when combined with a disease-

associated allele of SEC23A (F382L), SAR1A is more efficient than SAR1B at recruiting the Sec13-Sec31 complex that comprises the COPII outer coat. Because the primary defect of the F382L-SEC23A allele appears to be a diminished capacity to recruit Sec31, we therefore hypothesized that the differences observed between the Sar1 paralogs may be due to their specific interactions with Sec31. Using membrane budding assays in semi-intact cells, we have identified three amino acid residues sufficient to restore the efficiency of Sar1b to that of Sar1a in the presence of F382L-SEC23A. Through a photo-crosslinking assay we have found that these amino acids bind the proline rich active fragment of Sec31, and that this active fragment stimulates Sar1 GTPase activity. These data illuminate the relationship between Sar1 paralogs and the COPII outer coat, and suggest a possible mechanism for the paralog specific defects seen in CMRD.

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The COG complex coordinates vesicular trafficking via SNARE interactions.

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Membrane trafficking within the eukaryotic cell is a highly organized process that utilizes a set of regulatory components including SNARE's, Rab's, and tethers. The conserved oligomeric Golgi (COG) complex, a multi-subunit peripheral protein complex, has been implicated in the tethering of retrograde intra-Golgi vesicles. The COG complex consists of 8 subunits, grouped into two subcomplexes: COG 1–4 (Lobe A) and COG 5–8 (Lobe B). Two of the eight COG subunits have been shown to interact with SNARE proteins. In this study, we have extended our initial observation of COG-SNARE interactions to include all COG subunits with 15 Golgi localized SNARE proteins. We have found that COGs 4, 6, and 8 are capable of interacting with the SNAREs STX5, STX6, STX16, GS27, and SNAP29 as determined by yeast two hybrid and native Co-immunoprecipitation assays. Further analysis of the COG4-STX5 and the COG8-STX16 interactions by a COG mitochondrial re-localization assay reveals that COG4 and COG8 proteins are re-routing two classes of Golgi trafficking intermediates to the mitochondria vicinity. These results suggest that the COG subunits and/or sub-complexes can initiate the formation of two different tethering platforms for the proper delivery of two populations of Golgi vesicular carriers.

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Sedlin controls the ER export of procollagen by regulating Sar1 cycle.

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Genetics defects occurring in the Sedlin gene, a conserved component of TRAPP complex, cause the Spondyloepiphyseal Dysplasia Tarda (SED), a rare progressive condition characterised by impaired chondrogenesis resulting in short stature, flattening of vertebrae and premature osteoarthritis. The role of Sedlin in the pathogenesis of SEDT disease is still unknown. Prompted by the consideration that Sedlin mutations cause cartilaginous-restricted dysfunctions, we analysed the role of Sedlin in membrane trafficking generally but in particular in the transport of chondrocyte-specific cargoes, such as procollagen type II. We found that

Sedlin is selectively required for PCII to exit the ER, while it is not essential for ER exit of small soluble and membrane-associated cargoes.

Neosynthesized proteins exit the endoplasmic reticulum (ER) via COPII vesicles. Procollagen (PC), however, forms rigid 300 nm prefibrils that are too large to fit into standard 60-90 nm COPII vesicles and thus needs megacarriers.

TANGO1 binds PC and COPII and assists PC packing, but how it promotes the growth of megacarriers is not known. We show that TANGO1 recruits Sedlin and that Sedlin binds and promotes the inactivation of Sar1, a GTPase that in its active form can constrict membranes, and thus allows nascent carriers to grow and to incorporate PC prefibrils.

Sedlin depletion and/or mutation in SEDT patients slow-down the Sar1 cycle and prolongs the membrane association of Sar1-GTP at the ER exit sites, inducing constriction and premature fission of nascent carriers which fail to incorporate the large PC protofibrils but are still competent for smaller cargoes. All together these findings provide new insights not only into understanding the role of Sedlin but also shed new light on the molecular mechanisms underlying SEDT.

2248

Scyl1 functions as a scaffold for multiple components of the COPI machinery.

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COPI-coated vesicles mediate retrograde trafficking from the Golgi apparatus and the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) to the ER in the early secretory pathway. We recently demonstrated that Scyl1-like 1 (Scyl1), a member of the Scyl1-like family of catalytically inactive protein kinases binds COPI coat proteins and that loss of Scyl1 disrupts COPI trafficking. We now demonstrate that Scyl1 interacts with class II Arfs, most notably Arf4, but does not bind class I Arfs. Moreover, Scyl1 co-localizes with Arf4 at the ERGIC. Scyl1 interacts with membranes through a C-terminal helical domain that is distinct from its non-overlapping binding sites for COPI and Arf4, suggesting that Scyl1 couples Arf4 to COPI on ERGIC membranes. Additionally, gel filtration chromatography reveals that Scyl1 undergoes trimerization mediated by HEAT repeats found in the central region of the protein. HEAT-repeat assemblies generate binding sites for proteins and remarkably, through the HEAT repeats Scyl1 also binds to GBF1, a guanine-nucleotide exchange factor for class II Arfs. Thus, Scyl1 appears to be a scaffold for multiple components of COPI vesicle formation and consistently, over expression of Scyl1, which will disrupt the scaffold function leads to tubulation of the ERGIC. Our data reveal Scyl1 as the first known protein to interact specifically with class II Arfs and place Scyl1 as a key organizer of the COPI protein machinery.

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The structure of Sec12 implicates potassium ion coordination in Sar1 activation.

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COPII-coated vesicles traffic proteins and lipids from the endoplasmic reticulum to the Golgi. Crucial for the initiation of COPII coat assembly is Sec12, a guanine nucleotide exchange factor responsible for activating the small G protein Sar1. Once activated, Sar1-GTP binds tightly to ER membranes and recruits COPII coat components (Sec23/24 and Sec13/31). Here, we report the 1.35-Å resolution crystal structure of the catalytically active, 38-kDa cytoplasmic portion of

Saccharomyces cerevisiae Sec12. Sec12 adopts a β propeller fold, as previously predicted. Conserved residues cluster around a loop we term the “K loop”. Structure-guided site-directed mutagenesis, in conjunction with in vitro and in vivo functional studies, reveal that this region of Sec12 is catalytically essential, presumably because it makes direct contact with Sar1. Strikingly, the crystal structure also reveals that the K loop coordinates a potassium ion; bound potassium is, moreover, essential for optimum guanine nucleotide exchange activity in vitro. Thus, our results reveal a novel role for a potassium binding loop in catalyzing guanine nucleotide exchange, while at the same time deepening our understanding of the initiation of COPII vesicle budding.

2250

Luminal Calcium Regulates ER-to-Golgi Transport Efficiency Through ALG-2/Sec31 Interactions.

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Luminal calcium released from secretory organelles has been suggested to play a regulatory role in vesicle transport early in the secretory pathway. In ER-to-Golgi transport, specific depletion of luminal calcium leads to unidentified enlarged clusters of intermediate compartment markers (IC) at ER exit sites. In vitro, blocking luminal calcium egress leads to release of residual (post-budding) COPII subunit sec31 and unregulated COPII vesicle fusion, suggesting a role for residual COPII coat retention in the regulation of ER-to-Golgi transport. The calcium sensor apoptosis-linked gene 2 (ALG-2) stabilizes residual sec31 on COPII vesicles in response to calcium and restricts fusion, suggesting that it may be part of a signaling cascade by which luminal calcium exerts effects on ER-to-Golgi transport (Bentley, et al., 2010, *Mol. Biol. Cell* 21:1033). Here we demonstrate using an intact-cell morphological transport assay that specific luminal calcium depletion leads to a significant decrease in ER-to-Golgi transport rates for transmembrane cargo proteins. Furthermore, ultrastructural analysis revealed that luminal calcium depletion is accompanied by increased accumulation of IC proteins in COPII buds and clusters of unfused COPII vesicles at ER exit sites--no enlarged fusion structures were observed. Finally, in over-expression studies, we demonstrate that disruption of interactions between ALG-2 and the sec31 proline rich region (PRR) loop cause severe defects in ER-to-Golgi transport, and that these interactions regulate sec31 function on the membrane rather than initial targeting to ERES. Though ALG-2/sec31 interactions may regulate sec31 interactions with its other binding partners, their most rate-limiting consequence does not appear to be regulation of sec31A GAP-stimulating activity toward Sar1. This work identifies for the first time a functional requirement for luminal calcium at a demonstrably post-protein-folding step in ER-to-Golgi transport in intact cells. The kinetic and ultrastructural analyses combined indicate that luminal calcium may be involved in promoting efficient budding and/or post-ER sorting of transmembrane cargo between anterograde and retrograde directions of movement. For the first time, ALG-2 interactions with sec31 are shown to possess a functionally critical role in anterograde transport, indicating that ALG-2 is at least one of the calcium sensors that effect luminal calcium's regulatory role in vesicle trafficking.

2251

Roles of GOLPH3 and GOLPH3L in Establishing Golgi Organization

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We have shown that GOLPH3 homologs from yeast (Vps74p) to humans function as PI4P effectors (Dippold, et al., Cell 2009). GOLPH3 localizes to the trans-Golgi via its interaction with PI4P. GOLPH3 also interacts with MYO18A, linking the Golgi to the actin cytoskeleton, and imparting a tensile force that participates in vesicle budding and in establishing the Golgi's characteristic extended ribbon morphology. Unexpectedly, GOLPH3 is also an oncogene commonly amplified in human cancers and capable of enhancing growth factor signaling. Among vertebrates there exists an additional gene in the GOLPH3 family, GOLPH3L, which has been minimally characterized previously. Interestingly, although we find that GOLPH3 is ubiquitously expressed at high levels in all tissues and cell types, we find that GOLPH3L expression levels are low and restricted to secretory cells and tissues. We find that GOLPH3L shares some features of GOLPH3. GOLPH3L binds tightly and specifically to PI4P and localizes to the Golgi. However, we also find surprising differences between GOLPH3 and GOLPH3L with important implications for understanding the role of PI4P in organizing the Golgi.

2252

The role of CK2 in Sec31 phosphorylation and membrane trafficking.

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Protein export from the endoplasmic reticulum (ER) is an initial and rate-limiting step of molecular trafficking and secretion. This is mediated by coat protein II (COPII)-coated vesicles, whose formation requires small GTPase Sar1 and 6 Sec proteins including Sec31 and Sec23. Sar1 initiates and promotes COPII vesicle formation, but the mechanism that regulates coat assembly is largely unknown. The outer layer component of COPII coat Sec31 has been identified as a phosphoprotein. Here, we show that Sec31 phosphorylation regulates COPII vesicle formation. Sec31 phosphorylation reduced its membrane association whereas its non-phosphorylatable mutant remained at ER exit sites for longer. We identified the phosphorylation sites in the middle linker region of Sec31. Sec31 is phosphorylated by CK2, and thus, Sec31 phosphorylation is reduced by CK2 depletion and enhanced by CK2 expression. CK2 knockdown increased affinity of Sec31 for Sec23 and decreased ER-to-Golgi trafficking. These results suggest that Sec31 phosphorylation by CK2 controls the duration of COPII vesicle formation to regulate ER-to-Golgi trafficking.

2253

Molecular analysis of trafficking of collagen in the early secretory pathway.

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The early secretory pathway is highly conserved among all the metazoans and is considered crucial for several cellular processes including development and function of the immune, neuronal, endocrine and skeletal systems. This pathway is tightly regulated and contributes to the trafficking of a considerable fraction of the whole proteome in and around the urbane environment of a cell. It involves designated export machinery, consisting of COPII coat proteins involved in sorting of the cargoes in membrane bound vesicles emanating from the endoplasmic reticulum (ER). The flexibility of the coat machinery to accommodate diverse size and shaped cargoes in order to meet the sophisticated demands of the cell is long-debated and is still under

consideration. Some post-translational modifications or protein-protein interactions of the coat proteins are speculated to contribute to the coat conformational changes resulting in different size transport vesicles, but the mechanism involved in lugging large cargo, e.g. collagen remains to be determined. Collagen fibrils are the principal source of tensile strength in metazoans and define the shape and form of tissues in which they occur. Type I collagen is the major component of fibrils in tendon, bone and skin. There are numerous human skeletal and connective tissue disorders associated with the defects in the early secretory pathway which involve aberrations in COPII protein subunits or accessory proteins, which in turn perturbs the ability of collagen to sort correctly in trafficking machinery. Some of the pathological conditions associated with these diseases results from collagen deposition defect. The work described here resulted in the novel identification of ubiquitination in regulating ER-Golgi trafficking of collagen. This study would provide framework to advance our basic understanding of the early secretory pathway involved in collagen transport and its emerging connection with human skeletal diseases.

2254

Dissecting the Role of the Novel Interactor Ssp120 in the Emp46/Emp47 Complex.

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Correct localization is crucial for the function of proteins. It is achieved by conserved, membrane-associated, trafficking machinery that recognizes cargo and facilitates movement between compartments. Recently, a genome-wide proteomics project in *Saccharomyces cerevisiae* (Babu, M. *et al* in press) successfully identified many novel components of membrane-associated complexes by performing affinity purification in the presence of detergents followed by mass spectrometry. We investigated one such interaction, that of Ssp120, a protein of unknown function, with Emp46/Emp47, a complex that traffics glycosylated proteins from the ER to the Golgi. The inclusion of Ssp120 in the established Emp46/Emp47 complex was validated by both microscopy and co-immunoprecipitation. Ssp120 was found to localize to the Golgi, similar to Emp46/Emp47, in an Emp47-dependent manner. Homology searches revealed that Ssp120 resembles human MCFD2, a binding partner of the human homolog of Emp46/Emp47, LMAN1. In humans MCFD2 acts as a cargo-specific adaptor for LMAN1 that is necessary for the trafficking of a subset of glycosylated proteins from the ER to the Golgi. Mutations in MCFD2 often prevent secretion of coagulation factors V and VIII, resulting in hemophilia. While the calcium binding EF hands of MCFD2 mediate the LMAN1-MCFD2 interaction, we found that the C-terminal domain of Ssp120 mediates the Emp47-Ssp120 interaction. However, the calcium-binding EF hands may be important as *ssp120* strains have an equally strong calcium sensitivity phenotype as *emp47* strains. While we have yet to identify cargo for Ssp120, our work supports the model that Ssp120 acts as a cargo-specific adaptor for Emp46/Emp47.

2255

Modulation of membrane rigidity by human and yeast homologs of the vesicle trafficking protein Sar1.

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Understanding how proteins manipulate the shape and form of membranes is crucial to intracellular cargo trafficking, yet the mechanical activities of trafficking proteins remain poorly understood. Using an optical-trap based assay involving dynamic membrane deformations and

fluorescence recovery after photobleaching (FRAP) to measure protein mobility on *in vitro* endoplasmic reticulum mimic membranes, we examined the behavior of the two human paralogs of Sar1, a key component of the COPII family of vesicle coat proteins. Like their yeast (*S. cerevisiae*) counterpart, the human Sar1 proteins can lower the mechanical rigidity of the membranes to which they bind. Unlike the yeast Sar1, the rigidity is not a monotonically decreasing function of concentration. At high concentrations, we find increased bending rigidity and decreased protein mobility. These features imply a model in which protein clustering influences membrane mechanical properties.

2256

Membrane rigidity, vesicle formation and the ER quality control checkpoint.

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Eukaryotic secretory proteins exit the endoplasmic reticulum (ER) via transport vesicles generated by the essential COPII coat proteins. This step represents a critical quality control checkpoint in that misfolded proteins are generally excluded from COPII vesicles. In a search for yeast mutants that have defective ER retention of misfolded proteins, we identified several bypass-of-sec-thirteen (bst) mutants, where the otherwise essential COPII protein, Sec13, becomes dispensable. A genome-wide screen for the full complement of bst mutants identified nine "core" proteins that generally contribute to the packaging of asymmetrically distributed cargo proteins, including the particularly abundant GPI-anchored proteins. Our genetic and biochemical analyses suggest that within the outer coat scaffold formed by Sec13 and Sec31, Sec13 acts to rigidify the COPII cage, increasing its membrane-bending capacity. This structural integrity is not required when a bst mutation renders the membrane more amenable to deformation by depleting cargoes. We propose that the lapse in ER quality control that occurs in bst mutants reflects the altered biophysical properties of the membrane and coat itself to create structures that are more permissive to the inclusion of aberrant proteins. Restoring rigidity to the outer coat layer reverses the quality control breakdown of bst mutants, linking vesicle architecture to ER quality control.

2257

Functional characterisation of the golgin GMAP-210.

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Golgins are coiled-coil proteins localised to the cytoplasmic face of the Golgi apparatus. Members of the golgin family are thought to be involved in the long-range attachment or tethering of membranes that in some cases are required for Golgi structure and/or protein trafficking. GMAP-210 is a golgin localised to the cis-Golgi. GMAP-210 has two membrane-interacting domains, namely a curvature-sensitive amphipathic lipid-packing sensor (ALPS) motif at the N terminus and a GRIP-related Arf binding (GRAB) domain at the C terminus that interacts with the small GTPase Arf1. A truncated GMAP-210 comprising the ALSP motif, GRAB domain, and a short (one-third of original protein) middle region that is mainly comprised of coiled-coil domains between these two regions was shown to asymmetrically tether curved and flat lipid membranes *in vitro*; however its cellular function is poorly understood. To investigate the *in vivo* role of GMAP-210, we have carried out a functional study of GMAP-210 using HeLa cells. Surprisingly, when GMAP-210 is depleted by RNA interference (RNAi), the Golgi becomes compact. This phenotype is observed using four different siRNA oligos and is rescued by the exogenous expression of Myc-tagged GMAP-210, indicating that this morphological alteration is

specific. Electron microscopy revealed that the Golgi ultrastructure is also disturbed. Yeast two hybrid and pull down experiments revealed that GMAP-210 specifically binds to Rab2 GTPase in a nucleotide dependent manner, and RNAi rescue experiments with truncated GMAP-210 constructs indicated that not only the ALPS motif but also interaction with Rab2 is important for the function of GMAP-210 in vivo. Moreover, the transport of VSV-G protein from the endoplasmic reticulum to the Golgi is delayed by the knockdown of GMAP-210. These results indicate that GMAP-210 has key roles both in the maintenance of Golgi structure and in the secretory trafficking.

2258

Deciphering protein sorting itineraries at the trans-Golgi network of polarized epithelial cells.

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The trans-Golgi network (TGN) is thought of as the diverging point for the sorting of apical and basolateral proteins into their subsequent itineraries through the rest of the secretory pathway. However, how the membrane structure or lipid and protein composition of the TGN provides for the specific sorting of such itineraries remains unclear. Our preliminary studies, which rely on analysis of fluorescently labeled, cargo protein-enriched, fractionated TGN membranes show that much of the apical and basolateral cargo mass is independent of either the enzymatic trans-Golgi cisternae (sialyltransferase) or the cycling TGN (TGN38) markers, indicating that most of the cargo that accumulates in the TGN delineates an independent functional compartment that is essentially devoid of known compartmental markers. Furthermore, our studies show that ~70% of apical DPPIV and basolateral VSVG cargo proteins are present in distinct TGN-entities after a cargo accumulation step at 20°C. This indicates that a significant segregation of apical and basolateral proteins has occurred within the 20°C compartment, but additional protein sorting step(s) must occur to result in the release of distinct apical and basolateral carriers in MDCK cells. Currently, we are attempting to elucidate the mechanisms and the stringencies of the “coarse” and “fine” sorting events for the different cargo types in the TGN.

2259

Substrate driven regulation of GBF1 recruitment to cis-Golgi membranes.

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Guanilyl nucleotide exchange factors (GEFs) catalyze the activation of small GTPases such as ADP-ribosylation factors (Arfs) by facilitating the exchange of bound GDP for GTP. Arf activation allows recruitment and/or activation of several effectors critical for membrane trafficking at the Golgi. At *cis*-Golgi membranes, Golgi-specific BFA resistance factor 1 (GBF1) is the primary GEF responsible for Arf activation. Recruitment of cytosolic GBF1 to Golgi membranes represents a critical step, since activation can only occur at the membrane surface following association of both GBF1 and inactive Arf•GDP. Recently, we have elucidated a novel mechanism for regulation of recruitment of active GBF1 onto *cis*-Golgi membranes. Several laboratories previously reported that treatment with Brefeldin A (BFA) causes rapid accumulation of GBF1 on Golgi membranes, an effect attributed to formation of an Arf•BFA•GBF1 membrane-bound complex. We have now demonstrated that several manipulations that result in increased cellular Arf•GDP, including Exo1 treatment and ArfGAP1 over-expression, also caused accumulation of endogenous GBF1 onto Golgi membranes.

These results are consistent with our previous report that trapping of Arf in a ternary Arf•BFA•GBF1 complex is not detected in vivo, and suggest instead that accumulation of Arf•GDP substrate promotes recruitment of GBF1. The possibility that substrate Arf•GDP positively regulates GBF1 association with membranes was tested using inactive Arf T31N mutants. We observe that expression of low levels of several Arf T31N isoforms caused dramatic accumulation of GBF1 onto membranes. This Arf•GDP dependent enrichment of GBF1 occurred specifically on *cis*-Golgi membranes. Moreover, accumulated GBF1 resulted in increased Arf•GTP production and was therefore active. These results provide the first clear function for “inactive” Arf•GDP. Accumulation of GBF1 onto Golgi membranes occurred independently of PtdIns4P levels and required association of Arf•GDP with membranes through its myristoylated N-terminal helix. This result indicates that Arf•GDP likely regulates a membrane-associated factor found specifically on *cis*-Golgi membranes, potentially a GBF1 receptor. Together, our results suggest a novel mechanism in which Arf•GDP regulates recruitment of GBF1 to *cis*-Golgi membranes to facilitate maintenance of homeostatic levels of Arf•GTP.

2260

Plasmolipin is associated with targeting membranes and proteins to the apical and myelin membranes.

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The myelin and epithelial apical membranes are involved in forming barriers. While the myelin sheath insulates axonal microenvironment to allow fast signal propagation, the apical membrane faces and controls harsh extracellular environments. Myelin and apical epithelial membranes have a similar composition of liquid ordered phase-promoting lipids such as cholesterol and sphingolipids. The protein content of both membranes shows significant resemblance as well. Among these is plasmolipin (PLLP), an 18kDa protein comprised of the MARVEL four trans-membrane spanning domain. The function of PLLP in both localizations is unknown. Sequence analysis of human PLLP revealed a 29% identity to MAL, an apical targeting machinery protein previously shown by us to facilitate membrane domains formation. PLLP shares the same intramembrane dimerization WVMF motif ($\phi XX\phi$) and contains another three intramembrane helix-helix interaction motifs.

Fluorescent protein tagged-PLLP localized to the PM, TGN and an unidentified tubular membrane compartment in non-polarized COS7 or MDCK cells. In the Golgi, PLLP was segregated from the Golgi marker GalT but co-localized with GPI-anchored fluorescent protein (FP). Golgi to PM transport of the VSVG membrane cargo protein was blocked by overexpression of PLLP in COS7 cells. Elongation of the VSVG trans-membrane domain by 4 amino acids eliminated this effect while the shortened VSVG mutant ($\Delta 7$ -aa's) reached the PM rather in a significantly decreased transport rate. These data demonstrate there is an association of the TMD length of cargo proteins, which correspond to PLLP's TMD and the membrane lipids environment, and transport rate. This leads to a suggested trafficking and sorting capacity of PLLP and its effect on the transport machinery of cargo proteins. The hypothesis is that through hydrophobic mismatch interactions, PLLP serves as a “gate keeper”, blocking unmatched cargo proteins from passing through the Golgi.

Acceptor photobleaching demonstrated a substantial fluorescence resonance energy transfer (FRET) between cerulean- and YFP-tagged PLLP, demonstrating that PLLP forms oligomers when in the TGN and PM.

Together, our data support the premise that PLLP interacts with its membrane lipid environment and is part of the machinery that forms and maintains the unique composition of the apical and myelin membranes.

2261

Division of the intermediate compartment at the onset of mitosis provides a mechanism for Golgi inheritance.

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As mammalian cells prepare for mitosis the Golgi ribbon is first unlinked into its constituent stacks and then transformed into spindle-associated, pleiomorphic membrane clusters in a process that remains enigmatic. Also, whether Golgi inheritance involves the incorporation of Golgi enzymes into a pool of coat protein I (COPI) vesicles, or their COPI-independent transfer to the endoplasmic reticulum (ER) remains unclear. Based on the observation that the intermediate compartment (IC) at the ER-Golgi boundary is connected to the centrosome, we examined its mitotic fate and possible role in Golgi breakdown. The use of multiple imaging techniques and markers revealed that the IC elements persist during the M phase, maintain their compositional and structural properties and remain associated with the mitotic spindle, forming circular arrays at the spindle poles. At G2/M transition, the movement of the pericentrosomal domain of the IC (pclC) to the cell center and its expansion coincide with the unlinking of the Golgi ribbon. At prophase, coupled to centrosome separation, the pclC divides together with recycling endosomes, providing novel landmarks for mitotic entry. We provide evidence that the permanent IC elements function as way stations during the COPI-dependent dispersal of Golgi components at prometa- and metaphase, indicating that they correspond to the previously described Golgi clusters. In addition, they continue to communicate with the vesicular "Golgi haze" and thus are likely to provide templates for Golgi reassembly. These results implicate the IC in mitotic Golgi inheritance, resulting in a model that integrates key features of the two previously proposed pathways.

2262

Rab1 and its GAP TBC1D20 are recruited to Lipid droplets by the Hepatitis C Virus Nonstructural Protein 5A.

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Replication and assembly of Hepatitis C virus (HCV) depends on the host's secretory and lipid-biosynthetic machinery. Viral replication occurs on endoplasmic reticulum (ER)-derived modified membranes while viral assembly is thought to occur on lipid droplets (LDs). The ER and LD-binding non-structural protein 5A (NS5A) is a candidate for interfacing with the host membrane as well as secretory and lipid synthesis machineries. The interaction of GFP-tagged NS5A with host cell membranes and binding partners was characterized in living cells. The binding of NS5A to LDs is apparently irreversible, both in HCV-infected cells and when ectopically expressed. In HCV-infected cells, NS5A fluorescence was observed around the LDs and in perinuclear structures that were incorporated into a highly immobile platform superimposed over the ER membrane. The GTPase activating protein (GAP) TBC1D20 and its cognate GTPase Rab1 are recruited by NS5A to LDs. The NS5A-TBC1D20 interaction was previously shown to be essential for the viral life cycle. In cells, expression of the Rab1 dominant negative (Rab1DN) GTPase mutant abolished steady state LDs. In infected cells, Rab1DN induced the elimination of NS5A from viral replication sites. Currently, we are mapping the interaction between NS5A

and TBC1D20 using NS5A mutagenesis. We hypothesize that TBC1D20, via its GAP activity dissociates Rab1 from secretory membranes thereby facilitating its recruitment to LD membranes to promote LD biogenesis. Thus, we speculate that the HCV NS5A diverts lipid droplet metabolism to sustain the viral life cycle via activation of TBC1D20.

Kinases and Phosphatases II

2263

Identification of novel Wnt signaling-associated protein kinases.

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The Wnt pathway is an evolutionarily conserved signaling network that is critical for mammalian development and adult tissue maintenance. In addition, aberrant activation of the Wnt signaling is implicated in driving the formation of various human cancers, particularly those of the digestive tract. Inhibition of aberrant Wnt pathway activity in cancer cell lines efficiently blocks their growth, highlighting the great potential of therapeutics designed to achieve this in cancer patients. In this study, we try to identify novel protein kinases that are associated with canonical Wnt signaling pathway by using TOPflash reporter assay system and human protein kinase (~600 genes) library. As the result, we identified numbers of protein kinases that positively regulate Wnt signaling pathway. The novel protein kinases would be possible and valuable target for regulating Wnt pathway in cancer and mammalian development.

2264

Role of conserved NDR kinase Orb6 in the control of polarized cell growth.

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The conserved NDR kinase Orb6 controls cell morphogenesis in different organisms, ranging from yeast to neuronal cells. We previously found that Orb6 kinase regulates cell polarity by spatial control of Cdc42 GTPase. (Das et al, Curr Biol, 2009) Here we report that Orb6 kinase has a genetically separable function in the control of cell growth that is mediated by the RNA-binding protein Sts5.

We found that Sts5 is a cytoplasmic protein that rapidly localizes to the P-bodies, sites of RNA storage and degradation, following nutritional stress. In addition, a synthetic genetic array screen (SGA) revealed genetic interactions between Orb6 and several signaling pathways involved in nutritional response. Consistent with our finding that Sts5 is a substrate of Orb6 kinase, loss of Orb6 kinase activity leads to localization of Sts5 to the P-bodies.

We have identified several mRNAs that associate with Sts5 by microarray and q-PCR analysis. These mRNAs encode for conserved signaling proteins involved in morphology and bipolar growth activation, as well as cargos with a role in cell wall formation. The cellular levels of Sts5-associated mRNAs increase in *sts5* deletion mutants, suggesting that Sts5 may promote mRNA degradation, and decrease upon Orb6-kinase inhibition.

Our findings suggest that Orb6 kinase promotes polarized cell growth by preventing Sts5 localization to the P-bodies and consequent mRNA degradation and indicate that the Orb6 kinase function plays a role in modulating cellular responses to nutritional stress.

2265

Angiomotin family proteins are both targets and regulators of the Hippo pathway kinase Lats2.

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Angiomotin (AMOT) proteins were originally identified as regulators of angiogenesis, cell polarity, and cell migration. More recently AMOT-family proteins (AMOT, AMOTL1, and AMOTL2) were shown to also regulate cell growth and proliferation through the Hippo tumor suppressor pathway. We previously showed that a conserved N-terminal region of the AMOT proteins promotes the activity of the Hippo pathway kinase Lats2. The N-terminus of the AMOT proteins also localizes to actin stress fibers and promotes their assembly. Our experiments suggest that the AMOT N-terminus may act as a scaffold for Hippo signaling since it binds the three core Hippo components Mst2, Lats2, and YAP. Several studies have shown that the Hippo pathway is regulated by the actin cytoskeleton, but the mechanism is not known. To determine how AMOT proteins activate the Hippo pathway and whether the effects of actin on the Hippo pathway (and vice versa) are mediated by AMOT, we have begun a structure/function approach to analyze the conserved AMOT N-terminus. Data identifying binding sites for Hippo pathway components and regions required for actin localization and bundling will be presented. Although we showed that AMOT proteins can activate the Lats2 kinase, we find that the actin bundling activity of AMOT proteins may in turn be regulated by Lats2. We have identified a single conserved phosphorylation site (S175) in AMOT (130 kDa isoform) that is directly phosphorylated by Lats2. Phosphorylated AMOT130 is unable to bundle actin, and instead localizes to large vesicles. We propose that the Hippo pathway is not only activated by AMOT proteins, but also affects their ability to promote cell migration, cell polarity, and angiogenesis.

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Formation of GPCR Docking Site on GRK2: Role of the Extreme Amino Terminus and Active Site Tether.

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G protein-coupled receptor (GPCR) kinases (GRKs) were identified by their ability to specifically phosphorylate agonist-activated GPCRs leading to their desensitization. The seven GRKs are members of the AGC kinase superfamily, but their mechanism of activation is poorly defined. Agonist-activated receptors dramatically stimulate the catalytic activity of GRKs; thus, we sought to identify GRK2 residues located outside the active site that play a role in interactions with their substrate receptors. In a previous study, we showed that kinase carboxyl-tail (C-tail) residues are required for GRK activation. Genetic and antibody inhibition studies suggested that the amino terminus plays a role in receptor phosphorylation by GRK2 and GRK1, respectively. A crystal structure of GRK6 in a closed conformation and biochemical crosslinking of GRK1 suggest that N-terminal helix and the kinase C-tail interaction is important for GRK activation. In this study we carried out systematic mutagenesis of residues 3-18 and more exhaustive mutagenesis of a sub-region of the kinase C-tail, the active site tether (AST), of GRK2. Of the N-terminal mutants, Tyr¹³ has the most dramatic effect on receptor phosphorylation, but many residues appear to make contributions. N-terminal and AST mutants exhibited parallel

deficiencies in receptor-mediated activation in vitro and β_2 -adrenergic receptor phosphorylation in intact cells. Many, but not all, of the N-terminal and AST phosphorylation-defective mutants harbor defects in the agonist-induced recruitment to an activated α_2 -adrenergic receptor, measured by a cellular BRET-based assay. These results suggest that the N-terminus and AST, both intrinsically disordered in the inactive kinase, play important roles in formation of the docking site for activated receptors.

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RGS domain-mediated dimerization regulates GRK5 plasma membrane localization and function.

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G protein-coupled receptor (GPCR) kinases (GRKs) phosphorylate activated GPCRs at the plasma membrane (PM) to both terminate further activation of G proteins and promote G protein-independent signaling. Our goal is to elucidate the mechanisms of PM targeting of GRKs. Despite high sequence similarity, GRK5 and GRK6 constitutively localize at the PM whereas expressed GRK4 is predominantly cytoplasmic. GRK5/GRK4 chimeras and point mutations in GRK5 identified a short sequence within the Regulator of G protein Signaling (RGS) domain in GRK5 that is critical for GRK5 PM localization. Interestingly, this region of the RGS domain of GRK5 coincided with a region of GRK6 shown to form a hydrophobic dimeric interface (HDI) in the published GRK6 crystal structure. Co-immunoprecipitation (co-IP) and bimolecular fluorescence complementation (BiFC) assays suggested that expressed GRK5 wt, self-associated in cells, whereas GRK5-M165E/F166E (GRK5-EE), containing hydrophilic mutations in this RGS region, showed greatly decreased co-IP and BiFC interactions. Moreover, forced dimerization of GRK5-EE, via fusion to leucine zipper motifs, or appending an extra C-terminal membrane binding region to GRK5-EE (GRK5-EE-CT) recovered PM localization. In addition, we showed that GRK5-EE displayed a decreased ability to inhibit Par-1-induced calcium release compared with GRK5 wt. In contrast, PM-localized GRK5-EE-Caax or GRK5-EE-CT showed a comparable ability to GRK5 wt to block Par-1-induced calcium release. The results in this study suggest a novel model in which GRK5 dimerization is important for its plasma membrane localization and function.

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Protease-activated receptor 2 performs the protein secretion through the phosphorylation of CAMK II in rat parotid gland acinar cells.

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Protease-activated receptors (PARs) represent a novel class seven transmembrane domain G-protein coupled receptors. Recent studies have reported that PARs are present in a variety of cells and have been implicated in the regulation of a number of vital functions. The issue of whether the stimulation of PARs induces responses in parotid glands was examined; with special reference to intracellular Ca^{2+} ($[Ca^{2+}]_i$) dynamics during PARs stimulation. In the present study, PAR2 mRNA was expressed strongly in the parotid glands. Following vascular perfusion with HR buffer containing PAR2-activating peptide (PAR2-AP:SLIGRL-NH₂), the parotid acini showed a wide lumen and massive compound exocytosis in electron microscopy. In parotid acinar cells, PAR2-AP induced an increase in $[Ca^{2+}]_i$. Both removing of extracellular Ca^{2+} and using of Ca^{2+} channel blockers did not inhibit the PAR2-AP-induced $[Ca^{2+}]_i$ increase. The response to PAR2 activation was mainly caused by Ca^{2+} mobilization from intracellular Ca^{2+}

stores. This peptide induced Ca^{2+} release and entry were partially inhibited by the nitric oxide (NO) synthase inhibitor, L-NAME. The NO donor, GEA 3162, but not 8-bromo-cGMP, mimicked the effects of PAR2 in activating non capacitative calcium entry (NCCE). Both KN93 and W7 completely blocked a Ca^{2+} release from intracellular Ca^{2+} store and a Ca^{2+} influx from extracellular spaces. And when the cells were preincubated with KN93 and then were stimulated with PAR2-AP, the KN93 completely inhibited the PAR2-induced release of amylase. These results indicate that PAR2-AP activates a Ca^{2+} release from intracellular Ca^{2+} stores and following a NCCE pathway. And we proposed that an effect of PAR2 is dependent on CAMKII and CaMKII lies upstream of PAR2-induced Ca^{2+} release from intracellular stores in parotid gland.

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Effects of protease-activated receptors (PARs) on intracellular calcium dynamics of acinar cells in rat lacrimal glands.

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The lacrimal gland is responsible for secretion of the aqueous portion of the tear film. The functional unit of the lacrimal gland is the acinus structure, which consists of polarized cells connected around a central lumen via tight junctions.

Protease-activated receptors (PARs) represent a novel class seven transmembrane domain G-protein coupled receptors, which are activated by proteolytic cleavage. To date, four members of this class of receptors have been identified. The mechanism by which PARs are activated involves the proteolytic unmasking of an N-terminal sequence that acts as a tethered ligand. Recent studies have reported that PARs are present in a variety of cells and have been prominently implicated in the regulation of a number of vital functions. The issue of whether the stimulation of PARs induces responses in lacrimal glands was examined; with special reference to intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) dynamics during PARs stimulation.

In the present study, mRNAs for all known PAR subtypes were checked by a reversetranscriptase polymerase chain reaction. PAR2 mRNA was only detected in the lacrimal glands. In lacrimal gland acinar cells, both trypsin and PAR2-activating peptide(PAR2-AP), SLIGRL-NH₂, induced an increase in $[\text{Ca}^{2+}]_i$. Both removing of extracellular Ca^{2+} and using of Ca^{2+} channel blockers, did not inhibit the PAR2-AP-induced $[\text{Ca}^{2+}]_i$ increase. U73122 and Xestospongin C failed to inhibit PAR2-induced increases in $[\text{Ca}^{2+}]_i$. And we checked the origin of the calcium influx after Ca^{2+} release from intracellular Ca^{2+} stores. The NO donor, GEA 3162 mimicked the effects of PAR2 in activating non capacitative calcium entry (NCCE). Both low concentration of Gd^{3+} (5 μM) and calyculin A, did not completely block PAR2-AP-induced $[\text{Ca}^{2+}]_i$ increase.

These results indicate that the response to PAR2 activation was mainly caused by Ca^{2+} mobilization from intracellular Ca^{2+} stores and PAR2-AP activates both NCCE and CCE pathways. PAR-2 may function as a key molecule, under pathophysiological conditions such as tissue injury or inflammation.

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The mechanism study of spironolactone-induced Ca^{2+} increases in rat testicular arteriole smooth muscle cells.

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Arterioles play pivotal roles in controlling blood supply in various tissues. We have previously reported that spironolactone (SP) induced intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) increase in rat testicular arteriole smooth muscle cells. SP-induced dynamics of $[\text{Ca}^{2+}]_i$ can be caused by either a Ca^{2+} influx from extracellular fluid or Ca^{2+} mobilization from internal Ca^{2+} store, with the former being dominant. However, this $[\text{Ca}^{2+}]_i$ increasing mechanism is not so clear. This time we have investigated the alteration of $[\text{Ca}^{2+}]_i$ in testicular arterioles with respect to SP and some other drugs using a real-time confocal laser scanning microscope.

We isolated arterioles from testis of rats and soaked in HEPES-buffered Ringer's Solution (HR)(pH7.4). Connective tissues were digested by purified collagenase (100 units/ml) for 2 hours, then the specimens were loaded by Indo-1/AM (5 μM) for 1 hour at room temperature. They were placed on cover slides in chambers which were coated with Cell-Tak® and continuously perfused with HR containing some drugs. We used a real-time confocal microscope (Nikon RCM/Ab).

When SP (300 μM) was used as a stimulus, an increase of $[\text{Ca}^{2+}]_i$ in the smooth muscle cells was observed. The response was considerably inhibited under either extracellular Ca^{2+} -free conditions, the presence of Gd^{3+} , or with a treatment of diltiazem. U73122 failed to inhibit SP-induced increases in $[\text{Ca}^{2+}]_i$. 2-APB (a potent IP3 receptor antagonist) showed almost same as above. The protein kinase A inhibitor, H89, partially inhibited this increase, whereas, it had no effect using PKC inhibitor, GF109203X. In the presence of either suramin (a G protein antagonist) or NF449 (a $\text{G}\alpha$ inhibitor), SP-induced increases in $[\text{Ca}^{2+}]_i$ is partially blocked. When we used mifepristone, a glucocorticoid receptor antagonist, SP-induced Ca^{2+} increase was partially blocked.

In present study, we propose that the SP-induced dynamics of $[\text{Ca}^{2+}]_i$ can be caused by both a Ca^{2+} influx from extracellular fluid and Ca^{2+} mobilization from internal Ca^{2+} store, with the former being dominant. We suggested that there is some possibilities that SP is interact with both extracellular (i.e. a G protein-coupled type) and intracellular (i.e. glucocorticoid receptors, etc) receptors, and then rises in intracellular Ca^{2+} and causes the smooth muscle contraction in rat testicular arterioles.

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Calcineurin regulates $\text{Ca}_v3.2$ T-type Ca^{2+} channel by NFAT-like direct interaction.

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Re-expressed $\text{Ca}_v3.2$ T-type Ca^{2+} channel is required for the pressure overload-induced cardiac hypertrophy and is involved in the activation of calcineurin/NFAT pathway in heart. In this study, we investigated the interaction between $\text{Ca}_v3.2$ and calcineurin and the functional consequences of their interaction. We showed that calcineurin interact with $\text{Ca}_v3.2$ through two conserved motifs, PCISVE and LTVP, on the C-terminal region of $\text{Ca}_v3.2$. The interaction between $\text{Ca}_v3.2$ and calcineurin were both calmodulin and Ca^{2+} dependent. Interestingly, the dose response curve of Ca^{2+} dependence of $\text{Ca}_v3.2$ /calcineurin interaction is similar to that of calcineurin phosphatase activity. This indicates that the binding of $\text{Ca}_v3.2$ may affect calcineurin phosphatase activity. Indeed, $\text{Ca}_v3.2$ bound calcineurin had a reduced phosphatase activity

compared to the unbound one. The calcineurin/NFAT signaling pathway can also be enhanced by the disruption of $\text{Ca}_v3.2$ /calcineurin binding. We also investigated the effects of calcineurin binding on $\text{Ca}_v3.2$ T-channels and showed that the disruption of calcineurin binding to $\text{Ca}_v3.2$ was able to increase the current densities of $\text{Ca}_v3.2$. Lastly, we showed that the phenylephrine-induced cardiac hypertrophy was suppressed by a cell permeable peptide containing the $\text{Ca}_v3.2$ PCISVE motif. Our findings provide evidences for a novel reciprocal modulation of $\text{Ca}_v3.2$ T-type Ca^{2+} channel and calcineurin in regulating calcineurin/NFAT signaling pathway.

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ABA signaling in guard cells entails a dynamic protein-protein interaction relay from the ABA receptors to SLAC1 ion channel.

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Plant hormone abscisic acid (ABA) serves as an integrator of environmental stress such as drought, to trigger stomatal closure by regulating specific ion channels in guard cells. We previously reported that SLAC1, an outward anion channel required for stomatal closure, was regulated via reversible protein phosphorylation events involving ABA signaling components including protein phosphatase 2C members and a SnRK2-type kinase (OST1). In this study, we reconstituted the ABA signaling pathway as a protein-protein interaction relay from the PYL/RCAR type receptors, to the PP2C-SnRK2 phosphatase-kinase pairs, to the ion channel SLAC1. The ABA receptors interact with and inhibit PP2C phosphatase activity against the SnRK2-type kinase, releasing active SnRK2 kinase to phosphorylate and activate the SLAC1 channel, leading to reduced guard cell turgor and stomatal closure. Both yeast-two hybrid and bi-molecular fluorescence complementation assays were used to verify the interactions among the components in the pathway. The biochemical assays demonstrated the activity modifications of phosphatases and kinases by their interaction partners. The SLAC1 channel activity was used as an end-point readout for the strength of the signaling pathway depending on the presence of different combinations of signaling components. Further study using transgenic plants overexpressing one of the ABA receptors demonstrated that changing the relative level of interacting partners would change the ABA sensitivity.

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Regulation of connexin hemichannels by protein kinase C.

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Introduction: Hemichannels are located at the cellular surface of most mammalian cells. They are formed by connexin proteins and are recognized as a membrane pathway for paracrine/autocrine signals. Our objective is to determine the regulation of connexin43 (Cx43) hemichannels by protein kinase C (PKC). **Methods:** To determine changes in hemichannel activity we measured dye uptake in real time and unitary conductance by patch-clamp in whole cell modality. The HeLa cells stably transfected with Cx43-EGFP were exposed to an inhibitor (Bisindolylmaleimide) or activator (PMA) of PKC. Changes in the plasma membrane level of Cx43 were determined by confocal microscopy. **Results:** It was found that incubation with a PKC inhibitor increased dye uptake, an effect that was blocked by lanthanum ions (La^{3+}). The effect was characterized by two slopes, initially 0.96 AU/min, followed by a secondary slope of 0.37 AU/min. This effect was also characterized by a plateau after 10 minutes. Changing to

Ca²⁺/Mg²⁺ free medium (a stimulus activating hemichannels) in the plateau did not modify the dye uptake. The slope in the absence of PKC inhibitor was 0.01 AU/min. Treatment with PMA, a PKC activator, did not modify the membrane permeability. The electrophysiological results showed that the unitary conductance more prevalent in normal condition was 220±2 pS and increased to 440±4 pS in the presence to the PKC inhibitor. Confocal microscopy observations revealed that the PKC inhibitor induced internalization of Cx43 after about 5 min. **Conclusions:** The inhibition of PKC induces a rapid increase in plasma membrane permeability mediated by Cx43 hemichannels, a phenomenon that begins to decline after 5 minutes, and is probably mediated by internalization of hemichannels. Supported by grants from FONDECYT (3120006 to JLV) and the Heart and Stroke Foundation of Canada (to CCN).

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Identification and Characterization of a Novel Substrate of Protein Kinase C that Promotes Motility of Human Breast Cells.

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Engineered over-expression of protein kinase C- α (PKC α) is known to promote motility of non-transformed human breast MCF-10A cells. Additional PKC isoforms may collaborate in selecting the same or different substrates to produce the motility phenotype. In an effort to compare the phosphorylation patterns of individual PKC isoforms and to identify PKC substrates that lie on the motility pathway, we applied the traceable kinase method to three PKC isoforms (α , δ , and ζ). By use of a non-radioactive version of this method that entailed co-immunoprecipitation of high affinity substrates, we analyzed phospho-protein profiles by Western blot with an antibody that recognizes the phosphorylated PKC consensus site. Profiles generated by PKC- α and δ were similar and differed markedly from that of PKC- ζ . Mass spectrometry of selected bands revealed known PKC substrates and several potential substrates that included the small GTPase-associated effector protein Cdc42 effector protein-4 (CEP4). Of the potential substrates tested (PAK2, ROCK, and CLASP), only CEP4 was phosphorylated by pure PKC- α , δ , and ζ isoforms in vitro, and by endogenous PKC isoforms in MCF-10A cells treated with diacylglycerol (DAG)-lactone, a membrane permeable PKC activator. Following DAG-lactone stimulation of MCF-10A cells, the stoichiometry of CEP4 phosphorylation was 3.2 + 0.5 (mol phospho-CEP4/mol CEP4). After knock-down with isoform-specific shRNA-encoding plasmids, phosphorylation of CEP4 was substantially decreased in response to silencing of each of the three isoforms. These findings identify CEP4 as a novel intracellular PKC substrate of multiple PKC isoforms. The significance of phosphorylation at one PKC consensus site (Ser-18) was analyzed by preparing CEP4 as either the pseudo-phosphorylated mutant (S18D) or phosphorylation-resistant mutant (S18N). The pseudo-phosphorylated mutant was found to stimulate motile behavior of non-motile MCF-10A cells by 3-fold thereby recapitulating the role of DAG-activated PKC in these cells. This mutant also stimulated the motility of metastatic human breast cells (MDA-MB-231 cells) by 3-fold, whereas the phosphorylation-resistant CEP4 mutant suppressed motility of these highly motile cells by 2-fold. These studies identify CEP4 as a PKC substrate whose phosphorylation promotes the motility phenotype, thereby implicating Cdc42-related pathways in this behavior.

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il-32 θ , a new il-32 isoform, inhibited il-1 β and tnf- α productions through interaction with pkc δ and suppressing mapk activities in pma-treated human monocyte.

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various il-32 isoforms have been discovered as pro-inflammatory cytokines in immune response. however, il-32 β has an anti-inflammatory effect as in our previous report. in this study, we have identified a new il-32 isoform, il-32 θ , which exhibited anti-inflammatory effect in its capacity as an intracellular mediator. we constructed thp-1 (il-32 θ) human monocyte cells which stably express il-32 θ . stimulation of pma but not lps and poly i:c suppressed the expression levels of pro-inflammatory cytokines in thp-1 (il-32 θ) human monocyte cells. pma directly influences novel pkcs activities because diacyl glycerol only is required for activation of novel pkcs in contrast with classical pkcs. interestingly, il-32 θ interacted with only pkc δ and also pkc ϵ to a lesser degree among the novel pkc isoforms under pma stimulation. additionally, pan-pkc inhibitor g δ 6850 and pkc δ -specific inhibitor rottlerin, inhibited these interaction of il-32 θ , but classical pkc inhibitor g δ 6976 weakly altered interaction between pkc δ and il-32 θ . furthermore, pma induced-phosphorylations of mitogen-activated protein kinases such as erk and p38, were inhibited in the presence of il-32 θ . these results suggest that interaction of il-32 θ and pkc δ is required for inhibiting pma induced il-1 β and tnf- α productions through suppressing mapk activities.

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Spatial regulation of the Greatwall kinase in the cell cycle.

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Mitotic entry depends on the phosphorylation of cyclin B-Cdk1 substrates, whereas proper mitotic exit requires their dephosphorylation. In metazoans, reversal of Cdk1-dependent phosphorylation largely depends on Protein Phosphatase 2A in complex with its B55/Twins adaptor subunits (PP2A-B55). Inactivation of PP2A-B55 at mitotic entry promotes the accumulation of phosphorylated Cdk1 substrates, and is mediated by the Greatwall kinase (Gwl), which phosphorylates and activates endosulfine and Arpp19 as inhibitors of PP2A-B55. Later, PP2A-B55 activity must rise back to allow mitotic exit. How Gwl is regulated through the cell cycle is poorly understood. We found that the subcellular localization of Gwl changes dramatically through the cell cycle. Gwl is nuclear in interphase and suddenly becomes mostly cytoplasmic in prophase, before nuclear envelope breakdown. We have identified two critical nuclear localization signals (NLS) in the central, poorly characterized region of Gwl. Moreover, we found that the Polo kinase associates with and phosphorylates Gwl in the same region. Polo activity promotes the cytoplasmic localization of Gwl. We show that a gain of Polo function in embryos with reduced Gwl levels leads to frequent failure of mitotic entry in syncytial nuclei. We propose that Gwl activity is regulated by changes in its subcellular localization through the cell cycle via a mechanism that involves the inactivation of its NLSs in its central region by Polo-dependent phosphorylation.

Signaling Receptors (RTKs and GPCRs) II

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Semaphorin-plexin signaling: a tale of two dimers and two small GTPases.

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Plexins are cell surface receptors that bind to semaphorins and transduce signals for regulating neuronal development, immune response and many other essential processes. Signaling through plexins has been proposed to rely on its GTPase activating protein (GAP) activity specific for R-Ras and M-Ras. Activation of this GAP activity appears to require simultaneous binding of semaphorin and a RhoGTPase to the plexin extracellular and cytoplasmic regions, respectively. However, the plexin GAP activity has eluded detection in several recent studies, and the mechanisms by which semaphorin and RhoGTPase activate plexins remain elusive.

We discovered that, surprisingly, purified plexin cytoplasmic regions act as GAPs specific for Rap but not for R-Ras or M-Ras. Mutational analyses suggest that plexins utilize a catalytic mechanism distinct from the canonical RapGAPs. The RapGAP activity of plexins is normally autoinhibited, which can be robustly activated by induced dimerization. The crystal structure of PlexinA1 cytoplasmic region bound to a RhoGTPase was also determined. Structural and biochemical analyses demonstrate that binding of the RhoGTPases does not contribute to plexin RapGAP activation directly. Finally, semaphorin activates the RapGAP activity of full-length plexin in cells, which is required for plexin-mediated neuronal growth cone collapse.

In addition, we solved the crystal structure of PlexinA4 cytoplasmic region, which reveals a novel conformation that mediates the formation of a dimer. In the dimer structure the GAP active sites of plexin are completely buried, suggesting that plexin is kept inactive by the formation of an inhibitory dimer. Furthermore, this structure presents an elegant mechanism by which the binding of semaphorin and RhoGTPase promote the dissociation of the inhibitory dimer and simultaneously induce the formation of an activating dimer, leading to plexin activation and downstream signal transduction.

To summarize, these findings together define an essential pathway for semaphorin-plexin signaling, and provide a framework for understanding the molecular mechanisms of signal transduction through the plexin family receptors.

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Imaging of Fc_γ receptor signaling complexes dynamics by TIRF.

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IgG-presenting supported lipid bilayers were prepared to study the maturation of signaling complexes downstream of Fc_γ receptor engagement in macrophages. Time-lapse multicolor total internal reflection (TIRF) microscopy was used to demonstrate that mobile IgG caused macrophages to bind to the lipid bilayer and extend pseudopodia. Fc_γR/IgG complexes formed at the leading edge of the advancing pseudopod. These complexes then moved toward the center of the cell to form a structure reminiscent of the supramolecular complex observed in the immune synapse. Colocalization of Grb2 with nascent clusters of IgG indicated that phosphorylated receptor complexes underwent maturation as they trafficked toward the middle of the cell. Localization of AktPH domain indicated that 3'-phosphoinositides were distributed

throughout the pseudopod and were not enriched near activated receptors. This system should provide new insight into the remodeling of single receptor-associated signaling complexes.

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The expression and localization of betaglycan in the prostate of the adult brushtail possum (*Trichosurus vulpecula*).

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Benign prostatic hyperplasia is a common disease in men that is thought to be related to an abnormality of prostate growth. Understanding of the disease is hampered by a lack of a good animal model and a clear knowledge of the normal regulation of prostate growth. The brushtail possum (*Trichosurus vulpecula*) has a prostate that structurally resembles the human prostate gland and also exhibits seasonal growth and regression, thus making it a useful model to explore factors involved in physiological growth regulation. The transforming growth factor betas (TGF- β 1-3) are important growth inhibitory cytokines, that have been implicated in the regulation of prostate growth. TGF- β exerts its action as a growth regulator through inhibiting cell proliferation, activating differentiation, inducing apoptosis [1, 2] and initiating growth arrest [3, 4]. Betaglycan, the type III transforming growth factor- β receptor (TGF- β RIII), is an accessory co-receptor in the TGF- β superfamily. Betaglycan is present as a membrane bound and a soluble form. The membrane bound form presents TGF- β to the TGF- β RII thereby promoting cellular responsiveness to TGF- β , whereas the soluble form can sequester TGF- β , reducing the effect of the ligand.

The current study investigates the expression and localization of betaglycan in the possum prostate during seasonal growth and regression. Groups of three to seven animals were sacrificed at two monthly intervals during the year. The prostate was removed, weighed and bisected with half of the tissue frozen for Western blot analysis and the remaining tissue fixed for immunohistochemistry. Prostate tissue sections were incubated with TGF- β RIII goat polyclonal antibody raised to the ectodomain portion of betaglycan (Abcam, Sapphire Bioscience Pty Ltd, UK), negative controls were incubated with goat IgG. Western blotting was executed using the same antibody and expression quantified using the Odyssey (Li-cor Biosciences, Nebraska, USA) system.

Immunoreactivity for TGF- β RIII was localized predominantly to the epithelial and basal cells of the periurethral acini and the epithelial cells of the glandular regions. No immunopositive cells were observed in the adjacent stromal tissue. No change in the localization of TGF- β RIII was observed during the year. Western blotting demonstrated two immunoreactive bands with molecular size of 60 kDa and 80 kDa.

Expression of TGF- β RIII was highest in the glandular and periurethral regions of the prostate at the end of the main breeding period ($p < 0.05$) and immediately in advance of prostate regression. This corresponds with our earlier finding that changes in TGF- β 3 expression also increase at this time. This present study suggests that TGF- β RIII may be involved in promoting regression of the possum prostate.

Key words: betaglycan; TGF- β RIII; prostate

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Role of the truncated form of the human growth hormone receptor (GHR) in regulating GH effects.

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It is well known that the binding of human Growth Hormone to its receptor (GHR) on target cells leads to activation of three major intracellular signaling pathways: JAK2/STAT, PI3K/AKT and MAPK/ERK. However, in addition to the full length (FL) GHR, there exist two truncated (Tr) GHR isoforms, GHR1-279 and GHR1-277, that lack the majority of their intracellular domain as a result of alternative splicing of GHR mRNA. Tissue levels of these isoforms vary considerably, with the 1-279 form predominating over the 1-277 by ~10-fold. While both *in vitro* and *in vivo* studies suggest that the Tr GHR isoforms have a dominant negative effect on FL GHR by forming stable heterodimers, little is known about their normal physiological functions and their effects on the three major GH signaling pathways.

To address this, we have analyzed the changes in a target cell's response to GH as the ratio of Tr/FL GHR shifts. We hypothesized that Tr GHR isoforms play a functionally significant role in human cells and tissues, fine-tuning the ability of GH to activate intracellular signaling and, thus, its biological effectiveness.

We examined the effects of GH stimulation on its signaling pathways through western blot analysis of phosphorylated vs. non-phosphorylated forms of STAT5b, AKT and ERK1. In HEK293 cells, where RT-PCR and western blot analyses revealed abundant FL GHR and undetectable amounts of Tr GHR, GH stimulation resulted in rapid and transient effects on pSTAT5b, pAKT and pERK1. Increasing the Tr/FL GHR ratios, by transient transfection of a Tr GHR1-279 expression vector, resulted in a significant dose-related inhibition of GH's ability to phosphorylate STAT5b compared to non-transfected cells: a 1:1 ratio resulted in a ~2-fold decrease ($p < 0.05$), and a 3:1 ratio resulted in a ~6-fold decrease ($p < 0.05$). Investigations of Tr GHR's effects on the AKT and MAPK pathway are ongoing.

These data suggest that Tr GHR can limit the target cell's signaling in response to GH, revealing a possible physiological role for Tr GHR in modulating GH's biological activity differentially in its target tissues.

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A synthetic T cell receptor can activate Jurkat cells.

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T cells are activated when the T-cell antigen receptor (TCR) binds to an antigenic peptide bound in the major histocompatibility complex (pMHC) expressed on the surface of antigen presenting cells (APC). TCR signaling is mediated by tyrosine phosphorylation but the receptor itself has no intrinsic kinase activity. The molecular mechanism by which TCR-pMHC engagement is 'read-out' to trigger T-cell activation remains unclear. Previously we developed a synthetic

monomeric receptor that can act in a similar manner to the TCR. This receptor consists of the cytoplasmic phosphorylation sites from CD3zeta fused to an extracellular FKBP domain, which in the presence of the drug rapamycin can bind to FRB presented on the surface of an APC. Here, we report that this synthetic receptor is sufficient to activate the TCR signaling cascade in immortalized Jurkat T cells. Through live cell assays using T-cell-APC conjugates and functionalized supported lipid bilayers, we demonstrate that ligand binding to our synthetic receptor is sufficient to induce cell spreading and the formation of signaling micro-clusters. The downstream kinase effector ZAP70 and the inhibitory phosphatase CD45 are recruited and excluded, respectively, from these micro-clusters. Furthermore, Jurkat T cells are able to dynamically reorganize these signaling domains into a pattern that is analogous to an immunological synapse. Our synthetic receptor does not trans-activate the TCR complex, indicating it functions in an orthogonal manner to the TCR complex. This synthetic and inducible receptor provides a useful new tool to dissect the biophysical and molecular details of T cell activation.

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Rin1, via activation of Rab5, is a key regulator of preadipocyte 3T3-L1 differentiation.

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Ras interference 1 (Rin1) is a multifunctional protein involved both in endocytosis and signal transduction of the Insulin Receptor. In this study we used the preadipocyte 3T3-L1 cells to determine the effects of Rin1 (and its domains) during the differentiation process. Several point mutations were done on key amino acids that affect the function of each domain in order to analyze their effects on preadipocyte 3T3-L1 cells differentiation. Specifically, lipid droplet formation was quantified with ORO staining and adipogenic markers (i.e. PPAR γ , CEBP α , AMPK, MAPK, and AKT) were analyzed via western blotting. The data indicate that expression of Rin1 wild type has a dramatic inhibitory effect on preadipocyte differentiation as evidenced by the decrease of lipid droplet formation and the expression of selective adipogenic markers. Similarly, the expression of several Rin1 domains (i.e., VSP9 and SH2 domains) showed significant inhibition of preadipocyte 3T3-L1 cell differentiation. Taken together, these data suggest not only an important inhibitory effect of Rin1 on 3T3-L1 differentiation, but that all its functional domains are necessary for the negative effect in adipocyte formation.

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Action potentials induce Ca²⁺ signals in Schwann cells in myelinated axons: dynamic measurements using transgenically targeted YC 3.60 cameleon Ca²⁺ indicator.

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At the nodes of Ranvier, the axon, the myelin sheath and the myelinating glial cell are organized to operate as an integrated ensemble, the axoglial apparatus. During action potential conduction, the axonal specializations at the node, together with the adjacent paranodal terminations of the myelin sheath interact at the nodal complex. We have previously shown that a number of proteins involved in Ca²⁺ signaling cascade are concentrated in areas of close contact between axons and Schwann cells in the axoglial apparatus. It is likely that this architecture supports mutual signals between the axon and the Schwann cell. We tested this hypothesis in isolated sciatic nerve preparations using nerves from transgenic mice expressing the Ca²⁺ indicator, YC 3.60 discretely in Schwann cells. Isolated sciatic nerves freed of the perineurium were held between suction electrodes and positioned under a 2-photon microscope.

YC 3.60 fluorescence was imaged at 805 nm excitation and emission was collected at 450 nm (CFP) and 560 nm (YFP) and were represented as YFP/CFP ratios. Stimulation of nerves (25V at 100 Hz, 100 μ s pulses for 60s) elicited robust action potentials which were associated with Ca^{2+} signals in cytoplasmic regions of the Schwann cells. While in some instances, the Ca^{2+} signals occurred simultaneously throughout the Schwann cell cytoplasm, in others the signals occurred in a linearly propagated manner, and in some others as Ca^{2+} oscillations. Pretreatment with tetrodotoxin (1 μ M) abolished both action potentials and the associated Ca^{2+} signals. Similarly, pretreatment with purinergic receptor antagonists (MRS 2179, Suramin or reactive blue) reversibly blocked these Ca^{2+} signals. Interestingly, blockade of K^+ channels with 4-aminopyridine caused significant action potential widening but significantly reduced the amplitude of Schwann cell Ca^{2+} signals. This data might suggest a K^+ accumulation mediated cellular swelling leading to volume activated anion channel gating, and ATP release. Functional purinergic receptors exist in Schwann cells that ensheath sciatic nerve axons since exposure of nerves to purinergic agonists elicited Ca^{2+} signals in Schwann cells. Using the lack of sensitivity to the absence of extracellular Ca^{2+} , and pharmacological profiling we tentatively identify the receptor type on Schwann cells to be P2Y2. Such action potential driven Schwann cell Ca^{2+} signals may serve to maintain the integrity of the myelin sheath.

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Effect of PARs on $[\text{Ca}^{2+}]_i$ dynamics of sympathetic ganglia of rats.

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Protease-activated receptors (PARs) mediate cellular responses to various proteases in numerous cell types, including nerve cells. The issue of whether stimulation of PARs induces responses in neurons and satellite cells of sympathetic superior cervical ganglia (SCG) of rats was examined with special reference to PAR mRNA levels and to intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) changes, since $[\text{Ca}^{2+}]_i$ is a key factor in intracellular signaling. SCG whose essential structural integrity was maintained intact, were used. RT-PCR showed that SCG expressed mRNAs encoding PAR1, 2 and 3, among which, PAR2 expression was the highest. Confocal microscopic analysis indicated that thrombin and trypsin induced an increase in $[\text{Ca}^{2+}]_i$ in both some neurons and many satellite cells; initially, these proteases elicited a $[\text{Ca}^{2+}]_i$ increase in satellite cells, and subsequently, a $[\text{Ca}^{2+}]_i$ change in neurons was observed. Synchronized $[\text{Ca}^{2+}]_i$ changes in satellite cells were often observed. Neither the removal of extracellular Ca^{2+} nor Ca^{2+} channel blockers, affected trypsin- or PAR2-activating peptide (PAR2-AP)-induced $[\text{Ca}^{2+}]_i$ changes in satellite cells, suggesting that these changes were caused by Ca^{2+} mobilization from an internal store, but not by Ca^{2+} influx. However, neither the phospholipase C inhibitor U73122 nor the IP3 receptor antagonist heparin could inhibit the $[\text{Ca}^{2+}]_i$ changes of satellite cells, whereas the changes of neurons were considerably inhibited. This is the first study to demonstrate the presence of PARs in sympathetic nervous tissue, and to show that proteases induce $[\text{Ca}^{2+}]_i$ changes in both neurons and satellite cells via Ca^{2+} mobilization.

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ICAM-1 Ring Expression and Role in Transmigration of Leukocytes on Endothelial Cells.

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Vascular diseases, including stroke and atherosclerosis, remain the leading cause of death in the world. Intracellular adhesion molecule type 1 (ICAM-1) plays a crucial role in the evolution of vascular diseases. It has been found that risk factors such as diets high in cholesterol,

smoking, diabetes, cytokines and chemokines primes arteries for vascular disease, and increase ICAM-1 expression. It is also known, with aging and vascular disease, arteries lose their elasticity and become stiffer. What remains clear is how arterial stiffness, activation of endothelial cells and transmigration of leukocytes effect the expression and distribution of ICAM-1. To elucidate the effects of endothelial activation on the distribution of ICAM-1 we used an in vitro model of the human aortic endothelium treated with TNF- α (25 μ g/mL) for 0, 2, 4 and 24 hrs and stained for ICAM-1. We found an ICAM-1 expression increase proportional to the activation time. At 24 hrs there is uniform expression along with ICAM-1 rings, which were clusters of ICAM-1 in a ring shape around a circle of membrane that was absent of ICAM-1 expression. While typically ICAM-1 forms clusters around leukocytes, we observed ICAM-1 cluster in the absence of any cell interacting with the endothelial surface. These ICAM-1 rings could play a role in transmigration prior to the presence of leukocytes as a response to inflammation. However, ICAM-1 expression was localized to the nucleolus and no rings were observed when the monolayer was not treated with TNF- α . To determine the effects of arterial stiffness on ICAM-1 expression we added TNF- α to HUVEC monolayers on either soft (1 kPa), physiological (5 kPa) or stiff (70 kPa) substrates and found that the ICAM-1 rings were larger with softer surfaces. While it is known that ICAM-1 plays a role in adhesion of leukocytes to vascular surfaces, our research is observing if ICAM-1 also plays a role beyond adhesion, and actually travels with the leukocyte throughout diapedesis. We stained for ICAM-1 prior to leukocyte transmigration and post-transmigration, to observe any variations in the ICAM-1 apical-basal ratio, suggesting that ICAM-1 is involved in more than just adhesion. A full understanding of how ICAM-1 is involved in transmigration could give us a better understanding of the effects of vascular disease.

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Cbl and Cbl-b attenuate macrophage proliferation and facilitate macrophage colony stimulating factor receptor traffic into nascent macropinosomes.

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Casitas B-cell lymphoma (Cbl) family E3 ubiquitin ligases are critical negative regulators for receptor tyrosine kinase signaling. Cbl and Cbl-b double deficient (Cbl/Cbl-b dKO) bone marrow cells are hypersensitive to multiple cytokines. Cbl promotes M-CSF receptor (M-CSFR) multiubiquitination and endocytosis, and can significantly attenuate bone marrow macrophage proliferation in response to M-CSF. Here we show that Cbl/Cbl-b dKO bone marrow cells are hypersensitive to M-CSF, producing approximately 50 times more macrophages at day 7 compared to wild type cells. Immunofluorescence staining against plasma membrane M-CSFR revealed that activated M-CSFR was internalized at similar rate in both wild type and Cbl/Cbl-b dKO macrophages. However, the endocytic trafficking of internalized receptor is significantly changed. Previously, we demonstrated that nascent macropinosomes are important repositories for activated M-CSFR and may play a role in suppressing M-CSFR signaling. In wild type macrophages, most of the receptor traffics to large-dextran positive macropinosomes by 15 minutes after exposure to M-CSF. Strikingly, in Cbl/Cbl-b dKO macrophage there is almost no receptor trafficking into macropinosomes. Therefore, we propose that Cbl and Cbl-b mediate targeting of activated M-CSFR to macropinosomes as a novel mechanism of receptor down-regulation.

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Connexin-mediated release of pro-fibrotic ATP and hydrolysis of ATP to anti-fibrotic adenosine regulates the fibrogenic set-point of cardiac fibroblasts.

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Cardiac fibroblasts (CFs) maintain the extracellular matrix (ECM) in the heart, contributing to both basal formation of ECM and the enhanced ECM generation that occurs with cardiac remodeling after injury. Myocardial stress or injury can trigger excessive deposition of collagens and other ECM proteins by CFs, resulting in cardiac fibrosis, a response that may involve signaling by extracellular nucleotides. Cellular stretch and swelling release nucleotides from rat CFs via connexin (Cx) hemi-channels. siRNA knockdown of Cx43 reduced swelling-induced release of ATP in CFs by 47% ($p < 0.001$); similar results were seen with Cx45 knockdown. ATP stimulates α -smooth muscle actin (α SMA) and collagen synthesis via P2Y₂ receptors, but nucleotide hydrolysis, achieved by adding apyrase, substantially blunts production of α SMA protein and collagen (>90%, $p < 0.001$ and 40%, $p < 0.001$, respectively). Release of nucleotides thus likely plays a major role in both activating CFs during injury and maintaining the "set-point" of resting CFs. Because rat CFs express at least two ectonucleoside triphosphate diphosphohydrolase isoforms, ENTPD-1 and -2, which convert ATP to AMP/adenosine, we hypothesized that release of ATP and subsequent hydrolysis by ENTPDs represents counterbalancing pro- and anti-fibrotic pathways via nucleotide/P2Y and adenosine/P1 signaling, respectively. We found that inhibition of ENTPDs in CFs by polyoxometalate (POM)-1 increased α SMA protein expression and collagen accumulation by 90% ($p < 0.05$) and 2-fold ($p < 0.001$), respectively. Furthermore, POM-1 increased CF-mediated collagen gel contraction. Adenosine signaling in CFs is anti-fibrotic, via Gs-coupled P1 receptor activation and generation of cAMP. We find that degradation of extracellular adenosine by adenosine deaminase (ADA) increases basal collagen accumulation by 67% ($p < 0.01$) and α SMA protein expression by ~2-fold ($p < 0.05$). Moreover, ATP treatment was additive with ADA in increasing α SMA expression. Thus, basal nucleotide signaling is pro-fibrotic in CFs but is blunted by ENTPDs that generate adenosine. We conclude that cellular release of ATP, which activates pro-fibrotic P2Y₂ receptors, also signals via anti-fibrotic receptors for adenosine, which is generated by endogenous ENTPDs. Nucleotide signaling, degradation and adenosine generation thus regulate the set-point of CFs, and the rate and extent of ATP release and hydrolysis may determine the fibrotic response of CFs during injury or in pathological states.

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Adenosine-Induced Intracellular Signaling in Rabbit Lacrimal Gland Cells.

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The objective of the current study was to study the intracellular signaling events following adenosine stimulation of rabbit lacrimal gland (LG) cells. The LG produces fluid and proteins for the tear film aqueous layer. Dry eye conditions can be caused by LG dysfunction, leading to high discomfort, corneal infections and damage to the ocular surface. To understand the pathophysiology of dry eye, the normal physiology of the LG must be studied. In the current study, signal transduction pathways of the protein secretion process in LG acinar cells were investigated. Adenosine and carbachol have been shown to act synergistically on rabbit lacrimal gland cell protein secretion. To assess the intracellular mechanism of this synergy, rabbit lacrimal acinar cells in primary culture were stimulated with carbachol and adenosine. Activity, levels of key elements were measured as well as secretory response. The results of the study include a significant increase of cAMP levels in cells co-stimulated with adenosine and

carbachol compared to cells treated with only one of them, with adenosine and carbachol yielding a 2-fold increase in cAMP concentration compared to adenosine alone, and an approximate 8-fold increase compared to cAMP levels found following carbachol stimulation alone. Adenosine is traditionally known to affect cAMP levels, while carbachol is not. Studies with specific adenosine receptor agonists reveal that the effect is mainly due to the A₂ receptors. However, preliminary results of treatment with an adenylate cyclase inhibitor reveal that the changes of cAMP levels are not connected to the secretory responses. Phosphorylation of ERK1/2 has previously been shown to be important for the corresponding synergy in rat LG. Our results from Western blotting show no change in ERK1/2 phosphorylation following co-stimulation with adenosine and carbachol. Another MAPK, p38, has recently been shown important for carbachol signaling in the lacrimal gland. However, the results of this study indicate that p38 have minor or no importance in the synergy of adenosine and carbachol. The study concludes that cAMP formation and ERK1/2 and p38 phosphorylation do not have major roles in the adenosine-carbachol synergy in rabbit LG cells.

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CB1 Cannabinoid Receptor Regulation of Focal Adhesion Kinase Signaling in Neuronal Cells.

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Our laboratory examined the signaling pathways that regulate CB1 cannabinoid receptor (CB1R)-stimulated FAK tyrosine phosphorylation (Tyr-P) in murine N18TG2 neuroblastoma cells that express endogenous CB1Rs. FAK regulation involves phosphorylation at multiple Tyr residues including Tyr 397 (autophosphorylation site) and Tyr 576/577 (for maximal catalytic activity). Site-specific antibodies were used in immunoblotting experiments to examine the time-course of CB1R-stimulated FAK phosphorylation at Tyr 397 and Tyr 576. The synthetic CB1R agonist WIN55212-2 (WIN, 10 nM) and the endogenous cannabinoid 2-arachidonoylglycerol (1 μM) produced three distinct phases of 576/577 Tyr-P: Phase I (0-5 min) involved maximal Tyr-P, Phase II (5-20 min) involved a decline in Tyr-P, and Phase III (> 20 min) involved a plateau in Tyr-P at submaximal levels. In contrast, 397 Tyr-P was sustained and was less robust in magnitude compared to Tyr 576/577. Phase I 576/577 Tyr-P was blocked by the CB1R antagonist SR141716A (1 μM) and pertussis toxin (100 ng/mL) which indicates CB1Rs and Gi/o proteins are required for maximal FAK activation. The Src inhibitor PP2 (2 μM) abolished Phase I 576/577 Tyr-P which demonstrates 576/577 Tyr-P is Src-dependent. Experiments confirmed N18TG2 cells express Flk-1 vascular endothelial growth factor receptors (Flk-1 VEGFR) and epidermal growth factor receptors (EGFR). Phase I 576/577 Tyr-P was inhibited by the Flk-1 VEGFR antagonist SU5416 (1 μM) and EGFR antagonist AG 1478 (2 μM) which suggests maximal FAK activation involves Flk-1 VEGFR/EGFR transactivation. Studies confirmed N18TG2 cells express fibronectin (α5β1) and laminin (α6β1, α7β1) integrin receptors. Phase I 576/577 Tyr-P was blocked by the integrin antagonist RGDS peptide (100 μM). CB1R agonists did not stimulate FAK Tyr-P in the absence of integrin activation in suspended N18TG2 cells. Cells grown on fibronectin and laminin displayed increased FAK Tyr-P that was augmented by CB1R agonists and blocked by PP2 (2 μM). Finally, the actin disrupting drugs Cytochalasin D (2 μM) and Latrunculin A (1 μM) abolished Phase I 576/577 Tyr-P which indicates maximal FAK activation depends on the integrity of the actin cytoskeleton. Co-immunoprecipitation experiments revealed WIN (10 nM) induces β-actin association with FAK during Phase I, while FAK was associated with the actin polymerizing protein neuronal Wiskott-Aldrich syndrome protein (N-WASP) during Phase I. Given FAK's role in cell migration and cell survival, this cellular model provides a better understanding of the signaling mechanisms that mediate the

neuroprotective and neurodevelopmental effects of endocannabinoids. Supported by NIDA grants R01DA003690 (ACH), F32DA026295 (GDD).

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CB₁ Cannabinoid Receptors Induce Akt Phosphorylation At Serine 473 Via An RGD Integrin/FAK/PI-3K Pathway In Neuronal Cells.

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Our laboratory investigated the signaling pathways utilized by CB₁ cannabinoid receptors (CB₁Rs) to activate the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway in murine N18TG2 neuronal cells. Immunoblotting experiments revealed a maximally efficacious concentration of the synthetic CB₁R agonist WIN55212-2 (WIN, 10 nM) produces an increase in Akt phosphorylation/activation at Serine 473 (Akt-pSer) that peaked maximally between 0-5 min and declined to near basal levels by 10-20 min. It is well established that focal adhesion kinase (FAK) is an important regulator of Akt phosphorylation/activation. In N18TG2 cells, the time-course of WIN-stimulated FAK tyrosine phosphorylation (Tyr-P)/activation exhibited similar kinetics to WIN-induced Akt-pSer. WIN-stimulated Akt-pSer and FAK Tyr-P were both blocked by the CB₁R antagonist SR141716A (1 μM) and pertussis toxin (100 ng/mL) which indicates CB₁Rs and G_{i/o} proteins are required for Akt/FAK activation. The Src inhibitor PP2 (2 μM) also abolished WIN-stimulated Akt-pSer and FAK Tyr-P which demonstrates Akt/FAK activation are Src-dependent. Previous studies have shown the p85 subunit of PI-3K binds directly to FAK that is phosphorylated at Tyr residue 397 and this initiates activation of the major downstream PI-3K effector Akt. In N18TG2 cells, WIN stimulated an increase in FAK phosphorylation at Tyr 397, while co-immunoprecipitation experiments confirmed WIN induces a 43% increase in the association of the p85 regulatory subunit of PI-3K with FAK. Additionally, the PI-3K inhibitor LY294002 (20 μM) abolished WIN-stimulated Akt-pSer, but did not affect WIN-stimulated FAK Tyr-P. Immunoblotting experiments confirmed N18TG2 cells express RGD fibronectin (α5β1) integrin receptors. WIN-stimulated Akt-pSer and FAK Tyr-P were inhibited by the integrin antagonist RGDS peptide (100 μM). WIN did not stimulate Akt-pSer or FAK Tyr-P in the absence of integrin activation in suspended N18TG2 cells, but this response was restored in cells plated on fibronectin (10 μg). Finally, studies have shown that Akt activation requires Akt recruitment to the plasma membrane. In N18TG2 cells, disruption of the actin cytoskeleton by the actin depolymerizing agents, Cytochalasin D (2 μM) and Latrunculin A (1 μM), abolished WIN-stimulated Akt-pSer and FAK Tyr-P which indicates Akt/FAK activation require an intact actin cytoskeleton. In summary, these studies demonstrate that CB₁Rs utilize RGD α5β1 integrin receptors to activate FAK, which functions upstream of PI-3K, and transduces a signal that ultimately activates Akt in neuronal cells. Supported by NIDA grants R01DA003690 (ACH), F32DA026295 (GDD).

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ET-1 induces COX-2 expression via an ET_{A/B} receptor/c-Src/PDGFR/PI3K/Akt/ERK1/2/AP-1 pathway in Murine osteoblast-like MC3T3-E1 cells.

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Bone structure and metabolism are maintained by bone remodeling cycle in the process of the osteoblasts formation and resorption. Induction of COX-2 expression and PGE₂ secretion through various intracellular signaling pathways by endothelin-1 (ET-1) may play an important role in inflammation and directly stimulate osteoblastic activity. However, the molecular mechanisms underlying ET-1-induced COX-2 expression in murine osteoblast-like cell line

(MC3T3-E1) were largely unknown. Here, we found that ET-1-induced COX-2 protein and mRNA expression, and PGE₂ synthesis which were attenuated by the inhibitor of ET_A receptor (BQ123), ET_B receptor (BQ788), Gi protein (GPAnt2), Gq protein (GPAnt2A), c-Src (PP1), PDGF receptor (AG1296), PI3K (LY294002), Akt (SH-5), or MEK1/2 (U0126) and transfection with siRNA of ETA, ETB, Gi, Gq, Akt, or ERK2. In addition, ET-1-stimulated phosphorylation of Src, Akt, and ERK1/2 was attenuated by PP1. Inhibition of PI3K/Akt by LY294002 and SH-5 attenuated Akt and ERK1/2 phosphorylation, but had no effect on Src phosphorylation, suggesting that Src may be an upstream component of PI3K/Akt and ERK1/2 in these responses. Pretreatment with U0126 attenuated the ERK1/2 phosphorylation stimulated by ET-1, but had no effect on Akt activation, suggesting that Akt may be an upstream component of ERK1/2 in these responses. Moreover, ET-1-stimulated c-Fos expression and c-Jun phosphorylation in the nuclear fraction was also attenuated by PP1, AG1296, LY294002, SH-5, and U0126. These results suggested that ET-1-induced COX-2 expression and PGE₂ synthesis mediated through an ET_{A/B} receptor/c-Src/PDGFR/PI3K/Akt/ERK1/2/AP-1 pathway in MC3T3-E1 cells.

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Suppression of neurite elongation in PC12 cells due to NF-YA and NF-YC knockdown.

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Heterotrimeric transcription factor NF-Y controls various genes, through interaction with the CCAAT-boxes, and various cellular events: cell growth, aging, survival, cyto-architecture etc. For the analysis of its functions, we examined effects of NF-Y knockdown on nerve growth factor (NGF)-induced neurite outgrowth in PC12 rat adrenal pheochromocytoma cells. For the tetracycline-dependent gene expression-regulatory system, PC12 and HeLa cells stably expressing a tetracycline repressor were employed. Constructs used were pCMV-GFP (a green fluorescent protein) plasmids constitutively expressing GFP-NF-Y fusion proteins, pTRE-GFP plasmids expressing GFP-NF-Y fusion proteins under tetracycline control, pRNATin plasmids expressing shRNA targeting human or rat NF-Y mRNA under tetracycline control and also bearing a GFP reporter gene, and their related plasmids. These were introduced into the cells by lipofection to examine for neurite elongation by microscopy and for mRNA amounts by real-time RT-PCR. Subcellular localization of NF-Y subunits was analyzed by using their GFP-fusion proteins: NF-YA was found in the nucleus, whereas NF-YB and NF-YC were distributed mainly to the cytoplasm but also present in the nucleus to a lesser extent. It is considered that under reductive intracellular conditions NF-YB forms a dimer with NF-YC and moves into the nucleus, followed by association with NF-YA to form a functional heterotrimer. Also, NF-Y has been suggested to enter the nucleus by the action of p38 MAP kinase and to lead to p53 elevation and SHP-1 suppression, which keeps the NGF receptor TrkA active. Herein, upon treatment with hydrogen peroxide or a p38 MAP kinase inhibitor SB203580, NF-YA appeared to be diffused in the cytoplasm, suggesting disturbance in its nuclear localization. Since neurite formation is known to involve MEK/ERK signaling, effects of shRNA targeting GRB2 (an adaptor protein working at the upper part of MEK-ERK pathways) were checked as a control experiment: the GRB2 shRNA suppressed NGF-induced neurite elongation in Dox-treated cells, but not in Dox-untreated cells. Next, with NF-Y subunit shRNA, targeting NF-YA and NF-YC was found to suppress neurite elongation. Targeting NF-YB did not much affect the neurites, presumably reflecting a difference in the limiting effects of the NF-Y subunits. These observations showed NF-Y control of the NGF and MEK-ERK signaling, which might be modulated by its nuclear localization, implying the mechanisms for physiological fine-tuning through such subcellular conditions as redox and stress affecting the NF-Y functions.

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Evidence for a Cell Fate Refinement Mechanism in Sensory Neurons.

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The olfactory receptors (ORs), which are G-protein coupled receptors, number more than 1,000 and comprise the largest gene family in the mammalian genome. ORs are expressed both monogenically and monoallelically in olfactory sensory neurons (OSNs) and the mechanism that controls their regulation is largely unknown. ORs reside in constitutive heterochromatin and selection of one OR and from one allele occurs stochastically. To ensure singular monoallelic OR expression, a negative feedback mechanism is elicited by the first selected OR to suppress non-selected ORs in a given neuron.

Here we describe results for mice with a 'monoclonal' nose that express one OR, M71, in 95% of all mature OSNs. The M71 transgene suppresses expression of endogenous ORs, and remaining endogenous expression is mostly restricted to immature neurons in the olfactory epithelium. We show that the endogenous OR repertoire are expressed prior to suppression by M71 transgene expression, contrary to current models. When we introduced a second transgene into M71 mice that expressed another OR in most mature OSNs, OSNs uncharacteristically expressed both of the ORs. We hypothesize that unresolved OR competition compromised the neuron's ability to express only one receptor. We further show that suppression of endogenous ORs by M71 is not reversible, and that M71 does not need to be continuously expressed for endogenous ORs to remain suppressed.

In these experiments, we have engineered OSNs to express more than one OR in an OSN at a time, which is normally a low probability event. We believe that these experiments reveal the existence of a backup pathway that ensures only one OR will be expressed per neuron. It is possible that mammals have evolved similar mechanisms in other examples of stochastic, monoallelic gene expression, such as in random X-chromosome inactivation or lymphocyte receptor expression.

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Sphingolipids cause release from meiotic arrest and onset of apoptosis in *Xenopus* oocytes.

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Neutral sphingomyelinase (SMase) and progesterone (PG) each bring about both release from meiotic arrest and the onset of apoptosis in *Xenopus laevis* oocytes. These events are transduced by a G protein-coupled cell surface receptor and are oxidant-dependent. *Xenopus* oocyte maturation has been studied for many years, and it has been established that the G protein-coupled receptor activates the MAP kinase pathway. More recently, evidence has accumulated that demonstrates the activation of a parallel pathway involving the generation of ceramide, the activation of JNK, and the phosphorylation of Bad, culminating in caspase-3 activation and apoptosis in oocytes that are not quickly fertilized. Both oocyte maturation and apoptosis are sensitive to the redox environment of the cell; oxidative stress (e.g. H₂O₂ treatment, or simply the passage of time in media lacking nutritional supplements) result in apoptosis. Adding glutathione (GSH, 10 mM) to the medium inhibits apoptosis, but also blocks the ability of PG or SMase to cause maturation. The apoptotic death that is triggered by release from meiotic arrest correlates with an increased phosphorylation of Bad on residue S128, as evidenced by western blotting with an anti-S128 Bad phospho-specific antibody. We are

currently using both pharmacologic and genetic tools to study the relationships between sphingolipid metabolism, JNK activity, Bad S128 phosphorylation, redox status, and caspase-3 activity in *Xenopus* oocyte maturation and cell death.

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Lipopolysaccharide-induced 5-lipoxygenase expression in THP-1 monocytes is regulated by Akt mediated NF- κ B and Sp1 pathways.

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Monocyte adhesion to the vascular endothelium of blood vessels is key early event from inflammatory responses that lead to the development and progression of atherosclerosis in cardiovascular disease. However, the underlying mechanisms of monocyte adhesion to endothelium are still not completely understood. This study investigated the effects of LPS on monocyte adhesion to endothelial cells and the potential role for 5-LO in LPS-induced monocyte adhesion to endothelial cells. LPS-stimulated monocytes led to an increased adhesion to endothelial cells in a concentration-dependent manner. LPS-induced monocyte adhesion to endothelial cells were blunted by inhibition of 5-LO with MK886, a 5-LO inhibitor as well as monocytes from 5-LO deficient mice (5-LO^(-/-)). These results suggest that 5-LO is a pivotal role for LPS-induced monocyte adhesion to endothelial cells. Furthermore, to determine the potential role for LPS on the regulation of 5-LO expression, the molecular mechanisms of 5-LO was evaluated in LPS-stimulated THP-1 monocytes. LPS induced 5-LO mRNA and protein expression in associated with an enhanced promoter activity. The transcription factor of 5-LO promoter region including Sp1 and NF- κ B were associated with up-regulation of LPS-induced 5-LO expression. Linked to these results, the LPS-induced 5-LO expression was attenuated by AI, an Akt inhibitor. In parallel, LPS exclusively increased the phosphorylation of Akt in a time-dependent manner, suggesting that Akt pathways is a major element for LPS-induced 5-LO expression. Collectively, these data suggest that 5-LO expression is regulated by NF- κ B and Sp1 pathways through Akt activation in LPS-induced monocyte adhesion to endothelial cells. This mechanism may participate in the initiation of atherosclerosis in cardiovascular disease.

Signaling from the PM/Cytoplasm to the Nucleus

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Single-cell signaling dynamics reveal the logic of early response gene transcription driven by NF- κ B.

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NF- κ B is a family of dimeric transcription factors that regulate many genes→ associated with inflammation, cellular proliferation and survival as well as carcinogenesis. In the absence of extracellular signals, NF- κ B exists in complexes with inhibitory I κ B proteins. The I κ B/NF- κ B complexes are actively exported from the nucleus and NF- κ B is therefore predominantly localized to the cytoplasm. Inducers of the canonical NF- κ B pathway promote rapid degradation of I κ B proteins, releasing NF- κ B. The unmasked nuclear localization sequence of free NF- κ B directs it to the nucleus, where it can access the promoter regions of its target genes. For this reason, the degree of NF- κ B pathway activation is often equated with the amount of nuclear NF- κ B even though the correlation between NF- κ B localization and the NF- κ B-dependent

transcriptional response has not been determined in single cells. What does an individual cell 'read' from a translocation event? Does the transcriptional response reflect a simple ON/OFF switch, or is the response proportional to a specific aspect of this dynamic translocation event? In this work we examine the relationship between tumor necrosis factor (TNF)-induced NF- κ B translocation dynamics and the associated transcriptional response. We have developed a live-cell to fixed-cell workflow where we first image TNF-induced NF- κ B (p65/RelA) translocation dynamics and then count, in the same cells, the number of mRNA transcripts for NF- κ B target genes detected by single-molecule fluorescence in situ hybridization (smFISH). Correlating NF- κ B translocation dynamics to the number of transcripts for three rapidly induced NF- κ B-driven genes, we find that the quantity of nuclear NF- κ B at any single time point provides little information about its activation state. Instead, cells interpret changes in NF- κ B localization, measuring fold-change of nuclear NF- κ B. Each cell retains a 'memory' of its pre-ligand state and evaluates translocation events relative to this previous state. We found that the relationship between fold-change of nuclear NF- κ B and transcription in individual cells could not be predicted from existing computational models of the NF- κ B pathway. These observations have led us to a new model that provides insight into the regulatory circuitry that quantitatively controls NF- κ B-dependent early response gene transcription. Our results strongly suggest that competitive protein-DNA interactions provide a 'memory' required for fold-change detection.

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Inhibition of NF κ B activity upon loss of calreticulin function.

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Transcription factor NF κ B is activated by several processes including inflammation, endoplasmic-reticulum (ER) stress, increased Akt signaling and enhanced proteasomal degradation. Calreticulin (CRT) is an ER Ca²⁺ binding chaperone, which regulates many of cellular processes. Previously, we showed that CRT deficient cells are under ER stress and have elevated proteasome activity. A role for CRT has also been described in the regulation of immune response. These data collectively led us to hypothesize that loss of CRT function results in the activation of NF κ B signaling leading to enhanced resistance to apoptosis of these cells.

Wild type and CRT knockout mouse embryonic fibroblast were used to examine changes in the NF κ B signaling pathway. In contrast to our hypothesis, reporter gene assays showed a significant reduction in the basal NF κ B transcriptional activity. Furthermore, treatment with lipopolysaccharide increases the transcriptional activity of NF κ B in both the wild type and CRT deficient cells, however, the transcriptional activity of NF κ B was still significantly lower in the CRT deficient cells. Our data also shows that the reduced NF κ B activity in CRT deficient cells is not due to decreased p65 or p50 protein levels. To determine the mechanism of decreased NF κ B activity we examined changes in I κ B protein stability and showed a significant increase in the I κ B α protein level, which was abolished by Okadaic acid treatment.

We conclude that in the absence of CRT NF κ B signaling is inhibited due to decreased I κ B degradation and decreased NF κ B p65 nuclear translocation.

2298

Neuregulin1 Signaling Targets SRF and CREB and Activates the Muscle Spindle-specific Gene Egr3 Through a Composite SRF-CREB-binding Site.

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Muscle spindles are sensory receptors embedded within muscle that detect changes in muscle length. Each spindle is composed of specialized muscle fibers, known as intrafusal muscle fibers, along with the endings of axons from sensory neurons that innervate these muscle fibers and that convey length information to the CNS. Formation of muscle spindles involves neuregulin1 (NRG1) being released by sensory axons and activating ErbB receptors in muscle cells that are contacted, thereby triggering these cells to become intrafusal fibers. A critical component of the intracellular pathway that acts downstream of ErbBs is the transcription factor Egr3, which is transcriptionally induced as part of NRG1-ErbB signaling and in turn activates various target genes involved in formation of muscle spindles. The signaling relay within the NRG1-ErbB pathway that acts to induce Egr3 is presumably critical for muscle spindle formation but for the most part has not been determined. In the current studies, we examined, using cultured muscle cells, transcriptional regulatory mechanisms by which Egr3 responds to NRG1. We identified two adjacent upstream regulatory elements in the Egr3 gene, conforming to binding sites for cAMP response element binding protein (CREB) family members and for serum response factor (SRF), that are each involved in NRG-1 induced transcription. In myotube nuclear extracts, these elements bind protein complexes containing CREB and SRF. NRG1 signaling does not alter the DNA binding activity of CREB or SRF but instead targets CREB and SRF to presumably stimulate their transcriptional activity. To target SRF, we show that NRG1 signaling acts on the SRF coactivators myocardian-related transcription factor (MRTF)-A and MRTF-B, which are known to activate SRF-mediated transcription, by stimulating their translocation from the cytoplasm to the nucleus. CREB is phosphorylated in response to NRG1, which is consistent with it becoming activated. These results suggest that NRG1 induces expression of the muscle spindle-specific gene Egr3 by stimulating the transcriptional activity of CREB and SRF.

2299

Protein inhibitors of activated STAT (PIAS) proteins are new negative regulators of Runx2.

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Protein inhibitors of activated STAT (PIAS) proteins were initially identified as specific cofactors inhibiting DNA binding and transcriptional activation by the STAT family of transcription factors. PIAS family genes are expressed in osteoblasts. However, it still remains unknown whether any member of the PIAS family has any specific action on osteoblast differentiation. Here we report that PIAS proteins regulate the transcription factor Runx2, a master regulator of osteoblast differentiation. PIAS proteins, Pias1, Pias3 and Piasy, inhibit Runx2 transactivation activity. PIAS proteins and Runx2 showed colocalization to the same intranuclear regions in cotransfected COS1 cells. Piasy sequesters Runx2 from nuclear foci to the nuclear periphery. Histone deacetylases (HDACs) are also known to directly interact with Runx2 and inhibit its transactivation activity; however, PIAS-mediated repression was unaffected by the HDAC inhibitor Trichostatin A, suggesting that the repression was independent of HDAC catalytic activity. These results indicate that PIAS proteins function as new negative regulators of Runx2.

2300

Modulation of Cell Cycle Progression of Spermatocytes by Cigarette Smoke Condensate (CSC) via AHR-NRF2 Pathway.

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Aryl hydrocarbon receptor (AHR) is a ligand dependent transcription factor that regulates a battery of down stream targets in response to endogenous growth factors and exogenous environmental pollutants. *In vitro* and *in vivo* studies from our lab have demonstrated that AHR is expressed in murine spermatocytes and that its activation by CSC regulates several antioxidant enzymes. In this study, we show that exposure of a spermatocyte cell type [GC-2spd(ts)] to CSC *in vitro* induces the expression of nuclear factor erythroid 2-related factor 2 (NRF2), and translocation into the nucleus, where it is believed to modulate AHR expression by feedback mechanism. In addition, we demonstrate that the expression of NRF2 coincides with the activation of AHR as demonstrated by its prototypical indicator protein, CYP1A1. Results from pharmacological inhibition by AHR-antagonist (CH223191), as well as transfection of spermatocytes with *Ahr*-and *Nrf2*-siRNA and analysis of *Ahr*-KO mice testis by laser microdissection implicate the *Ahr-Nrf2* pathway in DNA damage and cell cycle arrest. *Ahr*-and *Nrf2*-siRNA transfection followed by q-RTPCR revealed the difference in the expression profiles of *Ahr*, *Nrf2*, *Cyp1a1*, *Ahr-R*, *Hsp90*, *Sod2*, *P21* and *Gadd45a* in the CSC treated spermatocytes. The regulatory role of AHR-NRF2 signaling in cell cycle progression was assessed through the regulation of cyclin D1 expression with or without CSC. Western blot and confocal localization of CSC exposed spermatocytes demonstrated that the cyclin D1 protein though unaltered in expression undergoes nuclear translocation, which was inhibited by AHR-antagonist and *Ahr*-siRNA. AHR knockdown by siRNA transfection significantly reduced the basal level of cyclin D1 expression. This observation was further strengthened with the identification of two AHR elements in the 5' UTR region of the *Cyclin D1* gene. Immunoprecipitation analysis of CSC treated spermatocytes demonstrated the interaction between AHR, NRF2 and cyclin D1 proteins. Thus the data obtained from these studies suggest that the cigarette smoke condensate has several AHR-agonists that are capable altering the growth pattern of spermatocytes through the AHR-NRF2 combined signaling mechanism.

2301

Nuclear translocation of the canonical Wnt-regulator Jade-1 is phosphorylation-dependent.

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Canonical Wnt signaling regulates cell-fate determination during embryogenesis, and more recently has been shown to be vital for a regenerative injury response in adult tissue. However, Wnt signaling must be strictly regulated as a lack of repression is associated with uncontrolled cell division leading to cyst formation or cancer. Jade-1 has recently been identified as a novel E3 ubiquitin ligase that targets the canonical Wnt effector beta-catenin for proteasomal degradation. Previous work in our laboratory demonstrated that Jade-1 localizes to the primary cilium, a hair-like organelle which projects from the apical surface of virtually every eukaryotic cell and is a critical regulator of several signaling pathways including Wnt. The ability of Jade-1 to negatively regulate canonical Wnt reporter activity was enhanced by nephrocystin-4 (NPHP4), a member of the nephronophthisis (NPH) protein complex, which co-localizes with

Jade-1 at the primary cilium. NPH is a cystic kidney disease and a leading genetic cause for renal failure in children. NPHP4 stabilized protein expression and nuclear translocation of Jade-1, and additively increased negative regulation of canonical Wnt reporter activity. Our results indicated that NPHP4 influences the phosphorylation status of Jade-1, and that inhibiting phosphatase activity blocked Jade-1 from entering the nucleus and blocked NPHP4-mediated Jade-1 stabilization. New findings confirm phosphorylation of Jade-1 using mass spectrometry, and identify a candidate kinase and corresponding phosphorylation site. A Jade-1 mutant lacking this phosphorylation site displays altered intracellular localization and WNT reporter activity. Currently, we address the role of Jade-1 phosphorylation in cilia biology and the pathogenesis of polycystic kidney diseases.

2302

MAP Kinase-Ternary Complex Factor exchange regulates a transcriptional switch to mediate adhesion-regulated proliferation.

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Cooperative signaling between cell adhesion to the extracellular matrix (ECM) and growth factors plays a central role in regulating immediate early gene expression and proliferation. Growth factors regulate the activity of the ternary complex factors (TCFs; Elk1, Net, and Sap-1), which act as transcription co-factors of serum response factor (SRF) that are activated by map kinase (MAPK) phosphorylation to regulate immediate early gene transcription. Although cell adhesion also can regulate immediate early gene expression and proliferation, it is unclear how adhesion to the ECM impinges upon MAPK/TCF signaling. Restricting adhesion and spreading of G₀-synchronized cells on substrates with decreasing size of micropatterned islands of fibronectin suppressed serum-induced immediate early gene expression and S-phase entry. Knockdown of Sap-1 decreased expression of the immediate early genes *egr1* and *fos* and subsequent proliferation normally present with high adhesion. Conversely, knockdown of Net rescued *egr1* and *fos* expression and proliferation normally suppressed by low adhesion. ChIP studies showed increased occupancy of *egr1* and *fos* promoters by Sap-1 with high adhesion, while low adhesion increased Net occupancy. This switch in TCF promoter binding was regulated by an adhesion-mediated switch in MAPK activity. Increasing adhesion enhanced serum-induced JNK activity while suppressing p38 activity, leading to increased Sap-1 phosphorylation and Net dephosphorylation, and switching Net with Sap-1 at *egr1* and *fos* promoters to support proliferation. Microarray studies confirmed this switch in TCF regulation of proliferative genes and uncovered novel gene targets and functions co-regulated by Sap-1 and Net. These data demonstrate a key role for the TCFs in adhesion-induced transcription and proliferation, and reveals a novel MAPK/TCF transcriptional switch that controls this process.

2303

Early Growth Response-1 Accelerates Liver Regeneration after Partially Hepatectomized Mice through GGPPS/MAPK signaling.

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Abstract

Background & Aims: Liver regeneration comprises a series of complicated processes, in which several transcription factors play an important role. Egr-1 is rapidly induced as part of the

immediate early gene response of the regenerating liver. Previous studies indicated that EGR-1^{-/-} mice exhibit delayed hepatocellular mitotic progression after partial hepatectomy. Whereas, the mechanism that Egr-1 in regulating regeneration progress is still unknown. Here we revealed a novel and important signal pathway mediated by Egr-1 in this process.

Methods: 70% partial hepatectomy (PH) were performed on mice after tail vein injection of adenoviral vectors, which expressing GFP (Ad-GFP), Egr-1(Ad-Egr-1) or Dominant negative Egr-1(Ad-DNEgr-1) to increase or down-regulate hepatic Egr-1 levels. Animals were sacrificed at intervals after the surgery, and the remnant livers were harvested and analyzed. In vitro study, serum starvation recovery model was selected to mimic liver regeneration process in normal human hepatic cells. The activities of proliferative- and cell-cycle-related signaling pathways were measured by Western blotting. The downstream of GGPPS was examined in the regenerating liver and human hepatic cells.

Results: Compared with wild mice, Loss of Egr-1 significantly inhibited liver recovery 5 days after PH. Consistent with the observation, the expression levels of key cell-cycle regulatory proteins, including CyclinD1, CyclinE and PCNA were markedly altered. The expression of GGPPS and the activity of downstream RAS/ERK/signaling pathway were inhibited by the DN-Egr-1 after partial hepatectomy, which is consistent with the serum-induced cell model. In addition, loss of Egr-1 aggravates the liver function with the increase serum ALT and AST level.

Conclusions: Egr-1 plays an important role in regulating the cell cycle of hepatocytes induced by partial hepatectomy. Egr-1 induced the expression of GGPPS and thus positive the regulation RAS/MAPK signaling, which could be an important mechanism underlying the regulated liver regeneration of Egr-1 in the liver.

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SmgGDS antagonizes BPGAP1-induced Ras/ERK activation and neuriteogenesis in PC12 cells differentiation.

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The process of cellular differentiation involves the intricate integration and control of multiple signaling networks. The Rho and Ras family of small GTPases and its regulators such as GAPs and GEFs play important roles in controlling cellular processes. BPGAP1 is a multidomain RhoGAP that regulates cell migration, endocytosis and ERK signaling via the concerted action of its proline-rich region (PRR), RhoGAP domain and the BNIP-2 and Cdc42GAP Homology (BCH) domain interacting with cortactin, endophilin, Rho, Cdc42, Mek2 and Pin1. Here, we further show that the BCH domain of BPGAP1 specifically targets K-Ras to induce a robust activation of ERK, leading to the differentiation of PC12 cells. However, such stimulatory effect was inhibited by both dominant negative mutants of Mek2 (Mek2K101A) and K-Ras (K-RasS17N) and also by the Small G-protein GDP Dissociation Stimulator (SmgGDS). Using live imaging to track the differentiation of PC12 cells, we have established that the interaction of BPGAP1 and SmgGDS affects not only the initiation of neurite outgrowth but also growth cone maturation and dynamics. These results demonstrate the versatility of the BCH domain of BPGAP1 in regulating ERK signaling by interacting with K-Ras and SmgGDS and support the unique role of BPGAP1 as a dual regulator for Ras and Rho signaling in cell morphogenesis and differentiation. This work was supported by a grant from the Ministry of Education, Singapore and in part by the Mechanobiology Institute, co-funded by the National Research Foundation and the Ministry of Education, Singapore.

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Modulation of Hedgehog signaling in renal cystogenesis is associated with changes in intracellular Ca²⁺ levels.

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Primary cilia are slender cellular extensions that are present on most vertebrate cells and regulate signal transduction pathways. Defects in primary cilia are associated with severe human conditions such as cystic kidney disease, cystic liver disease, cognitive impairment, blindness and obesity. THM1 is a ciliary protein required for proper cilia structure and is a negative modulator of Hedgehog (Hh) signaling (Tran et al. *Nat. Genet.* 2008). Deletion of murine *Thm1* during late embryogenesis results in large, fluid-filled cysts in the adult kidney. Importantly, deletion of *Thm1* together with *Gli2*, the primary transcriptional activator of the Hh pathway, significantly attenuates renal cyst formation, indicating a causal role for elevated Hh activity in *Thm1* renal cysts. Polycystic kidney disease (PKD) is a common and widely studied renal cystic pathology caused by genetic alteration of Polycystin 1 (PC1) or of Polycystin 2 (PC2) proteins. Since PC1 and PC2 (a GPCR and a Ca²⁺ channel, respectively) localize to primary cilia and regulate intracellular Ca²⁺ levels, the current hypothesis is that cilia-mediated changes in intracellular Ca²⁺ levels underlie cyst formation in the kidney ductal epithelium. To determine whether Hh signaling induces changes in intracellular Ca²⁺, we are using M1 mouse renal medullary collecting duct cells. Our initial characterization of M1 cells shows that ciliary proteins, THM1, Arl13B, Polaris, IFT52, PC1 and PC2, localize to primary cilia. M1 cells respond appropriately to small molecule Hh modulators; Hh agonist, SAG, increased expression of Hh target genes, *Ptch1* and *Gli1*, by 2.3 fold and 4.4 fold, respectively, while addition of Hh antagonists, Sant2 or Gant61, inhibited this up-regulation. Sant2 and Gant61 attenuated renal cyst formation in an embryonic metanephric organ culture assay, and caused 3- and 6-fold increases, respectively, in intracellular Ca²⁺ levels in M1 cells. We are currently investigating the source of increased intracellular Ca²⁺ by Hh antagonists and the effect of THM1 deficiency on intracellular Ca²⁺ levels using shRNA knock-down of *Thm1*. Together, our data suggest a role for Hh signaling in renal cystogenesis by possibly altering levels of intracellular Ca²⁺.

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Masparadin Effects on BMP Signaling Molecules.

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The hereditary spastic paraplegias are a group of upper motor neuron disorders characterized by continuous degradation of motor neurons trailing through the motor cortex, down the spinal cord, and out to the periphery. Mast syndrome (SPG21) is an autosomal-recessive complicated form of HSP that originates from a mutation of the ACP33/masparadin gene.

Bone Morphogenetic Proteins (BMPs) are a group of multifunctional growth factors that, along with forming bone and cartilage, influence axonal synaptic growth and function. Upon activation by BMP ligand, type II BMP receptors phosphorylate type I receptors. This activates intracellular signaling molecules Smad 1, 5 and 8. These bind Smad 4 and the Smad complex enters the cell nucleus to drive gene transcription.

Previous studies have shown multiple HSP-associated proteins to be inhibitors of BMP signaling. Therefore, our objective is to prove that masparadin is an additional BMP inhibitor. Veritably, masparadin-depleted neurons have exhibited increased axonal branching.

Preliminary results have demonstrated increases in BMP signaling in knockout MEFs over a series of five timepoints as compared to wildtype. BMP4 ligand stimulation will be done over the same series of timepoints for a more accurate comparison. Results have also demonstrated increases in knockout MEFs stimulated with BMP4 ligand for 1 Hr as compared to wildtype. Phosphorylated Smad 1/5 levels will be examined via western blot and compared to total Smad levels after stimulation with BMP4 ligand.

GFP-masparidin will be overexpressed in wildtype MEFs and compared against knockout MEFs to determine whether overexpression of masparidin changes BMP signaling levels and restore knockout levels to those found in wildtype. GFP-masparidin will also be overexpressed in Cos-7 cells and compared to control. Comparable experiments will be done using primary neuron cultures from wildtype and knockout mice.

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A Wnt-Id1 signaling pathway maintains self-renewal capacity in adult mouse neural stem cells.

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It is critical for populations of adult tissue-resident stem cells to maintain self-renewal capacity in order to avoid progressive cell depletion with increasing age. However, little is currently known regarding the signaling pathways and transcriptional networks that govern stem cell self-renewal in-vivo. Id family transcriptional regulators are expressed throughout life in multiple adult stem cell niches, including the subventricular zone (SVZ) of the mammalian brain. Induced loss of function of the Id homologues Id1 and Id3 leads to significant loss of self renewal capacity in primary cultures of adult mouse neural stem cells, along with increased propensity toward neural differentiation and increased expression of p16-INK4a and p21-CIP1. We examined the consequences of induced overexpression of Id1 in mouse primary cultures derived from transgenic animals and observed a simultaneous decrease in proliferation and increase in self-renewal capacity. This cellular phenotype is similar to that seen in adult neural stem cells treated with Wnt3a, an activator of canonical Wnt signaling. Wnt3a treatment strongly activated expression of Id1 in cultured cells, suggesting that Id may be a key mediator of the self-renewal program downstream of Wnt signaling in adult neural stem cells. Id factor loss of function blocked the ability of Wnt3a to up-regulate self-renewal capacity, and treatment with the small-molecule Wnt-signaling inhibitor JW74 led to a clear decrease in self-renewal capacity and Id1 expression level. Ongoing in-vivo experiments will explore the significance of this regulatory relationship in the SVZ of adult mice and in a mouse model of spontaneous glioma.

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The p66^{Shc} adapter protein regulates the morphogenesis and epithelial maturation of fetal mouse lungs.

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Multiple growth factor signaling pathways are mediated by Shc adapter proteins that, in turn, are expressed as three transcriptional and translational variants with distinct and opposing functions. The p66^{Shc} isoform has anti-mitogenic properties, regulates oxidative stress responses, and participates in mitochondria-mediated apoptosis. It is also highly expressed in the canalicular but not the saccular or alveolar stages of mouse lung development, suggesting an important morphogenetic function. Moreover, p66^{Shc} expression persists in the lungs of

infants with bronchopulmonary dysplasia, a chronic lung disease that is induced by premature birth. However, constitutive p66^{Shc} deletion yields no morphologic phenotype and the structure of the Shc gene precludes the development of an inducible isoform-specific knockout mouse. To elucidate its role in lung development, we microinjected Oligofectamine (Invitrogen) suspensions containing 6.6 nM isoform-specific p66^{Shc} or nonsilencing siRNA into the tracheae of E12 mouse lungs. The lung explants were maintained in serum-free organ culture for three days, then subjected to morphometric analysis. To assess cellular proliferation and differentiation, sections were also immunostained for p66^{Shc}; smooth muscle actin (SMA); proliferating cell nuclear antigen (PCNA); the proximal airway differentiation antigens Clara cell 10-kDa protein (CC10) and thyroid transcription factor (TTF)-1; and the alveolar surfactant proteins (SP) -A, B, and C. Explants injected with nonsilencing siRNA demonstrated specific epithelial uptake and normal morphological development relative to uninjected control explants. In contrast, explants injected with p66^{Shc} siRNA had significantly increased luminal cross-section areas, decreased branching, and increased epithelial proliferation ($p < 0.05$ for all) as well as decreased epithelial p66^{Shc} expression. Relative to controls, the expression of SP-B, SP-C, CC10, and TTF-1 were decreased by p66^{Shc} knockdown. SP-A was not expressed in lungs transfected with either p66^{Shc} or control siRNA, and SMA expression was not altered by p66^{Shc} silencing. These data suggest that p66^{Shc} attenuates epithelial proliferation while promoting both distal and proximal epithelial maturation during normal and pathological lung development.

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Recombinant OA/gpnmb induces differentiation and Src/ERK/AKT activation in osteoblast.

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Osteoactivin (OA)/gpnmb is a type I transmembrane protein (576 amino acids) containing an extracellular domain with an RGD motif, and intracellular domain with an immunotyrosine inhibitory motif (ITIM) and has been linked to osteoblast and osteoclast differentiation and function. Our laboratory previously showed that OA induces osteoblast differentiation and function in vitro and bone formation in vivo. In this study, we investigated the effects of recombinant OA (rOA) on osteoblast differentiation and signaling. First, we examined the effects of rOA treatment on osteoblast proliferation and survival. MC3T3-E1 osteoblast-like cells or primary osteoblast cultures were treated with different doses of rOA and proliferation and survival were evaluated at different time points. rOA treatment had no significant effects on proliferation and survival. Next, we examined the effects of rOA on markers of osteoblast differentiation. rOA treated cells showed a significant increase in Runx2, Osterix, Osteocalcin, Alkaline Phosphatase and Type I Collagen expression at days 7 and 14 in culture. These data suggest that the effect of rOA on osteoblast differentiation is independent of cell proliferation and/or survival. Furthermore, we examined OA signaling in osteoblasts and determined whether rOA treatment causes activation of Src, ERK1/2 and AKT in osteoblasts. These signaling molecules have been shown to be involved in differentiation and proliferation in osteoblasts. MC3T3-E1 cells were treated with rOA (50 ng/ml) at different time points. ERK phosphorylation reached its highest between 10 and 30 minutes following OA treatment, while AKT was maximally phosphorylated after 30 minutes of OA induction. In addition, Src phosphorylation increased gradually and reached a maximum after 1 hour of treatment. These results show that OA is acting through an out-side-in signaling that activates Src/ERK/AKT signaling pathways. Taken together, these data suggest that OA induces osteoblast differentiation and function, at least in part, through Src/ERK/AKT activation. Further studies are directed towards examining whether OA regulates osteoblast differentiation via Erk/Runx2 pathway.

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YAP translocation in human trabecular meshwork cells.

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Objective: Yes-associated protein (YAP) is a transcriptional co-activator and a component of the Hippo pathway. It is only transcriptionally active while in the nucleus, and can be sequestered in the cytoplasm for degradation. Little is known about YAP in ocular tissue with the current literature primarily focusing on its role in organ size control, tumorigenesis, and proliferation. Glaucoma, a major cause of irreversible blindness, has been associated with loss of cellularity in the human trabecular meshwork (HTM). Additionally, soluble cytoactive factors are known to be altered in the aqueous humor of patients with glaucoma. Serum, used for cell culture *in vitro*, is a rich reservoir of soluble factors and its presence has a direct impact on various cellular functions such as proliferation, migration and stress response. Previous research has demonstrated that serum starvation induces YAP phosphorylation and cytoplasmic retention across a range of immortalized cell lines, and that nuclear localization can be induced with serum. In order to better understand the regulation of this important proliferative pathway in HTM cells, we wished to investigate the role of serum-induced YAP regulation by examining the expression and localization of YAP in SV40 immortalized HTM cells (TM-1) and primary (HTM) cells.

Methods: TM-1 cells and HTM cells from normal donors were cultured in DMEM containing 10% fetal bovine serum. They were then serum starved for 24 hours and then treated with either 10% serum containing media or serum free media as a control. The treatment lasted for 3 hours, after which they were fixed in and immuno-stained for YAP. YAP localization was then determined using immunofluorescence microscopy. Additionally, we serum starved the primaries for extended periods (1-8 days) before fixation and staining.

Results: In control (serum-free) TM-1 cells, YAP localized predominantly to the cytoplasm. With serum treatment, the TM-1 cells exhibited strong YAP nuclear localization. Primary HTM cells demonstrated nuclear localization of YAP in both the presence and absence of serum. To confirm if this was a lingering effect of serum during initial culture, we also fixed and stained HTM cells at 1, 4, 6, and 8 days. At all timepoints, primary HTM cells exhibited strong nuclear localization of YAP.

Conclusions: The serum-starved TM-1 cells are consistent with previous reports of cytoplasmic retention of YAP and provide further evidence for YAP nuclear translocation following serum treatment. These results demonstrate that there are serum-derived factors that modulate the activation of YAP in TM-1 cells. Furthermore, these results indicate that this is not the dominant regulatory mechanism in primary HTM cells *in vitro*. Additional research will investigate the significance of YAP nuclear localization in HTM cells under serum-free conditions *in vitro*. Such differences are critical in considering choices of cell type for investigating signaling pathways pertaining to glaucoma or other diseases. Overall, these results provide insight on the function of YAP in the HTM and have broader implications in the study of glaucoma.

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Palladin Cycles Between the Stress Fibers and the Nucleus to Regulate Transcription During Myofibroblast Differentiation.

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Fibrosis is a pathological process that resembles exaggerated wound-healing. The primary cellular mediator of fibrosis is the myofibroblast, which can arise through multiple mechanisms. Myofibroblast differentiation is regulated by the transcription factor Serum Response Factor (SRF) and its coactivator Myocardin-Related Transcription Factor (MRTF). Monomeric G-actin associates with MRTF in the cytoplasm and prevents its translocation to the nucleus. When the G to F-actin ratio is low, MRTF is released and can shuttle into the nucleus where it modulates transcription of SRF target genes. MRTF has recently been shown to bind directly to palladin, implicating this actin-crosslinking protein as a novel player in the SRF/MRTF molecular pathway, possibly as a transcriptional co-factor. To investigate the role of palladin in pancreatic fibrosis, we performed immunoblot analysis on human pathological specimens, and found that palladin is significantly upregulated in fibrotic vs normal pancreas. To probe the molecular function of palladin in fibrosis, a line of immortalized human pancreatic myofibroblasts was used as an in vitro model system. Cellular fractionation combined with immunoblot analysis revealed that palladin is detected in both the stress fibers and the nucleus of these cells, suggesting that it may participate in signaling between the two compartments. To investigate the role of palladin in regulating gene expression in pancreatic myofibroblasts, we used lentiviral expression of shRNA to generate two sub-lines of cells in which palladin was stably knocked down. Total RNA from knockdown and control cells was isolated and subjected to transcriptomic analysis (RNAseq). The results showed that the levels of expression of multiple genes involved in establishing a mesenchymal phenotype were significantly altered when palladin was knocked down in pancreatic myofibroblasts. As this pathway is likely to be conserved among the different cell types that are capable of differentiating into myofibroblasts, the current project could yield benefits for the entire field of fibrosis-related disorders. Supported by NIH and NSF.

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Biological roles of Carabrone originated from Carpesium A. as an immune modulator.

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This study was being tried to reveal the biological roles of Carabrone originated from Carpesium abrotanoides as a modulator of immune activation. To reveal the Carabrone function as NF-κB modulator, ELISA, RT-PCR, promoter analysis, Western blot and confocal were carried out. The results obtained from ELISA showed that an induced IFN γ in the activated NK92 cells was dramatically reduced with treatment with Carabrone. And RT-PCR analysis suggested that Carabrone has effects on the modulation of transcriptional regulation for cytokine, IFN γ expression. The promoter analysis for NF-κB also showed that Carabrone have an effect of modulation of NF-κB transcriptional activity. The intra-cellular role of Carabrone as modulator of NF-κB signal pathway was further investigated through an analysis of phosphorylated signal molecules, which showed that Carabrone was not able to affect on the modulation of

phosphorylated ERK, IKK and I κ B degradation. And extraction of nucleus from cytosol was analyzed for the purpose of identifying NF- κ B complex, p65 and p50 translocate cytoplasm to nuclear. This analysis suggested that Carabrone did not have an effect on the translocation of p65 from cytosol to nucleus. Therefore, Carabrone function as a modulator of NF- κ B may be involved in interference of DNA binding to NF- κ B transcriptional factors. As summary, results from ELISA, promoter analysis, RT-PCR and western blot analysis indicate that Carabrone might be working as a potent NF- κ B inhibitor in the activated NK92 cells via blocking DNA binding to p65 and p50 and also could be used as a possible therapeutic material for modulating inflammation.

Keywords: Carabrone, NF- κ B, IFN γ , Immune modulator

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Nrf2 deficiency promotes RANKL-induced osteoclast differentiation and bone resorption.

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Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor, which regulates the expression of a variety of antioxidant and detoxification genes through an antioxidant-response element. Reactive oxygen species (ROS) have known to act as a signaling mediator for osteoclast (OC) differentiation. Thus we here examined the effect of Nrf2 deficiency on OC differentiation. In Nrf2-null bone marrow derived macrophage cells, the expression of several antioxidant enzymes including peroxiredoxins I, V and VI, thioredoxin 1, thioredoxin reductase 1 and sulfiredoxin profoundly decreased, and intracellular ROS level in both basal and receptor activator of NF- κ B ligand (RANKL)-exposed conditions was much higher compared with wild-type cells. Nrf2 deficiency augmented RANKL-induced OC differentiation, actin ring formation, and osteoclastic bone resorption. Nrf2 deficiency promoted RANKL-induced activation of mitogen-activated protein kinases including c-Jun N-terminal kinase, extracellular signal-regulated kinase and p38, and induction of c-Fos as well as consequent induction of nuclear factor of activated T cell, cytoplasmic 1 that is a pivotal determinant of OC differentiation. In addition, antioxidant N-acetylcysteine pretreatment considerably reduced the acceleration of RANKL-induced OC differentiation in Nrf2-null cells by decreasing intracellular ROS. Thus, our results suggest that Nrf2 may regulate RANKL-induced OC differentiation by modulating intracellular ROS, and provide a new strategy for treating osteoporosis and other bone diseases.

2314

Molecular hydrogen alters signaling pathways and gene expression profiles in multiple mouse organs.

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Recent studies have indicated that molecular hydrogen is a promising anti-oxidative and anti-inflammatory agent for medical applications. However, the molecular mechanism for these

therapeutic effects of hydrogen is yet to be elucidated. Molecular hydrogen is administered mainly by inhalation of air containing 2–4% hydrogen gas (hydrogen-containing air, HCA) or by oral intake of hydrogen-dissolved water (hydrogen-rich water, HRW). To investigate its mechanism of action, we first examined hydrogen concentrations in blood and organs after its in vivo administration to Wistar rats, and found that oral intake of HRW rapidly but transiently increased hydrogen concentrations in the liver and atrial blood, while hydrogen concentrations in arterial blood and kidneys were only one-tenth of those in the liver and atrial blood. In contrast, we observed that inhalation of HCA increased hydrogen concentrations equally in both atrial and arterial blood. We next examined whether molecular hydrogen modifies gene expression levels in mouse livers by DNA microarray analysis to determine the biological functions of hydrogen other than scavenging hydroxyl radicals. We identified 140 mouse genes that were upregulated (31 genes) or downregulated (109 genes) by administration of hydrogen in the form of HCA and HRW. Ingenuity Pathway Analysis revealed that hydrogen influenced expression of NF- κ B- and NFAT-regulated genes. Western blot analysis showed that hydrogen attenuated Erk, p38 MAPK, and NF- κ B signaling in mouse livers. Finally, we evaluated whether the change in gene expression was influenced by the route of hydrogen administration, and found that both HRW and HCA had the most potent suppressive effects on signaling pathways and gene expression in systemic organs, suggesting that hydrogen may be a systemic signaling modulator that acts in a concentration-independent manner.

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Identification of biological pathways that mediate fungal response to occidiofungin exposure.

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Azoles are the most widely used antifungal agents. However, the rise in azole-resistant fungal pathogens has increased the demand for new antifungals. A unique antifungal compound, occidiofungin, was recently identified as a non-ribosomally synthesized cyclic glycolipopeptide produced by *Burkholderia contaminans* MS14. Previous work has determined that the peptide is fungicidal and stable against environmental extremes. Although the biological target of occidiofungin is unknown, it is clear that occidiofungin does not function by binding to ergosterol, or inhibiting enzymes required for ergosterol or 1,3- β -glucan synthesis, all common mechanisms of action for clinically available antifungals. To further compare occidiofungin with other antifungal compounds, the role of two cellular pathways previously shown to be involved in modulating antifungal activity were evaluated: the cell wall integrity pathway (CWI) and calcium signaling. We found that cells treated with subinhibitory concentrations of occidiofungin showed increased levels of chitin as measured by Calcofluor White staining. Elevated chitin suggests occidiofungin exposure results in cell wall disruption. To directly measure whether occidiofungin activates the cell wall integrity pathway, the phosphorylation status of the p44/p42 MAP kinase, Slt2p, was analyzed by Western blot analysis. In *S. cerevisiae*, *C. albicans*, and *C. glabrata*, Slt2p phosphorylation was activated in both a dose- and time-dependent manner. The importance of the CWI pathway in occidiofungin bioactivity was tested using haploid *S. cerevisiae* mutants deleted for components of the CWI pathway. For all mutants tested, occidiofungin based MIC values were similar to wild type. The addition of extracellular calcium (3mM) to standard yeast media has been shown to reduce the activity of azole compounds in a calmodulin and calcineurin dependent manner. To determine a role for calcium in occidiofungin bioactivity, MIC assays were carried out. Similar to azoles, we found that low levels of extracellular calcium resulted in a 2X decrease in occidiofungin activity, although the importance

of calmodulin and calcineurin in this response is currently unclear. Together these data suggest that while occidiofungin activates the CWI pathway in yeast, it is likely an indirect response to the antifungal. The 2-fold reduction in activity of occidiofungin is similar to the reduction observed with azoles. Further studies are needed to determine whether the classic calcium-calmodulin signaling pathway is mediating calcium resistance to occidiofungin.

Gene Structure and Transcription

2316

Single-molecule dynamics of transcription by human RNA Polymerase II.

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Gene expression is regulated primarily at the transcription step – the synthesis of RNA on DNA template. Transcription of messenger RNA in eukaryotes is carried out by RNA polymerase II (Pol II). The machinery involved in Pol II transcription initiation is very complex (six General Transcription Factors, several regulatory complexes, >50 polypeptides in total), and the mechanism of assembly of the Pol II transcription initiation machinery on promoters remains poorly understood. We have developed the first single-molecule system for studying the mechanism of assembly of the Pol II pre-initiation complex in a fully reconstituted in vitro system. We used this system to detect consecutive Pol II transcription initiation rounds from single DNA templates, to detect transcriptional modulation by the promoter sequence, and to detect transcriptional activation by a human activator protein. We found that consecutive rounds of transcription from the same DNA molecule were independent of each other (that is, a given DNA molecule did not have a memory of whether it has been transcribed before). This result suggests that the Pol II pre-initiation complex disassembles after each transcription round, and assembles at the promoter again for the next round to occur. We are in the process of deciphering the mechanism of assembly of the Pol II pre-initiation complex at the single-molecule level. The work required development of new imaging instruments, new surface chemistry, new RNA detection probes, and new data analysis algorithms.

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Direct quantification of mRNA production in living embryos suggests a novel mechanism for noise reduction between nuclear transcriptional activity and cytoplasmic mRNA distributions.

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Noise in gene expression has been shown to play an important role in various organisms. In particular, transcriptional bursts are thought to be a common mode for mRNA output. Yet, the final patterns of gene expression, especially in the case of multicellular organisms such as *Drosophila melanogaster*, are highly precise and reproducible. In order to address this apparent contradiction, we present a strategy to measure transcriptional dynamics in living fly embryos by monitoring the production of mRNA at their sites of transcription on DNA loci in real time simultaneously in hundreds of individual nuclei. In particular, we measure the transcriptional activity of the hunchback promoter as a function of the position along the embryo throughout the first three hours of development. We observe “waves” of transcription associated with the progressing mitotic cycles, where transcription rises steadily in the beginning of the nuclear

cycle, peaks mid-way through it and disappears during mitosis. Although the overall shape of these transcription waves is similar, we see a high degree of variability among nuclei. However, we see no evidence of transcriptional bursting. Single molecule mRNA FISH reveals that this variability in transcription rate does not translate into noise in the cytoplasmic mRNA distribution suggesting the need for a mechanism of noise rectification. In fact, we observe an overall 4-fold noise reduction between nuclear activity and cytoplasmic mRNA levels. Using a simple stochastic model we demonstrate that our noise measurements can only be explained through a combination of both temporal and spatial averaging.

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Local and origin specific determinants modulate global DNA replication controlsC. Richardson¹, J. J. Li¹;¹Microbiology and Immunology, University of California, San Francisco, San Francisco, CA

Replication control is fundamental to genomic stability as aberrant re-initiation from replication origins within a single cell cycle can induce high rates of segmental amplification, chromosomal aneuploidy, and possibly other genomic instabilities. Current models for how eukaryotic cells prevent such re-initiation focus on the global cell-wide inhibition of replication proteins involved in loading the Mcm2-7 replicative helicase at origins (e.g. cyclin dependent kinase, CDK, inhibition of ORC, Cdc6, Cdt1, Mcm2-7). By preventing this initial step of initiation from reoccurring once S phase begins, re-initiation can be effectively prevented. Such models, however, treat origins as generic interchangeable elements and cannot account for the diverse efficiencies with which origins re-initiate when global control mechanisms are disrupted. These varied re-initiation efficiencies also cannot be explained by the well-documented diversity in origin timing and efficiency observed during normal S phase initiation. Instead, we now have evidence of a novel mechanism that contributes to the diversity in origin re-initiation efficiency.

Disrupting the regulation of Mcm2-7 and Cdc6 in *Saccharomyces cerevisiae* results in preferential re-initiation at a select group of origins in the genome. We have identified novel genetic elements near two of these origins (ARS317 and ARS1238) that confer preferential re-initiation upon these and other origins. These elements do not confer any detectable change in the replication efficiency or timing of adjacent origins, suggesting that their regulatory effect is specific to origin re-initiation. Hence, we refer to these elements as Re-Initiation Promoters (RIPs). The two RIPs mapped are AT rich sequences 40-50bp in size and exert their effects on adjacent origins in an orientation and distance dependent manner. Analysis of Mcm2-7 association with origins suggests that RIP elements allow local escape from the residual CDK inhibition of helicase loading when global CDK inhibition of Mcm2-7 and Cdc6 is disrupted. We are currently examining whether RIP elements recruit proteins that can antagonize the inhibitory action of CDKs at adjacent origins.

Such local modulation of origin control suggests that there is a complex genomic landscape of re-replication potential, particularly when mechanisms preventing re-replication are partially or sporadically disrupted. Hence, if re-replication does contribute to genomic alterations, as has been speculated for cancer cells, some regions of the genome may be more susceptible to these alterations than others.

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Genomics of Root Architecture.

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In recent years, great strides have been made in understanding the root growth and development of *Arabidopsis Thaliana* but have been mostly focused on a physiological, proteomic or transcription factor network level. Our approach takes a look at root development through a genomic perspective utilizing the tissue specific expression of the reporter gene, Green Florescence Protein (GFP), in a subset of the mutant *Arabidopsis Thaliana* plants known as the GAL4-GFP enhancer trap lines. Given the mostly unknown genomic nature of our selected mutated plants, characterizing the tissue expression organization at the genomic level will expand established models of gene expression spatiotemporal maps and cell fate by gradients. Thermal Asymmetric Interlaced PCR is being used to recover the promoter sites of the T-DNA inserts used to create the tissue specific expression in the GAL4-GFP enhancer trap lines. Following mapping, respective GFP expressing root cell protoplasts will be harvested to examine the retention of the cell identity in relation to the location within the genome. The study looks at how the overall *Arabidopsis* genome is being orchestrated during development for the final tissue specific expression of a select number of enhancer trap lines as a model for understanding root development. (This research was funded by the NIH T34 GM 08395-22 grant.)

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Gene Gap Investigation of Chalcone Synthase in California Tarweeds and The Hawaiian Silverswords.

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Complex organisms such as plants have many different metabolic pathways that influence substrate specific reactions. Changes in these lead to phenotypic differences related to speciation events. The anthocyanin biosynthetic pathway (ABP), which is a part of the Phenylpropanoid pathway, controls many internal and external mechanisms that regulate temperature, UV protection, microbial invasion, fruit, seed and flower coloring. Anthocyanin production is important to attract pollinators for the proliferation and evolution of the species. In the ABP, Chalcone synthase (CHS) is an enzyme that is responsible for catalyzing the first condensation reaction and essential for other genes further down the pathway such as, dihydroflavonol-4-reductase (DFR), which is a middle gene, and anthocyanidin synthase (ANS), which is a late gene in the pathway. The Hawaiian Silverswords are of great interest due to the fact that the species under went adaptive radiation and have large phenotypic differences but little genotypic differences from their ancestor, the California Tarweeds. In previous studies copies of CHS in *Dubautia scabra*, *Dubautia linearis*, and *Wilkesia gymnoxiphium* in the Hawaiian Silverswords as well as *Madia elegans*, *Madia gracilis*, and *Holocarpha macradenia*, which are apart of the California tarweeds, have been partially sequenced and analyzed. The gaps that remain are the focus of this study looking at sequence included in exon 1 and 2 of the CHS gene for *Wilkesia gymnoxiphium*, and *Dubautia linearis*. The current strategy will be to use polymerase chain reaction, cloning, sequencing, and further analysis of genotypic differences will be done using bioinformatics.

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Glucocorticoid reduces the gene expression of neuronal PAS domain 4 (Npas4) in the brain.

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>Objective: The expression levels of neuronal PAS domain 4 (NPAS4) mRNA are decreased in the hippocampus of socially-isolated or restricted mice, which is accompanied by impairments of memory and emotional behavior with a reduced hippocampal neurogenesis. The reduction of NPAS4 expression induced by psychosocial stress may play a role in mental disorders, since NPAS4 has recently been shown to regulate the development of GABAergic inhibitory neurons. In this study, to investigate the transcriptional regulation of NPAS4 expression by stress, we focused on the effect of corticosterone (CS) on NPAS4 transcription. **>Methods:** Effect of CS on Npas4 expression in restraint stressed mice was evaluated by using a glucocorticoid receptor (GR) antagonist, RU486. Npas4 expression level in the hippocampus of ICR mice were measured 2 hours after the CS (10mg/kg) injection or 1 week after the adrenalectomy. The effect of GR on the Npas4 promoter activity in Neuro2a cells was determined by a luciferase assay. Interaction of GR and Npas4 promoter was confirmed by a chromatin immunoprecipitation assay. **>Results:** Npas4 mRNA levels were reduced by restraint stress for 3 h, but pretreatment with RU486 prevented this reduction. Restraint stress for 3 h increased serum CS concentration compared with controls, while RU486 had no effect upon CS concentration. Acute CS treatment significantly decreased the expression level of Npas4 mRNA in the hippocampus of mice, while the expression level was increased by adrenalectomy. Reduced NPAS4 expression was also observed in CS-treated Neuro2a cells. Putative GREs were found at -2 kb to -1 kb upstream of the transcription initiation site of Npas4 promoter. The Npas4 promoter activity was increased by deletion of GREs rich sequence or treatment with RU486. Moreover, chromatin immunoprecipitation assay revealed the binding of ligand-bound GR to Npas4 promoter region. **>Conclusion:** These results suggest that psychosocial stress reduces the Npas4 gene expression via the binding of CS/GR complex to GREs located on the promoter region of the gene.

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TATA-Binding Protein (TBP)-Like Protein Is Required for p53-Dependent Transcriptional Activation of an Upstream Promoter of the *p21^{Waf1/Cip1}* Gene.

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TBP-like protein (TLP) is involved in development, checkpoint, and apoptosis through potentiation of gene expression. TLP-overexpressing human cells, especially p53-containing cells, exhibited a decreased growth rate and increased proportion of G1-phase cells. TLP stimulated expression of several growth-related genes including *p21* (*p21^{Waf1/Cip1}*). TLP-mediated activation of the *p21* upstream promoter in cells was shown by a luciferase promoter reporter assay. A point mutation at the p53-binding sequence located in the *p21* upstream promoter diminished the TLP-responsivity. In the luciferase promoter reporter assay conducted in p53-null cells, TLP could not activate *p21* promoter. These results showed that the p53-binding sequence located in the *p21* upstream promoter and p53 itself are required for TLP-mediated transcriptional activation. TLP specifically activated transcription from the endogenous upstream promoter, and p53 was required for this activation. We showed that TLP and p53 bound to each other *in vitro/in vivo*, so it is easy to imagine synergistically enhanced activity of the upstream promoter by TLP and p53. Etoposide, a representative cell stress agent, also resulted in activation of the upstream promoter as well as nuclear accumulation of TLP and p53.

Moreover, chromatin immunoprecipitation assay revealed that the upstream promoter was associated with endogenous p53 and TLP, and the p53 recruitment to p53-binding sequence was enhanced by TLP. The results of the present study suggest that TLP mediates p53-governed transcriptional activation of the *p21* upstream promoter.

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Characterization of the miR-125b1 promoter and its deregulation in breast cancer cell lines.

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In cancer cells, abnormal transcriptional gene silencing has been associated with genetic and epigenetic defects. The disruption of DNA methylation patterns and covalent histone marks has been associated with cancer development. Until recently, microRNA (miRNA) gene silencing was not well understood. In particular, miR-125b1 has been suggested to be a miRNA with tumor suppressor activity, and it has been shown to be deregulated in various human cancers. In this study, we characterized the promoter of the miR-125b1 and the epigenetic deregulation associated with its silencing.

To define miR-125b1 promoter region we analyzed in silico the locus of the gene. Then, we characterized the promoter activity by luciferase assay, cloning a fragment in the 5' extreme close to the transcriptional start site of the miR-125b1 gene. We found that the sequence in the 5' extreme close to the transcriptional start site of the miR-125b1 has promoter activity and it is unidirectional. Subsequently, we analyzed the DNA methylation in the CpG island promoter and found that in breast cancer cell lines, it is methylated compared to the same region in non-transformed breast cell line. Also, we evaluated the covalent histone modifications associated with transcriptional activation (H3K4me2) and repression (H3K9me3 and H3K27me3). In the breast cancer cell lines, there is an enrichment in the H3K9me3 compared with the non-transformed breast cell line. To determine the effect of DNA methylation at the CpG island and the covalent histone repression marks of miR-125b1 on the expression of this gene, we performed a expression assay. We observed a significant reduction on the expression of miR-125b1 in cancer cells lines suggesting that the epigenetic deregulation in the CpG island promoter might reduce miR-125b1 expression.

Our data suggest that the DNA fragment in the 5' extreme close to the transcriptional start site of the miR-125b1 gene is a functional and unidirectional promoter. Also, the CpG island in this region is aberrantly DNA methylated and it has an enrichment in the histone repression marks in breast cancer cell lines. Finally, this epigenetic changes promote the silencing of the miR-125b1 gene. Grants: PAPIIT (IN213311) and CONACYT (83959).

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DNA-protein interactions around the NPY2R -224 A/G variant in the Neuropeptide Receptor Y2 (NPY2R) Gene in Predisposition to Hypertension.

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Essential hypertension (HTN) is a consequence of an interaction between environmental and genetic factors. Previous work in animal models and human populations identified Neuropeptide Y receptor 2 (NPY2R) as a candidate gene for HTN. In two independent Japanese populations, the GG genotype single nucleotide polymorphism (SNP) located 224 bp upstream of the transcription start site was associated with an increased risk of HTN. The aim of this study was

to assess the DNA-nuclear protein interactions surrounding the NPY2R -224 A/G SNP. Human Embryonic Kidney (HEK) 293 cells were transfected with pGL4.10 DNA constructs containing the promoter of NPY2R with either an A or G nucleotide at the -224 position upstream of the luciferase reporter gene. Two versions of double stranded oligonucleotide probes corresponding to the sequence flanking NPY2R -224 A/G were synthesized and incubated with several amounts of nuclear protein fractions for an electrophoretic shift assay (EMSA). The DNA/protein reactions were loaded on a 5% non-denaturing polyacrylamide gel. Interestingly, the luciferase activity induced by the NPY2R promoter with the G allele in position -224 was reduced by 34% compared to the promoter containing an A in that position. Differences in the level of relative Luciferase activity between A and G constructs were statistically significant ($p < 0.01$). The EMSA showed an allele-specific binding with the oligonucleotide containing an A in -224 position. The data provide strong evidence for a functional role of NPY2R in genetic predisposition to HTN.

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Synergy Between Chromatin Remodeling complexes (SWI/SNF) and Splicing.

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Pre-messenger RNA splicing plays a critical role in gene expression. In spite of the considerable progress that has been made in understanding the mechanism of splicing, an important question that remains far less well understood is how splicing is coordinated with RNA synthesis and, more specifically, how changes in the chromatin template affect splicing. Recent studies in mammals show that nucleosome occupancy and specific histone modifications are enriched in the exonic regions of genes, which raise the possibility that specific histone marks may be associated with splicing signals. For example, proteins that bind to methylated histones, facilitate the recruitment of snRNPs to nascent transcript and influence the efficiency of splicing. Recently we have shown that GCN5-dependent histone acetylation is critical for cotranscriptional spliceosome assembly and spliceosomal rearrangements. To gain further mechanistic insights into this observation, we have explored the possibility of “communication” between the chromatin remodeling machinery that interact with acetylated histones and the splicing machinery using both genetic and biochemical approaches. One such chromatin remodeling complex is SWI/SNF complex, which binds to the acetylated histones. Further, previous studies show that SWI/SNF regulates alternative splicing in human. In order to understand the role of SWI/SNF complex in splicing we performed an in vivo copper sensitive splicing assay using ACT1-CUP1 reporter constructs. Interestingly, the assay reveals that deletion of SNF2, the ATPase component of SWI/SNF complex, significantly suppress the growth defects of the 3' splice site mutants. Consistent with the growth assay, the primer extension of ACT1-CUP1 constructs also indicate the improved splicing in SNF2 deleted cells as compared to WT. Furthermore, in vivo interaction studies demonstrate physical interactions between the SWI/SNF complex and the snRNP components. Together, these studies suggest a synergistic relationship between the SWI/SNF complex and the splicing machinery in gene expression.

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Digitor, an Essential Protein with Homology to Mammalian ATMIN, is Involved in Brain Development and Oxidative Stress Pathways in *Drosophila*.

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In *Drosophila* we have used yeast two-hybrid interaction assays to identify Digitor (CG14962) a zinc-finger protein with six TQT motifs that interacts with the carboxy-terminus of Skeletor. Skeletor localizes to interband regions of polytene chromosomes during interphase and transient expression studies of GFP-tagged Digitor suggested a nuclear localization for Digitor as well. Furthermore, pulldown assays confirmed a physical interaction with Skeletor as well as with Cut-up, a Dynein light chain protein of 8 kDa. Multiple sequence alignments indicated that Digitor may be the *Drosophila* homolog of the ATM-kinase interacting protein ATMIN, sharing the zinc-finger and SQ/TQ motifs that are hallmarks of DNA damage response proteins. In order to initiate functional studies of Digitor we identified a P-element insertion that based on RT-PCR analysis is a likely null allele. Homozygous mutants for Digitor give rise to late pupal lethality with no escapers. Interestingly, while the size of Digitor mutant larvae are indistinguishable from wild-type larvae their brain size is specifically affected, being significantly smaller. By TUNEL assays we show that this small brain phenotype is associated with increased apoptosis. Furthermore, heterozygous mutant flies have increased susceptibility to acute exposure to paraquat implicating Digitor in signaling pathways combating acute oxidative stress. Supported by NIH grant GM62916 and NSF grant MCB0817107.

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Elucidating the Transcription Networks in the *Drosophila melanogaster* Salivary Gland.

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Various transcription factors contribute to the development of the *Drosophila* salivary gland. To fully understand the formation of this organ, we must know the role of these transcription factors, identify their downstream targets, and learn if target gene regulation is direct. This study focuses on five transcription factors: CrebA, Fork head (Fkh), Hucklebein (Hkb), Senseless (Sens), and Sage, and the identification of all genomic targets to which they bind in the salivary gland of *Drosophila melanogaster*. The transcription factors listed are particularly important due to their functions in the salivary gland, and the finding that, if mutated, significant defects occur. Although previous studies have revealed important functions for these proteins, little is known regarding the genes they bind and regulate within a given tissue. We plan to use "ChIP-Seq" with green fluorescent protein (GFP) tagged versions of each transcription factor to find everything each protein binds to in the genome of the salivary gland. Our goal was to build constructs for expressing GFP-tagged versions of each protein in the fly salivary gland. PCR amplification products of the open reading frame of each protein were first subcloned into the TOPO entry vector and then into a Gateway UAS-GFP fly expression vector. The Gateway constructs were then to be injected into the germ cells of the *Drosophila* embryos to establish lines that will be used for salivary gland-specific expression of each tagged protein. Each transcription factor is at a different stage in this process. CrebA, Fkh, and Hkb open reading frames (ORF) have been PCR amplified. Sens has undergone TOPO cloning and Sage has been injected into embryos and we are in the process of generating and establishing Sage-GFP transgenic lines. Once we have established lines for expressing each of these proteins, we will work with the *Drosophila* ModEncode project to do the ChIP-Seq. Once the binding sites are

identified, we will have genome-wide view of which genes each of these transcription factors regulate.

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Analysis of extra-transcriptional functions of RNA polymerase III bound sites in *Saccharomyces cerevisiae*.

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RNA polymerase III (Pol III) is primarily responsible for the transcription of transfer RNA (tRNA), 5S ribosomal RNA and several other non-coding RNA molecules. Moreover, Pol III transcription complex (TFIIIC, TFIIIB and Pol III) assembled on eukaryotic chromosomes can exert extra-transcriptional effects on chromosome function. Previous findings point out that bound Pol III complexes or even partial complexes can exert significant effects on expression levels of neighboring genes. These bound complexes can also influence other chromosomal processes like directing integration of Ty elements, acting as pause sites for replication fork progression, overriding of nucleosome positioning sequences, transcriptional repression of neighboring RNA Polymerase II (Pol II) genes and conversely the protection of neighboring pol II genes from repressive chromatin spreading. Transfer RNA genes (tDNAs) have also been shown to act as insulator-like enhancer blockers in yeast.

In *Saccharomyces cerevisiae*, a tDNA [*tV(UAC)*] lies between divergently transcribed *CIS1* and *SES1* genes. Microarray analyses indicated that *SES1* is transcribed at ~70-fold higher levels than *CIS1* under normal growth conditions. We speculated that tDNA [*tV(UAC)D*] acts as an insulator that prevents inappropriate activation of *CIS1* promoter by regulatory elements responsible for high transcription of *SES1*. In addition to increased *CIS1* transcript levels, we observed that deletion of this tDNA resulted in an extended *CIS1* transcript. Further investigation of the extended transcript illuminated that this longer transcript is due to readthrough from an untranslated transcript *SUT467* originating near the *SES1* upstream activating sequence (UAS). It appears that *tV(UAC)D* blocks the progression of this intergenic transcript, to allow the normal *CIS1* promoter to escape transcriptional interference. This suggests a unique barrier function of *tV(UAC)D* that prevents advancement of Pol II.

Previous studies on tDNA heterochromatin barrier function identified that in addition to the Pol III complex genes *TFC3* and *BRF1*, the chromatin proteins Nhp6, Htz1, Bdf1, Rpd3, the chromosome assembly proteins condensin and cohesin, and genes coding for components of Sas, Rsc and Isw remodeling complexes also participate in heterochromatin barrier activity. Likewise, our Northern analysis on individually mutated yeast strains in *TFC3*, *BRF1*, *NHP6*, and *SAS2* showed the longer *CIS1* transcript. Whereas, mutation in *HTZ1*, *BDF1*, *RPD3*, *RSC* and *ISW2* failed to show the extended transcript. Subtle appearance of this longer transcript was observed in condensin and cohesin mutants.

Our results indicate that bound Pol III (partial or complete) complexes along with other chromatin proteins at the tDNA can also function as barriers to Pol II progression, which could be important in protecting gene promoters from deleterious effects due to cryptic intergenic transcription.

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Intricate regulation of Gcr1 expression drives metabolic flux in response to glucose starvation in *S. cerevisiae*.*J. M. Claggett¹, M. A. Hossain¹, T. L. Johnson¹; ¹Division of Biological Sciences, Molecular Biology Section, University of California, San Diego, La Jolla, CA*

In a dynamic environment, cells must undergo precise metabolic changes that are determined by a series of tightly regulated gene expression reactions. Since the transcription factor Gcr1 is extensively involved in the expression of cyclins, ribosomal protein genes, and glycolytic genes, we hypothesized that regulation of Gcr1 might play a critical role in determining the metabolic changes required for the cellular response to glucose starvation. Here we show that total Gcr1 levels are regulated in a manner that is glucose-dependent and that contributes to metabolic flux. Unexpectedly, we also discovered that *GCR1* generates multiple protein isoforms when cells are grown in a glucose-rich environment and that the expression of each isoform is minimally dependent on regulation at the level of transcription, splicing and decay. Remarkably, exclusive expression of each isoform causes overlapping and distinct effects on genome-wide RNA expression by RNA-seq analyses suggesting that changing the ratio of Gcr1 isoforms provides a means by which the cell can elicit highly specific changes in gene expression. Since we find that the Gcr1 isoforms are differentially regulated in response to glucose depletion, our data support a model whereby the cell alters the Gcr1 isoform ratio to enable gradual metabolic adjustment when exposed to a fluctuating glucose environment.

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Regulation of VWF expression in response to hypoxia.*A. Mojiri¹, M. Nakhaii-Nejad¹, S. Kulak¹, W. Phan¹, E. Michelakis¹, N. Jahroudi¹; ¹Medicine, University of Alberta, Edmonton, AB, Canada*

Background: Von Willebrand factor (VWF) is a procoagulant molecule, exclusively expressed in endothelial cells and megakaryocytes. In lung VWF expression is primarily restricted to larger vessels with low or non-detectable levels in capillaries. In response to injury, such as pulmonary hypertension (PH), elevated levels of VWF in lung are detected while the mechanism is not known. VWF transcription is regulated by a complex mechanism including distinct regions that are necessary for VWF promoter activation in brain and lung. Transacting factors YY1 and NF-IB, functioning as activator and repressor respectively, participate in lung specific expression of the VWF gene. We investigated the mechanism of VWF activation in response to lung injury using a hypoxia induced model of pulmonary hypertension in mice.

Method: Transgenic mice expressing LacZ transgene under the regulation of brain-lung specific VWF regulatory sequences were exposed to hypoxia and levels, as well as expression patterns of endogenous VWF and LacZ transgene were determined using RT-PCR and immunofluorescent microscopy analyses. Additionally cultured lung microvascular endothelial cells were exposed to hypoxia and levels of VWF transcript, transacting factors NF-IB and YY1 levels, as well as their nuclear distribution and chromatin binding were determined. YY1 inhibition through siRNA transfection was used to determine its role on VWF expression in response to hypoxia. Furthermore, platelet activity was measured under shear stress in both control and hypoxia exposed cells.

Results: VWF and LacZ transgene expression in hypoxia treated mice were significantly upregulated and specifically there was de novo activation of VWF transcription in lung microvascular endothelial cells. Hypoxia-induced increase in VWF transcription was

concomitant with decreased NF-IB and increased YY1 interaction with their respective binding regions on the VWF regulatory sequences. Additionally, increased nuclear localization of YY1 in hypoxia exposed endothelial cells in vivo as well as in vitro was demonstrated. Increased levels of VWF were accompanied with a significant increase in platelet binding as was shown in real time experiment. Inhibition of YY1 expression has abolished hypoxia-induced increase of VWF.

Conclusion: Hypoxia results in upregulation and specifically microvascular directed de novo activation of VWF transcription in lung endothelial cells leading to increased platelet binding. This process is associated with modulation of functions of specific transacting factors namely NF-IB and YY1 that participate in VWF transcription regulation in lung.

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Efficient chromosomal integration in avian cells by a transposon-based approach, in which helper-independent *piggyBac* plasmid was transfected by lipofection, electroporation, sonoporation, and a combination of electroporation and sonoporation.

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The chromosomal integration of genes in avian cells by a transposon vector was evaluated. The *piggyBac* transposase was obtained from the moth *Trichoplusia ni*. Helper-independent *piggyBac* plasmid containing EGFP nucleotide sequence, *pmhyGENIE-3* was used to determine the efficiency in QT6 cells, a quail fibroblast cell line. As a reference, a plasmid containing EGFP nucleotide sequence, *pCX* was used.

The QT6 cells were cultured in DMEM/F12 and approximately 3.5×10^5 cells in a culture dish with a 35-mm diameter were transfected with *pmhyGENIE-3* using four different transfection techniques: lipofection, electroporation, sonoporation, and a combination of electroporation and sonoporation. The number of replicates was six for the experiment. At 2 days and 14 days after transfection, cells were fixed in 10% formalin and nuclei were stained with Hoechst33342. The number of cells emitting EGFP green fluorescence and the number of cells emitting Hoechst33342 blue fluorescence were determined to calculate the relative transfection efficiency. From the relative transfection efficiencies at Day 2 and at Day 14, the efficiency of chromosomal integration was calculated.

With *pmhyGENIE-3*, the relative transfection efficiency by lipofection was 6.3% at Day 2 and 4.0% at Day 14 to yield the integration efficiency of 63.4%. With *pCX*, it was 19.3% at Day 2 and 0.5% at Day 14 to yield the integration efficiency of 2.7%. Overall, the integration efficiency by *piggyBac* transposon ranged from 63.4% by lipofection to 48.9% by the combination of electroporation and sonoporation with the average of 54.9%. However, no statistically significant difference was observed among four transfection techniques.

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Position-specific impact of small RNAs on alternative pre-mRNA splicing.

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Modulation of aberrant pre-mRNA splicing using antisense oligonucleotides (ASOs) is a fast developing technology with a tremendous therapeutic potential. In addition, an ASO-based approach could be employed to define "splicing cis-regulators" that are otherwise difficult to uncover using conventional methods. Employing ASO-based approaches, we have reported position-specific role of intronic residues downstream of the 5' splice site of *SMN2* exon 7,

skipping of which is associated with spinal muscular atrophy (SMA), one of the leading genetic causes of infant mortality. Our study revealed intronic splicing silencer N1 (ISS-N1) and an overlapping GC-rich sequence as the possible therapeutic targets for splicing correction in SMA. ISS-N1 and GC-rich sequences occupy 10th-24th and 8th-15th positions of *SMN2* intron 7, respectively. Our finding that an 8-mer ASO targeting GC-rich sequence is able to fully correct aberrant splicing in SMA represents the first such report of therapeutic efficacy of a short ASO. Our subsequent study revealed that an unpaired cytosine residue at the 10th intronic position (¹⁰C) serves as a negative regulator of splicing in the context of the structural changes brought about by an ASO targeting immediately downstream sequences. Our results underscore the utility of an ASO-based approach as an immensely powerful tool to capture unique long-distance interactions that play critical role during dynamic process of pre-mRNA splicing.

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An embryonic stem cell factor essential for maintenance of self-renewal regulates PRC2 target genes.

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Mammalian genomic DNA is functionally packaged in the nucleus so that a large number of genes are dynamically regulated in a temporal and cell type-specific manner. How this is accomplished is a fundamentally important question that has wide implications in biology from stem cell function to disease. We focused on specialized genomic sequences characterized by a high propensity to become unpaired, termed base-unpairing regions (BURs). BURs can be found throughout the mammalian genome. SATB1 was previously identified as a protein which binds specifically to BURs. SATB1 functions as a genome organizer by regulating three-dimensional folding of chromatin through binding BURs, recruiting chromatin remodeling and transcriptional factors to its target genes, and establishing region-specific epigenetic modifications. Through such function, SATB1 is capable of reprogramming the expression profiles of genes. We searched for other BUR-binding proteins in mouse embryonic stem cell (ESC) using BUR affinity chromatography and protein mass spectrometry. Our study led to identification of a number of nuclear proteins that bind to BURs in the ESC genome, one of which associates with Suz12, a component of the Polycomb repressive complex 2 (PRC2). This factor is located adjacent to heterochromatin and regulates gene expression to prevent ESC differentiation. When expression of this particular protein was knocked down, ESC spontaneously differentiated, activating genes that are normally repressed by the PRC2 complex. Chromatin immunoprecipitation followed by quantitative PCR and deep sequencing showed that the genomic occupancy of Suz12 was greatly reduced at the target gene loci when this protein was knocked down. These studies suggest an important mechanism, at the level of higher-order chromatin architecture, which is crucial for the maintenance of ESC self-renewal and pluripotency.

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Enhancers of transcription active in mouse embryonic stem cells.

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Identifying regulatory regions that regulate cell-type specific and developmentally regulated gene expression programs in mammalian genomes remains a challenge as these regions can be located at great distances from the genes they regulate. Understanding how these regulatory regions function in regulating specific genes is critical in understanding how the

genome directs the process of cellular differentiation and cell fate restriction that occurs during development. We identified enhancer regions and used chromosome conformation capture (3C) data to investigate which genes these enhancers regulate. Initially we used ChIP-Seq and genomic feature data from mouse embryonic stem (mES) cells to predict enhancers active in this cell type. Using features that were most predictive of enhancers (EP300, H3K4me1, MED12, NIPBL) We identified 1277 high probability enhancer regions (≥ 0.8) in the mouse genome that are linked with increased transcriptional output of surrounding genes in mES cells (Chen et al. 2012). Interestingly some of these enhancer regions are clustered in the genome at enhancer hubs similar to the well characterized β -globin locus control region. While increased sequence conservation compared to promoter regions was not predictive of individual enhancers the enhancer hubs show conservation of sequence and epigenetic features marking the human genome in hES cells. Furthermore, these epigenetic features are specific to undifferentiated ES cells and lost upon differentiation. In addition, investigation of HiC data, which identifies chromatin-chromatin interactions genome wide, revealed that enhancer clusters contact transcribed genes within about 250 kb of surrounding chromatin with some examples of contact extending beyond this local region. Further investigation using 3C into an enhancer hub 70-100 kb downstream of *Sox2*, a transcription factor critical for pluripotency maintenance in ES cells, revealed that this cluster region contacts the *Sox2* promoter in mES cells but not in fibroblast cells that do not express *Sox2*. Taken together these data suggest a critical role for this distal enhancer hub downstream of *Sox2* in maintaining the increased expression of *Sox2* in ES cells. Furthermore, enhancer hubs throughout the genome, which are bound by several transcription factors critical in maintaining pluripotency (OCT4, SOX2, NANOG, KLF4), likely have a central role in regulating the gene expression program in ES cells.

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Covalent and non-covalent SUMO interactions regulate Maf1 function.

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Maf1 is a novel transcription repressor that directly represses the transcription of RNA polymerase (pol) III- and select RNA pol II-dependent promoters. Importantly, Maf1 negatively regulates the expression of genes that promote oncogenic transformation and increased Maf1 expression inhibits anchorage independent growth. Recent studies have identified the PI3 kinase signaling pathway as a negative regulator of Maf1. Our current studies have identified an important new role for sumoylation in mediating the ability of Maf1 to repress RNA pol III-dependent transcription. As increased expression of either SUMO1 or SUMO2 repressed the expression of tRNA and 7SL RNA genes, we further examined whether Maf1 might be regulated by sumoylation. We find that Maf1 is covalently modified by both SUMO1 and SUMO2. Mutation of each of the eleven lysine residues to arginine revealed that K35 is the major site of sumoylation on Maf1. Compared to Maf1 wild type (WT), Maf1K35R is impaired in its ability to repress RNA pol III-dependent transcription and to suppress colony growth. While sumoylation does not alter Maf1 subcellular localization, Maf1K35R is defective in its ability to associate with RNA Pol III. This impairs the recruitment of Maf1 to promoters in vivo and the subsequent dissociation of RNA pol III. In addition to its covalent interaction with Maf1, SUMO1 and SUMO2 non-covalently associate with Maf1. However, covalent modification of Maf1 at K35 is not required for non-covalent SUMO interactions. Further mutational analysis identified S123 as a critical residue that controls non-covalent interactions of Maf1 with SUMO. Compared with Maf1WT, Maf1S123A displays an enhanced ability to interact with SUMO, and to repress transcription and colony growth. In contrast to Maf1S123A, Maf1S123D exhibits functions that

are comparable to that observed with Maf1WT. Furthermore, Maf1S123A displays an increase in its association with RNA pol III that is dependent upon enhanced SUMO expression. Together, our results demonstrate that both covalent and non-covalent interactions of SUMO with Maf1 positively regulate its interaction with RNA pol III and its ability to repress RNA pol III-dependent transcription and cell growth. Given that emerging evidence is revealing that desumoylases are aberrantly up-regulated in cancer, it is likely that the deregulation and desumoylation of Maf1 plays an important role in oncogenic transformation.

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Brain isoform of Glycogen Phosphorylase being a marker of undifferentiated hepatic progenitor cells and affecting progenitor cell differentiation.

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The liver progenitor cell (Lig-8 cell line)-derived monoclonal antibody has been proved specifically responding to liver progenitor cells. We used the monoclonal antibody to immunoprecipitate its target protein and we identified the brain isoform glycogen phosphorylase (GPBB) through liquid chromatography combined tandem mass spectrometry (LC-MS/MS) analysis. GPBB is known as the first present isozyme of glycogen phosphorylase during fetal development. In adult rat liver, liver isozyme of glycogen phosphorylase is the major predominant isotype, which catalyzes glycogen degradation. GPBB has never been detected in adult rat liver. In this study, our results demonstrate GPBB exists in identified liver progenitor cells, both Lig-8 and well known WB-F344 cell lines at RNA and protein level but both liver progenitor cell lines do not express liver form or muscle form of glycogen phosphorylase. More interestingly, expression of GPBB in liver progenitor cells declined as the cells were induced differentiation by sodium butyrate induction. When the GPBB gene of WB-F344 cells is silenced by short hairpin RNA (shRNA) interferences, the mature biliary epithelial marker CK19 decreases about 40% during sodium butyrate-induction model. We suspect that GPBB can not only be a novel marker of undifferentiated hepatic progenitor cells but also affect liver progenitor cells differentiation, which possibly provides energy to support hepatic progenitor cells differentiation to biliary cells. Furthermore GPBB facilitates identifying human liver stem cells.

Tissue Development and Morphogenesis II

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Cellular Stress and Protein Folding in Vascular Epigenetics: Chance and Necessity [Algorithms] in Adaption and Disease Reactions.

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Objective: From same genotype, different vascular phenotypes result by coded genetic [Mendelian] and epigenetic [non-Mendelian] reactions: Normal hierarchically organized, functionally efficient and abnormal, inefficient, leaky, mess-chaotic [tumor] blood and lymphatic vessel patterns may be formed by [hem-]angiogenesis, arteriogenesis and lymphangiogenesis. The folding of vascular epigenetic regulator proteins was investigated for regulatory principles on interaction with nascently transcribed RNA from cells subject to environmental chemical [metabolic, immunological] and physical [shear] stress chance [stochastic] and coded necessity [algorithm] reactions. **Methods:** Ann. N. Y. Acad. Sci. **1022**: 163-184, 2004; **1137**: 316-342,

2008; Biophys. J. **102** [3], 639a[3245], 2012. **Results:** In reticuloendothelial cells, environmental chance reactions induce proteins and regulatory RNAs [$<200n$] of defined sequences, like angiomorphogens [angiotropins]. These are RNPs formed by folding of a Mendelian-coded protein [S100-A12] upon interaction with non-Mendelian functional non-coding RNA bioaptamers and metal [Ca, Cu, Na, K] ions. A metal ion-structured $5'CUG^3$ -hairpin loop of RNA fits to a folded protein groove. Modified nucleosides essential for interaction are e.g. isoguanosine / crotonoside, adenosine-N1-oxide of 151Da base families. Biosynthetically, these result from Fenton-type redox-OH*-radical RNA modification induced by environmental chance factors [stress, hypoxia, metal ions, nutrition reductones/vitamins]. These intimately participate in coded processes resulting defined novel sequences: The non-Mendelian RNA are genomic DNA derivatives, but not anymore base-complementary and, therefore, not to retranslate completely to genomic sequences. Chemically, by a general search, a novel common structural relation could be disclosed for factors involved in angio-, arterio- and lymphangiogenesis. It suggested that RNA regulate phenotypic adaptation to environmental needs by complementary interaction with defined conserved homologous helix-nucleating Mendelian consensus domains shared in vascular epigenetic regulator proteins.

Conclusions: The results suggest epigenetic phenotyping being not just only stochastic [chance] and heuristic [trial/error] regulatory processes. But coded [algorithmic] necessity pathways include sequences of finite instructions in which probabilistic randomness may be incorporated by chance and heuristic reactions. Different associated intrinsic and extrinsic interactive complementary biomolecular imprints and factors are implied: **[1]** Non-Mendelian nucleic acid 3D-episcritps in helix-nucleating complementary interaction with **[2]:** Conserved Mendelian homologous domains in epigenetic regulator protein and other matrices which are genotype-derived, **comprising** variant, mutational, infectious [viral] and heritable disease implications. **[3]** Extrinsic and intrinsic factors upon which formation of [1] and interaction of [1] with [2] depends [e.g. splicing, redox- and metalloregulation]. Thus, epigenetic codes comprise more complexity, diversity and plasticity repertoires than genetic codes.

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Potential Contribution of SRY in Sexual Dimorphisms in Neurodevelopment.

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Genes on the Y chromosome are usually expressed in the testis and are tightly regulated during embryonic development and adult. Since the Y chromosome is present only in males, ectopic expression of Y-encoded genes could have significant male-specific effects on the affected tissues, thereby resulting in sexual dimorphisms in the development, physiology and diseases. The Y-encoded SRY is the founder of a family of transcription factors; called SRY-box (SOX) proteins harboring a conserved HMG box capable of binding to similar consensus sequences. SRY and SOX proteins are cell-fate determinants, involved in the development of numerous organs and tissues. The HMG boxes of SRY and SOX proteins are functionally interchangeable, but could differ in their gene regulation functions. SRY could be abnormally expressed in various non-gonadal tissues, thereby exerting a male-specific effect(s) on the development of the affected tissues. To address the issue on where and when SRY could be ectopically expressed, we have examined two transgenic mouse lines harboring an YFP reporter gene directed by the promoter of either the pig or human SRY promoter. We have focused our study on embryonic day 9.5 (e9.5) and embryonic day 11.5 (e11.5). Immunofluorescence and confocal microscopy analysis showed that the YFP transgene was primarily expressed in the developing neural tube, neural crest cells, dorsal root ganglia, neocortex, and enteric ganglia. Parallel analyses of various neural crest and neural markers, such as SOX9, SOX10, NeuN and p75, showed that the SRY promoters were capable of directing the expression of the YFP

transgene in cells expressing these same markers at these two developmental stages. In particular, SRY and SOX9/SOX10 harbor the highly conserved HMG box, the co-expression of SRY in the same cells as SOX9 and SOX10 raises the possibility that SRY could competitively bind to the same target genes for SOX9/SOX10 in the neural cells, thereby altering their gene regulatory programs and exerting a male-specific effect(s) on the neural development. Our results suggest that if ectopically expressed, SRY could be a male-specific genetic modifier and induce sexual dimorphisms in neural development and associated disorders.

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Nov/CCN3 is a novel prostaglandin E₂-induced secreted protein of the ductus arteriosus.

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[Objective] The ductus arteriosus (DA) is a fetal shunt that connects the pulmonary trunk and the descending aorta, and it closes immediately after birth. Not only functional closure (vascular contraction), but also anatomical closure (vascular remodeling such as neointima formation) is essential for permanent DA closure. Our previous study has demonstrated that prostaglandin E₂ (PGE₂)-EP4 signaling plays a critical role in neointima formation of DA closure by promoting migration of smooth muscle cells via secretion of hyaluronan. We aspired to comprehensively identify other secreted proteins of the DA induced by PGE₂. **[Methods and results]** In order to comprehensively detect secreted proteins, we performed liquid chromatography mass spectrometry (LC/MS/MS) analysis for the supernatant of cultured rat DA smooth muscle cells (DASMCs) stimulated with PGE₂. LC/MS/MS analysis revealed that the amount of four proteins, Nov/CCN3, biglycan, fibronectin, and follistatin-like 1 was increased when compared with the control supernatant. In order to confirm the result of LC/MS/MS analysis, enzyme linked immunosorbent assay (ELISA) revealed that concentration of Nov/CCN3 was increased in the supernatant of DASMCs stimulated with PGE₂ in a dose-dependent manner. Quantitative RT-PCR (qRT-PCR) analysis revealed that the expression levels of Nov/CCN3 mRNA were increased in DASMCs stimulated with PGE₂ and an EP4 agonist and that the expression levels of Nov/CCN3 mRNA were the highest in the rat DA tissue at embryonic day 21st among the samples from fetuses at embryonic day 19th and 21st, and neonates just after birth. Furthermore, we found that Nov/CCN3 significantly inhibited PGE₂-induced neointima formation in the ex vivo DA tissues. We also revealed that the amount of hyaluronan was significantly decreased in the supernatant of DASMCs by Nov/CCN3 stimulation. **[Conclusions]** Nov/CCN3 was detected as a novel secreted protein of the DA induced by PGE₂, and suppressed neointima formation of the DA at least in part via inhibiting hyaluronan production.

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Basal cell protrusive activity is a primary determinant of planar cell polarity in the *Drosophila* egg chamber.

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Coordinated cell behaviors are critical to form complex structures in a developing organism. We are using the *Drosophila* egg chamber as a highly tractable model for the study of morphogenetic mechanisms. The egg chamber is composed of germ cells encapsulated by an epithelial layer of follicle cells (FCs). The initially spherical egg chamber elongates along its anterior-posterior (AP) axis to form an elliptical egg. This morphogenesis requires an unusual

form of planar polarity at the basal FC surface in which cortical actin filaments, filopodial protrusions and fibril-like structures in the ECM all align perpendicular to the elongating axis. These structures are then thought to serve as a molecular corset to restrict growth to the AP axis. Recent work has shown that, during this same period, the FCs also undergo a dramatic form of collective cell migration, which causes the entire egg chamber to rotate within the ECM. We hypothesize that this global migration is possible because each FC actively migrates and contributes equally to net forward movement. Factors that provide the motile force for migration would thus be planar polarized at the basal epithelial surface, such that they are enriched at the leading edge of each migrating FC. We have found that the filopodia mark the front of the migrating FCs and their formation is dependent on Ena/VASP. Reducing Ena levels eliminates filopodia but does not influence FC migration, suggesting another actin network could be polarized at the front of the cell. The Arp2/3 activator Scar/WAVE fulfills this role by being planar polarized both in the presence and absence of Ena suggesting a branched actin network moves the FCs forward. Arp2/3 activity is cell autonomously required for protrusion formation. Reduction of Arp2/3 activity across the entire epithelium, though, results in global loss of FC planar polarity and migration. We have also identified Dystrophin as the earliest known marker of planar polarity in this tissue. Interestingly, Dystrophin polarization is never established when Arp2/3 activity is reduced. However, inhibiting Arp2/3 activity after migration begins blocks motility while leaving planar polarity intact. These data suggest that, in addition to driving FC migration, Arp2/3-dependent cell protrusive activity plays a primary role in establishing FC planar polarity, but that other factors stabilize this state once migration commences.

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Patterned cellular behaviors coordinating tissue morphogenesis in the developing *Drosophila* wing.

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How collective cellular behavior specifies the size, shape and polarity of an entire tissue is poorly understood. We address this question *in vivo* using the *Drosophila* wing. During pupal development, the wing epithelium undergoes significant reshaping: one part of the tissue – the hinge – undergoes a large scale contraction that determines tissue shape and global planar polarity in the wing blade. We have previously shown that pupal wing morphogenesis is characterized by a specific pattern of tissue deformation, referred to as shear, which results from an inhomogeneous flow of cells within the epithelium (Aigouy *et al.* Cell. 2010). While we know that the tissue shear pattern arises from coordinated cellular behaviors, including contraction of hinge cells, oriented cell divisions and oriented neighbor exchanges, the contribution of cellular behaviors to the shear pattern is not well understood. Understanding this process would require comprehensive quantitative data on the cellular behaviors that occur throughout the entire tissue during its development. Thus, we developed a method to image the entire wing (400x800 microns) at cellular resolution for 24 hours and built custom image processing tools to quantify cell shape, divisions, rearrangements, and extrusions to be able to correlate cellular behaviors with the shear pattern that shapes the wing. The resulting dataset includes all tracked cells and cell bonds of the tissue, and their description in term of size, shape, and destiny, totalizing around 650 millions of entries in our database for a single movie. We discovered that in the hinge, cell extrusions only play a minor role in the reduction of tissue size. Cells continue dividing but reduce their apical cross-section by half, resulting in a densely packed section of the tissue. Using laser ablation, we found that hinge contraction is likely to be internally driven by cell contractility, so that the hinge literally pulls on the wing blade to generate

cell flow in the neighboring tissue. Additionally, using "Particle Image Velocimetry" to compute the shear pattern in our high resolution movies, we found that tissue shear is constrained regionally by the wing veins and the hinge-blade interface. We are currently addressing how oriented cell divisions and oriented cell neighbor exchanges affect the shear pattern in these specific regions. Our approach bridges the cellular and tissue scales in the study of tissue morphogenesis and should ultimately provide important insight into how tissues acquire their final shape and size.

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The Atypical Cadherin Fat3 Conveys Retinal Ganglion Cell Signals Regulating Amacrine Cell Migration.

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In the vertebrate retina, neurons are organized by function into separate layers that communicate through precisely organized synaptic contacts. This laminar development and synaptic organization requires neuronal migration into the appropriate nuclear layer followed by dendrite extension into the correct plexiform layer. Previously it was shown that an important role for the atypical cadherin Fat3 is to control amacrine cell (AC) dendrite formation and AC distribution between the inner nuclear layer (INL) and ganglion cell layer (GCL). Fat3 is expressed by ACs and retinal ganglion cells (RGCs) and likely functions by regulating developmental interactions between these cell types. In the absence of *fat3*, the mouse retina is altered by the formation of two additional plexiform layers that are the result of changes in AC distribution between the INL and GCL, and the formation of ectopic AC dendrites. When *fat3* is selectively removed from ACs by *ptf1a*-Cre mutant ACs are faithfully retained in the INL but still form ectopic dendrites. Thus we propose that AC migration and dendrite formation are dependent upon separate Fat3 functions in RGCs and ACs respectively. Specifically a non-autonomous function of Fat3 in RGCs creates a "boundary" that prevents ACs from migrating across the inner plexiform layer (IPL) and into the GCL. This hypothesis was tested by generating RGC-specific CKOs using *brn3b*-Cre transgenic mice in which Cre is expressed in ~80% of RGCs. The effect of Fat3 signaling between RGCs and ACs was determined by measuring the distribution of the BHLHB5-positive class of ACs between the INL and GCL. When *fat3* is removed from RGCs there is a measurable increase in the number of BHLHB5-positive ACs located in the GCL. These findings are similar to the aberrant AC migration phenotype that occurs in *fat3* KOs. Together these results support a model in which Fat3 regulates AC development by acting autonomously in ACs to determine dendrite number and non-autonomously in RGCs to control AC movement. This unexpected commitment from a classical planar cell polarity signaling molecule in the control of cell migration likely reflects functional adaptations of this protein class that are unique to vertebrates.

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Wntless is required for cell survival in the developing chick spinal cord.

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The Wnt signaling pathway contributes to the regulation of important developmental and cellular events, such as cell survival, proliferation, and specification. Within vertebrates, one of the best-characterized examples of a Wnt gradient is found in the developing spinal cord. There, dorsally expressed Wnts form a dorsal to ventral gradient of proliferation and neuronal specification. For Wnts to function properly, they must first be secreted from Wnt-producing cells. Wntless (Wls), a

transmembrane protein, is necessary for the secretion of all known Wnt family members in vertebrates. Knockout of Wls in the mouse model system causes early embryonic lethality that coincides with the timing of the first known requirement for Wnt signaling. The goal of this project was to determine the role of Wls in Wnt gradient formation in the chick spinal cord.

We hypothesized that loss of Wls would disrupt the Wnt gradient and thereby cause defects in survival, proliferation, and patterning. To test this hypothesis, electroporation was used to achieve a transient siRNA-mediated knockdown of Wls. Transverse sections were analyzed for morphological changes, the most noticeable of which was a significant loss of cells on the electroporated side of the spinal cord accompanied by a significant delamination of cells. With respect to the patterning of the spinal cord, we stained for N-tubulin, a marker for mature neurons located in the mantle zone of the spinal cord. The electroporated side of the spinal cord showed a significant decrease in the number of mature neurons. However, upon normalizing this loss to the loss of cells in general, there was no significant change between the control and electroporated sides of the spinal cord, suggesting that the loss of cells is affecting progenitor cells. Current data suggests that this loss of cells is due to inappropriate apoptosis - as assayed by TUNEL. Though knockdown of Wls also decreased the total number of proliferative cells - as assayed by phosphohistone H3, the percentage of proliferative cells was not significantly diminished. Thus, this study suggests that Wls-dependent Wnt signaling is necessary for cell survival in the developing neural tube.

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Jak2 is essential for mouse ectoderm development and plays a critical role in neural function.

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Janus tyrosine kinase 2 (Jak2) is known to play a critical role in hematopoiesis. However, its role in neural function is poorly understood. We hypothesized that ectodermal deletion of Jak2 would result in neural dysfunction. To test this, we generated an ectoderm-specific Jak2 conditional knockout (cKO) mouse. In this model, a Jak2 flox/flox mouse was crossed with a transgenic mouse expressing Cre recombinase under the control of the Msx2 promoter. An R26R LacZ-reporter was used to monitor Cre activity. We found that Jak2 was deleted in ectoderm derived organs such as the nervous system, sensory organs, epidermis, and epidermal tissues of the fingernails, hair, and skin. Accordingly, this Jak2 cKO mouse presented with digital abnormalities including hyper-dactyly and hypo-dactyly. In addition, the Jak2 cKO mice were extremely hyperactive when compared to their littermate controls. The hyper-active phenotype began around weaning and lasted until 18 months of age. Lastly, the Jak2 cKO mice exhibited extreme alopecia and pre-mature aging. These data demonstrate that Jak2 is critical for ectodermic organ development and thus may provide additional insights into the role of Jak2 signaling in neural disorders.

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Jitterbug(jbug)/Filamin and Protein Tyrosine Phosphatase 69D (PTP69D) are required for tendon cell orientation and axon targeting in *Drosophila*.

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Mechanical stress generated by the interaction of cells/tissues during development regulates cell differentiation, growth and morphogenesis. Gain or loss of function (LOF) of genes that maintain the isometric tension of cells results in alterations of shape and polarity. Using *Drosophila* as a model system we found that LOF of chascon- jitterbug(jbug)/Filamin system, the protein tyrosine phosphatase 69D (PTP69D) and non muscular Myosin-II (MyoII), display similar shape and cell orientation phenotypes in tendon like cells. These cells differentiate from a monolayer epithelium and serve as bridge between the muscle and the exoskeleton of the fly. During metamorphosis, the dorsal flight muscles attach to the thoracic epithelium, and shorten pulling the differentiating tendon cells, which extend an axon like process that follows the muscle. Interestingly, we found that *jbug/Filamin* is required in photoreceptor cells for axonal growth and targeting, similarly to *PTP69D*, which is expressed both in tendon and photoreceptor cells. *jbug/Filamin* is expressed in photoreceptors under the control of the transcriptional regulator Hindsight and localizes at the membrane from apical to the axonal terminal. We propose that Jbug/Filamin, PTP69D and MyoII are components of an adaptation mechanism that maintains the isometric tension of tendon and photoreceptor cells during its interaction with other tissues during development.

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N-Cadherin regulates the proliferation and differentiation of ventral midbrain progenitors to dopaminergic neurons.

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Adherens junction between dopaminergic (DA) progenitors maintains the structure of ventricular zone and polarity of radial glia cells in the ventral midbrain (vMB) during embryonic development. We have previously shown that removal of β -catenin, which is an essential component of adherens junction and canonical Wnt signaling pathway, controls multiple steps in the neurogenesis of DA neurons in vMB. In addition, our results also indicate that N-cadherin and β -catenin show extensive colocalization in the neural progenitors in the ventricular zone of vMB. However, it is unclear how loss of N-cadherin might influence the integrity of the adherens junction and the process of DA neurogenesis.

Here, we use conditional gene targeting approaches to perform region-specific removal of N-cadherin in the neurogenic niche of DA neurons in the vMB. Similar to the conditional β -catenin mutants, removal of N-cadherin in vMB using Shh-Cre disrupts the adherens junctions of DA progenitors and radial glia processes in vMB. Surprisingly, in contrast to the β -catenin mutants, loss of N-cadherin in vMB leads to a significant expansion of DA progenitors, including those expressing Sox2, Ngn2, Otx2, and Lmx1a. Cell cycle analyses reveal that the cell cycle exit in the progenitor cells was decreased in the mutants from E11.5 to E12.5. The efficiency of progenitors to differentiate into DA neurons is decreased from E10.5 to E12.5, leading to a marked reduction in the number of DA neurons at E11.5, E12.5, and E17.5. The loss of N-cadherin leads to a diffuse distribution of β -catenin proteins from the adherens junction to the

entire cytoplasm in the neuroepithelial cells, suggesting that the canonical Wnt signaling might be activated in the progenitors in the vMB. Consistent with this notion, the expression of canonical Wnt target genes, *Otx2*, *Lmx1a*, and *cyclin D1*, show a significant increase in the N-cadherin conditional mutants.

Taken together, these results support the notion that N-cadherin regulates the proliferation of DA progenitor cells and the differentiation of DA neurons via the canonical Wnt- β -catenin signaling in the vMB.

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Rbfox3-regulated Alternative Splicing of Numb Promotes Neuronal Differentiation during Development.

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Alternative pre-mRNA splicing is a major mechanism to generate diversity of gene products. However, the biological roles of alternative splicing during development remain elusive. Here we focus on a neuron-specific RNA binding protein, *Rbfox3*, recently identified as the antigen of the widely used anti-NeuN antibody. SiRNA-mediated loss-of-function studies using the developing chicken spinal cord revealed that *Rbfox3* is required to promote neuronal differentiation of post-mitotic neurons. *Numb* pre-mRNA encoding a signaling adaptor protein was found to be a target of *Rbfox3* action and *Rbfox3* represses the inclusion of an alternative exon via binding to the conserved UGCAUG element in the upstream intron. Depleting a specific *Numb* splice isoform reproduces similar neuronal differentiation defects. Forced expression of the relevant *Numb* splice isoform is sufficient to rescue, in an isoform-specific manner, post-mitotic neurons from defects in differentiation caused by *Rbfox3* depletion. This study provides a novel mechanism by which *Rbfox3*-dependent *Numb* alternative splicing regulates progression of neuronal differentiation during vertebrate development.

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Nodal signaling regulates endodermal cell motility and actin dynamics via Rac1 and Prex1

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During organ morphogenesis, fate specification and differentiation decisions are translated into dynamic cell behaviors in order to form the three-dimensional shape of the organ. In the zebrafish embryo, endodermal cells are specified prior to gastrulation and subsequently undergo a series of complex movements to bring initially dispersed cells into a coherent epithelium that will ultimately line gastrointestinal tract. In order to investigate the molecular mechanisms underlying these early morphogenetic movements, we generated a novel transgenic line that labels the endodermal actin cytoskeleton, which allowed us to track actin dynamics and cell motility at high resolution in vivo. We observed that during early gastrulation, endodermal cells first migrated in a random, non-persistent manner and exhibited a high degree of actin dynamics. However, at late gastrulation, actin protrusions were more persistent and migration became more oriented as endodermal cells converged on the dorsal side of the embryo. We show that the dynamic actin and random motility characteristic of early gastrulation are dependent on Nodal signaling, and that Nodal signaling regulates activity of the Rho GTPase Rac1. Furthermore, we identified the Rac exchange factor Prex1 as a Nodal target and found that Prex1 is also required for random motility. Reducing Rac1 activity in endodermal cells caused them to bypass the random migration phase and resulted in cells aberrantly

contributing to mesodermal tissues. Together our results reveal a novel role for Nodal signaling in regulating actin dynamics and migration behavior, which are crucial for endodermal morphogenesis and cell fate decisions.

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Quantitative analysis of cytokinesis *in situ* using the *C. elegans* vulval precursor cells.

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Single cell and tissue-level morphogenetic events involve remodeling of the cytoskeleton and cellular junctions. Cytokinesis, the final step of cell division, is executed by an actomyosin ring, which constricts to physically separate a cell into two. The molecules that regulate cytokinesis have been well established using various model organisms including yeast, cultured cells and invertebrate embryos, such as that of *Caenorhabditis elegans*. However, it is unknown whether molecular requirements are the same in an intact developing organism, and how the tissue setting affects cytokinesis.

We developed a novel model to characterize cytokinesis *in situ*, using *C. elegans*. Specifically, we study the vulval precursor cells (VPCs). These cells undergo three rounds of division and several morphogenetic changes to form the worm's egg-laying organ. Using high-resolution 3D time-lapse imaging and computational analysis, we first determined the kinetics and geometry of cytokinesis in the VPCs. As we have observed in human and fly cultured cells and the *C. elegans* zygote, contractile ring closure in the VPCs accelerates during the first half of cytokinesis, before decelerating until closure. The maximum speed of furrowing is 15% per minute, similar to that of human and fly cultured cells. The progressive cell size decrease during VPC development allowed us to determine that contractile ring dimensions scale with cell size. The contractile ring closes asymmetrically, towards the apical membrane of VPCs, as seen in mammalian epithelial cells. We hypothesized that apical junctions cause this asymmetry by inhibiting furrowing of the apical cortex. Depleting E-cadherin did not perturb the kinetics and geometry of cytokinesis, most likely due to redundancy from the AJM-1 based junctions for maintaining epithelial integrity. We will next test the effects of disrupting both types of apical junctions. In investigating the roles of conserved cytokinesis proteins in the VPCs, we find that amongst those, ZEN-4 and Anillin are needed for proper vulval morphogenesis. Notably, depletion of Anillin results in slower contractile ring closure as was seen in mammalian cultured cells injected with an anti-anillin antibody.

Currently available anti-mitotic cancer therapies have unexplained tissue specificity. We are optimistic that our novel model for studying cell division *in situ* will help address this difficulty. Furthermore, cytokinesis could serve as a target for novel cancer chemotherapies. Since cytokinesis requires remodeling of the cell cortex, characterizing it in the context of an intact tissue should aid the development of future therapies and predict their effectiveness in various tissue contexts.

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Roles of Dlg5, a Crohn's disease associated protein, as a regulator of TGF- β signals and epithelial to mesenchymal transition.

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Crohn's disease (CD), which is one of the two major forms of inflammatory bowel disease, is characterized by chronic inflammation of the gastrointestinal tract. Dlg5 polymorphism is one of

the risk factors for CD. Dlg5 is a member of MAGUK (membrane associated guanylate kinase) adaptor family proteins. Dlg5 localizes at cell-cell adhesion sites in epithelial cells, and Dlg5 gene knockout mice showed defects in the kidney and brain. However, these functions cannot explain the association with CD. Thus, it is important to clarify Dlg5 functions to develop therapeutic strategy and understand the pathogenic mechanism of CD.

Using LLC-PK1 epithelial cells, in which Dlg5 expresses at high level, we have found that TGF- β -induced epithelial to mesenchymal transition (EMT) suppressed Dlg5 expression in LLC-PK1 epithelial cells. Depletion of Dlg5 expression by knockdown promoted the expression of the mesenchymal marker proteins, fibronectin and α -smooth muscle actin (SMA), and suppressed the expression of E-cadherin, suggesting that Dlg5 suppresses EMT of LLC-Pk1 cells. Furthermore, we found that induction of fibronectin and SMA expression by Dlg5 depletion was inhibited by pharmacological T β RI inhibitor (ALK5 inhibitor II) and physiological T β R inhibitor, Smad7. These observations suggest that Dlg5 suppresses T β R-dependent signals, resulting in suppression of EMT.

We next examined the mechanism of Dlg5 regulation of T β R-dependent signals. Immunoprecipitation assay showed that Dlg5 was co-immunoprecipitated with both T β RI and T β RII in 293T cells. Dlg5 and T β RI were co-localized at plasma membrane. In addition Dlg5 overexpression promoted T β RI degradation. These findings suggest that Dlg5 forms a complex with T β Rs and enhances their degradation. This mechanism may explain how Dlg5 suppresses T β R-dependent signals and EMT.

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The maternal-zygotic transition reinforces the actin cytoskeleton to make morphogenesis robust.

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Robustness is a property built in to biological systems to ensure normal function, even in the face of internal and environmental fluctuations. During development, robustness safeguards embryos against mistakes that underlie miscarriage, shortened gestation, and structural and functional birth defects. Yet the mechanisms that make development robust are poorly defined, and are completely unknown on the cell biological scale. Our goal for this study is to understand how morphogenesis is made more robust during cellularization, the first tissue-building event in *Drosophila* development. We have identified Spitting Image (Spt; CG8247) and its homolog Serendipity- α (Sry- α) as novel actin regulators that share a redundant actin building function during cellularization. We find that Spt is maternally loaded into embryos, while Sry- α is zygotically expressed in a pulse just before cellularization. Spt activity alone is sufficient for development at an optimum temperature, but Spt and two copies of Sry- α are required for cellularization at extreme temperatures and when the actin cytoskeleton is genetically compromised by a reduced dose of profilin. Thus, zygotic Sry- α augments maternal Spt, to ensure that actin accumulates to a level required for reliable cellularization over a range of conditions. This work has two significant implications: First, our data reveals a new paradigm whereby zygotic proteins are not necessarily instructive, but instead add to the maternal contribution to make development more robust. Second, to our knowledge, we provide the first demonstration that the complex spatiotemporal dynamics of morphogenesis can be made more robust by reinforcing the actin cytoskeleton.

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Non catalytic domains of membrane type-1 matrix metalloproteinase (Mmp14) are involved in mammary gland branching morphogenesis.

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Invasion of epithelial cells through the surrounding stromal extracellular matrix (ECM) is a key event in branching morphogenesis. Whereas epithelial invasion (penetration) during glandular development is well-controlled, the invasive properties of cancer cells in tissues are aggressive and disorganized. To understand how the malignant behavior usurps the signaling pathways from the normal tissues, it is important to understand the regulatory pathways in the normal gland. Elucidating the mechanism by which mammary epithelial cells (MECs) invade the fat pad to bring about exquisite branching morphogenesis may provide valuable clues to target mammary cancer cell invasion and metastasis.

Mmp14 is known to have a crucial role in allowing cancer cells to invade by degrading ECMs, activating other proteases and shedding cell surface molecules. We hypothesized that Mmp14 is also a key player in normal mammary gland branching morphogenesis. Mmp14 profiling in different developmental stages of mammary gland indicated that it was highly expressed during mammary branching morphogenesis in nulliparous mice; the expression was particularly intense in MECs at the tip of the growing branches. To investigate the function of Mmp14 in this context, we used three dimensional cultures of mammary organoids and genetically manipulated MECs, as well as Mmp14 transgenic mice. We discovered that MECs employ both proteolytic activity-dependent and -independent cellular invasion in collagen-1 (CL-1) gels. The cells utilize an association between Mmp14 and an ECM receptor as part of the non-proteolytic activity during the invasion/branching. We have identified the non-catalytic domains that are responsible for signaling in less dense CL-1 and will discuss the different mechanisms Mmp14 utilizes in different modes of cellular invasion during mammary branching morphogenesis.

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The Role of Slit Signaling in Asymmetric Self-Renewal of Mammary Stem Cells.

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Mammary gland regeneration following each estrus cycle and pregnancy relies on the self-renewal of a population of multipotent mammary stem cells (MaSCs). Despite their importance in maintaining tissue homeostasis, how MaSCs generate progenitors and self-renew remains poorly understood. Recent work in the developing mouse retina and epidermis suggest that stem cells balance self-renewal with expansion through closely regulated asymmetric and symmetric divisions, and I have recently identified both of these divisions in the gland. SLITs are a family of extracellular guidance cues that signal through the Roundabout (ROBO) receptors. During pubertal outgrowth, ROBO1 is expressed exclusively in the basal cell population, which is thought to contain the stem cells. Loss of *Robo1* at this stage of development leads to increased expression of the ACD regulator *Inscuteable* (mINSC), and we find that this increase in mINSC is associated with a decrease in ACDs in MaSCs and dividing cells in the end buds. However, in basal cells along the ducts, the increased mINSC expression leads to an increase in apico-basal divisions. As the effects are context-dependent, these results suggest that loss of SLIT signaling may deregulate the spindle machinery and cause randomized cell divisions. Given these collective data, I hypothesize that SLIT signaling regulates the renewal and development of the mammary gland by controlling the plane of cell division in stem and progenitor cells through mINSC. Furthermore, our results suggest a mechanism by which

extracellular cues, such as SLITs, regulate the process of MaSC/progenitor cell self-renewal. As the effects of loss of SLIT signaling depend on the biological context of the dividing cell, our results also emphasize the importance of the stem cell niche in governing the growth and regeneration of epithelial tissues.

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Revealing the consequences of cell-to-cell variability in Ras activity on the collective behavior of mammary epithelial cells.

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To study the consequences of cell-to-cell variability on multicellular processes such as morphogenesis, we used DNA-programmed assembly to construct three-dimensional mammary epithelial microtissues that are mosaic for low-level expression of activated H-Ras. We found two emergent behaviors in mosaic microtissues: cells with activated H-Ras are basally extruded or lead motile multicellular protrusions that direct the collective motility of their wild-type (WT) neighbors. Remarkably, these behaviors were not observed in homogeneous microtissues where all cells expressed the activated Ras protein, indicating that heterogeneity in Ras activity, rather than the total amount of Ras activity, is critical for these processes. Our results directly demonstrate that cell-to-cell variability in pathway activation can drive emergent behaviors during epithelial morphogenesis.

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Perturbation of Cellular Clock Affects Breast Acinar Morphogenesis.

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Most physiological and biological processes are regulated in a circadian fashion through an endogenous clock that acts both at the organism level and the cellular level. The cellular clock consists of a set of core clock genes that oscillate with a 24-hour period and eventually control non-clock genes involved in fundamental cellular processes. Both disruption of circadian rhythm and altered clock gene expression have been associated with breast cancer. By using a method to induce circadian oscillations in cultured cells, we found that the expression of key clock genes, such as PER2 and BMAL1, oscillate in a circadian fashion in breast epithelial cells (HME1), but not in breast cancer cells. HME1 cells can form mammary gland-like acinar structures in three-dimensional (3D) culture. Thus, we exploited the HME1 model to test whether decreasing the circadian expression of PER2, one of the clock genes found affected in breast cancer that perturbs the cellular clock, can impair 3D acinar development. By using a mechanistic approach, we demonstrated that PER2 knock down, by negatively affecting the level of expression/amplitude of BMAL1 transcript oscillation, which in turn exacerbates PER2 downregulation, severely hampers the development of HME1 acini. This study provides the first evidence of clock gene-mediated regulation of the breast acinar morphogenetic process.

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Prostaglandin E₂-EP4 Signaling Inhibits Vascular Elastic Fiber Formation in the Rodent Ductus Arteriosus.

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Background Elastic fiber formation in the arterial wall developmentally begins mid-gestation and increases rapidly during the last trimester, just as blood pressure and cardiac output increase steeply. The ductus arteriosus (DA), a fetal arterial bypass artery between the aorta and the pulmonary artery, exhibits poor elastic fiber formation compared to the neighboring elastic arteries. However, the DA and its connecting elastic arteries are exposed to essentially the same mechanical forces and hemodynamics. The molecular mechanism regarding poor elastic fiber formation in the DA remains unknown. **Methods and Results** We found that the prostaglandin E₂ (PGE₂) specific receptor EP4-disrupted DA exhibited aorta-like elastic fiber formation and then resulted in persistent patent DA after birth. On the other hand, the wild-type DA exhibited poor elastogenesis and normally closed after birth. PGE₂ and a selective EP4 agonist significantly attenuated elastic fiber formation in rat DA smooth muscle cells (SMCs). Because lysyl oxidase (Lox) catalyzes cross-links in elastin assembly, we examined the expression of Lox. PGE₂-EP4 stimulation significantly down-regulated Lox protein, but not mRNA in DA SMCs. We further analyzed the downstream signals responsible for the EP4-mediated decrease in Lox protein. The c-Src-PLC_γ signal pathway, but not cAMP pathway played a primary role in the PGE₂-EP4-induced degradation of Lox protein in lysosomes. **Conclusion** The present data suggest a new signal pathway in which PGE₂-EP4-c-Src-PLC_γ stimulation promotes the degradation of Lox protein, thereby impairing elastogenesis in the rodent DA. The findings regarding the regulation of elastogenesis by PGE₂-EP4 stimulation may provide new insight into elastic fiber formation and strategies against related elastin deficiency diseases.

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Connective Tissue Growth Factor is a Key Regulator of Oxygen-Induced Retinopathy.

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Connective tissue growth factor (CTGF/CCN2) is an inducible matricellular protein that plays a major role in connective tissue remodeling during development and diseases. We found that CTGF/CCN2 was dynamically expressed in the developing murine retinal vasculature and was abnormally increased and localized within neovascular tufts in the mouse eye with oxygen-induced retinopathy (OIR). Ectopic expression of the CTGF/CCN2 gene through lentivirus-mediated gene transfer further accelerated neovascularization while lentivirus-mediated loss-of-function or -expression of CTGF/CCN2

significantly reduced ischemia-induced neovessel growth in OIR mice. The neovascular effects of CTGF/CCN2 were mediated, at least in part, through increased expression and activity of matrix metalloproteinase (MMP)-2. In cultured cells, CTGF/CCN2 activated MMP-2 promoter through increased expression and tethering of the p53 transcription factor to a highly conserved p53 binding sequence within the MMP-2 promoter. Concordantly, the neovascular effects of CTGF/CCN2 were suppressed by p53 inhibition which culminated in reduced enrichment of the MMP-2 promoter with p53 and decreased MMP-2 gene expression. Our data identified new

gene targets and downstream effectors of CTGF/CCN2 and provided the rationale basis for targeting the p53 pathway to curtail the effects of CTGF/CCN2 on neovessel formation associated with ischemic retinopathy.

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PI3K/Akt1 plays an essential role in retinal angiogenesis.

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Phosphoinositide 3-kinase (PI3K) and Akt, downstream target molecule of PI3K, regulate multiple cellular functions including cell proliferation, cell cycle progression, tumor growth, and angiogenesis. In this study, we investigated the role of Akt during retinal angiogenesis. Vascular endothelial growth factor (VEGF)-induced capillary-like tube formation was significantly abolished in Akt1 knock-down Human Umbilical Vein Endothelial Cells (HUVECs), whereas Akt2 knock-down cells were not affected. In addition, VEGF-stimulated corneal angiogenesis was completely inhibited by lacking Akt1. Retinas from WT or Akt1 deficient mice were stained with endothelial cell marker, isolectin B4. Interestingly, retinas from Akt1 knock-out mice showed tortuous, irregular and clustering retinal vessels and delayed vessel development compared to that of WT mice. In addition, retinas from both EC and SMC-specific Akt1 knock-out were shown delayed vascular development compared to retinas from control or EC or SMC-specific Akt2 knock-out mice. It represented that loss of Akt1 caused vascular morphological alterations. Given these results, we suggest that Akt1 requires for vessel development and vascular morphological alterations during retinal angiogenesis.

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How to make and eye: Non-apical progenitors substantially contribute to retinal neurogenesis.

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The retina as an outpocketing of the central nervous system (CNS) has proven to be an excellent model for studying brain development. Understanding general developmental programs in the retina generates important insights in understanding other, less accessible parts of the CNS.

In this study, we present a new pool of progenitor cells occurring at late stages of zebrafish retinal development. Until recently it was believed that all retinal neurons derive from apical division of multipotent progenitors. A couple of years ago however, non-apical divisions (a phenomenon so far mainly explored in the rodent neocortex) were observed in the outer plexiform layer (OPL) and shown to give rise to the horizontal cell (HC) layer (Godhino et al. 2007). Our lab has extended on this finding by showing that these precursors can a) divide at multiple locations between the OPL and the inner plexiform layer (IPL) and b) seem to have the potential to divide more than once before differentiating into HCs. We also show that HCs are not the only cells in the retina arising from subapically dividing precursors but that also a subset of bipolar cells (BCs) does so. These BC precursor divisions occur more apically than the ones of HCs. Both precursor pools are unipotent and give rise to one cell type only. Careful statistics on fixed samples elucidated the birth order of cells arising from this new class of progenitors: BC progenitors are the first cells to divide at sub-apical locations followed by divisions of HC progenitors. This analysis sheds new light onto the general birth order of retinal neurons and expands on the findings merely based on apically dividing cells.

To understand the need for these additional progenitor types we take tissue and cell morphology into account. One reason for the emergence of retinal basal progenitors could be

space constraints within the tissue. The zebrafish retina, unlike the neocortex, is not generated in an “inside-out” fashion, as the most apically located photoreceptors are born rather early in development. Therefore, BC progenitors might divide subapically mainly because they cannot reach the apical surface anymore. Our current data substantiates this assumption.

To understand the morphological basis of the more basally dividing HC precursors we take a different approach: knock down of the transcription factor Ath5 leads to a depletion of retinal ganglion cells, the most basally located cell population, whose space is then taken up by amacrine cells (ACs) (Kay et al. 2005). Interestingly, upon Ath5 knock down we observe an increase of divisions in more basal locations than ever detected in the wild type scenario. We now investigate if these divisions give rise to ACs a cell type that is usually born exclusively at the apical side.

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RFX transcription factors and their direct target genes (in ciliogenesis).

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Evolutionarily RFX transcription factors (TFs) are present only in the unikonts: animals, fungi, choanozoa and amoebzoa. RFX TFs have a unique DNA binding domain with which they bind to the X-box promoter motif (RFX = Regulatory Factor binding to the X-box). Thereby they directly regulate their target genes. It has been shown experimentally that in fungi RFX TFs regulate genes involved in cell cycle control, while in animals RFX TFs regulate genes involved in the immune response and in cilia formation and development. We have carried out searches for X-box promoter motifs in several animal genomes (*C. elegans*, *Drosophila*, mouse and humans) and have successfully identified direct RFX TF target genes, many of which we confirmed to be involved in ciliogenesis, for example by using GFP assays in transgenic *C. elegans* worms or luciferase assays in transfected mammalian cell lines. Accordingly we were able to assign some of these ciliary genes – upon malfunction – to being at the root of a human disease class termed ciliopathies. We are presently working on cell biological aspects of human brain-related, suspected ciliopathies. We are also trying to tie together the different biological functions (in ciliogenesis) of direct RFX TF target genes by cross-comparing a large number of candidate X-box regulated genes in a variety of different genomes. With these approaches we will be able to track the RFX TF target gene module from basic biological function to disease states in humans.

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LIM kinase-mediated regulation of cytoskeletal dynamics in mouse submandibular salivary gland branching morphogenesis.

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Branching morphogenesis in the mouse submandibular gland is a complex and dynamic process involving several structural changes in the epithelium, including dynamic regulation of cytoskeletal stability, cell migration, and cell-cell adhesions to control formation of clefts, or indentations in the epithelial buds. In this study, we report that LIM kinase (LIMK), a dual-specificity serine/threonine kinase that is regulated by Cdc42 and Rac GTPase in early embryonic salivary glands, is required for branching morphogenesis and is a master regulator of these structural changes. We utilized LIMK I/II siRNA and pharmacological LIMK inhibitors – BMS-3 and BMS-5 - to show that LIMK I/II functions in a myosin-independent pathway to increase cofilin phosphorylation thereby inactivating it, and hence stabilize F-actin filaments in

embryonic day 13 salivary glands. LIMK I/II inhibition was previously reported to stabilize microtubules through interaction with p25/tubulin polymerization promoting protein (TPPP). Accordingly, use of TPPP siRNA caused a decrease in branching and a similar increase in stabilized microtubules. Interestingly, 24 hours of incubation with LIMK I/II siRNAs caused destabilization of clefts. Cleft destabilization was also observed in mesenchyme-free epithelial cultures treated with a LIMK inhibitor (BMS-3) that negatively impacts both the actin- and microtubule-dependent effects of LIMK, whereas an inhibitor that only affects only F-actin organization (BMS-5) prevented initiation of new clefts, as detected by time-lapse microscopy. Whereas BMS-3 drastically reduced cell-cell adhesions and cell proliferation, BMS-5 did not affect cell-cell adhesions or cell proliferation. We utilized computational analysis to determine that BMS-5 significantly reduced cell area relative to control while BMS-3 did not. Using time-lapse confocal microscopy to track movement of nuclei in BMS-5 inhibitor-treated, mesenchyme-free epithelial rudiments, we found that LIMK also inhibited epithelial migration in an actin-dependent manner. We propose a mechanism whereby LIMK regulates actin polymerization and microtubule stability to control epithelial cell-cell adhesion and cell migration to affect both cleft formation and cleft stability in developing salivary glands. Supported by NIH/NIDCR RO1 DE019244.

Stem Cells and Pluripotency II

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The Nuclear Dynamics of Pluripotency Gene Loci During Embryonic Stem Cell Differentiation.

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Mammalian embryonic development proceeds in a highly organized fashion, orchestrating the creation of a complex multi-cellular organism from just one cell. Embryonic Stem (ES) cells, which are derived from the inner cell mass (ICM) of a blastocyst stage embryo, are an excellent *in vitro* model for the study of pre-implantation mammalian development. During the process of differentiation, ES cells undergo dramatic changes in gene expression/repression, chromatin structure, and histone modifications. Little is known about how such drastic changes are coordinated in time and space in the complex nuclear environment. Chromosomal movements/interactions may be an integral event in the transition from the ES cell transcriptional program to cell-type specific gene expression. In addition, gene interactions with nuclear bodies may mediate the observed changes in chromatin organization and gene expression. We were therefore interested in investigating the nuclear position of critical ES cell genes during the transition of ES cells from a pluripotent to differentiated state.

Murine ES cells were differentiated by withdrawal of leukemia inhibitory factor (LIF) or with retinoic acid (RA) and harvested at 12-24 hour intervals following the onset of differentiation, utilizing methods to preserve the 3-D structure of the nucleus. Fluorescence *in situ* hybridization (FISH) was used to examine the 3-D nuclear position of genes including Nanog, Sox2, and Oct4 in pluripotent and differentiating ES cells. Interestingly, we observed transient colocalization of the Oct4 gene alleles during early ES cell differentiation. The timing of Oct4 allelic associations coincides with the repression of the Oct4 gene, suggesting a role for Oct4 gene movements in Oct4 gene repression. Further, we have identified a 3.4kb DNA element at the Oct4 locus, which is able to mediate pairing of a transgenic locus with endogenous Oct4 loci during ES cell differentiation. Ongoing experiments seek to identify proteins that bind to this critical 3.4kb

genomic region, thereby elucidating mechanisms that initiate and/or mediate transient pairing of Oct4 alleles and subsequent gene repression.

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Identification and Applications of a Genome Locus with High Homologous Recombination Frequency in Mouse Embryonic Stem cells.

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Gene targeting by homologous recombination (HR) provides a powerful means for studying gene function by a reverse genetic approach. This technology also offers a potential tool for gene therapy. However, gene targeting frequencies are very variable for reasons that largely remain unclear. In the course of generating genetic replacement mouse models we found for the first time that the gene targeting locus, a 6 kb sequence in the region of exon 2 of the Myh9 gene, displays an extremely high and repeatable frequency of HR in mouse embryonic stem (ES) cells (95% in this case vs 1-10% in most cases; Wang A, et al. Proc Natl Acad Sci 2010, 107:14645-50). To our knowledge this is the highest rate that has been reported to date. Interestingly, there is no evidence for a specific DNA sequence that is responsible for this high targeting efficiency since a gradual shortening of the homologous arms results in a corresponding reduction of targeting frequency. This finding provides a useful entry point for studying the mechanisms of HR in mammalian cells, and further it may have important applications. One benefit of the Myh9 locus is that it can provide a safe harbor for transgene insertions in the absence of the influence of the integration site, allowing multiple transgenic lines to be more accurately compared. In our current study we took advantage of the high frequency of HR at the Myh9 locus and carried out a series of site-specific transgenic projects including: I) generating genetic replacement mouse models to study the isoform and domain specificity of nonmuscle myosin IIs (Ibid.; Zhang Y et al., see poster this meeting). So far, at least 5 mouse models have been produced for these purposes. II) Creating Myh9 related disease (Myh9-RD) mouse models. Further investigation confirmed that these mouse models successfully mimic the Myh9-RD phenotype found in humans (Zhang Y, Blood. 2012, 119: 238-50). III) Obtaining high purity cardiomyocytes derived from mouse embryonic stem cells. To this end, a construct of Puromycin controlled by a cardiac-specific promoter was integrated into the Myh9 locus. In each case, the high HR frequency facilitated isolation of the desired ES cell clones. Currently, we are expanding the applications of this useful targeting site.

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Gene expression analyses of a heart injury model system in *Ciona intestinalis*.

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Ciona intestinalis is a useful animal model system for studying developmental processes. It is particularly helpful in studies of heart development since many of the developmental steps and genes are conserved in *Ciona* and replicate early heart development in other Chordates, such as vertebrates. However, in contrast to most vertebrates, *Ciona* hearts have the capacity for regeneration. In order to identify important regeneration factors in the *Ciona* heart, microarray analysis was conducted on RNA from adult *Ciona* hearts with normal or damaged myocardium using custom Affymetrix GeneChips. Hearts were injured via ligation or cryoinjury to stimulate regeneration. After a 24 or 48 hour recovery period, total RNA was isolated from damaged and control hearts. Initial results indicate significant changes in gene expression in hearts damaged by ligation in comparison to cryoinjured or control hearts. Ligation injury shows differential expression of 223 genes as compared to control (fold change >2, p<0.01, Student's t-test) with

limited false discovery (5.8%). Among these 223 genes, 117 have known orthologs of which 68 were up-regulated and 49 were down-regulated. Altered orthologous genes that were discovered via microarray analysis were validated by RT-PCR. Currently, gene expression patterns in the *Ciona* hearts are being identified via in situ hybridization, including FGF 9/16/20, Foxo, and a non-orthologous heart-specific gene. In combination, these studies will help to elucidate the regulatory mechanisms of cardiac myocyte proliferation by the identification of orthologous genes involved in the development and regeneration of the *Ciona* myocardium.

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Ultrastructure and Immunohistochemical Analyses of a Regenerative Myocardium.

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Ciona intestinalis is an invertebrate animal model system that is well characterized and has many advantages for the study of cardiovascular biology. A striking difference between most vertebrates and *Ciona* is that the *Ciona* myocardium is capable of regenerating cardiac myocytes throughout its lifespan, which makes the mechanisms of cardiac myocyte proliferation in *Ciona* intriguing. In order to stimulate regeneration, hearts were injured via ligation around the middle of the heart. After a 24 or 48 hour recovery period, damaged and control hearts were fixed for immunohistochemical or ultrastructure analyses. Preliminary TEM studies show degradation of the myofibrils and changes in the organization of mitochondria in cardiac myocytes. Interestingly, in addition to damaged myocytes, dividing myocytes are also evident and undifferentiated cells populate the damaged myocardium. Furthermore, thickening of the extracellular matrix is apparent, particularly within the lumen of the heart. Histological studies using Mayer's Hematoxylin and Eosin as well as Movat pentachrome stains show basic organization of the matrix components within the *Ciona* heart. Specific labeling of the myocardium using the MF20 antibody (Iowa Hybridoma Bank) shows organization of the cardiac myocytes. Studies using immunohistochemistry to identify proliferation and apoptosis in cardiac myocytes of damaged hearts are currently underway. Taken together, these studies will coordinate differences in cellular organization to ultrastructural changes in cardiac myocytes within the regenerative myocardium of *Ciona*, which will help to elucidate the basic mechanisms of cardiac myocyte proliferation.

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FXR, a Novel Receptor in Bone Cell Differentiation and Function.

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Farnesoid X receptor (FXR), also known as NR1H4 (nuclear receptor subfamily 1, group H, member 4) is a bile acid receptor key in maintaining bile acids and cholesterol homeostasis. FXR is highly expressed in liver and small intestine, where bile acids are synthesized and secreted, respectively. As such, FXR plays an important role in fat metabolism. It has also been found that FXR is an upstream regulator of Fibroblast Growth Factor 21 (FGF-21), which has recently been shown to contribute to bone loss through the inhibition of osteoblastogenesis and stimulation of adipogenesis. While it has long been known that FXR is expressed in liver, intestine, kidney, and adipose tissue, its expression and function in bone are not well understood. In this study, we examined the expression of FXR in bone and demonstrate its key regulatory role in osteoblast and osteoclast differentiation and function. We performed qPCR on

mRNA collected from long bone and calvaria of WT mice, demonstrating the expression of FXR in normal bone development. Next, we examined the skeletal phenotype of FXR KO mice. Micro-CT analysis showed a significant increase in bone volume and trabecular thickness in FXR KO compared to WT mice. Cell survival and proliferation assays in primary osteoblasts collected from FXR KO and WT mice showed significantly less proliferation and survival in the FXR KO compared to WT. In contrast, when MC3T3-E1 osteoblast-like cells were differentiated in the presence of an FXR agonist (GW4064), they demonstrated significantly less matrix mineralization staining by day 21 compared to untreated culture. Osteoclast differentiation (in the presence of M-CSF and RANKL) of hematopoietic stem cells harvested from FXR KO and WT mice showed significantly fewer number of osteoclasts coupled with less TRAP staining and activity in the FXR KO compared to the WT. These results suggest that FXR is a negative regulator of osteoblasts and positive regulator of osteoclasts, and therefore a novel protein in bone cell differentiation and function. Taken together, further studies are warranted to demonstrate the mechanism of FXR in bone cell differentiation and function in vivo.

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Normal migratory epithelial stem cells express vimentin.

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Keratins, the largest group in the intermediate filament (IF) family, are broadly expressed in epithelial cells, while vimentin (Vim), which belongs to the type-III IF proteins, is found primarily in mesenchymal cells. However, Vim it is also found in epithelial cells during development, and in keratinocytes grown under low-calcium conditions, in the outgrowths of epidermal explants, and in some metastatic tumor cells. These results lead to consider Vim as a possible marker of oncogenic progression, but little is understood about its role in normal epithelial cells.

We describe, the coexistence, organization and function of Vim IFs and keratin IFs in diploid, non-transformed human epidermal keratinocytes cultured under conditions with normal Ca^{2++} levels that support proliferation, stratification and terminal differentiation. These conditions enabled us to identify and localize the epidermal stem and transient amplifying cells, either in the growing colonies and in confluent stratified epithelia. Vim^+ keratinocytes in confluent cultures were about $3.7\% \pm 0.15$ of total cells as determined by flow-cytometry analyses. We also show that the Vim^+ migratory keratinocytes correspond to the stem and/or transit amplifying cells of the epidermis; since they expressed epithelial stem cell markers such as cytokeratin14, p63, basonuclin and $\alpha 5\beta 1$ -integrin. Functional experiments designed to analyze the effect of EGF on the growth rate and the migration of keratinocytes showed that EGF promotes an increase in number of Vim^+ keratinocytes and augmented colony growth as compared to control cultures. The ratio between the number of Vim^+ cells and colony radius remained constant during time in EGF-treated cultures, suggesting a direct correlation between the number of Vim^+ keratinocytes and the rate of colony expansion. The results also show that after wounding a confluent cultured epithelium, the number of Vim^+ cells increases at the wounded edge in less than 20-24 hours, before proliferation would take place.

There are several conditions in human skin that could involve Vim expression in basal keratinocytes, amongst them morphogenetic movements. Cultured HEK form confluent layers, undergo pattern formation producing ridges that form complex structures resembling the primate dermatoglyphs. By 39 days post-confluence, Vim⁺ keratinocytes oriented in curves and swirls suggesting that they undergo extensive cell movements and migration.

Our results suggest that in the proliferative epidermal keratinocytes a subset of the stem and/or transit amplifying cells would become migratory through the presence and organization of Vim IFs, integrating the mechanical functions of the cytoskeletal elements for keratinocyte migration, proliferation and re-epithelization.

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Temporally-Regulated Mechanotransduction Controls Stem Cell Cardiomyogenesis.

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As cells migrate and differentiate throughout development, they secrete and assemble extracellular matrix (ECM), giving rise to time-dependent, tissue-specific stiffness, i.e. cardiac muscle originates from soft mesoderm, <500 Pa (Pascal; a unit of stiffness), and stiffens ~10-fold as the myocardium matures. When mimicked in vitro with a hyaluronic acid (HA) hydrogel, myocardial matrix stiffening enhanced cardiac specific gene expression and myofibril organization in immature, isolated pre-cardiac mesoderm. With less specified mouse embryonic stem cells (mESCs), mechanical cues alone were insufficient to induce cardiogenesis, but a combination of precisely-timed developmentally-relevant chemical cues (e.g. BMP4, Activin A, DKK-1) and presentation of dynamic matrix mechanical cues at the cardiac mesoderm induction stage enhanced maturation of mESC-derived cardiomyocytes versus growth factors alone. Measurement of sarcomere length shows mESC-derived cardiomyocytes plated on dynamic HA hydrogels exhibited greater than 30% more mature myofibrils compared to cells plated on static, stiffer matrices. While active mechanotransduction aided maturation, the specific proteins responsible for responding to time-dependent stiffness remain unknown. In order to assess matrix-mediated mechanotransduction across cells of varying myocardial commitment, we examined the expression and phosphorylation state of 800+ protein kinases, as well as organization of mechanosensitive proteins, e.g. focal adhesion kinase (FAK) and vinculin, of pre-cardiac mesoderm or mESC-derived cardiomyocytes plated on matrices with either dynamic or static cardiac tissue-specific stiffness. Microarray analysis of protein kinases showed differential expression as a function of mechanics, confirmed by ratiometric western blotting. Many focal adhesion proteins exhibited time-dependent upregulation on dynamic versus static matrices, including CAS, FAK, Paxillin and Src. These data indicate that mechanically driven maturation is at least partially achieved via active mechanosensing at focal adhesions. Identifying mechanosensitive pathways that are active in cardiomyogenesis can lead to a better understanding of how stem cell differentiation and development are mediated by extracellular matrix properties.

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Regulated subnuclear gene positioning restricts neural stem cell competence in *Drosophila*.

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Progressive loss of stem/progenitor competence to specify earlier-born cell fate is a fundamental process of development whose mechanisms are little understood. Using a combination of DNA fluorescent *in situ* hybridization (FISH) and immunostaining in intact *Drosophila* embryos, we visualized changes in genome architecture *in vivo* as neuroblasts (neural stem cells) undergo rapid (~1hr) transcriptional transitions within the developing central nervous system. We tracked the subnuclear position of the gene locus encoding the transcription factor Hunchback (Hb), which specifies early-born temporal identity. We found that the *hb* gene progresses through three states: transcriptionally active, transcriptionally inactive but inducible, and finally, permanently silenced. We found that when the *hb* gene is transcriptionally active or inducible, the *hb* gene locus is positioned in the nuclear interior, and the neuroblast is competent to specify early-born identity. In contrast, when the *hb* gene is permanently silenced, we found its locus is positioned at the nuclear lamina, a highly repressive domain. Strikingly, the timing of this repositioning correlates precisely with the time neuroblast competence is lost. When the nuclear lamina is disrupted, the *hb* locus is positioned away from the nuclear envelope, and the competence window, as defined by the duration *hb* is transcriptionally inducible, is extended. Furthermore, we identified a neuroblast nuclear factor, Distal antenna (Dan) whose expression perfectly matches the neuroblast competence window. Prolonging Dan expression prevented repositioning of the *hb* locus to the nuclear lamina and extended neuroblast competence, suggesting it may play a role in regulating gene architecture in neuroblasts. We propose that gene reorganization is a mechanism by which stem cell competence to specify particular fates becomes restricted during development. These data may provide insight into mechanisms that can be harnessed to reverse loss of stem cell competence for tissue replacement therapies.

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Escargot blocks niche cell-stem cell conversion in the *Drosophila* testis.

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Stem cells are the building blocks during development and serve as a reservoir of cells for maintaining tissues throughout life. They reside within specialized microenvironments, or niches, that control many aspects of stem cell behavior. Our lab utilizes the *Drosophila* testis and intestine as models to explore the molecular mechanisms regulating adult stem cell behavior. Quiescent somatic hub cells reside at the apical tip of the *Drosophila* testis and regulate the behavior of two populations of stem cells, the germline stem cells (GSCs) and somatic cyst stem cells (CySCs). As such, hub cells are a critical component of the stem cell niche in the testis.

The transcriptional repressor Escargot (Esg) is expressed in GSCs, CySCs, and hub cells. *Drosophila* males homozygous for a hypomorphic allele of *esg*, *shutoff* (*shof*), begin to lose hub cells during development, followed by subsequent loss of both stem cell populations. Cell type-specific reduction of *esg* function revealed that hub cells lacking *esg* acquire CySC

characteristics and undergo differentiation as cyst cells, eventually resulting in testis that lose all hub cells and eventually CySCs and GSCs. We have identified Esg-interacting proteins and confirmed an interaction between Esg and the co-repressor C-terminal binding protein (CtBP), which is also required for maintenance of hub cell identity. Our results indicate that differentiated niche cells can acquire stem cell properties upon removal of a single transcription factor *in vivo*, revealing the importance of defining networks that maintain the balance of cell fates within the stem cell niche. Esg is also expressed in other *Drosophila* stem cells; therefore, we propose that investigating Esg will reveal conserved mechanisms utilized to regulate stem cell behavior in multiple tissues.

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Gap Junction Communication in the Adult Neural Stem Cell Niche.

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Only recently has the discovery of adult neurogenesis been able to overturn the long-standing dogma describing the adult mammalian brain as a static organ. The subventricular zone (SVZ) represents the largest documented neurogenic region within the adult brain. This bone fide neural stem cell (NSC) niche provides a specialized microenvironment that integrates both local and systemic signals to potentiate endogenous NSC activity. The vasculature and associated extracellular matrix are among key components that not only comprise niche architecture, but serve to support and regulate its homeostasis and regeneration. In fact, putative NSC and their most immediate progenitor progeny, transit-amplifying cells, are intimately associated with the vascular plexus within the SVZ. Furthermore, this vascular association is exaggerated during niche regeneration in experimental models. Although some evidence points to secreted factors mediating cross-talk between and among SVZ cells, the role of direct contact in stem cell signaling, maintenance and differentiation has yet to be determined. Gap junction intercellular communication (GJIC) represents a direct means for cell-cell communication normally mediated through physical contact. However, the connexin family of proteins that comprises gap junctions has evolved beyond their traditional role of allowing small molecules, ions and metabolites to pass between adjoining cells. Recent studies have implicated unpaired connexins in intracellular cascades that regulate cell growth, survival, and migration. To address the potential role that gap junctions play in modulating the phenotype and function of NSC, we have developed a novel computational method to detect and quantify connexin expression in specific subsets of stem and progenitor cells in the SVZ. Furthermore, combining confocal microscopy with this image analysis platform has allowed us to correlate connexin expression patterns to blood vessel distance measurements within the niche. Using these systematic tools and methods of analysis, we have characterized the cell specific distribution of connexins among neural stem and progenitor cells (NSPC) as well as the vascular endothelium in the SVZ.

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Effects of Heavy Metals on a Mouse Embryonic Stem Cell Model of Developmental Neurotoxicity.*C. E. McDonald¹, M. Zagzoog¹, M. El Majdoubi¹; ¹Dominican University of California, San Rafael, CA*

Neurotoxic effects of heavy metals on the developing brain (developmental neurotoxicity) are a major public health concern. Because of the limitations of animal-based models and traditional cell culture models of neuronal development, the mechanisms of developmental neurotoxicity are poorly understood. In recent years, embryonic stem cell (ESC)-derived neuronal models have been developed and offer distinct advantages over traditional in vivo and in vitro model systems for investigating the effects of neurotoxins directly on the developing neuron. ESCs are undifferentiated cells that can proliferate indefinitely and also be induced to differentiate into functional adult neurons in culture. This in vitro neuronal differentiation recapitulates several critical processes involved in the development of the nervous system such as proliferation, migration, differentiation, and synaptogenesis. In the present study, we cultured feeder-independent mouse embryonic stem cells (ES14 stem cell line) and directed their differentiation into neurons using retinoic acid, a powerful induction factor of stem cell neuronal differentiation. Using this model of developing neurons, we assessed developmental neurotoxicity of four heavy metal compounds found in the environment: mercury, cadmium, lead, and manganese. Changes in cell viability, replication rates and migration were monitored at different steps of the developmental process of ESCs. The efficiency of neuronal differentiation was determined by calculating the proportions of cells that are immuno-positive for MAP-2, a cytoskeleton protein unique to neurons. Undifferentiated ESCs were generally more sensitive to higher physiological doses of all four compounds, which inhibited cell proliferation and induced apoptosis. In contrast, lower physiological doses of these compounds did not impact ESCs proliferation but significantly decreased neuronal migration and differentiation. These results are consistent with findings in animal-based models and show that the directed neuronal differentiation of ESCs is a useful model system for investigating the effects of heavy metals and endocrine disruptors as well as their mechanism of action directly on developing neurons.

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Fast formation of neurodiscs from adherent rat neural stem cell culture via multi-parametric microenvironment control.*B. Miller¹, A. Zayac¹, T. Gaige¹, P. Hung¹, C. Chen¹; ¹R&D, CellASIC/EMD Millipore, Hayward, CA*

It is known that neural stem cells are sensitive to their respective microenvironment including cell-cell contact, cell-ECM interaction, nutrient and waste transport, and oxygen microenvironment. However, how these parameters in the microenvironment affect the stem cells' morphology, proliferation, and pluripotency remains unknown.

In this study, we adopted a commercially available live cell imaging microfluidic platform capable of multi-parametric microenvironment control on an adult rat hippocampus neural stem cell line. For cell-ECM interaction, protocols were developed to coat the #1.5 coverglass substrate with polyornithine/laminin, poly-D-lysine or poly-L-lysine. For nutrient and waste transport, a pneumatically driven mechanism was applied to provide low-shear steady-state perfusion through the neural stem cell cultures. Since the device is made of gas-permeable materials, by inoculating the culture with different mixtures of gases, the oxygen microenvironment can be designated ranging from severe anoxia to hyperoxia. To achieve different cell-cell contact scenarios, seeding densities were optimized to allow the culture to start growing from low to

high cell concentrations. By attempting various combinations of these microenvironment parameters, we found that the rat neural stem cells exhibited different morphologies and proliferated best under physiologic mild hypoxia conditions as reported by other groups. Through the combination of high seeding density at 200,000/mL, polyornithine/laminin coating, continuous perfusion at 5 μ L/hour, and 3% oxygen mild hypoxia gas microenvironment, the rat neural stem cells formed a 3mm x 3mm x 0.1mm neurodisc (neurosphere-like tight structures) in 72 hours. Through live cell imaging on a fluorescent microscope, we further discovered that while the peripheral cells around the neurodisc were successfully immunostained for two pluripotency markers, Nestin and Sox2, the image of the neurodisc itself showed condensed bright spots of Sox2 but no Nestin. The ability to form these neurosphere-like tight structures quickly in non-suspension culture as well as the ability to image them with fluorescent microscopy could facilitate assay development for neural stem cells as well as provide a model system for neurogenesis and neural development research.

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The role of Smad4 mediated signaling in mouse trophoblast lineage development.

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The maintenance and differentiation of trophoblast lineages is essential for implantation, embryonic patterning, fetal growth and nutrition. Mouse trophoblast stem (TS) cells established from the outgrowth of the polar trophoblast of blastocysts or the extraembryonic ectoderm of post-implantation embryos are the precursors of trophoblasts and can contribute to all trophoblast lineage derivatives in vivo, providing a powerful in vitro system for the study of trophoblast stem cell self-renewal and differentiation. Although it is known that Transforming Growth Factor (TGF- β)/Nodal-related signaling together with FGF4 signaling is critical for TS self-renewal, the function of Smad4, the central mediator of the TGF- β related signaling pathways, in TS cells has not been fully investigated, partially due to the early lethality of Smad4 null embryos. To investigate the role of Smad4-dependent signaling in trophoblast development, we derived smad4 null TS cells. Although loss of Smad4 does not overtly affect cell proliferation, Smad4 null TS cells display enhanced motility and invasiveness and undergo epithelial-mesenchymal transition. Consistent with the changes in cellular properties, transcriptome profiling reveals misregulation of genes associated with tumorigenesis and cancer progression in Smad4 null TS cells. Moreover, Smad4 deficiency also alters the differentiation kinetics and trophoblast lineage distribution of the mutant TS cells. We will present in vitro and in vivo evidence linking the differentiation defects seen in the Smad4 null TS cells to alterations in several signaling pathways, including the Wnt pathway, whose role in TS cell biology has not been appreciated previously. Taken together, our studies revealed a novel requirement for Smad4 in maintaining the morphology and homeostasis of TS cells through Smad4-dependent signaling and cross-talks with other developmentally important signaling pathways. Our analysis will provide insights into the molecular mechanisms that regulate trophoblast lineage development in vivo.

2376B1239

Proapoptotic effect of valproic acid on progenitors of ES-derived glutamatergic neurons.

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We improved and established the culture system involving the generation of essentially pure (more than 97%) populations of glutamatergic neurons from mouse embryonic stem (ES) cells. Although we first tried establishing this culture system in our laboratory according to the previous report, these ES-derived neurons did not survive as long as reported previously under

the same conditions that were originally described. We mainly arranged the medium condition without decreasing the purity of glutamatergic neurons, and finally established the efficient and reproducible method.

Valproic acid (VPA) is a branched-chain saturated fatty acid with a long history of clinical use as an antiepileptic drug (AED). It is well known that VPA is also a powerful histone deacetylase inhibitor (HDACi) and relieves HDAC-dependent transcriptional repression by hyperacetylating histones. There are many contradictory reports that show VPA's neuroprotective or neurodestructive effects on neuronal progenitor cells and neurons. And the mechanisms underlying those effects are explained by the function of VPA either as an AED or an HDACi. There is no report examining the VPA's effect on homogeneous neurons and their progenitors.

Here, we report that VPA has a proapoptotic effect not on glutamatergic neurons but on their progenitors at the therapeutically employed concentrations through its function as an HDACi, not as an AED. We treated ES-derived glutamatergic neurons and their progenitors with two famous HDACis including trichostatin A (TSA) and sodium butyrate (NaB), VPA, and VPM (a structural analogue of VPA which has the same antiepileptic effect as VPA, but lack the HDAC inhibitory activity), and examined the apoptosis ratio and acetylated histone levels. TSA, NaB, and VPA showed remarkably similar levels of acetylating and proapoptotic effects on progenitors, but not on neurons. In contrast, VPM showed neither acetylating nor proapoptotic effect regardless of cell types. These findings indicate that inhibition of HDACs may be the mechanism underlying progenitors' apoptosis induced by VPA. Taken together with the fact HDACis prevent proliferation and induce apoptosis of malignancies, the cell specificity of HDACis' proapoptotic effect may be due to the division potential. Fetal exposure of animals and humans to VPA is reported to produce congenital malformations and cognitive impairment. VPA's inhibition of HDACs which play an important role during the embryogenesis of numerous organisms can be the mechanism underlying such phenomena.

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ES-specific proteins are degraded via autophagy in hESCs.

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Human embryonic stem cell (hESC) pluripotency is regulated by several ES-specific proteins such as OCT4, SOX2, and NANOG and pluripotency-related proteins. However, the mechanisms underlying the control of protein homeostasis in hESCs remain elusive. Here, we demonstrate that autophagy acts to modulate the levels of ES-specific proteins critical for hESC pluripotency. Autophagy inhibition reduced hESC pluripotency despite the upregulation of ES-specific proteins. In cells cultured under starvation conditions, autophagy inhibition significantly increased the levels of ES-specific proteins and LC3 II. The colocalization of OCT4 with an ubiquitin-binding receptor for autophagy (SQSTM1) in the cytoplasm indicated a direct interaction of ES-specific proteins with autophagosomes. Autophagy was involved in the degradation of cytoplasmic and nuclear ES-specific proteins. The present results suggest that autophagy regulates homeostasis of ES-specific proteins.

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The role of a voltage-gated calcium channel in controlling stem cells in a non-excitabile tissue like skin

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Adult stem cells are multipotent cells that possess the capacity for programmed organ replacement and carry the promise of induced organ repair in response to injury or damage. Voltage gated calcium channels (VGCC) are found in excitable cells such as neurons and cardiomyocytes and they respond to electrical signals and work as pacemakers. Although VGCCs are expressed, the role for VGCCs in non-excitabile cells such as adult stem cells is poorly understood.

Murine hair follicle is a great system to study stem cells due to its short, genetically controlled, repeated cycles of growth (anagen), regression (catagen) and quiescence (telogen). Intense recent focus has come to elucidate the controls of stem cell quiescence and the timing of re-entering the proliferative anagen stage.

In this study, we find that Cav 1.2 is an L-type VGCC that is expressed highly in hair follicle stem cells. According to calcium imaging and patch clamping experiments, hair follicle stem cells lack voltage dependent calcium currents. To understand the role of this calcium channel that works independently of calcium, we looked at different mutants and small molecule inhibitors that mimic the different stages of the channel. Gain of function mutation in this channel (mimicking the open state of the channel) causes Timothy Syndrome (TS) in humans associated with baldness in the first two years of life. Expression of Cav1.2^{TS} in hair follicle stem cells causes late entry to anagen consistent with the TS phenotype. Loss of function mutation removing the pore region of the channel (mimicking the closed state of the channel) in hair follicle stem cells also causes late entry to anagen. Treatment of mice with various L type channel blockers (mimicking the inactive state of the channel) causes early entry to anagen. In conclusion, we find that Cav1.2 does not act as a classical voltage gated calcium channel but it provides a voltage-independent, two-state signal (open and inactive) in hair follicle stem cells that inhibits quiescence and promotes tissue regeneration and may be a target for inducing regeneration in other non-excitabile tissues.

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Biophysical regulation of pluripotency and embryonic development.

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We are studying how cell and nuclear mechanics regulate the exit from pluripotency in mouse embryonic stem cells, and how these same biophysical factors manifest similarly in controlling the development of the early mouse embryo. Towards these ends, we have developed several techniques to study stem and progenitor cells both in vitro and in vivo (i.e. in the embryo); these techniques are physics-based and include optical trapping, microrheology and microfluidics for studying cell and nuclear mechanics. Moreover, we are also studying the connection between chromatin structure and nuclear mechanics with quantitative microscopy and temporal spectroscopy. We found first, using optical stretching and microfluidics, that embryonic stem

cells and their nuclei become significantly softer when they make their first fate decision towards a lineage specification. Second, we applied quantitative microscopy techniques to show that the reason the nuclei of these cells become softer as they exit pluripotency is a global decondensation of chromatin. This chromatin decondensation changes the viscoelastic properties of the nucleus. It is noteworthy that our results, at first sight, seem to contradict previous results showing that, as stem cells differentiate, the nucleus becomes stiffer and chromatin condenses. However, we emphasize that we are studying the very first stages of embryonic stem cell differentiation, i.e. when the stem cell first exits the pluripotent state. Finally, we are applying microrheology to study the mechanics and structure of the cytoskeleton and the nucleus within cells in the very first stages of embryonic development. It is in these very first stages that pluripotency is established in the mouse embryo, and the dynamics of this embryonic stage have profound implications on how pluripotency is programmed in development. What we have found is that the same considerations of how biophysics regulates pluripotency in embryonic stem cells have similar consequences in the embryo. Cell and nuclear mechanics are modulated when cells are internalized in the embryo to form the inner cell mass. The inner cell mass then segregates, according to the specific mechanical properties of the cells within the mass, into extraembryonic tissue and the pluripotent epiblast from which embryonic stem cells are harvested. As an outlook, we will explore how the biophysical mechanisms we are observing may play a significant role in feedback loops between transcription and intracellular forces. Our findings could have significant implications for understanding how differentiation and reprogramming are regulated in stem cell biology.

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Identification of non-platelet RNA-containing particles in human umbilical cord blood.

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For many years, platelet-rich plasma (PRP) has been considered to have regenerative function. In addition, evidences indicate that exosomes released by stem cells are therapeutic for cardiovascular disease. To clarify the mechanism of the true factors that function on tissue regeneration or repair, we have studies PRP in human umbilical cord blood and mouse blood. In the cultural condition, a group of small particles in PRP can grow from less than 500 nm to 5 μ m. Under electron microscopy, at least 5 varieties of small non-platelet RNA-containing particles (NPRCPs) were identified. Unlike platelets, these particles do not contain α -granules, cell organelles or glycogen granules. Most NPRCPs are between 1 μ m to 5 μ m, composed of nuclear granules and proteins that locate in a thin bi-layer membrane. Analyses of the contents of the nuclear granules indicate that NPRCPs contain only small RNAs with sizes less than 250 nt; about 30% of them are microRNAs, which is similar to that described to stem cell released exosomes. MicroRNA array analyses indicate that about 40 microRNAs are significantly up-regulated in NPRCPs. Immunofluorescent studies confirm that NPRCPs contain Oct4, sox2, integrin β 1, DDX4, tubulin, and actin. We also have found that GFP-transgenic mouse (GFP was constructed downstream to actin promoter)-derived NPRCPs express GFP. When co-culturing with nucleated cells, the number of NPRCPs decreased, while the number of the new stem cells increased. Immunofluorescent studies indicate that these small new cells express oct4 and sox2. Our data provide strong evidences that NPRCPs are the regenerative factor in PRP. NPRCPs can grow in cultural condition and possibly are from the stem cell exosomes. In addition, NPRCPs are rich in umbilical cord blood and fetal blood, which could explain the better regenerative function of using umbilical cord blood than using adult peripheral blood.

Ubiquitin and Proteasome Function

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A Temperature-sensitive Mutation in the Ubiquitin-activating Enzyme E1 and its Relation to the Maintenance of Chromosome Integrity.

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A temperature-sensitive (ts) CHO-K1 mutant tsTM3 exhibits chromosomal instability and cell cycle arrest in S to G₂ phases with decreased DNA synthesis at the nonpermissive temperature, 39°C. Complementation tests with other mutants characterized previously showed that the tsTM3 did not complement with DNA replication-defective ts mutant ts-131b and the ubiquitin-activating enzyme E1 (Uba1) defective ts mutant ts85, suggesting that these mutants harbor same genetic defect. Uba1 catalyze the first step in ubiquitin conjugation to mark cellular proteins for degradation. In addition to regulating protein stability, ubiquitination also participates in a variety of non-proteolytic function including DNA replication and DNA damage repair. To identify the causative mutation, we cloned and sequenced the *Uba1* gene from wild-type and mutant cells. Comparison of these sequences revealed that the mutant phenotype is caused by a G-to-A transition that yields a Met-to-Ile substitution at position 256 in hamster Uba1. The temperature-sensitive defects in tsTM3 were complemented by the expression of the wild-type of Uba1 tagged with green fluorescent protein. We conclude that a ts-mutation of *Uba1* found in tsTM3 cells appears to be a novel mutation which affects the maintenance of chromosome integrity, reflecting the important roles of Uba1 in cellular function.

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Novel small molecule inhibitors of p97 as potential cancer therapeutics.

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The ubiquitin-proteasome system (UPS) sustains cancer cell viability by regulating proteins that enable pro-survival mechanisms and by alleviating proteo-toxic stress, caused by an imbalance of protein synthesis and degradation. Bortezomib, a proteasome inhibitor approved for the treatment of multiple myeloma and mantle cell lymphoma, has validated the UPS as a target for pharmaceutical intervention. Because of Bortezomib's limited use in the cancer setting, the discovery of novel drugs that target non-proteasomal targets within the UPS will provide novel approaches to exploit cancer cells addiction to protein homeostatic mechanisms.

The AAA-ATPase p97 functions to reshape the proteome by converting chemical energy into mechanical force. p97 oversees several facets of protein homeostasis, including ubiquitin-dependent protein degradation, endoplasmic reticulum-associated degradation (ERAD), protein secretion and autophagy. In pre-clinical cancer model systems, increased autophagic flux has been described as a potential escape mechanism from proteasome inhibition¹. Moreover, the dependency of certain late-stage tumors on autophagy highlights the importance of this pathway as a pro-survival mechanism². p97's role in the UPS and autophagy suggests that pharmacologically targeting p97 may provide another means to inhibit tumor growth.

N2,N4-dibenzylquinazoline-2,4-diamine (DBeQ) was recently identified from a high-throughput screen as a selective and moderately potent inhibitor of p97³. Through structure-activity relationship-based efforts, we have discovered several analogues of DBeQ with increased

potency to 20nM. These compounds also show anti-tumor activity in myeloma and non-small cell lung carcinoma cells. To verify on target activity of this class of inhibitors, we have implemented several cellular reporter assays that readout out specific functions of p97, including ubiquitin-dependent degradation, ERAD, protein secretion and autophagy. Surprisingly, when our DBeQ analogues were tested in this suite of cellular assays, differential inhibition amongst these reporter assays was observed. These findings suggest that chemical inhibition can preferentially block distinct functions of p97, possibly due to separate affinities for p97 in complex with different cofactors. We are currently undertaking biochemical and cellular approaches to further characterize these specific inhibitor effects. Additionally, we are testing these molecules in vivo to determine which cellular activities p97 are most critical for inhibiting tumor growth.

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2383

The organisation and evolution of the Muskelein/RanBPM/CTLH complex.

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Ubiquitination is a major form of post-translational modification that regulates protein stability, trafficking and signalling processes in eukaryotes. Substrate specificity is governed by E3 ubiquitin ligases of which there are several hundred in the human genome. Some ubiquitin ligases act as single proteins, others are participants in multi-protein complexes. The goal of our investigations has been to understand the organisation and evolution of a little-studied, multiprotein RING E3 ligase complex, the muskelein/RanBPM/CTLH complex. This complex is conserved between mammals and budding yeast, and five of the components (Rmnd5, Maea, TWA1, RanBPM and muskelein) have a related domain organisation, all containing LisH and CTLH domains. Rmnd5 contains a RING domain and confers E3 ligase activity. However, functions of the complex in yeast and mammals appear distinct. In yeast, the complex acts to degrade fructose 1, 6, bisphosphatase to switch cells to glycolytic metabolism. In mammalian cells, over-expression or knockdown of several components impacts cell morphology and actin organisation. To understand this complex in more depth, we undertook co-immunoprecipitation and protein localisation studies with wild-type or domain deletion forms of the five proteins, in mammalian cells under high, normal, or low glucose conditions. These data have enabled an initial map of organisation of the mammalian complex, in which TWA1 appears central to complex assembly. We also examined the molecular phylogeny of the five components to understand their evolutionary relationships. Muskelein is specific to the opisthokont lineage, whereas RanBPM, Rmnd5, Maea and TWA1 are present in all eukaryotic supergroups. Sequence similarities between the LisH/CTLH domains of Rmnd5 and Maea, and RanBPM and TWA1, respectively, support a model that the encoding genes evolved through a series of gene duplications on the eukaryotic stem lineage, indicating the ancient origin and likely central roles of these proteins in complex organisation and function. These data add to knowledge of the complexity of the ancestral eukaryote.

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The MARCH8 E3 ubiquitin ligase N-terminus plays an important role in CD44 specificity.

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Ubiquitination of plasma membrane (PM) proteins can signal changes to their internalization, recycling, or degradation rates and thus plays a critical role in regulating cell surface composition and turnover of PM proteins. E3 ubiquitin ligases are primarily responsible for conferring substrate specificity to ubiquitination reactions. Eleven membrane-associated RING-CH (MARCH) E3 ligases have been identified in humans that ubiquitinate and down-regulate a number of PM proteins. MARCH1 and MARCH8 are closely related multi-pass membrane proteins with similar subcellular distributions at the PM and in endosomes. MARCH1 and MARCH8 can ubiquitinate multiple PM proteins, although little is understood about what dictates their substrate specificities. Here we consider two substrates, CD98 and CD44, which occupy the same subcellular compartments but diverge in their ability to be ubiquitinated by MARCH1 and MARCH8. CD98 and CD44 are PM proteins that are internalized by clathrin-independent endocytosis (CIE) and recycled back to the PM via CIE recycling tubules. In HeLa cells, CD44 and CD98 are long-lived PM proteins. Over-expression of either MARCH1 or MARCH8 elicits the loss of cell surface CD98 through ubiquitination, and its rapid lysosomal degradation. In contrast, while both MARCH1 and MARCH8 co-localize with CD44 in HeLa cells, only MARCH8 causes its degradation. MARCH1 and MARCH8 have U-shaped membrane topologies, with a short ectodomain confined by two transmembrane domains (TMDs) and both N- and C- terminal tails extending into the cytosol. The N-terminus contains a highly conserved catalytic RING domain just upstream of the first TMD. These proteins share ~55% amino acid sequence homology, with the highest variance in the most distal N- and C- terminal sequences. The N-terminus, TMDs, and C-terminal tail have separately been implicated in determining MARCH substrate specificities. To determine the region of MARCH8 responsible for CD44 specificity, we made chimeric MARCH proteins, exchanging portions of the N-terminus, TMDs, and C-terminus of MARCH8 with MARCH1. For all chimeras, CD98 degradation served as a positive control for ubiquitin ligase activity. Surprisingly, the MARCH8 C-terminus was not involved in CD44 recognition. Instead, we found that the N-terminal cytosolic juxta-membrane region of MARCH8, including its RING domain, was important for regulating CD44 degradation. Differing in only 8 amino acids between MARCH1 and MARCH8, this region appears to be involved in conferring CD44 substrate specificity.

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Obscure links and cullin(g) ends.

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The sarcomere, one of the most regular macromolecular assemblies in nature, is the force-generating apparatus in all cross-striated muscles. Despite the seemingly static nature of the sarcomere, its myofibrillar components undergo constant turnover. Sarcomeric proteins like titin or α -actinin have been known to interact with muscle specific E3-ubiquitin ligases for their subsequent degradation through the ubiquitin-proteasome system. We observed that cullin-RING proteins, ubiquitously expressed E3-ligases that may have distinct properties in cross-striated muscles, also exhibit a sarcomeric localization that is altered in models of cardiac and skeletal myopathy. Recent reports and evidence from experiments on knockout mouse models

indicate that several cullin E3-ligases, their substrate adaptors and regulators, may be linked to obscurin proteins, a family of large sarcomeric proteins that include obscurin, and its close homologue Obsl1.

Using the obscurin knockout mouse model we were able to elucidate the protein turnover mechanism of sAnk1.5, a muscle specific isoform of ankyrin 1.

sAnk1.5 ubiquitylation is tightly regulated by posttranslational modifications, its interaction with the BTB/POZ-domain containing protein KCTD6, and its subcellular compartmentalization by obscurin. KCTD6 represents a novel substrate adaptor for the Z-disc associated cullin-3 E3-ubiquitin ligase. Lack of obscurin reduced sAnk1.5 levels and lead to mislocalization of the sAnk1.5/KCTD6 complex within the sarcomere. Scaffolding functions of obscurin may therefore prevent activation of the cullin-mediated protein degradation machinery and ubiquitylation of sAnk1.5, through sequestration of sAnk1.5/KCTD6 at the sarcomeric M-band, away from the Z-disc associated cullin-3. Regulatory functions of obscurin proteins on cullin-dependent protein turnover may not be restricted to sAnk1.5, as cullin proteins, their substrate adaptors and regulators are altered in muscles of obscurin knockout mouse models.

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Ubiquitination regulates the turnover of Parkinson disease-linked UCH-L1 protein

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Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1/PARK5) is a neuronally expressed deubiquitinase that has been implicated in familial and sporadic Parkinson disease (PD). UCH-L1 cleaves C-terminal adducts of ubiquitin and has been shown to play an important role in neuronal survival and in maintaining the monomeric ubiquitin pool. Indeed, loss of UCH-L1 expression in mice results in progressive axonal degeneration, learning and motor defects, shortened life span, and reduced monomeric ubiquitin level. However, little is currently known about UCH-L1 function or regulation within the nervous system. Here we report that UCH-L1 is post-translationally modified by ubiquitination. We observed UCH-L1 ubiquitination in cultured cells, mouse brain tissue, and in a cell free *in vitro* system. Our studies have also identified a novel interactor of UCH-L1, an E3 ubiquitin-protein ligase, dubbed UCH-L1 interacting protein (UCHIP), which mediates, at least in part, the ubiquitination of UCH-L1. We found that neither the PD-associated UCH-L1 mutation (I93M) nor the reportedly neuroprotective polymorphism (S18Y) alter interaction between UCH-L1 and UCHIP. Additionally, western blotting analysis of brain tissue from mice lacking UCHIP expression revealed an elevation in the total UCH-L1 protein level, suggesting that UCHIP-mediated ubiquitination may regulate UCH-L1 protein turnover. Our studies have important implications for understanding the role of UCH-L1 in normal neuronal physiology as well as in neurodegenerative disease states.

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SUMO-dependent regulation of the yeast transcription factor Cin5.

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Cin5/Yap4/Hal6 has crucial roles in the responses to presence of oxidative agents, hyperosmolarity, metals, DNA damage, etc. Yet, *CIN5* was originally isolated in a genetic screen for mutants that exhibit chromosome loss and sensitivity to microtubule inhibitors, phenotypes that are frequently associated with deficient regulation of mitosis. As expected from its many roles, Cin5 functions within an intricate and dynamic network of interactions with

several other transcription factors, regulating expression of hundreds of genes. However, very little is known about how this transcription factor itself is regulated. Expression of Cin5 is induced early in the stress response and phosphorylation by the PKA and GSK3 kinases has been proposed to positively influence its protein stability. However, non-phosphorylatable *cin5* mutants grow as well as wild type in the presence of high salt, suggesting there are other regulatory mechanisms.

In order to learn more about its functions and regulation, we undertook a two-hybrid screen designed to identify proteins that interact with Cin5. Among the proteins identified, several harbored SUMO-interacting motifs and bind to proteins that carry that modification. Thus, these results suggest that Cin5 might be regulated by sumoylation and we have identified two lysine residues as putative targets. These residues were mutated to prevent sumoylation of Cin5 and the mutants have been characterized for changes in localization, protein stability and other phenotypes.

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Plant CULLIN3-based E3 ligases and their function in transcriptional processes.

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E3 ubiquitin protein ligases play important regulatory roles in cell division, cell cycle control and general development in eukaryotic organisms. Cullin3 (CUL3) and MATH-BTB/POZ domain (BPM) proteins form Cullin3-based ubiquitin E3 ligase (CRL3s) complexes that facilitate ubiquitylation of proteins, which can lead to their degradation via the 26S proteasome. The plant *Arabidopsis thaliana* encodes for six BPM proteins (BPM1 to 6) and two CUL3 proteins (CUL3a and 3b). Here we show that the MATH domain mediates the interaction of BPMs with members of the ERF/AP2 (ethylene-responsive factors/ APETALA2) transcription factors, a large protein family with more than 140 members in *Arabidopsis*. ERF/AP2 proteins are widely involved in developmental processes as well as tolerance towards abiotic stress conditions such as cold, drought, or salt stress. We hypothesized that in plants BPM proteins target a large number of these transcription factors, and that this interaction leads to their ubiquitylation and proteolytic degradation. This hypothesis is supported by our findings that selected members are highly unstable in a 26S proteasome-dependent manner. By using detailed biochemical and genetic analysis, we demonstrate that the observed instability depends on the activity of CUL3-based E3 ligase complexes that employ BPM proteins as their respective substrate adaptors. These results define CUL3-based E3 ligase as central and novel regulators in controlling transcriptional processes in plants.

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Identification and characterization of an ENU-induced mutation in *Usp14*.

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The Ubiquitin Proteasome System (UPS) plays a critical role in many aspects of neurodevelopment, including neurite outgrowth, axon guidance, and synaptogenesis. Work from our laboratory has demonstrated that mutation of the proteasomal deubiquitinating enzyme (DUB) *Usp14* is responsible for the neurodevelopmental defects observed in the *ataxia (ax¹)* mice. The *ax¹* mice present with reduced muscle development, motor-end plate disease, loss of synaptic function and premature death. **Therefore, we hypothesize that study of *Usp14***

deficient mice allows for the discovery of new pathways required for proper structural and functional NMJ development and possible identification of new drug targets for the treatment of motor neuron diseases. In this study we identified and characterized a new mouse line with an ENU-induced mutation in *Usp14* (*nmf375*). Positional cloning and genetic complementation assays established that the causative mutation in the *nmf375* mice resides in *Usp14*. Sequence analysis of *Usp14* transcripts and genomic DNA revealed a point mutation in the splice site donor region of exon 9 of the *Usp14* gene. Using quantitative PCR and western blot analysis, we determined that the *nmf375* mutation causes mRNA splicing defects leading to a reduction in both the mRNA and protein for Usp14. Instead of exhibiting the NMJ developmental deficits observed in the *ax^l* mice, the *nmf375* mutant mice display adult onset neuromuscular disease. To determine possible differences between the phenotypes of these two Usp14 mutant mice, we examined genetic background as a contributing factor. While the *ax^l* mice are maintained on a C57BL/6J background, the *nmf375* arose on BALB/c. C57BL/6J mice containing the *nmf375* mutation are dead at birth and indicate that the *nmf375* mutation is likely to result in a greater reduction in Usp14 levels than *ax^l* and that the effects of the loss of Usp14 are sensitive to genetic background. Interestingly, we found age dependent differences in proteasomal activity in BALB/c versus C57BL/6J mice that do not appear to be coupled with differences in proteasomal protein levels. These results demonstrate that Usp14 is required for the development and maintenance of the NMJ and that differences in proteasome activity may be responsible for the structural and functional changes seen in the Usp14 deficient mice.

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The deubiquitinating enzyme Ataxin-3 regulates the activity of the ubiquitin ligase Hrd1 in Endoplasmic Reticulum-Associated Degradation.

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Protein Quality Control (PQC) comprises cellular pathways that regulate the turnover of short-lived, misfolded proteins. Aberrations in PQC have been linked to various malignancies and neurological diseases. A main component of PQC is Endoplasmic Reticulum-Associated Degradation (ERAD), which controls the turnover of proteins synthesized in the Endoplasmic Reticulum. During ERAD, misfolded proteins are recognized, ubiquitinated, extracted into the cytosol and degraded by the proteasome. While significant progress has been made in understanding ERAD, it is not entirely clear how substrate ubiquitination and degradation are regulated. Two proteins associated with ERAD are the E3 ubiquitin ligase HRD1 and the deubiquitinating enzyme (DUB) ataxin-3. Ataxin-3 is of particular interest because mutations in it cause the dominantly inherited neurodegenerative disease Spinocerebellar Ataxia Type 3, one of at least nine polyglutamine-related diseases that include Huntington's Disease and several SCAs. Normal ataxin-3 suppresses neurodegeneration in several *Drosophila* models of polyglutamine disease and protects mammalian cells from proteotoxic stress. The cellular functions of ataxin-3 or how it causes disease are unclear. Here, we present evidence that ataxin-3 and HRD1 functionally interact in ERAD. Ataxin-3 and HRD1 co-immunoprecipitate from mammalian cells and co-localize at the Endoplasmic Reticulum. Ataxin-3 counteracts the positive effect of HRD1 on ERAD substrate degradation in cells. HRD1 makes different types of poly-ubiquitin chains in reconstituted systems *in vitro*, depending on the molecular complex with which it associates. Recombinant ataxin-3 opposes poly-ubiquitin chain formation by HRD1 in a manner that depends on its DUB activity. Ataxin-3 with mutated ubiquitin-interacting motifs does not counteract HRD1 as well as wild type ataxin-3. Also, this DUB can cleave ubiquitin chains formed by HRD1 after their synthesis. These data support a model where ataxin-3 binds ubiquitin chains formed by HRD1 to cleave them, instead of inhibiting the ability of HRD1 to

synthesize poly-ubiquitin *in vitro*. Our work suggests that ataxin-3 balances HRD1 activity, providing clues into the decision between substrate degradation or rescue in ERAD.

2391

Protein phosphatase PP6 modulates the phosphorylation status of beta-catenin.

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Phosphorylation status of beta-catenin is a key determinant to the Wnt/beta-catenin signaling pathway. It was reported that protein phosphatase PP2A, a member of the type 2A serine/threonine phosphatase family, promotes the Wnt/beta-catenin signaling through dephosphorylation of beta-catenin. In this study, we found that another type 2A phosphatase PP6C physically interacted with beta-catenin and this interaction was enhanced upon activation of the Wnt signal. A PP6C-containing holoenzyme dephosphorylated phosphorylated forms of beta-catenin. Overexpression of PP6C enhanced the Wnt/beta-catenin signaling to a similar degree when PP2A was overexpressed. Taken together, our data suggested that PP6 is involved in the Wnt/beta-catenin signaling pathway through modulation of the phosphorylation status of beta-catenin.

2392

Brazilin induces p62-mediated autophagy by ubiquitin-proteasome inhibition in rheumatoid fibroblast-like synoviocytes.

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Brazilin (7, 11b-dihydrobenz[b]indeno[1,2-d]pyran-3,6a,9,10 (6H)-tetrol) isolated from *Caesalpinia sappan*, which possesses chemotherapeutic potential through its ability to suppress inflammation. The objective of this study was to investigate the effect or mechanism of brazilin in fibroblast-like synoviocytes derived from patients with RA.

RA FLS were isolated from fresh synovial tissue biopsy samples obtained from RA patients at total knee arthroplasty. The underlying molecular mechanism for autophagy formation, NF- κ B signaling was examined by analyzing transmission electron microscope and immunoblotting.

Treatment with brazilin resulted in non-apoptotic cell death in a caspase-independent manner. In contrast, brazilin in RA FLS robustly elicited accumulation of autophagosome and upregulated p62 expression in RA FLS. Interestingly, such activation of autophagy pathway by brazilin was mediated by enhanced production of ubiquitinated proteins, which mainly accumulated in insoluble fractions. Furthermore, treatment of brazilin drastically suppressed IL-1 β and LPS-induced NF- κ B activation under the condition of autophagy formation.

The results suggest that brazilin activates p62-mediated autophagy pathway, which in turn results in controls inflammatory process through suppressing NF- κ B activation.

2393

Autophagy and Ubiquitin-Proteasome Pathway are the Keystones of Sperm Mitochondrial Degradation following Mammalian Fertilization.

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Autophagy and the ubiquitin-proteasome system (UPS) are the major protein degradation pathways responsible for the removal of outlived proteins, protein-aggregates, and organelles, including mitochondria. Autophagy comprises several processes which ensure selective

degradation of various cellular structures by sequestering them in autophagosomes. In almost all eukaryotes, mitochondria and mitochondrial DNA (mtDNA) are inherited from the maternal parent. This phenomenon cannot be explained solely through dilution of paternal mtDNA. The UPS has long been considered a degradation mechanism for the sperm-contributed paternal mitochondria. Recent studies in *C. elegans* suggest an interplay between proteasome-dependent degradation and mitophagy, but little is known about these events in mammals. Autophagy of sperm mitochondria could occur along three distinct degradation routes leading to autophagic clearance: 1) Autophagy-associated ubiquitin-receptor p62/SQSTM1 recognizes ubiquitinated cargo and interacts with autophagosome-binding ubiquitin-like modifiers, such as LC3 or GABARAP; 2) Ubiquitinated proteins are extracted from mitochondria and from aggresomes, the protein aggregates induced by ubiquitin-binding adaptor protein HDAC6 that transports them along the microtubules; 3) Mitophagy receptor BNIP3L binds to sperm mitochondria inside the fertilized oocyte and targets them toward autophagosome. We hypothesized that boar sperm mitochondria are recognized by the above ubiquitin-binding receptors and specifically degraded by autophagic machinery inside the fertilized porcine oocyte. Oocytes were inseminated with the MitoTracker tagged boar spermatozoa for 6 hrs and cultured with/without the addition of a specific proteasomal inhibitor MG132 for an additional 24 hrs. The GABARAP-positive autophagosomes formed a halo around the sperm nucleus and mitochondrial sheath at 30 hrs post insemination; treatment with MG132 slowed down the degradation of sperm mitochondria and caused the accumulation of the GABARAP in the vicinity of the sperm mitochondria. A change in GABARAP protein band density was observed by Western blotting of MG132-treated oocytes. Other components of the autophagy/mitophagy pathways, including LC3, HDAC6, and BNIP3L, were detected and immunolocalized in the boar spermatozoa and porcine zygotes by immunofluorescence and Western blotting. Immunoprecipitation of GABARAP from porcine zygotes identified two potential co-precipitating proteins, FABP3 and Profilin-1. These preliminary results indicate that autophagy-associated ubiquitin-like protein modifiers could cooperate with ubiquitin-proteasome system during the degradation of boar sperm mitochondria after fertilization.

2394

Impairment in neuronal development with disruption of polyubiquitin gene *Ubb*.

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Disruption of polyubiquitin gene *Ubb* leads to hypothalamic neurodegeneration and metabolic disorders including obesity and sleep abnormalities in mice. However, it has yet to be identified why the reduced Ub levels due to loss of *Ubb* lead to these neuronal phenotypes and whether these phenotypes are due to the neuronal cell autonomous defects to maintain ubiquitin (Ub) levels. To determine whether the reduced Ub levels in neurons *per se*, but not their cellular microenvironment, are the cause of the neuronal phenotypes observed in *Ubb* knockout mice, we investigated the characteristics of cultured neurons isolated from *Ubb* knockout mouse embryonic brains, particularly on neuronal development and the formation of synapse. We first found that, during the culture of neurons, although wild-type neurons maintained their cell numbers up to 13 days *in vitro*, they were reduced in *Ubb* knockout neurons due to increased apoptosis, with concomitant increase of pro-inflammatory cytokines, such as IL-1 β and TNF- α . By immunofluorescence analysis using various neuronal markers, such as β III-tubulin, α -internexin, and microtubule-associated protein 2, we found that neuronal morphology, neurite outgrowth and axonal development were significantly impaired in *Ubb* knockout neurons. Furthermore, we demonstrated that, although an early neuronal marker (α -internexin) levels decreased during development in wild-type neurons, they remained similar in *Ubb* knockout neurons, suggesting the delayed or impaired neuronal maturation. In addition, we found that β III-tubulin levels were reduced and the synaptogenesis was impaired in *Ubb* knockout neurons

with decreased synapsin I levels. Intriguingly, we found that Ub conjugates and total Ub levels were significantly reduced in *Ubb* knockout neurons, but not the free Ub levels, which were similar between wild-type and *Ubb* knockout neurons. Despite the upregulation of *Ubc* in *Ubb* knockout neurons, due to the most significant contribution of *Ubb* toward total Ub levels, compensation by the other polyubiquitin gene *Ubc* was not sufficient to bring up total levels of cellular Ub to those of wild-type neurons. However, we were able to restore Ub conjugates and total Ub levels in *Ubb* knockout neurons to those of wild-type neurons by providing extra Ub using lentivirus-mediated delivery. When *Ubb* knockout neurons restored their cellular Ub levels, their behaviors were comparable to wild-type neurons. Combined together, these results suggest that reduced Ub levels in neurons are the intrinsic cause of neuronal phenotypes observed in *Ubb* knockout mice.

2395

Effect of cellular ubiquitin levels on the oxidative stress response system regulated by the Nrf2-Keap1 pathway.

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Upon exposure to oxidative stress, cellular stress response pathway is activated and the cytoprotective genes are upregulated to increase the likelihood of survival. Among these are polyubiquitin genes *Ubb* and *Ubc*, which were shown to be upregulated by treatment with NaAsO₂. Here, we found that ubiquitin (Ub)-deficient cells (mouse embryonic fibroblasts and cultured neurons), in which cellular Ub levels were reduced due to disruption of *Ubb* or *Ubc* exhibited reduced viability under oxidative stress induced by NaAsO₂. Therefore, we hypothesized that the maintenance of cellular Ub levels is important for oxidative stress response and we tested our hypothesis by investigating whether the activation of Nrf2-Keap1 pathway, which is the major oxidative stress responsive pathway in cells, is dependent on cellular Ub levels. We first confirmed that *Ubb* and *Ubc* were indeed upregulated upon treatment with tert-butylhydroquinone (tBHQ), a well-known activator of Nrf2, in a dose-dependent manner. We then found that the levels of Nrf2 were significantly increased in wild-type cells with tBHQ treatment for 5-10 h, but their levels were much lower in Ub-deficient cells. Furthermore, upon treatment with emetine to block the protein synthesis, we demonstrated that Nrf2 was degraded in a time-dependent manner in wild-type cells, which was completely abolished by co-treatment with tBHQ. However, in Ub-deficient cells, this stabilization of Nrf2 was hardly observed. Reduced Nrf2 levels upon tBHQ treatment in Ub-deficient cells were also confirmed by immunofluorescence analysis. When Nrf2 was stabilized, we found that Nrf2 was almost exclusively localized to the nucleus of wild-type cells, however, the Nrf2 immunoreactivity in the nucleus and the percentage of cells with nuclear Nrf2 were reduced in Ub-deficient cells. Furthermore, we found that, although target gene expression levels, such as *Gstp1* and *Nqo1*, were increased upon activation of Nrf2 in wild-type cells, this increase was less significant in Ub-deficient cells. We believe that, although degradation or stabilization of Nrf2 is tightly regulated in wild-type cells in the absence or presence of stress, this tight regulation seems to be weakened when ubiquitin-proteasome system is impaired due to the reduced cellular Ub levels. In sum, these results suggest that reduced viability of Ub-deficient cells under oxidative stress could, at least in part, be due to impaired activation of Nrf2, which results in the diminished cytoprotective response of cells.

2396

The essential role played by FAT10 promoter in the pathogenesis of Mallory-Denk bodies (MDBs) formation.

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Mallory-Denk bodies (MDBs) are aggresomes composed of undigested ubiquitinated short lived proteins which have accumulated because of a decrease in the rate of their degradation by the 26s proteasome. The decrease in the activity of the proteasome is due to a shift in the activity of the 26s proteasome to the immunoproteasome triggered by an increase in expression of the catalytic subunits of the immunoproteasome which replaces the catalytic subunits of the 26s proteasome. This switch in the type of proteasome in liver cells is triggered by the binding of IFN γ to the IFN γ sequence response element (ISRE) located on the FAT10 promoter. To determine if either FAT10 or IFN γ are essential for the formation of MDBs we fed both IFN γ and FAT10 knock out (KO) mice DDC added to the control diet for 10 weeks in order to induce MDBs. Mice fed the control diet and Wild type mice fed the DDC or control diet were compared. MDBs were located by immunofluorescent double stains using antibodies to ubiquitin to stain MDBs and FAT10 to localize the increased expression of FAT10 in MDB forming hepatocytes. We found that MDB formation occurred in the IFN γ KO mice but not in the FAT10 KO mice. Western blots showed an increase in the FAT10 and ubiquitin smears in the Wild type mice fed DDC but not in the FAT10 KO mice. To conclude we have demonstrated that FAT10 over expression is essential to the induction of MDB formation in the DDC fed mice.

2397

Cdc48 and its adaptor Ubx4 promote mitotic progression by properly localizing the proteasome in the nucleus.

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Cdc48 is a member of the AAA-ATPase family and has diverse functions in membrane fusion, protein degradation, and cell cycle. These functions are mediated by specific adaptors of Cdc48. We found that the adaptor Ubx4 was not essential for cell viability in the budding yeast *Saccharomyces cerevisiae*, but its deletion was synthetic-lethal with *cdc48-3* temperature-sensitive mutant. Repressing Ubx4 expression through the GAL promoter in combination with *cdc48-3* mutation caused mitotic arrest with sustained mitotic regulators Clb2 and Cdc20 that are normally degraded in anaphase through the E3 ubiquitin ligase Anaphase Promoting Complex (APC). The mitotic arrest was independent of spindle checkpoint and any defect in APC. Interestingly, the cell growth and proteolytic defects of *ubx4 cdc48-3* can be partially rescued by overexpression of Rpn4, the transcriptional activator for proteasome subunits, indicating that the proteasome function is compromised. Indeed, the proteasome visualized by GFP-tagged subunit Pre6 forms a nuclear aggregate in *ubx4 cdc48-3*, instead of its normal distribution in the nucleus. Furthermore, Ubx4 associates with the proteasome through its amino-terminal UBL domain. Our study reveals that mitotic progression requires properly localized proteasome that is mediated by the chaperone Cdc48-Ubx4.

2398

ZBTB20 interacts with the SUMO-conjugating enzyme UBC9 and is a novel target for SUMOylation.

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Autism spectrum disorder (ASD) and intellectual disability (ID) are the two most frequently reported, often co-morbid, neurodevelopmental disorders that affect children all over the world. Recently, we identified mutations in the ZBTB20 gene which were associated with susceptibility to ASD and ID. The gene is highly expressed in developing brain and encodes a protein of a BTB (broad complex, tramtrack, bric-a-brac) – zinc finger family of transcription factors. The ASD and ID-associated ZBTB20 missense mutations affected dendritic and synaptic structure of pyramidal neurons.

To elucidate the potential molecular links and pathways by which the ZBTB20 protein functions in neuronal cells, we looked for brain-expressed proteins that interact with ZBTB20. Using a yeast-two-hybrid screen, we identified several proteins that interact with ZBTB20 including the E2 SUMO conjugating enzyme UBC9, suggesting that ZBTB20 likely undergoes SUMOylation. The interaction of ZBTB20 and UBC9 was confirmed by co-immunoprecipitation and co-localization analyses of both proteins expressed in transfected HEK293H cells. Subsequently, we confirmed that ZBTB20 contains two putative conserved SUMOylation (ΨKXE) motifs. We further showed that ZBTB20 undergoes SUMOylation in transfected cells, binds SUMO-1, and the N-terminal region of ZBTB20 is critical for its interaction with UBC9. Mutations altering either of the two putative SUMO motifs had no effects on the subcellular localization of ZBTB20 in mammalian cells. However, deleting the conserved lysine residue (K330) in motif 1, but not the conserved lysine residue (K371) in motif 2, adversely affected SUMO-1 binding to ZBTB20. These findings suggest that residue K330 is the primary target for *in vivo* SUMOylation. The post-translational modification SUMOylation is a major regulator of functional properties of many proteins implicated in human neurological disorders and plays a key role in neuronal function. Further studies will help understand the effect of SUMOylation in the regulation of ZBTB20 physiological functions in neuronal cells and its potential role in ASD and ID.

Oncogenes and Tumor Suppressors III

2399

Nuclear transport of cell surface EGF receptor and ErbB-2 is regulated through integral membrane-bound trafficking.

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Multiple cell surface receptor tyrosine kinases (RTKs), such as FGF receptor-1 (FGFR-1), IGF receptor, cMet, VEGF receptor, and the four members of EGF receptor (EGFR) family, have been reported to localize in the nucleus. Among these, both full-length nuclear EGFR and ErbB-2 have been shown to be involved in transcriptional regulation, cell proliferation, DNA repair, DNA replication, and chemo- and radio-resistance. Recent studies have demonstrated that

endocytosis and importin β are involved in the nuclear transport of cell surface RTKs, including EGFR, ErbB-2, and FGFR-1. We previously showed that cell surface EGFR is translocated to the nucleus via a retrograde route from the Golgi to the endoplasmic reticulum (ER) and then EGFR is targeted to the inner nuclear membrane (INM) through the INTERNET (integral trafficking from the ER to the nuclear envelope transport) pathway. Nevertheless, the nuclear trafficking mechanisms of other membrane RTKs, apart from EGFR, remain unclear, which dampened progress of this important area that has been overlooked for decades. To expand our knowledge of the trafficking mechanisms of various nuclear RTKs, we compared the nuclear transport of EGFR family proteins with that of FGFR-1, since FGFR-1 has an atypical transmembrane domain, which is different from EGFR. In this study, we used the digitonin permeabilization system and sub-nuclear fractionation assays to investigate the difference between the two pathways of nuclear trafficking in EGFR/ErbB-2 and FGFR-1. We interestingly found that the membrane-bound INTERNET mechanism regulates the nuclear trafficking of EGFR/ErbB-2 but not that of FGFR-1. We further demonstrated that the ER translocon Sec61 β localized in the INM is involved in releasing ErbB-2 from the INM to the nucleoplasm, which is similar to the nuclear trafficking of EGFR. This novel discovery as location and function of translocon Sec61 in the INM may serve as a general mechanism for nuclear translocation of other cell surface receptors. Together with previous studies, our proposed model timely provides a comprehensive membrane-bound trafficking pathway for the nuclear translocation of cell surface EGFR/ErbB-2, but not for that of FGFR-1. Thus, at least two different pathways exist to translocate cell surface receptors into the nucleus. This provides a new direction for investigating the trafficking mechanisms of various nuclear RTKs. Understanding the trafficking mechanisms as to how RTKs are transported from the cell surface to the nucleus will significantly contribute to understanding the functions of nuclear RTKs and identifying potential therapeutic targets for clinical application.

2400

Activation of Epidermal Growth Factor Receptor Modulates Bmi-1 Protein Level through miRNAs in Lung Adenocarcinomas.

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Bmi-1 is a member of the Polycomb Repressive Complex 1 (PRC1) that functions in the epigenetic silencing of genes governing self-renewal, differentiation, and proliferation. Bmi-1 was originally identified as an oncogene cooperating with c-Myc in c-Myc-induced lymphomagenesis. Later, Bmi-1 upregulation was found in a wide spectrum of human cancer types. In lung adenocarcinomas (LAC), ectopic expression of Bmi-1 shows an inverse correlation with the INK4 locus proteins (p16/p14ARF), and is associated with tumor progression. Although Bmi-1 has been suggested to regulate cancer cell growth and metastasis, how Bmi-1 is regulated in LAC remains unclear. Here, we show that Bmi-1 is specifically overexpressed in protein, but not in mRNA level, in LACs with EGFR mutations. In LAC cells with wild-type EGFR, addition of EGF ligand or overexpression of mutant EGFR enhanced Bmi-1 protein level within 24 hr. In contrast, overexpression of mutant K-Ras did not alter the Bmi-1 level. Further investigations identified that EGFR enhanced the Bmi-1 protein level through modulation of miRNAs including miR-200. Knockdown of Bmi-1 or overexpression of miR-200 severely blocked the colony forming and migration/invasion activities, and downregulated the (epithelial-mesenchymal transition) EMT markers in LAC cells. In conclusion, these results showed that in LACs, EGFR signaling modulates Bmi-1 protein level through miRNAs, which in turn drives the cell growth, EMT, and metastasis.

2401

Flotillin mediated stabilization of the receptor tyrosine kinase ErbB2.

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The receptor tyrosine kinase ErbB2 is a member of the epidermal growth factor receptor (EGFR) family. Amplification and overexpression of ErbB2 occur in up to 30% of all human breast cancers and high ErbB2 levels are correlated to poor prognosis for breast cancer patients. In contrast to the EGFR, ErbB2 receptors have low rates of endocytosis and are continuously recycled back to the cell surface. The low internalization rate results from Hsp90 mediated stabilization of ErbB2 at the membrane. Inhibition of Hsp90 by geldanamycin (GA) or analogues (e.g. 17-AAG/tanespimycin) leads to efficient downregulation and degradation of ErbB2. We elucidated a new function of flotillins, involved in the stabilisation of ErbB2 at the plasma membrane. In SKBR3 breast cancer cells and breast cancer tissue, a positive correlation between flotillin and ErbB2 expression levels could be demonstrated. Moreover, tissue microarray analyses of biopsies from 194 patients diagnosed with carcinomas of the breast showed that flotillin-2 emerged as a potential predictor of prognosis in breast cancer. Interestingly, depletion of flotillin-1 and flotillin-2 leads to internalisation and degradation of ErbB2. Furthermore, flotillin-1 and -2 were found to be in a molecular complex with ErbB2 and Hsp90. The depletion or functional inhibition of one of these proteins results in disruption of this complex, followed by destabilisation of ErbB2 at the membrane, its internalisation and degradation. As a consequence, phosphorylation of ErbB2 is reduced and ErbB2-triggered downstream signalling is inhibited. In summary, our data demonstrate a novel mechanism for interfering with ErbB2 signalling, which potentially can have clinical impact.

2402

ErbB4 is a glycoprotein that is required for PI3K-Akt-Mdm2 signaling to degrade androgen receptor.

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Here we characterized ErbB4 (JMb/CYT1) as an N-glycosylated protein. With similar organization of extracellular domains to EGFR, of which the N-glycosylation sites were determined; accordingly we defined asparagine at the 358th amino acid of ErbB4 as an N-glycosylation site. The glycosylation is required for tyrosine autophosphorylation of ErbB4 that then stimulates PI3K/Akt/Mdm2 pathway resulting in AR polyubiquitylation/proteasome-mediated degradation. Hence ErbB4 can abolish the proliferation of prostate cancer cells. In this study, we provide the mechanisms of ErbB4 (JMb/CYT1) down-regulates proliferation rates of prostate cancer cells via PI3K-Akt-Mdm2 signalings. Additionally, 358th (Asn) of ErbB4 is an important N-glycosylation site for ErbB4-inducing PI3K-Akt-Mdm2 activation.

2403

FAM83A confers EGFR-TKI resistance in breast cancer.S. Furuta¹, S.-Y. Lee², R. Meier³, M. E. Lenburg⁴, P. A. Kenny⁵, R. Xu⁶, M. J. Bissell¹;¹Department of Cancer and DNA Damage Repair, Lawrence Berkeley National Laboratory, Berkeley, CA, ²Department of Molecular Biology, Pusan National University, Busan, Korea,³Sanofi-Aventis, Geneva, Switzerland, ⁴Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA, ⁵Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY, ⁶Department of Molecular and Biomedical Pharmacology, University of Kentucky College of Medicine, Lexington, KY**Abstract**

Epidermal growth factor receptor (EGFR) is overexpressed often in breast carcinomas and correlates with poor prognosis. Therapeutic use of EGFR tyrosine kinase inhibitors (TKIs) for breast cancer treatment, however, is hampered by resistance, and the mechanisms remain largely unknown. EGFR mutations are rare in breast cancer, pointing to the need for further exploration of alternative mechanisms for EGFR-TKI resistance. We hypothesized that resistance may originate, at least in part, from molecular alterations that activate signaling pathways downstream of EGFR. Utilizing a novel screen based on 'reversion' of malignant breast cells into phenotypically non-malignant cells in three-dimensional gels (3D) after EGFR-TKI treatment (1), we identified FAM83A as a candidate cancer-associated gene capable of conferring resistance to the drug. FAM83A overexpression in breast cancer cells increased proliferation and invasion, and imparted EGFR-TKI resistance both in cultured cells and animals. Tumor cells that survived EGFR-TKI treatment in vivo had upregulated endogenous FAM83A levels. Additionally, FAM83A overexpression dramatically increased the number and size of transformed foci in cultured cells and anchorage-independent growth in soft agar. Conversely, FAM83A depletion in cancer cells led to reversion of the malignant phenotype, delayed tumor growth in mice and rendered cells sensitive to EGFR-TKI. Analyses of published clinical data revealed a significant correlation between high FAM83A expression and poor prognosis in breast cancer patients. We explored the molecular mechanism by which FAM83A exerts its effects on malignant transformation and found that FAM83A interacts with and causes phosphorylation of c-RAF and PI3K p85 subunit, upstream of MAPK and downstream of EGFR (2). These data provide a plausible additional mechanism by which tumor cells can become EGFR-TKI-resistant and suggest that targeting FAM83A (and some of its other family members (3)*) may increase EGFR-TKI's efficacy for breast cancer treatment.

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2404

Oncogenesis of Pancreatic Ductal Adenocarcinoma by Amplification of a Novel PEAK1/eIF5A/Hypusine Signaling Loop.R. L. Klemke¹, J. Kelber¹, K. Fujimura¹, T. Wright¹; ¹Pathology, UC San Diego and Moores Cancer Center, La Jolla, CA

We recently identified PEAK1 (pseudopodium-enriched atypical kinase one, SGK269) as a catalytically active, non-receptor tyrosine kinase, that associates with the actin cytoskeleton and focal adhesions, where it regulates cell migration and proliferation (PNAS 107:10920-5. 2010).

Herein, we assess the roles of PEA1 expression as a biomarker and potential therapeutic target in human malignancies, and determine its mechanism of regulation in cancer cells. PEA1 upregulation occurs in multiple human malignancies including pancreatic ductal adenocarcinoma (PDAC). We find that PEA1 is sufficient and necessary for PDAC tumor growth and metastasis *in vivo*. Importantly, activated KRas induces a robust PEA1-dependent Src/PEA1/ErbB2 tyrosine kinase amplification loop that drives PDAC growth and metastasis. Interestingly, PEA1 protein amplification in PDAC results from a unique mechanism involving KRas-induced amplification of the translation elongation factor, eIF5A, which is activated by hypusine modification of lysine 50. Hypusinated eIF5A is amplified in more than 80% of PDAC patients and correlates with poor patient survival. Inhibition of eIF5A function using shRNA knockdown or pharmacological agents that prevent eIF5A hypusination prevent PEA1 expression and suppresses Src/ErbB2 signaling activities leading to cell cycle arrest and PDAC cell death. Together our findings indicate that activating KRas mutations, seen in the majority of PDAC patients, drive amplification of an eIF5A/PEA1 signaling axis, which controls Src/ErbB2 kinase signaling, PDAC cell growth, and metastasis. We conclude that eIF5A and PEA1 are excellent biomarkers of PDAC and possible new therapeutic targets to treat PDAC patients.

2405

Bax Δ 2, A Novel Tumor-Generated Functional Bax Subfamily.

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The pro-death Bcl-2 family protein and tumor suppressor Bax is frequently mutated in tumors with microsatellite instability (MSI). The mutation often results in a "Bax negative" phenotype and therefore is generally thought to be beneficial to the tumor development. Here, we report the identification of a group of novel Bax isoforms, Bax Δ 2 sub-family, generated by a unique combination of MSI mutation and alternative splicing. Bax Δ 2 is tumor-specific and only detected in Bax MSI positive cell lines and primary tumors. Bax Δ 2 turns out to be a potent cell death inducer but, unlike the parental Bax α , does not target to mitochondria. In addition, Bax Δ 2 selectively sensitizes certain MSI tumor cells to a sub-set of chemotherapeutic agents. Thus, our data provide evidence that mutation and alternative splicing of tumor suppressors such as Bax can result in new functional isoforms that can be detrimental to tumor development. This finding may have important implications in cancer prognosis.

2406

Down Regulation of Gene expression in Cancer by Alteration of RNA Splicing.

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Fibroblast growth factor (FGF) signaling plays a key role in a variety of processes, including cellular proliferation, cellular differentiation, and carcinogenesis. The FGFR family of membrane-spanning tyrosine kinase receptors consists of four members (FGFR1�4) that differ in their tissue expression, specificity for ligand, signal pathways, and biological effects.

FGFR3 are a member of FGFR tyrosine kinase proteins that contain an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. The extracellular region, which contains two or three Ig-like domains, is important for FGF binding and the subsequent dimerization and activation of FGFRs. FGFR3 is the most frequently affected receptor

with numerous mutations localized in or near the third Ig-like domain or in the sequence linking Ig-like domains II and III.

Alternative mRNA splicing of the FGFR gene specifies the sequence of the COOH-terminal half of Ig-like-III domain, resulting in either the IIIb or IIIc isoform of the FGFR. This alternative splicing event is regulated in a tissue-specific manner and dramatically affects ligand binding specificity.

A nested RT-PCR analysis of FGFR3 from human carcinomas revealed novel mutant transcripts caused by aberrant splicing and activation of cryptic splice sequences. Two aberrantly spliced transcripts were detected with high frequency in 50% of 36 primary tumors and in 60% of 10 human colorectal cancer cell lines.

2407

Secreted IGFBP3 and SFRP1 mediate cellular senescence.

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Cellular senescence is implicated in tumor suppression and organismal aging, but signaling pathways mediating senescence are not well understood. By analyzing the proteins secreted from senescent cells, we identified two novel mediators of senescence.

1) Cancer cells can be reprogrammed to senesce in response to stresses such as chemotherapy. We observed that non-senescent MCF-7 breast cancer cells become senescent upon addition of the conditioned medium from senescent MCF-7 cells induced to senesce by doxorubicin treatment, which suggested the presence of secreted mediator(s) of senescence. Proteomic comparison of protein secretion from senescent and non-senescent MCF-7 cells revealed that IGFBP3 (insulin-like growth factor binding protein 3) is over-secreted from doxorubicin-treated senescent MCF-7 cells. IGFBP3 induced senescence through suppression of AKT and requiring p53 and Rb. We found that IGFBP3 can be cleaved and inactivated by t-PA (tissue-type plasminogen activator), which is in turn inhibited by another known secreted mediator of senescence, PAI-1 (plasminogen activator inhibitor 1). We have discovered a cascade whereby PAI-1 induces senescence through the elevation of IGFBP3 levels. Immunohistochemistry staining of human breast cancer tissues showed co-localization of PAI-1 and IGFBP3 in the tumor epithelium. RNAi suppression of PAI-1 or IGFBP3 inhibited senescence induction upon doxorubicin treatment of MCF-7, indicating that PAI-1 and IGFBP3 mediate doxorubicin-induced senescence in these cells.

2) Employing the quantitative proteomic analysis of secreted proteins, we determined that the Wnt signaling inhibitor SFRP1 (Secreted Frizzled-related Protein 1) is over-secreted from IMR-90 human primary fibroblasts induced to senesce by DNA damage (etoposide treatment). SFRP1 over-secretion occurred upon treatment with different DNA damaging agents or by oxidative stress, and was required for stress-induced senescence. We present evidence suggesting that secreted SFRP1 mediates senescence by inhibiting the Wnt signaling and activating the Rb pathway. Interestingly, cancer-associated SFRP1 mutants were defective for senescence induction.

These studies identified extracellular components of senescence signaling. Senescence mediators secreted from senescent cells may amplify the senescence response and provide a non-cell autonomous tumor suppression mechanism in precancerous cells as well as in chemotherapy-treated tumor cells.

1. Proc Natl Acad Sci U S A. 2012 Jul 24;109(30):12052-7.
2. Mol Cell Biol. 2012 Aug 27. [Epub ahead of print]

2408

microRNAs as potential regulators of EGFR therapy resistance in breast cancer cells.

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Signaling from epidermal growth factor receptors (EGFRs) is dysregulated in breast cancer, where it is associated with an aggressive phenotype and poor outcome. The success of targeted therapies for EGFR has been marred by *de novo* or acquired resistance. Gefitinib, a small molecule tyrosine kinase inhibitor specific for EGFR, has been used as therapy for advanced non-small cell lung carcinoma. However, clinical trials of gefitinib in breast cancer have failed due to therapy resistance and adverse toxic effects. We developed a gefitinib resistant syngenic variant of the gefitinib sensitive human breast cancer cell line SKBR3 (SKBR3.GR), with the objective of studying the role of microRNAs (miRNAs) in therapy resistance. A screening of miRNAs implicated in therapy resistance demonstrated that miRNA (miR-)200a was downregulated while miR-221 and miR-222 were upregulated in the SKBR3.GR cells compared to the parental cells. The miR-200 family plays a critical role in the suppression of epithelial-to-mesenchymal transition (EMT) by repressing key molecules involved in this process. Additionally, the miR-200 family can regulate tumor cell adhesion, migration, invasion and metastasis; and has been associated with EGFR inhibitor resistance. MiRs-221/222 promote EMT and have also been associated with cancer progression and acquisition of gefitinib therapy resistance in cancer cells. To elucidate the functional significance of the differential expression of miR-200a and miR-221/222 in therapy resistance, we compared the protein expression of known targets of these families in SKBR3.GR and parental cells. Our results show that in SKBR3.GR cells, EGFR expression was significantly increased while EGFR phosphorylation was inhibited by gefitinib treatment. The miR-200 targets Zeb1 and β -catenin, molecules that promote EMT, were significantly upregulated; while the miR-221/222 target PTEN, a tumor suppressor, was downregulated in SKBR3.GR cells. Our results suggest that decreased miR-200a levels, and thus, increased Zeb1 and β -catenin, a potent pro-cancer transcriptional activator; and elevated miR-221/222 expression, that downregulates the expression of tumor suppressors, are potential mechanisms of gefitinib resistance in aggressive breast cancer.

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Cooperative phosphorylation of two kinases for the phosphorylation of FADD-serine 194.

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Previous study [Mol. Cell, **19**:321-32, 2005; Cancer Res., **71**:7207-15, 2011] described that FADD can be phosphorylated at serine 194 by casein kinase 1 α (CK1 α) and/or polo-like kinase 1 (Plk1) upon taxol treatment. However, the regulatory mechanism between CK1 α and Plk1 in phosphorylating FADD is yet to be clarified. In this study, we demonstrate the presence of

physical interaction and functional interplay between CK1 α and Plk1 in response to taxol treatment. Phosphorylation of FADD by Plk1 was clearly inhibited in CK1 α -depleted cells, whereas that by CK1 α remained unchanged in Plk1 depleted cells. Therefore, our results indicate that CK1 α acts upstream of Plk1 in phosphorylating FADD. Moreover, the catalytic domain of Plk1 (a.a 1 - 308) bound CK1 α . Therefore, this study adds CK1 α to the list of upstream kinases of Plk1. Collectively, our data demonstrate the presence of cooperative action between CK1 α and Plk1 to phosphorylate FADD. [This research was supported by the grant (KDDF-201202-10) from the Korea Drug Development Fund, and by grant (2011-0031223) from the National Research foundation at the Ministry of Education, Science and Technology in Korea.]

2410

Critical role for Pim kinase-phosphorylated eIF4B in cellular transformation by v-Abl and Bcr-Abl oncogenes.

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Eukaryotic initiation factor 4B (eIF4B) is a multidomain protein that plays a critical role in the initiation of protein synthesis and has been known as a hyperphosphorylated protein. The Pim kinase family, which is consisted of three members: Pim-1, Pim-2, and Pim-3, has been implicated in various cellular processes including cell proliferation and survival and tumorigenesis. We demonstrated that Pim-1 physiologically interacts with eIF4B. Pim-1, Pim-2 but not Pim-3 could increase phosphorylation levels of eIF4B on Ser406 and Ser422 in HEK293T cell. Pim-1 and Pim-2 kinase directly phosphorylated eIF4B on Ser406 and Ser422 in vitro kinase assay. Interestingly, we found that Pim kinases in v-Abl transformed cells can enhance the expression and the phosphorylation of eIF4B protein. Our results also revealed that the expression and phosphorylation of eIF4B are Abl kinase-dependent because treatment with Abl kinase inhibitor disrupts these processes. Furthermore, phosphorylation of eIF4B was shown to be highly sensitive to Pim kinase inhibitor STI-4a and ectopic expression of eIF4B phosphomimetic mutants could promote survival of Abl-transformed cells treated with the Pim kinase inhibitor. In addition, eIF4B knockdown sensitizes v-Abl transformed cells to apoptosis and attenuated tumorigenicity of K562 cells. Abl transformation efficiency of bone marrow cells derived from eIF4B knockdown mice was greatly reduced as compared with wild-type mice. Together, we have identified eIF4B as a novel substrate of Pim kinases in Abl transformed cells. Our observations suggest that eIF4B may mediate some of the effects of the Pim kinases on hematopoietic cell transformation. The results reveal a novel Pim kinase-dependent eIF4B activation that plays a crucial role in Abl-mediated tumorigenesis.

2411

Hsc70 functions as a novel interaction protein of Cx43 in G1/S transition.

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Heat shock cognate protein 70 (Hsc70) is a member of the heat shock protein 70 family, which shuttles between the cytoplasm and the nucleus and serves as a molecular chaperone. Several reports show that Hsc70 is involved in cell cycle progression during G1/S transition. Connexin 43 (Cx43) is known to act as a tumor suppressor. However, the underlying mechanisms of Cx43-mediated tumor suppression are still poorly understood.

To clarify the role of Hsc70 in Cx43-mediated cell cycle suppression, in this study, we used a proteomics approach to identify the novel Cx43-interacting partners, which are involved in Cx43-

mediated suppression of the cell cycle. We have identified a Cx43-interacting protein, Hsc70, from whole cell lysate of Huh-7 cells, which express Cx43 abundantly.

We confirmed that the Hsc70 directly binds to the C-terminal domain of Cx43 in vitro and in vivo. Overexpression of Cx43 in Huh-7 cells significantly suppresses cell proliferation. This suppression is abolished by co-overexpression with Hsc70. Taken together, our findings reveal that the Cx43-Hsc70 interaction might play an important role in Cx43-mediated tumor suppression.

2412

Identification of the Molecular Machinery Regulating the Notch ligand Delta.

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Introduction:

The signaling receptor Notch plays a key role in stem cell self-renewal, cell proliferation, cell differentiation, and apoptosis. Consequently, it is involved in many diseases and cancers. Notch signaling is regulated by various cellular processes, including modulation of the activity of Notch ligands. These ligands need to be ubiquitinated, endocytosed, and in some cell types recycled back to the plasma membrane to trans-activate Notch. It has been suggested that the ligand Delta is processed while trafficking through endocytic compartments, but the nature of this putative modification is unknown. The aim of this project is to identify the molecular mechanisms regulating the activity of Delta, and subsequently modulating the signaling levels of Notch.

Methods and findings:

In order to identify new regulators of Delta, we performed a genome-wide shRNA screen using an in vitro Notch co-culture assay. We used the well characterized OP9-DL1 cell line as a signal-sending cell, and HeLa cells that natively express the Notch receptor as signal-receiving cells. Notch activity was monitored in HeLa cells using a luciferase reporter that we had cloned and stably transfected in these cells. We are currently developing various secondary assays in order to validate our screen. First, we will eliminate false positives, and then we will perform functional assays to identify which candidates are relevant for physio-pathological conditions. Some of the interesting hits that came out from the primary screen include endocytic regulators of Delta, components of the ubiquitination machinery, and genes involved in diseases/cancers.

Conclusion and relevance:

This project represents a unique opportunity to identify new regulators of Notch signaling, with potential application in the clinical field in order to develop new therapeutic targets for cancers involving Notch.

Tumor Invasion and Metastasis III

2413

The Nanomechanical Signature of Breast Cancer.

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Tumor mechanobiology is an important yet unresolved aspect of cancer progression. How the mechanical properties of cells evolve from a healthy stage to malignancy and manifest themselves in tissues is poorly understood. Here, we report on identifying individual stages of tumor progression in native human breast biopsies by means of their distinct nanomechanical signatures. Indentation-type atomic force microscopy (IT-AFM) analysis provided detailed maps of stiffness properties for normal, benign and malignant mammary tissues. Healthy ductal epithelium and benign lesions each exhibit uniform stiffness profiles with a distinct peak. Accounting for tissue heterogeneity, primary cancer lesions are characterized by a broad stiffness distribution with a prominent soft peak representing cancer cells. These findings are validated by the stiffness profiles from specific stages of tumor progression in MMTV-PyMT transgenic mice. Correlative analysis of extracellular matrix and cytoarchitecture show that invasive cancer tissues exhibit disorganized laminin-1, which indicates a loss of epithelial polarity typically associated with malignancy and invasiveness. In addition, collagen I was largely absent within nests of cancer cells and appeared far less structured in the nearby stroma compared to the tumor periphery. At the cellular level, a distinct co-expression of desmin and vimentin, both markers of epithelial-mesenchymal transition (EMT), was found in soft cancer cells. As a known promotor of aggressiveness and invasion, our nanomechanical data indicate that soft hypoxic regions have increased migration potential at the late cancer stage. Detecting the soft phenotype in lung metastasis suggests that the compliance of malignant cells in the primary tumor is correlated to the cancer metastasis. Our study reveals the clinical translational significance of nanomechanical signatures as potential diagnostic and prognostic markers of breast cancer.

2414

Electrically monitoring effects of transforming growth factor-beta on wound healing migration of breast cancer cells.

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The motility of cancer cells has been widely studied and considered as an intrinsic component of the metastasis process. Previous studies have indicated that transforming growth factor-beta (TGF- β) can activate downstream signaling pathways of breast cancer cells and facilitate their invasion and metastasis in vivo. The conventional methods to detect cell motion, however, are often qualitative and require human judgment or involve the acquisition of video images and subsequent processing of large amounts of data. The scratch assay is a widely used technique to investigate wound healing processes, but its reproducibility is low due to the lack of a defined wound gap between cells. Here, we applied electric cell-substrate impedance sensing (ECIS) to monitor and examine the effect of TGF- β on breast carcinoma cells. The ECIS technique is an

approach to monitor the impedance of a small gold electrode used as a substratum for cells in culture. Fluctuations in impedance measured with this approach have been experimentally related to cell motility. We took wound-healing and micromotion measurements on MCF-7 and MDA-MB-231 cell layers, a non-invasive and a highly invasive human breast carcinoma cell lines. Our data showed that the wound healing rate of MDA-MB-231 cells significantly increased after cells were treated with 10 ng/ml of TGF- β . In addition, an increase in the motile behavior of MDA-MB-231 cells was observed by the micromotion measurement for about 20-hr long though the effect decreased over time. On the contrary, exposure to TGF- β caused little effect on MCF-7 cells. The results suggest that treatment of MDA-MB-231 cells with TGF- β facilitates their scattering behavior. Treatment with an inhibitor of TGF- β receptor kinase, SB-431542, effectively blocked the effect of TGF- β on MDA-MB-231 cells.

2415

Atypical PKC phosphorylates Par6 and facilitates TGF β -induced EMT.

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Epithelial to mesenchymal transition (EMT) is controlled by cellular signalling pathways that trigger the loss of cell-cell adhesion and lead to the restructuring of the cell cytoskeleton. Transforming growth factor beta (TGF β) has been shown to regulate cell plasticity through the phosphorylation of Par6 on a conserved serine residue (S345) by the type II TGF β receptor. Here we show that atypical Protein Kinase C (aPKC) is an essential component to this signalling pathway in non-small cell lung cancer (NSCLC) cells. We observed that the aPKC, PKC ζ interacts with TGF β receptors through Par6, and that these proteins localize to the leading edge of migrating cells. Furthermore, Par6 phosphorylation on Ser 345 by TGF β receptors is enhanced in the presence of aPKC. aPKC kinase activity as well as association with Par6 was found to be important for Par6 phosphorylation. Moreover, gene silencing of aPKC reduces TGF β induced changes in cell morphology, E-cadherin loss, stress fibre formation and RhoA degradation. Finally, we report that introduction of a phospho-mimetic mutant of Par6 (Par6 S345E) into aPKC silenced cells rescues TGF β induced EMT implicating a role for both aPKC and TGF β in executing a full EMT phenotype. In conclusion, our results suggest that both aPKCs and the TGF β receptors regulate phospho-Par6 levels to drive EMT and cell migration.

2416

TWEAK functions as chemotactic factor for glioma cells via Lyn activation.

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The long-term survival of patients with glioblastoma multiforme is compromised by the tumor's proclivity for local invasion into the surrounding normal brain. These invasive cells escape surgery and display resistance to chemotherapeutic- and radiation-induced apoptosis. We have previously shown that tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor superfamily, can stimulate glioma cell invasion and survival via binding to the fibroblast growth factor-inducible 14 (Fn14) receptor and subsequent activation of the Rac1/NF- κ B pathway. In addition, we have reported previously that Fn14 is expressed at high levels in migrating glioma cells *in vitro* and invading glioma cells *in vivo*. Here we demonstrate that TWEAK can act as a chemotactic factor for glioma cells, a potential process to drive cell invasion into the surrounding brain tissue. Specifically, we detected a chemotactic migration of glioma cells to the concentration gradient of TWEAK using the Modified Boyden chamber assay. Because TWEAK has been shown to be expressed in normal astrocytes, microglia, and endothelial cells, we neutralized TWEAK using a recombinant soluble murine

Fn14-Fc decoy receptor and examined the effect on glioma cell invasion in the *ex vivo* rat brain slice model. Incubation of the brain slice with the Fn14-Fc decoy receptor reduced glioma cell invasion as compared to control IgG treated brain slice. Since Src family kinases (SFK) have been implicated in chemotaxis, we next determined whether TWEAK:Fn14 engagement activated these cytoplasmic tyrosine kinases. Our data shows that TWEAK stimulation of glioma cells results in a rapid phosphorylation of Src as detected by Western blot analysis using a pan-Src phosphorylation antibody. To determine which SFK member is phosphorylated by TWEAK, we applied the multiplex Luminex assay and identified Lyn as a candidate SFK. Immunoprecipitation of Lyn from TWEAK-treated cells verified that Lyn is phosphorylated as determined by phosphotyrosine immunoblot analysis. Immunodepletion of Lyn by siRNA oligonucleotides suppressed the chemoattractive effect of TWEAK on glioma cells. We hypothesize that TWEAK secretion by cells present in the glioma microenvironment induce invasion of glioma cells into the brain parenchyma. Understanding the function and signaling of the TWEAK-Fn14 ligand-receptor system may lead to development of novel therapies to therapeutically target invasive glioma cells.

2417

The *Mad1* 1673 G→A alters the function of the mitotic spindle assembly checkpoint and the sensitivity to treatment in patients with ovarian cancer.

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Background: MAD1, a protein of the mitotic spindle assembly checkpoint (SAC), recognizes MAD2 through two leucine zippers, transporting and activating MAD2, which promotes a metaphase arrest signal. A SNP of *Mad1* was found to affect the SAC function that could be involved in a bad response to therapeutic agents that alter the dynamics of microtubules. **Objective:** To investigate the relationship of the polymorphism *Mad1* 1673 G→A with the efficiency of the SAC and the generation of aneuploidies, and with the therapeutic response of patients with ovarian cancer. **Methods:** The polymorphism was evaluated in 144 healthy individuals and 91 patients. The mitotic arrest and the presence of errors in segregation were analyzed in cultured human lymphocytes treated with nocodazol. Errors in segregation were also evaluated in 27 biopsies of patients. **Results:** Allele frequencies in healthy individuals were G: 50, A: 50, while in the patients, they were G: 38%, A: 62% ($p < 0.05$). The percentage of cells with mitotic arrest was higher in GG cells ($p < 0.05$). The frequency of micronuclei and nondisjunction events increased in AA cells ($p < 0.05$). Tumors from polymorphic patients had a higher percentage of aneuploid cells ($p < 0.05$). The GG patients showed a higher biochemical response, optimal cytoreduction and sensitivity to the treatment. There were no differences in progression-free or overall survival between both groups. **Conclusions:** the polymorphism *Mad1* 1673 G→A affects the SAC functionality, increasing the frequency of aneuploid cells. This polymorphism modifies the response to agents that alter the dynamics of microtubules in patients with ovarian cancer.

2418

Changes in Cell Signalling and Colocalization of the OTR and AR in Prostate Cancer.*M. L. Gould¹, H. D. Nicholson¹; ¹Anatomy Department, University of Otago, Dunedin, New Zealand*

Background. Oxytocin (OT) is involved in regulating prostate growth. In normal prostate cells OT inhibits growth. In the androgen independent cancer PC3 cell line, OT and androgen increase cell proliferation. There is only a single isoform of the oxytocin receptors (OTR) and yet, the OT peptide is able to stimulate and inhibit proliferation in the same cell type.

In the cell membrane, there are cave-like invaginations called caveolae and flat lipid rafts. In normal prostate cells, OTR is thought to be localized to caveolae. Here, one of the main signalling pathways is through extracellular-signal-regulated kinases (ERK) phosphorylation resulting in inhibition of cell proliferation. However, in androgen independent prostate cancer, there is a loss of caveolae, so that OTR moves on to the flat part of the cell membrane [Gould et al, 2010]. In androgen independent cancer, androgen receptors (AR) also move from the cell nucleus to become incorporated into the cell membrane [Foradori et al, 2008].

The objective of this study was to investigate a) whether there is a change in ERK activation in PC3 cells and b) whether this is accompanied by colocalization of OTR and AR on the cell membrane.

Methods. Normal human prostate epithelial cells (PrEC) and androgen-independent (PC3) cancer cell lines were cultured in the presence of OT and androgens at varying concentrations for 4 days. The MTS assay measured cell proliferation. Changes in ERK phosphorylation were identified using the In-Cell-Western technique. OTR and AR expression was identified by immunocytochemistry. Colocalization of OTR with AR was identified using double immunolabelling. Colocalization was also measured by Duolink in situ.

Results. Low concentrations of OT alone ($P=0.026$) and OT combined with testosterone (T) ($P=0.044$) were able to stimulate cell proliferation in the PC3, but had no effect on PrEC cells. Treatment of PrEC cells with OT and T stimulated phosphorylation of the ERK signalling pathway, but in PC3 cells no activation of ERK was seen with any treatments. The PrEC cells expressed OTR in the cell membrane and AR in the nucleus, whereas PC3 cells expressed both OTR and AR in the cell membrane. OT or T alone increased colocalization of OTR and AR in the cell membrane of PC3 cells compared to PrEC cells. Colocalization was increased further following treatment with OT and T together.

Conclusion. This study provides evidence that OT and T were able to stimulate cell proliferation in prostate cancer cells and that these changes are accompanied by alteration of ERK phosphorylation. This study also shows an interaction between AR and OTR may occur

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Elevated expression of Fn14 in non-small cell lung cancer correlates with mutant KRAS or amplified MET and promotes tumor cell invasion and metastasis.

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Lung cancer remains the leading cause of cancer-related deaths in the world. Tumor invasiveness and therapeutic resistance strongly contribute to the low (<10%) five-year survival rate for advanced stage lung cancer; thus, identification of new targets affecting invasiveness and resistance for therapeutic intervention is a necessity. TWEAK (tumor necrosis factor-like

weak inducer of apoptosis) and its receptor, the fibroblast growth factor-inducible 14 (Fn14), are highly expressed in human cancers and play a role in cancer cell proliferation, tumor invasiveness and tumor survival. We have reported that Fn14 is highly expressed in non-small cell lung cancer (NSCLC) and Fn14 expression correlated with EGFR activation. In addition, H1975 cells, resistant to EGFR-tyrosine kinase inhibitors through a secondary mutation (T790M), retain elevated expression of Fn14, while suppression of Fn14 significantly inhibited cell invasion. Amplified c-Met and mutant kRas are also well known drivers of NSCLC aggressiveness and therapeutic resistance. We hypothesize that elevated Fn14 expression can be maintained by mutant kRas or c-Met activation, and that blockade of the TWEAK/Fn14 signaling axis can suppress NSCLC invasiveness and metastasis. Here we show that Fn14 correlated with c-Met activation, and kRas mutation in primary NSCLC tumors. Fn14 and c-Met were highly expressed in metastatic tumors, 86 and 92% respectively, compared to matched primary NSCLC tumors, suggesting a role in the metastatic phenotype. Expression of mutant kRas or amplified c-Met in lung epithelial cells and/or NSCLC cells enhanced Fn14 protein expression, while therapeutic suppression of these driver pathways decreased Fn14 protein expression. Increased expression of Fn14 induced by mutant kRas or c-Met activation was completely abrogated upon MEK inhibition, suggesting a common pathway of Fn14 modulation. Cancer cell migration and invasion driven by the activation of kRas or c-Met was significantly repressed by suppressing Fn14 expression levels. The knockdown of Fn14 in an activating kRas mutation cell line, A549, effectively suppressed *in vivo* metastasis. In the future, we aim to further understand the role and mechanisms governing TWEAK/Fn14 signaling downstream of known driver mutations in NSCLC to support rational therapeutic combinations and inform clinical patient selection for TWEAK/Fn14 inhibition. The TWEAK/Fn14 signaling axis appears to be a vulnerable target in NSCLC invasiveness and survival, including tumors resistant to frontline therapies such as EGFR-TKIs.

2420

Epithelial cells stimulate the formation of protrusions in invasive carcinoma cells.

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Cell membrane protrusions, including invadopodia and pseudopodia, are critical for metastatic cancer cells to invade basement membrane and migrate through the interstitial matrix of the stroma. However, what factors regulate protrusion formation of invasive carcinoma cells is not fully understood. Here, we provide evidence that proteins secreted by normal breast epithelial cells (MCF10A) dramatically enhance membrane protrusions of invasive breast cancer cells (MDA-MB-231), not non-invasive breast cancer cells (MCF7). After exposure to MCF10A conditioned medium (CM), MDA-MB-231 cells projected multiple thin branching protrusions, while in regular medium (RM) these cells displayed broad membrane ruffling and conventional lamellipodia. Confocal images showed that the CM-induced protrusions were mainly composed of microtubule bundles wrapped by cortical actin filament bundles, a cytoskeletal organization different from filopodia and lamellipodia in MDA-MB-231 cells. The formation of these long protrusions required cooperative assembly of microtubules and actin filaments as well as Src signaling. Depletion of beta 1 integrin in MDA-MB-231 cells made cells insensitive to MCF10A CM, indicating that long protrusions may be induced by soluble extracellular proteins in MCF10A CM. Indeed, the induction of long microtubule-rich protrusions was recapitulated by soluble laminin-332 uniquely secreted by MCF10A. These results reveal a critical role of non-transformed cells in the enhanced formation of protrusions in metastatic carcinoma cells.

2421

Vimentin – from a marker to an active contributor of EMT.

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Epithelial-mesenchymal transition (EMT) is involved in cancer progression and particularly metastasis, with epithelial cells detaching their cell-cell contacts and adopting fibroblast-like mesenchymal characteristics. The intermediate filament protein vimentin is an important marker of EMT and it also regulates cell migration. We have recently shown that vimentin is functionally involved in cancer cell invasion and EMT maintenance by upregulating several genes involved in EMT, e.g. tyrosine-kinase Axl (Vuoriluoto et al., 2011). This has prompted us to investigate the molecular details underlying the ability of vimentin to regulate EMT-linked proteins in cells.

Several transcription factors have been linked to EMT and cancer cell motility. Our recent data suggest that vimentin is required for the stability of EMT-related transcription factors on the protein level. This involves a positive feed-back loop whereby EMT induction results in upregulation of vimentin that is necessary and sufficient to enhance signalling by EMT-related kinases to phosphorylate specific transcription factors at novel sites.

Our aim is to investigate the interplay between vimentin, EMT-linked kinases and transcription factors in EMT induction and cancer cell migration, helping to unravel the details behind cancer cell metastasis.

2422

Regulatory mechanism of integrin $\beta 1$ expression in collectively migrating cancer cells.

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Cancer cells frequently invade as cell group which retains cell-cell junctions, so-called collective migration/invasion. Migrating cell cohort can be distinguished into "leading cells" in the front row that guide "trailing cells" at the rear. Although pace-setting role of leading cells has been suggested, the molecular mechanism how leading cells contribute to the motile behavior of cell group remains elusive. Here we show that expression of integrin $\beta 1$ is up-regulated in leading cells compared to trailing cells. Increase of integrin $\beta 1$ expression is crucial for effective migration in cells moving as a group. Two transcriptional regulators, RET Finger Protein (RFP) and Myocardin Related Transcription Factor-B (MRTF-B), form complex responding to the partial loss of cell-cell adhesion in leading cells and up-regulates the integrin $\beta 1$ expression. Knockdown of RFP or MRTF-B significantly impaired the collective invasion of squamous cell carcinoma cell line in organotypic culture model. These results suggest that up-regulation of integrin $\beta 1$ by RFP and MRTF-B contributes to the property of leading cells to lead the cancer cell group.

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Cystatin SN is upregulated in colorectal cancer and interacts with cystatin C for the regulation of cathepsin B activity.

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Cystatin SN (CST1) is one of salivary cystatins that form tight equimolar complexes with cysteine proteases such as the cathepsin B and D. Previously, we reported the upregulation of CST1 and a significant correlation between high expression of CST1 and pTNM stage in gastric cancer. Here we report that CST1 is significantly overexpressed in colorectal cancer tissues compared to nontumor regions. We also examined the role of CST1 in tumor growth and metastasis, using a CST1 overexpressed HCT-116 cell tumor xenograft model. Increased cell proliferation and adhesive morphological changes were observed in HCT116 cell lines stably transfected with CST1 cDNA. T-cell factor and E-cadherin promoters were activated by exogenous overexpression of CST1 or CST3 and by recombinant CST1 (rCST1) or rCST3 treatment. Furthermore, CST1 interact with CST3 (cystatin C), a potent CTSB (cathepsin B) inhibitor, with a higher affinity than did CST3 with CTSB in the extracellular space of HCT116 cells. CTSB-mediated cellular invasiveness and proteolytic activities were inhibited by rCST3 treatment, but addition of rCST1 disrupted this inhibition. These results suggest that CST1 upregulation in colon cancer might be involved in adhesive tumor cell proliferation as well as in the regulation of CTSB activity via strong interaction with CST3.

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The role of novel gene fad104 in migration and invasion of cancer cells.

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Fad104 (factor for adipocyte differentiation 104) is a novel gene expressed temporarily in the early stages of adipocyte differentiation. Previously, we showed that fad104 regulated cell proliferation, adhesion, spreading and migration. Since fad104 regulated cell migration, it was thought that fad104 regulated invasion and metastasis of cancer cells. In the present study, we clarified the role of fad104 in migration and invasion of A375 melanoma cells.

Using quantitative PCR and Western blotting, we first analyzed the expression levels of fad104 in highly metastatic A375SM cells and poorly metastatic A375C6 cells. The expression level of fad104 in A375SM cells was lower than that of A375C6 cells, implying that the expression level of fad104 in highly metastatic cancer cells is lower than that of poorly metastatic cancer cells. We next verified the effect of fad104 on migration and invasion of cancer cells using transwell assays. A375C6 cells transfected with fad104 siRNA increased in the number of migrated and invaded cells compared with control cells transfected luciferase siRNA, suggesting that the reduction of fad104 expression in A375C6 cells enhances migration and invasion of A375C6 cells. In contrast, fad104-expressing adenovirus-infected A375SM cells showed a reduction in migration and invasion compared with β -galactosidase-expressing adenovirus-infected A375SM cells (control cells). Furthermore, the expression of matrix metalloproteinase 2 in fad104 overexpressing A375SM cells was lower than that of control cells. These results suggest that fad104 contributes to suppression of migration and invasion in cancer cells.

Next, we investigated the molecular mechanism of fad104 in cancer cells. Our previous study revealed that fad104 suppressed osteoblast differentiation through the inhibition of bone morphogenetic protein (BMP)-Smad1/5/8 signal. BMPs are member of the transforming growth

factor- β (TGF- β) family. It is well known that TGF- β -Smad2/3 signal is critical for the regulation of invasion and metastasis in cancer cells. Therefore, we next assessed whether fad104 regulates TGF- β -Smad2/3. A375SM cells with overexpression of fad104 showed the decrease in phosphorylation of Smad2/3 after being treated with TGF- β . Taken together, we demonstrated that fad104 suppressed migration and invasion in cancer cells through the inhibition of TGF- β -Smad2/3 signal.

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FERM domain-containing proteins in prostate cancer progression.

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Most prostate adenocarcinomas have their origins in preneoplastic lesions in the acinar structures of the prostate. These lesions alter the regular pattern of the prostate epithelia involving cell migration, cell-cell and cell-substrate reorganisation. Interaction with the surrounding environment is required and it is proposed that crosstalk between cell and stroma determines the fate of a prostate cell.

In order to reproduce the structural and functional differentiation of human prostatic acini *in vivo*, we have set up a 3-D microenvironment in Matrigel™. In this model, normal prostate (RWPE-1), low metastatic (DU-145) and highly metastatic prostate (PC-3) cancer cell lines are able to organize either as spheroids (cell aggregates) or as acinar-like structures. With this model we aim to investigate the progression from normal prostate to adenocarcinoma.

Four-point-one, ezrin, radixin, moesin (FERM) domains are present in a variety of mammalian proteins. There are around 50 distinct FERM domain-containing proteins (FDP) encoded by over 30 genes in the human genome. FDP are at the heart of a network of proteins linking the actin cytoskeleton with membrane dynamics at the leading edge of migratory cells. We have recently identified radixin as a key player in promoting cell migration of prostate cancer cells, by regulating Rac1-mediated epithelial polarity and formation of adherens junctions through Vav GEFs.

Using our model of prostate cancer progression we are now using an RNA interference approach and confocal microscopy techniques to identify the role of the FDP family in the formation and/or disruption of prostate acinar structures.

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Mechanism of Connective Tissue Growth Factor Inhibited Peritoneal Metastasis in Gastric Cancer.

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Connective tissue growth factor (CTGF) has diverse functions involved in wound healing, inflammation, cell adhesion, chemotaxis, apoptosis, tumor growth, and fibrosis. However, recent studies showed that over-expression of CTGF in human oral squamous cell carcinoma reduces cell growth and tumorigenicity. Similar tumor growth inhibitory effects were observed in lung

cancer cells in which CTGF over-expression were less angiogenic and metastatic due to blocking of the VEGF-A signaling pathway. CTGF was also reported to be a key regulator of colorectal cancer invasion and metastasis, and it appears to be a good prognostic factor. This study aims to investigate the roles of CTGF in peritoneal metastasis in gastric cancer (GC) and the underlying mechanisms. Our data demonstrated that CTGF overexpression inhibited adhesion and down regulation enhanced adhesion in vitro. In vivo tumorigenicity showed CTGF overexpression inhibited peritoneal dissemination. CTGF expression modulates peritoneal dissemination and is an important factor in prognosis. Patients expressed higher CTGF level have higher survival probability. Moreover, blocking integrin $\alpha 3\beta 1$ prevented cell adhesion to recombinant CTGF and co-immunoprecipitation showed CTGF binding to integrin $\alpha 3\beta 1$. Co-inoculation of rCTGF and gastric cancer cells in mice demonstrated the effective inhibition of peritoneal dissemination and significantly increased survival probability. Together, these data demonstrated that GC peritoneal metastasis is mediated through cell surface integrin $\alpha 3\beta 1$ binding to laminin. CTGF effectively blocks this interaction by binding to integrin $\alpha 3\beta 1$. Therefore, recombinant CTGF demonstrated its therapeutic potential in preventing peritoneal metastasis.

Cancer Therapy II

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Indibulin inhibits proliferation of MCF-7 cells by binding at a unique site on tubulin.

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Indibulin (2-[1-(4-Chlorobenzyl)-1H-indol-3-yl]-2-oxo-N-pyridin-4-ylacetamide; D-24851), a synthetic inhibitor of tubulin assembly, has shown promising results in Phase I and Phase II clinical trials for cancer chemotherapy. In this study, indibulin was found to bind to pure tubulin with a dissociation constant of $0.5 \pm 0.1 \mu\text{M}$. Interestingly, indibulin did not affect the binding of several of the tubulin-binding agents including vinblastine, colchicine and curcumin to tubulin suggesting that it might have a unique binding site on tubulin. Indibulin inhibited the proliferation of MCF-7 cells in culture with a half maximal inhibitory concentration (IC_{50}) of $150 \pm 13 \text{ nM}$. It depolymerized interphase and mitotic microtubules in MCF-7 cells in a concentration dependent manner. Indibulin treatment led to the formation of aberrant mitotic spindles in MCF-7 cells. The reassembly of cold-depolymerized microtubules was found to be significantly delayed in the presence of 300 nM indibulin suggesting that indibulin inhibits microtubule growth in these cells. Using live cell imaging of EGFP-tubulin expressing cells, indibulin (150 nM, IC_{50}) was found to suppress the dynamics of individual microtubules in MCF-7 cells. The rates and extents of growth and shortening excursions of microtubules were reduced in the presence of indibulin. Further, the time spent by microtubules in the pause state increased strongly with a concomitant decrease in the time spent in both growing and shortening phases. The dynamicity of microtubules was also significantly reduced in the presence of 150 nM indibulin. In addition, indibulin perturbed the localization pattern of EB1, a microtubule plus end binding protein. In contrast to the control, where EB1 localized as a comet at the end of microtubules, EB1 was localized along the entire length of microtubules in cells treated with 75 and 300 nM indibulin. This study provides evidence indicating that indibulin binds to tubulin at a unique site and blocks mitosis by inhibiting microtubule dynamics.

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Caspase 3-independent Apoptosis and Autophagy Induced by the Oleanolic Acid in Human Malignant Melanoma Cells and in vivo Study of Anti-melanoma Effect of Oleanolic Acid.

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Objective: Malignant melanoma is extremely aggressive and highly resistant to current therapies. Oleanolic acid (OA), a naturally occurring triterpenoid widely distributed in food and medicinal plants, has drawn increasing attention due to its anti-inflammatory and anti-cancer effects. This study aimed to investigate the effects of OA on the induction of apoptosis and autophagy in human melanoma cells, identify the apoptotic pathway through which OA activates the apoptosis, and examine the potential therapeutic effect of OA on melanoma development in mouse xenograft model. Methods: MTT, colony formation, soft-agarose colony formation and wounding healing assays were employed to examine the effect of OA on the proliferation of human melanoma cell line WM1552C cells. The effects of OA on cell cycle progression and apoptosis were examined by the flow cytometry. Apoptosis and autophagy were visualized by Hoechst 33258 and MDC staining, respectively. The expression of apoptosis and autophagy related genes were measured by real-time qPCR and Western Blot. Subcutaneous inoculation and growth of melanoma cells in murine model were used for *in vivo* study of potential therapeutic effect of OA. Results: OA displayed a significant ($p < 0.05$) inhibitory effect on the proliferation of WM1552C human melanoma cells in time- and dose-dependent manners (IC_{50} at 24h= 27 microM). OA significantly ($p < 0.01$) decreased the colonogenic ability, anchorage-independent growth, motility and invasiveness of WM1552C cells. In addition, flow cytometry and Hoescht 33258 stain suggested that OA induced apoptosis. Z-VAD, a pan caspase inhibitor, significantly alleviated the cytotoxicity of OA on melanoma cells, suggesting the caspase-dependent nature of apoptosis induced by OA. However, caspase-3 inhibitor (Z-DEVD-fmk), did not significantly increase the viability of melanoma cells treated by OA. Western blot showed that OA did not significantly alter the expression of caspase 3 in OA-treated WM1552C melanoma cells. MDC staining showed autophagic vacuole and lysosome, and the expression of Light Chain-3 (LC-3) was up-regulated by the OA treatment. *In vivo* study showed that OA significantly reduced the tumor mass ($p < 0.05$) in C57 BL/6 mice. Conclusion: OA inhibits the growth and invasiveness of malignant melanoma cells and induces autophagy and caspase 3-independent apoptosis. *In vivo* study suggests that OA is a potential chemotherapeutic candidate that potently inhibits the melanoma development.

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Rosehip Extracts Prevent Glioblastoma Cell Proliferation by Regulating Retinoblastoma Phosphorylation.

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Glioblastoma multiforme (GBM) are malignant tumors that arise in the brain. The current system of clinical management for GBMs consists of surgical resection followed by radiotherapy and chemotherapy. However, this treatment regimen has not improved the lifespan of GBM patients in the past twenty years. Therefore, new therapeutic options are being aggressively investigated. Rosehip (*Rosa canina*) extracts have been used for centuries as alternative therapies. Recent studies demonstrate that rosehip extracts possess anti-tumor properties. Therefore, we tested the antiproliferative capacity of rosehip extracts against GBM cell lines. We

hypothesize that human GBM cell lines exposed to rosehip extract will demonstrate lower rates of cell proliferation as a result of cell cycle regulation. The human GBM cell lines, U-251 MG and U-1242 MG were treated with rosehip extracts (1 mg/mL – 25ng/mL) and demonstrated a decrease in cell proliferation. Utilizing a fluorescent-based labeling strategy (Live-Dead Assay), we examined whether rosehip extracts prevented cell proliferation by initiating apoptosis. Pretreatment of the GBM cells with rosehip extracts (1 mg/mL – 25ng/mL) induced the inhibition of cell proliferation without promoting apoptosis; whereas, cells treated with staurosporine (1 μ M), a known inducer of apoptosis, showed an increase in cell apoptosis. Furthermore, we assayed the phosphorylation level of the retinoblastoma (Rb) protein to determine the mechanisms by which rosehip extracts were preventing cell proliferation. Rb phosphorylation was decreased following exposure to the rosehip extracts, suggesting the extracts prevent cell cycle progression beyond the G1 phase. Taken together, these data suggest that rosehip extracts inhibit cell proliferation via a cytostatic mechanism that prevents cell cycle progression. Moreover, rosehip extracts may serve as an alternative, or supplement, to the current clinical management of GBMs.

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Apoptosis and Autophagy Induced by Zerumbone and in vivo and in vitro Study of Inhibitory Effect of Zerumbone on Human Malignant Melanoma.

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Objective: To investigate the underlying mechanisms by which sesquiterpene zerumbone (ZER) exerts its inhibitory effects on human malignant melanoma cells, and examine the potential therapeutic effect of zerumbone on melanoma development and lung metastasis in mouse xenograft model. Methods: the anti-proliferative effect of ZER on WM1552C and SK-MEL-28 cells was examined by MTS, soft-agarose colony formation and wound healing assays. Invasive ability of the cells was determined by Matrigel Transwell assay. The effects of ZER on cell cycle and apoptosis were examined by flow cytometry. The expression of apoptosis and autophagy related genes were measured by real-time PCR and Western Blot. Apoptosis and autophagy were confirmed by Hoechst and MDC staining, respectively. Subcutaneous inoculation and tail vein injection of melanoma cells in murine model were used for in vivo study of potential therapeutic effect of zerumbone. Results: ZER inhibited the proliferation of WM1552C and SK-MEL-28 cells in both time- and dose-dependent (IC₅₀ at 24h= 15.1 μ M and 10.8 μ M, respectively) manners. ZER significantly reduced the colony formation, anchorage-independent colony formation, motility and invasive ability of malignant melanoma cells. ZER caused a cell cycle arrest at G₀/G₁ phase and induced apoptosis. Caspase-3 and light Chain-3 (LC3) were significantly up-regulated by ZER. MDC staining showed autophagic vacuole and lysosome. In vivo study showed that ZER significantly reduced the tumor mass and lung metastasis (P < 0.05) in C57 BL/6 mice. Conclusion: ZER may exert potent anti-melanoma effects by causing cell cycle (G₀/G₁) arrest and inducing apoptosis and autophagy. The in vivo study suggests that zerumbone can be a potential chemotherapeutic candidate for deterring growth and metastasis of melanoma .

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Regulation of the cell cycle on progression in A549 cells and MCF-7 cells treatment by Gronwell seed.

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Gronwell seed(Coix lachryma-jobi seed)(GS) is a grass crop that has long been used and played a role in traditional medicine as a nourishing food, and for treatment of various ailments, particularly cancer. The pharmaceutical composition is expected to have an anti-neoplastic effect, anti-human papilloma viral disease effect, and effect against various cutaneous diseases, such that it is useful for chemoprophylaxis or therapy against tumors. Previously, we reported that extract essence of GS and including fatty type active compounds, Monoolein inhibited TPA-induced in vitro conventional assay and thereby acted as a chemopreventive agents against carcinogenic compounds, using mouse experimental systems. In this study, the purpose of other potency of this useful samples was to develop new active effect, cytotoxic function as an effective anti-proliferative test against cancer cells and cell cyclic phase was investigated by Western blotting analysis. GS and Monoolein had potent anti-proliferative effects against A549 cell and MCF-7 cell under usual condition. Exposure of the A-549 and MCF-7 cell lines to an increasing dose of inhibitor, GS (1 ug, 10 ug and 100 ug) resulted in a dose dependent inhibition in cell anti-proliferation (15.2%, 41.6% and 71.6% of untreated control, respectively). Furthermore, treatment with 10ug of Monoolein was associated with up to 30 % increase in the anti-proliferative potency on the both cell lines. The anti-proliferative effects of A549 and MCF-7 by Monoolein were found to be accompanied by a G1 arrest on cell cycle, following the expressions of cyclin D1 and cdk2. This notion was further supported by examination of other cell cycle-dependent expression and these results suggest that growth arrest induced by Monoolein blocks both cell in G1 phase of the cell cycle. Based on our these observation, it is tempting to speculate that GS and Monoolein may find its place as interesting agent for the prevention and/or treatment human cancer intervention.

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Cytotoxicity against natural killer cell resistant cancer cell can be increased by enhancing of NKG2D ligands expression.

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This study was designed to demonstrate the importance of the expression level of NKG2D ligands on cancer cells in enhancing NK cell cytotoxic activity. In this presentation, I introduce the relationship between natural killer (NK) cell susceptibility and the surface markers of cancer cells. I have demonstrated that the cytotoxic activity of human naïve NK cells against six different cancer cell lines. NK cells showed a high cytolytic effect against K562 and Jurkat, but showed low cytolytic effect against Ramos, Hep3B, and Raji. Through phenotypic analysis, I found evidence that more susceptible cancer cell lines (K562 and Jurkat) express more NKG2D ligands (Major histocompatibility complex (MHC) class I chain-related A/B (MIC-A/B) and UL16 binding protein (ULBP)-1, ULBP-2). As expected, the blockade of NKG2D dramatically attenuated NK cytotoxicity of K562 and Jurkat. Upon *in vitro* stimulation with quercetin, low susceptible cancer cells increased NKG2D ligand expression, leading to enhancement of NK cell cytotoxic activity. Taken together, these results demonstrate that NKG2D ligands are important ligands for NK cell cytotoxicity.

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Identification of coding single nucleotide polymorphisms that impair hBLM helicase function.*H. Mirzaei-souderjani¹; ¹CMMB, USF, Tampa, FL*

A conserved family of DNA helicases, the RecQ-like family, has been demonstrated to have an important role in the maintenance of genome stability from prokaryotes to eukaryotes. Mutations in three of the five human homologs of the ancestral RecQ of *E.coli* result in syndromes with one or all of the following characteristics: 1) premature aging 2) development and skeletal abnormalities 3) severe predispositions to cancer. Here we demonstrate that we have successfully engineered a functional human-yeast chimera by fusing the N-terminus of the yeast Sgs1 to the catalytic and conserved C-terminus domains of the human BLM. This chimera was produced aided by protein disorder prediction algorithms of Sgs1 and BLM. We have characterized this chimeric protein by assessing its ability to suppress defect observed in an *sgs1* null strain when expressed under the Sgs1 native promoter. We have demonstrated that the SGS1-BLM chimera protein can reduce the rate at which gross chromosomal rearrangements appear in an *sgs1* null strain. Moreover the chimeric protein is able to fully suppress sensitivity to DNA damaging agent hydroxyurea (HU) in the absence of a functional Sgs1. The chimeric protein has provided us with a tool to characterize BLM mutants in a yeast system. Here we have illustrated that both the helicase deficient chimera and Bloom's syndrome causing point mutations result in an inactive protein. Additionally we have examined *blm* missense polymorphisms available in the SNPdb and other sources and have been able to identify SNPs that have an effect on the functionality of the Blm protein.

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Hyper-O-GlcNAcation Supports Pancreatic Tumor Cell Survival and NF- κ B Activation.*Z. Ma¹, K. A. Vosseller¹; ¹Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA*

Cancer cells shift energy production from oxidative phosphorylation to the less efficient glycolysis even in the presence of oxygen (the Warburg effect). Cancer cells are also addicted to glutamine for proliferation and survival. This metabolic reprogramming necessitates greatly increased uptake of glucose and glutamine. A fraction of glucose and glutamine entering cells flux through the hexosamine biosynthetic pathway (HBP), the end-product of which is utilized for the carbohydrate post-translational O-linked β -N-acetylglucosamine (O-GlcNAc) modification, suggesting potential cancer cell "hyper-O-GlcNAcation". O-GlcNAc is dynamically added by the O-GlcNAc transferase (OGT) to serine/threonine residues of cytosolic and nuclear proteins and removed by O-GlcNAcase (OGA). O-GlcNAcation in a manner analogous to O-phosphorylation plays crucial roles in signal transduction linked to a variety of cellular processes such as cell cycle control, cellular stress responses, and transcription. O-GlcNAc modifies a number of cancer-associated proteins including p53, c-Myc, and Snail. However, the possible roles of hyper-O-GlcNAcation in cancer are not fully understood. Here, we observed increased HBP flux and hyper-O-GlcNAcation in human pancreatic ductal adenocarcinoma (PDAC), which is one of the most lethal cancers, with a 6 month median survival time after diagnosis and an overall 5-year survival rate of \sim 5%. Consistent with PDAC hyper-O-GlcNAcation, OGT levels were elevated and OGA levels were reduced respectively. Reducing hyper-O-GlcNAcation selectively suppressed PDAC cell proliferation, anchorage-independent growth, PDAC orthotopic xenograft mouse tumor growth, and triggered apoptosis, but did not affect the growth of non-transformed pancreatic cells. Conversely, increasing O-GlcNAc promoted PDAC cell survival in forced suspension culture. PDAC and many other cancers are supported by oncogenic constitutive NF-

κ B activity. NF- κ B transcriptional activity is regulated by posttranslational modifications such as phosphorylation and acetylation. Here, we showed that the p65 subunit of NF- κ B and upstream kinases IKK α and IKK β were O-GlcNAc modified in PDAC. Lowering hyper-O-GlcNAcation decreased IKK β expression and attenuated p65 activating phosphorylation (S536), nuclear translocation, NF- κ B transcriptional activity, and target gene expression. Conversely, elevating O-GlcNAc increased IKK α and p65 O-GlcNAcation and increased p65 nuclear localization, S536 activating phosphorylation and NF- κ B transcriptional activity. Finally, reducing O-GlcNAcation on p65 by mutating two O-GlcNAc sites (T322 and T352) to alanines attenuated the induction of cancer cell anchorage-independent growth. Our data suggest that elevated O-GlcNAcation contributes to pancreatic cancer cell survival and oncogenic NF- κ B upregulation and that suppression of hyper-O-GlcNAcation by targeting OGT may serve as a novel therapeutic intervention in PDAC.

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STAT3-RANTES autocrine signaling is essential for tamoxifen resistance in human breast cancer cells.

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The acquisition of tamoxifen resistance in breast cancer is a major therapeutic problem. We have developed tamoxifen resistance MCF-7 (TRM-7) cell line to elucidate the molecular mechanisms and factors involved in acquiring the resistance. In this study, we demonstrate that phosphorylation of STAT3 at tyrosine 705 (Y705) and RANTES expression are increased in response to tamoxifen in human breast cancer cells. Based on these results, we hypothesize that up-regulated STAT3 phosphorylation and RANTES may correlate with development of drug resistance. Here, we demonstrate that STAT3 and RANTES can contribute to maintenance of drug resistance. STAT3 phosphorylation is constitutively retained via RANTES autocrine loop, and this in turn up-regulates anti-apoptotic signals in TRM-7 cells. A STAT3-RANTES autocrine signaling affected expression of anti-apoptotic BCL-2 family genes and also prevented TRM-7 cells from the programmed cell death through inhibition of PARP and caspase-9 cleavage. Subsequently, blockade STAT3 and RANTES resulted in the reduction of anti-apoptotic signal in TRM-7 cells. Then reduced anti-apoptotic signal is rescued by exogenously treated RANTES protein, eventually drug resistance is rescued too. Taken together, our results suggest that STAT3-RANTES autocrine signal is essential to maintain the drug resistance and protect cell from programmed cell death. Introducing the mechanisms of STAT3-RANTES autocrine signaling can provide a new strategy to manage tamoxifen resistance tumor patients.

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Cells Resistant to Vorinostat Show Increased Hallmarks of ER Stress and Elevated Sensitivity to Bortezomib Induced Cell Death.

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Histone deacetylase inhibitors (HDACi) are anti-cancer agents, which have shown promising activity in hematological malignancies. However, only a small percentage of patients initially respond to treatment and all will eventually develop resistance. HDACi are known to inhibit the deacetylation of histones as well as various non-histone proteins. Revealing mechanisms of HDACi resistance will help elucidate their modes of action, as well as help overcome *de novo* and acquired resistance in the clinic. To study HDACi resistance in hematological malignancies we developed two vorinostat resistant cell lines by a dose escalation protocol from the

monocytic-like lymphoma cell line U937 and the diffuse-large B cell lymphoma cell line SU-DHL6. Molecular profiling of our resistant cells show that they express hallmarks of ER stress, such as (1) a dilated ER, (2) increased XBP1 mRNA, (3) cleaved ATF6, and (4) increased GRP78 protein and mRNA. Endoplasmic reticulum (ER) stress is caused by an accumulation of misfolded proteins in the ER lumen that causes a multifaceted response that is primarily cytoprotective called the unfolded protein response (UPR). The misfolded proteins that are deemed unsalvageable by the ER are targeted for degradation by the proteasome. Vorinostat resistant cells do show an accumulation of ubiquitinated proteins and an increase in proteasomal activity compared to their parental counterparts. We hypothesize that adaptive ER stress could be a mechanism of vorinostat resistance because the UPR induces the upregulation of pro-survival genes and autophagy, which we recently showed is required to maintain HDACi resistance. We show that vorinostat resistant cells die in response to treatment with the proteasome inhibitor bortezomib and this correlates with an upregulation of some ER stress markers. Understanding vorinostat resistance holds clinical relevance in terms of improving HDACi therapy and being able to identify subsets of patients who would benefit most from combination therapy such as vorinostat with bortezomib.

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Antitumor properties of acetoxychavicol acetate on glioblastomas.

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Acetoxychavicol Acetate (ACA), an antioxidant agent acts as an inhibitor of xanthine oxidase, which plays an important role in the catabolism of purines and catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid. By products of xanthine oxidase oxidation (uric acid) has been implicated in several abnormal physiological processes such as brain ischemia, vascular injury, inflammatory diseases, and most notably tumor lysis syndrome. Additionally, it was shown that brain tumors express increased xanthine oxidase levels as compared to normal brain tissue. Furthermore, early studies demonstrated that the xanthine oxidase inhibitor, ACA, was a chemopreventive agent that suppressed tumor formation in the oral cavity and colon, while subsequent studies revealed that ACA exerted positive anti-tumorigenic effects on leukemia, breast, colon, and myeloma cancers. We demonstrated in the present study that ACA induces anti-tumorigenic properties in glioblastomas by antagonizing glioblastoma cell proliferation and migration. Additionally, we assessed the involvement of reactive oxygen species (ROS) and the unfolded protein endoplasmic reticulum stress response for their mechanistic roles in ACA induced glioblastoma cell death.

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aPKC iota/lambda regulates Hh signaling during basal cell carcinoma growth.

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Basal cell carcinoma (BCC) initiation and expansion requires high levels of Hedgehog (Hh) signaling. While Smoothed (Smo) inhibitors are effective, early tumor resistance illustrates the need for additional downstream targets for therapy. Here we identify atypical Protein Kinase C iota/lambda (aPKC) as a novel BCC oncogene essential for Hh signaling. Genetic knockdown using shRNA against aPKC, or pharmacological inhibition of aPKC, inhibits proliferation, Hh signaling, and ciliogenesis of the murine BCC cell line ASZ001. Genome-wide transcriptome analysis of BCC cells reveals pharmacological inhibition of aPKC or Smo target similar pathways. aPKC is a Hh target gene that is overexpressed in mouse and human BCCs and functions downstream of Smo to bind and phosphorylate Gli1, resulting in maximal DNA binding

and Hh activation. Consistent with its role in regulating Hh signaling, application of a topical aPKC inhibitor suppresses Hh signaling and tumor growth in primary murine BCC tumors. As acquired drug resistance is a growing problem, we also demonstrate Smo antagonist-resistant BCC cells and human tumors overexpress active aPKC and pharmacological inhibition of aPKC suppresses proliferation. These results demonstrate polarity signaling is critical for Hh-dependent processes and suggest aPKC may be a new therapeutic target for the treatment of naïve and resistant BCCs.

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Extreme autophagy after high dose gamma irradiation or mixed modality irradiation due to a shift in autophagic flux.

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Radiation-induced cell death by different radiation modalities was investigated in human brain tumor cells, glioblastoma multiforme (GBM) by transmission electron microscopy (TEM). Essentially all cells contained autophagosomes but irradiated GBM cells showed an increase in number and size of autophagosomes indicating activation of autophagy. Gamma radiation induced autophagy has been previously reported but the activation of autophagy by irradiation with thermal neutrons or gadolinium neutron capture is presented here for the first time. Acridine orange staining confirmed the increase in autophagosomes observed by TEM relative to the different radiation treatments and increasing time after irradiation. The increased number of autophagosomes in irradiated cells could be due to either an increase in autophagosome formation or decrease in clearance of autophagosomes. The equilibrium between these two is defined as autophagosome flux. A defective autophagic flux is a prominent characteristic of cells irradiated with thermal neutrons or with gadolinium neutron capture. An extreme example of disruption in the autophagic flux is shown in cell profiles having massive accumulation of damaged intracellular components without proper disposal or recycling via lysosomal enzyme digestion. Indeed, it appears that the autophagosomes are engulfing other autophagosomes. Others suggest that the failure to degrade autophagosomes compromises cellular viability, so it is not unreasonable to expect that the representative cell received a lethal hit of radiation and will not survive the accumulation of the massive autophagosomes within the cytoplasm. The image of extreme autophagy also shows a flattened membrane extension that is part of the membrane wrapping around an autophagosome. The membrane extension appears to be in the process of searching out more cargo and engulfing that cargo to add to the pre-existing autophagosome. This morphological clue suggests that the defective steps in this cell irradiated with Auger electrons from Gd neutron capture are cargo recognition steps and membrane engulfment steps. It is suggested that these later steps in autophagy are compromised by the high dose gamma irradiation or mixed mode irradiations so that the GBM cells are unable to complete normal recycling by lysosomal enzyme digestion processes.

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2440

Trajectories of Organelle Morphology Changes During Malignant Transformation.

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Organelle morphology changes provide the basis for many histological screens for cancer. However, little is understood about how the molecular changes during malignant transformation affect organelle morphology or how these changes to organelle morphology in turn affect the proper function of the biochemical pathways they encapsulate. In this study, we overexpressed oncogenic Ras and oncogenic Myc either alone or in combination in primary mouse embryonic fibroblasts (MEFs). Using novel computational analyses, we extracted metrics of cell, nucleus, nucleolus, and mitochondrial morphology from individual cells. Malignant transformation of MEFs requires two oncogenic "hits" and typically, changes in organelle morphology are most pronounced in the Ras and Myc doubly transformed population. For instance, the nucleus in the doubly transformed cells is larger relative to cell size and rounder when compared to other conditions. Organelle morphology can also exhibit progressive changes, as in the case of nucleolus size which is smallest in the control cells, larger in either of the two single transformations, and largest in the double transformation. Multidimensional parameter analysis will reveal which morphological changes associate most strongly to the different genetic states. These data indicate that organelle morphology can indicate intermediate states during malignant transformation, and will inform development of future diagnostic and therapeutic tools.

2441

Engagement of cellular prion and heat shock organizing protein as a novel therapeutic target for glioblastoma.

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A main concern in cancer therapy is resistance of tumors such as glioblastoma to current treatments protocols. Here, we report that glioblastoma proliferation mediated by cellular prion protein (PrPC)- secretable STI1/hop (Stress inducible protein 1/hsp70-hsp90 organizing protein) interaction, is blocked by STI1/hop₂₃₀₋₂₄₅ peptide, which mimics PrPC binding site, in tumor cells in vitro and in malignant glioma in vivo. Both STI1/hop and PrPC are highly expressed and co-localize in glioblastoma cells (U87MG). STI1/hop is secreted by U87MG either cultured in vitro or xenografted orthotopically. In vitro, proliferative effect induced by recombinant STI1/hop was inhibited by STI1/hop₂₃₀₋₂₄₅, an irrelevant peptide from STI1/hop amino-terminal domain (STI1/hop₆₁₋₇₆) had no effect. Most importantly, local therapy of established U87MG orthotopic xenograft with STI1/hop₂₃₀₋₂₄₅ significantly delayed tumor growth and improved survival on nude mice. Consequences of STI1/hop₂₃₀₋₂₄₅ treatment in vivo were strongly decreased cell proliferation and U87MG cells sensitization for apoptosis, which can contribute to the observed anti-tumor effects. Remarkably, PrPC silencing by shRNA impaired the tumor growth in vivo and STI1 treatment had no effect upon proliferation in PrPC-silencing cells, indicating the critical role

of PrPC-STI1 interaction for tumor maintenance. Interestingly, real time RT-PCR and tissue microarray from surgical samples demonstrated that STI1/hop gene expression is significantly enhanced in high-grade gliomas (grades III and IV) when compared to non-tumoral tissues, which renders it a powerful tumor marker. Thus, STI1/hop₂₃₀₋₂₄₅ is promising candidate for cancer therapy by acting as adjuvant for currently established medications.

2442

A New Anti-Cancer Agent, Acetyltanshinone IIA, Inhibits Estrogen Receptor Positive Breast Cancer Cell Growth by Inducing ER α mRNA Reduction and Protein Degradation

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Acetyltanshinone IIA (ATA) is a new anti-cancer agent with good *in vitro* and *in vivo* anti-tumor activity. Recently, we found that ATA displayed a strong growth inhibition effect on breast cancer cells that are estrogen receptor positive (ER+). More importantly, ATA exhibited much stronger growth inhibition potency than the commonly used hormonal agent tamoxifen. The IC₅₀ values for ATA are 6.7 to 6.8 fold less than those for tamoxifen in MCF-7 and T-47D breast cancer cells. ATA was found to be metabolized into hydroquinone tanshinone IIA (HTA) in MCF-7 cells and HTA can dock at the same site where estrogen binds to ER. ATA was found to significantly reduce ER α but not ER β protein. The reduction occurred only in the nucleus, not in the cytosol, indicating that it is target and location specific. The ATA-mediated ER α reduction was blocked by a proteasome inhibitor, MG132. In addition, ATA also reduced the mRNA level of the ER α encoding gene, ESR1, which distinguishes ATA from another anti-estrogen drug, fulvestrant. Finally, real-time PCR revealed that ATA caused mRNA level reduction of GREB1, which is an ER-responsive gene. In summary, we propose that ATA may inhibit ER+ breast cancer cell growth by reducing ER α mRNA levels and causing ER α degradation via a proteasome dependent pathway. As ATA can selectively degrade ER α but not ER β , and ATA can reduce ER α gene expression, ATA may be used to treat ER+ breast cancer patients who are resistant or non-responsive to fulvestrant and tamoxifen.

2443B

Treatment targeting hypoxic tumor cells enhances therapeutic effect of sunitinib.

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Angiogenesis is a critical step during tumor progression. Although antiangiogenic therapy has shown early promise in cancer treatment, the modest benefits antiangiogenic therapy exerts in delaying tumor progression often do not translate into an overall prolongation of survival time. One possible explanation for this is that antiangiogenic therapy actually promotes the emergence of a more aggressive cancer cell phenotype by generating increased hypoxia in the primary tumor—a well-recognized promoter of tumor progression. TH-302 is a 2-nitroimidazole triggered hypoxia-activated prodrug (HAP), which has been shown in human tumor xenograft models to selectively target the hypoxic tumor compartment and reduce tumor volume. Here, we show that melanoma cells grown under hypoxic conditions exhibit increased resistance to a variety of chemotherapeutic agents and generate larger and more aggressive tumor xenografts than control cells grown under normoxic conditions. Melanoma cells grown under hypoxic conditions exhibit a pronounced sensitivity to TH-302—a phenotype exacerbated by the addition of sunitinib. Finally, TH-302 prolonged the overall survival of melanoma bearing transgenic mice (Tyr::CreER; BRafCA/+; Ptenlox/lox) in a series of chemotherapeutic and chemoprevention treatment regimens of varying duration—both alone and in combination with

sunitinib, and this correlated with a statistically significant reduction in tumor size and thickness as well as a reduction in the expression of hypoxia-induced genes. Interestingly, the combination of temozolomide with TH-302 did not exert the same effects on survival, suggesting that TH-302 requires sunitinib-dependent tumor hypoxia for its additive effects to be realized. These studies provide a translational rationale for combining TH-302 with antiangiogenics to enhance the treatment benefit of antiangiogenics in melanoma.

2444

Short synthetic double stranded RNA with dual activity - oncolytic and immune modulatory - for hepatocellular carcinoma.

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Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and a leading cause of cancer death. Current pharmacological approaches for the treatment of human HCC are very limited in their efficacy. A potential role for noncoding double stranded RNAs (dsRNAs) in the control of tumours has recently emerged in a variety of models with recognition of their ability to stimulate an immune response or directly effect cell death. We sought to determine the efficacy of dsRNAs of different molecular weights against human HCC cells in vitro. We utilized synthetic polyA:polyU of either 5 base pairs (low molecular weight [LMW]) or 70 base pairs (high molecular weight [HMW]) in length as well as 20'-methylated versions of these dsRNAs. LMW dsRNA in the culture supernatant had a rapid, pronounced, dose-dependent negative effect on cell viability as measured by MTT assays when tested against human HCC cell lines (HepG2, Huh7, PLC/PRF/5), primary human HCC cells, a murine HCC cell line (Tib-75), and a human monocyte cell line (THP-1), though this effect was less pronounced in a normal human hepatocyte cell line (THLE-2). Annexin V and propidium iodide staining revealed a role for apoptosis in cell death caused by LMW dsRNA. Multiplex analysis of culture supernatants revealed that exposure to LMW dsRNA was associated with the production of tumor necrosis factor-alpha and interleukin-6. In contrast, HMW dsRNA had no effect on cell viability and elicited production of different cytokines. These observations define a novel category of synthetic dsRNA of reduced size and dual activity, both oncolytic and immune modulatory. Altogether, these findings support further mechanistic and in vivo studies to explore the safety, effectiveness and utility of such dsRNAs with dual biological activity as novel therapeutic agents in HCC and other cancers.

2445

Cytotoxicity of Human Recombinant Arginase [Co]-PEG5000 in the presence of Citrulline is dependent on decreased Argininosuccinate synthetase expression in human cells.

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A number of cancers are auxotrophic for L-arginine. Depletion of extracellular L-arginine has been accomplished by dietary restriction and enzyme therapy including pegylated recombinant arginine deiminase, pegylated recombinant human arginase I, and pegylated cobalt substituted recombinant human arginase I-Co-ArgI-PEG. We tested the latter on tumor and normal cell lines in the presence and absence of L-citrulline and correlated sensitivity with cytosolic levels of argininosuccinate synthetase-ASS and ornithine transcarbamylase-OTC. Three day thymidine and leucine incorporation and seven day MTS assays were performed. Immunoblots were used

to quantify ASS and OTC. In the presence of supplemental citrulline, Co-ArgI-PEG was cytotoxic to tumors lacking ASS but not normal cells. In addition to the above cytotoxicity assays, Balb/c mice were treated with weekly or twice weekly doses of IP Co-ArgI-PEG. Animal toxicity, PK and PD were measured. Co-ArgI-PEG depleted serum L-arginine for 3-4 days from first injections. Co-ArgI-PEG was measurable for over a week in the blood with a beta phase half life of 3 days. Toxicities was limited to weight loss and marrow necrosis. We further developed an NSG mouse model of A375 melanoma cells, an ASS negative cancer. We treated the mice with the MTD of Co-ArgI-PEG in the presence or absence of citrulline. Dramatic tumor growth inhibition was observed. After one month, half of the relapsing tumors showed ASS induction. Co-ArgI-PEG has been produced under cGMP and vialled. Quality control tests are ongoing. GLP pharmacology/toxicology in mice has been initiated. We hope to begin phase I clinical studies with Co-ArgI-PEG in solid tumor patients that are ASS negative in 2013.

2446

A microscopy-based cell-microarray assay for quantifying cytotoxicity (ADCC/CDC) and cellular response at single cell resolution.

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A novel single cell microarray assay has been developed for the determination of complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC). And this single cell microarray assay can be applied to cellular response assay such as quantifying Fc-gamma receptors. Not only does this new cell microarray assay allow cytotoxicity analysis to be conducted on a single cell basis, but it also overcomes the need for hazardous radiochemicals. Additionally, fluorescence labeled antibodies can be used to easily identify individual cells with antibodies bound that remain alive. In other words, this assay can specify which cells are resistant to CDC/ADCC of a specific antibody. In addition, this assay provides real-time imaging and data of cell toxicity and other cellular responses over a period of time.

2447

Multicellular tumor spheroid models to explore cell cycle checkpoints in 3D.

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MultiCellular Tumor Spheroid (MCTS) mimic the organization of a tumor and is considered as an invaluable model to study cancer cell biology and to evaluate new antiproliferative drugs. We report how the characteristics of MCTS in association with new technological developments can be used to explore the regionalization and the dynamics of cell cycle checkpoints in 3D. Spheroids expressing fluorescent reporters were engineered and used to explore cell cycle distribution and regionalization during growth. In these models we monitored cell cycle arrest in response to checkpoint activation after growth factor starvation, DNA injury or treatment with drugs. The kinetics and regionalized aspects of the response were investigated both on fixed sections and on live spheroids using light sheet microscopy. All together, our data demonstrate the power of the combination of spheroids made of genetically modified cells expressing cell cycle reporters with both classical antibody-based detection and the use of innovative 3D imaging strategy. This study paves the way for the investigation of the molecular aspects of checkpoint response in 3D models and the dynamic studies of the 3D response to novel antiproliferative agents.

2448

Angiogenic and proliferative factors in the prostatic microenvironment: senescence and angiogenesis inhibition x lesions in the transgenic adenocarcinoma of mouse prostate (TRAMP) modelF. Montico¹, A. C. Hetzl¹, E. M. Cândido¹, L. A. Kido¹, R. M. Lorencini¹, V. A. Cagnon¹;¹Structural and Functional Biology, University of Campinas, Campinas, Brazil

Prostatic microenvironment in the senescence resembles cancer-associated reactive stroma, characterized by stimulated cell proliferation and angiogenesis. In this context, angiogenic inhibitors, such as SU5416 and TNP-470, represent promising alternatives in prostate cancer treatment. Thus, the aim herewith was to characterize proliferative and angiogenic aspects of the prostatic stroma in elderly mice submitted to antiangiogenic therapy in comparison with stromal features in TRAMP mice lesions.

Control groups received subcutaneous saline injections: Young (YNG), Prostatic Intraepithelial Neoplasia (PIN) and Cancer (PC) controls (18-week-old FVB, 8 and 18-week-old TRAMP mice, respectively). Experimental groups (52-week-old FVB mice) were: Senile (SEN): same treatment as controls; SU5416 (SU): intraperitoneal SU5416 injections; TNP-470 (TNP): subcutaneous TNP-470 injections; SU5416 + TNP-470 (ST): same treatments as SU and TNP groups. After 21 days of treatment, dorsolateral prostate was collected for light microscopy, immunohistochemical and Western blotting analyses for vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and insulin-like growth factor receptor-1 (IGFR-1).

Senescence was associated with proliferative lesions similar to those verified in TRAMP mice, such as PIN and well-differentiated adenocarcinoma, even though with lower frequency. Prostatic molecular features in the senescence also resembled those in the TRAMP lesions and were characterized by increased VEGF, FGF-2 and IGFR-1 immunolabelling and protein levels. Antiangiogenic therapy interrupted the progression and/or led to regression of the lesions observed in the SEN group, mainly following SU5416 and TNP-470 association. In addition, angiogenesis inhibition, especially in the TNP and ST groups, resulted in reduction of VEGF and FGF-2 levels in a similar pattern, whereas this was not observed for IGFR-1.

The findings indicated that senescence predisposes to prostatic malignancies, considering the morphological similarity with PIN and PC groups, as well as the activation of molecules involved in angiogenesis and cell proliferation, leading to a reactive stroma-like microenvironment in this life period. The decrease pattern of VEGF and FGF-2 levels following angiogenesis inhibition showed the effectiveness of this therapy and also pointed to a crosstalk among molecular pathways involving these pro-angiogenic factors. However, the maintenance of increased IGFR-1 levels after antiangiogenic treatment suggests that this therapy demand further analysis, considering the proliferative role of this molecule and its involvement in several pathways which can activate angiogenesis.

2449

Tumor-specific silencing of COPZ2 gene encoding coatomer protein complex subunit ζ 2 renders tumor cells dependent on its paralogous gene COPZ1.

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Anticancer drugs are effective against tumors that depend on the molecular target of the drug. Known targets of cytotoxic anticancer drugs are involved in cell proliferation; drugs acting on such targets are ineffective against nonproliferating tumor cells, survival of which leads to eventual therapy failure. Function-based genomic screening identified the coatomer protein complex ζ 1 (COPZ1) gene as essential for different tumor cell types but not for normal cells. COPZ1 encodes a subunit of coatomer protein complex 1 (COPI) involved in intracellular traffic and autophagy. The knockdown of COPZ1, but not of COPZ2 encoding isoform coatomer protein complex ζ 2, caused Golgi apparatus collapse, activation of unfolded protein response, blocked autophagy, and induced apoptosis in both proliferating and nondividing tumor cells. In contrast, inhibition of normal cell growth required simultaneous knockdown of both COPZ1 and COPZ2. COPZ2 (but not COPZ1) was down-regulated in the majority of tumor cell lines and in clinical samples of different cancer types. Reexpression of COPZ2 protected tumor cells from killing by COPZ1 knockdown, indicating that tumor cell dependence on COPZ1 is the result of COPZ2 silencing. COPZ2 displays no tumor-suppressive activities, but it harbors microRNA 152, which is silenced in tumor cells concurrently with COPZ2 and acts as a tumor suppressor in vitro and in vivo. Silencing of microRNA 152 in different cancers and the ensuing down-regulation of its host gene COPZ2 offer a therapeutic opportunity for proliferation-independent selective killing of tumor cells by COPZ1-targeting agents.

Host-Pathogen/Host-Commensal Interactions

2450

Apical surface dynamics during early EPEC attachment to intestinal epithelial cells.

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Enteropathogenic *Escherichia coli* (EPEC) is a common food-borne pathogen and one of the leading causes of infantile diarrhea and death in third world nations. EPEC colonizes the intestine by hijacking the actin cytoskeleton of host enterocytes and forming an actin-rich pedestal under the site of attachment while causing effacement of surrounding microvilli. The signaling cascade leading to pedestal formation has been extensively studied; however, events surrounding initial EPEC contact with an enterocyte are not well characterized. Using live cell and scanning electron microscopy on Caco2_{BBE} intestinal epithelial cells in culture, we show here that microvilli play a critical role in early EPEC attachment. Immediately following first contact of an EPEC cell, nearby microvilli extend and orient towards the bacterium, in some cases “tethering” the bacterium. Microvilli also cluster together around the bacterium, an event likely mediated by the adhesion molecule protocadherin-24 (PCDH24) which is concentrated at microvillar tips. Following these events, which occur within the first few minutes of bacterial

contact, we observed a rapid depletion of PCDH24 and other apical proteins around sites of attachment concomitant with microvillar effacement and actin pedestal formation. Strikingly, assays using PCDH24-overexpressing and knockdown cells show that EPEC attachment is inversely proportional to the amount of PCDH24 present in the brush border. Together, these data suggest that, as in early brush border formation, PCDH24 functions to stabilize microvilli through inter-microvillar adhesion during infection. The host cell could use this function as a means to maintain brush border integrity and prevent or slow intimate bacterial attachment. In the absence of PCDH24, brush border integrity is compromised, allowing greater access to EPEC and subsequently higher levels of bacterial colonization.

2451

Cell-to-cell spread of *Listeria* and *Rickettsia* displays distinct morphokinetics and utilizes different bacterial and host factors.

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Intracellular bacterial pathogens invade host cells and spread from cell to cell to promote replication and avoid elimination by the host. Several species, including *Listeria monocytogenes* and *Rickettsia parkeri*, spread by hijacking the host cell's actin cytoskeleton to form actin tails, which propels them into the plasma membrane and induces protrusions that are engulfed by neighboring cells. Previous work showed that *L. monocytogenes* and *R. parkeri* use distinct bacterial and host factors to polymerize and organize actin, resulting in different motility characteristics and actin tail architectures. This suggests they might also utilize distinct pathways for spread. Here, we show that the cell-to-cell spread process differs morphologically and kinetically between the two pathogens. Using live cell imaging, we found that *L. monocytogenes* forms protrusions that are 3.6 times longer than *R. parkeri*. Spread is initiated at 3-4 h post infection (hpi) for *L. monocytogenes* versus 16 hpi for *R. parkeri*. The delayed initiation of spread for *R. parkeri* is likely due to its longer doubling time compared to *L. monocytogenes* (8 h versus 40 min). Once spread is initiated, the overall kinetics for an individual bacterium are similar between the two pathogens (25 min). However, *L. monocytogenes* is slower than *R. parkeri* in the timing of protrusion engulfment into a recipient cell. This kinetic difference, along with the morphological differences seen for protrusion formation, suggests the two pathogens are internalized into neighboring cells by distinct mechanisms. To characterize the pathways that regulate cell-to-cell spread, two independent screens are underway to isolate host and bacterial genes important for this process. We have identified several factors, including *Rickettsial* Sca2 actin polymerizing protein and host capping protein (CAPZB) as critical components for *R. parkeri* cell-to-cell spread. Future work will identify and characterize additional bacterial and host factors critical for regulating spread with the ultimate goal of defining shared and divergent spread pathways exploited by different pathogens.

2452

Integrated analysis by RNAi screening of mammalian cell invasion by the bacterial pathogen *Listeria monocytogenes*.

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The gram-positive bacterium *Listeria monocytogenes* is the causative agent of human listeriosis, a food-borne disease characterized by gastroenteritis, meningitis and abortions. A crucial stage in infection is the bacterial internalization and intracellular replication in phagocytic and normally non-phagocytic cells. Invasion of epithelial cells requires the interaction of host cell

receptors with surface *L. monocytogenes* proteins from the internalin family, characterized by the presence of leucine-rich repeats involved in protein/protein interactions; in particular, internalin A (InIA) is able to bind the adherens junction molecule E-cadherin while the hepatocyte growth factor receptor (also known as Met) recognizes InIB. The InIB-Met interaction leads to the activation of a signaling cascade which results in an actin- and clathrin-dependent uptake of *L. monocytogenes*.

In order to understand the global regulation of the *L. monocytogenes* internalization process in an integrated approach that involves the characterization of the invasion processes by other bacterial and viral pathogens, our laboratory joined the InfectX (www.infectx.ch) -a consortium belonging to the SystemsX.ch initiative. The aim of InfectX is to define a 'human infectome' network for several bacterial and viral pathogens by using a comparable RNAi screening and data analysis approach for pathogen entry into human cells. In the case of our particular *L. monocytogenes* screening protocol, we established an immunofluorescence-based assay relying on the cytosolic detection of InIC, a bacterial protein from the internalin family that is highly expressed and secreted by intracellular *L. monocytogenes*, allowing for a robust quantification of the infection. Results of kinome screens confirmed the functionality and stability of our assay and we are currently upscaling the screen to a genome-wide level.

2453

Cytological Changes Due to Chlamydial Infection are Not Conserved Among Species.

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Chlamydiae are obligate intracellular bacteria that cause a variety of diseases in both humans and animals. Previous studies have established that *C. trachomatis* L2 causes shortened mitosis, declustering of amplified centrosomes and cytokinesis failure resulting in multinucleation, all potential mechanisms for transformation of cells. Here, we have investigated the cytological changes that have been established for *C. trachomatis* and performed a comparison to *C. muridarum* and *C. caviae* to determine the conservation of these effects between different Chlamydia species. First, we observed a greater percentage of multinucleated cells due to *C. trachomatis* infection than the other Chlamydia species. Therefore, we aimed to determine if the other established cytological changes are conserved among Chlamydia species. All three species were able to cause a decrease in mitotic index and amplified centrosomes. However, we observed the greatest spindle multipolarity in *C. trachomatis*, with an intermediate phenotype in *C. muridarum* and the least multipolarity in *C. caviae*. The spindle multipolarity follows the trend in multinucleation that was first observed.

Furthermore, when cells were infected with *C. trachomatis*, the cell was unable to cluster centrosomes as efficiently as the other species. These results suggest Chlamydia species may interact differently with host centrosomes. We hypothesize that different cytological changes among species may correlate with the ability to cause transformation.

2454

The *Pseudomonas aeruginosa* N-Acylhomoserine Lactone Quorum Sensing Molecules Target IQGAP1 in Human Epithelial Cells.

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Quorum sensing (QS) signaling allows bacteria to control gene expression once a critical population density is achieved. The Gram-negative human pathogen *Pseudomonas aeruginosa*

uses *N*-acylhomoserine lactones (AHL) as QS signals, which coordinate the production of virulence factors and biofilms. There is increasing evidence suggesting that AHLs can induce changes in phenotype in neighboring cells, including other bacteria and eukaryotic host cells. Still, rather little is known about the exact mechanisms of action of AHL on eukaryotic cells and their direct targets or receptors.

In the present study, we have designed and validated new 3O-C₁₂-HSL probes, which we utilize to find out the human protein target of 3O-C₁₂-HSL in epithelial Caco-2 cells.

Using 3O-C₁₂-HSL biotin- and fluorescein-based probes for LC-MS/MS and confocal imaging, respectively, we demonstrated for the first time that 3O-C₁₂-HSL interacts and co-localizes with the IQ-motif-containing GTPase-activating protein IQGAP1 in Caco-2 cells. The interaction between IQGAP1 and 3O-C₁₂-HSL was further confirmed by pull-down assay using a GST-tagged protein with subsequent Western blot of IQGAP1 and identification of 3O-C₁₂-HSL with a sensor bioassay. Moreover, 3O-C₁₂-HSL induced changes in the phosphorylation status of Rac1 and Cdc42 and localization of IQGAP1 as evidenced by confocal and high resolution STED microscopy and Western blots. Rac1 and Cdc42 are the upstream effectors of filamentous actin remodeling, and thereby of cell shape and motility in mammalian cells. The RasGAP homology domain in IQGAP1 directly interacts with the Rho-family GTPases, Rac1 and Cdc42 in their phosphorylated and GTP-bound state.

Our findings imply that the IQGAP1 is a novel partner for *P.aeruginosa* 3O-C₁₂-HSL and, likely an integrator of Rac1 and Cdc42- dependent altered cell migration. We propose that the targeting of IQGAP1 by 3O-C₁₂-HSL can trigger essential changes in the cytoskeleton network and thereby be an essential component in bacteria – human cell communication.

2455

Identifying Interactions between *Legionella pneumophila* Effector Proteins and Endocytic Rabs.

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The *Legionella pneumophila* bacterium is a respiratory pathogen phagocytized by alveolar macrophages. Through the use of a Dot/Icm type IV secretion system, *Legionella* is able to inject effector proteins into a host cell to ensure its survival. Secreted effector proteins allow the bacteria to evade degradation within the endocytic pathway as well as enable intracellular replication. Inhibiting lysosomal fusion for removal from the cell suggests an interaction between endocytic cell proteins and *Legionella* effector proteins. Previous studies have identified interactions between Dot/Icm substrates, bound to the membrane of a *Legionella*-containing vacuole, and Rab proteins localized to the late endosome and lysosome. In our lab, yeast two-hybrid assays have been used to examine interactions between early endosomal Rab proteins, Rab4a, Rab5a, Rab15, Rab22a, and Rab35, and various *Legionella* effector proteins. LegC7, known to disrupt vesicular trafficking and the endolysosomal pathway, was one of many *Legionella* effector proteins found to bind to Rabs in the yeast two-hybrid assay. I am examining the interaction between *Legionella* LegC7 and Rab5 proteins. Methods used to characterize these interactions include: amplifying genes of interest using PCR, GC Cloning, gel extractions, ligations, bacterial transformation, protein expression and DNA sequencing. Analyses of protein-protein binding will be investigated by GST- and Nickel-pull-down assays. Confirming the interactions between *Legionella* proteins and Rab GTPases will allow for a better understanding of how *Legionella* is able to modify the endocytic pathway using a Dot/Icm secretion system. This may provide insight into how bacteria manipulate host cells for survival.

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Cellular aspects of the infection by *Acanthamoeba castellanii*.

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Acanthamoebiasis in the human causes amebic keratitis and granulomatous encephalitis (Marciano-Cabral et. al. 2000). Particularly, *A. castellanii* induce human infection, this process require amoebas viability and actin microfilaments. In *A. castellanii* little know on surface proteins and actin cytoskeleton during host cell interaction (Yang et. al. 1997). In this work, we hypothesize that *A. castellanii* express ligands on their surface that binds to a receptor on host cell. Moreover, analyzed the actin filaments during parasite-host cells interaction. Operationally, the trophozoites of *A. castellanii* were cultured in Chang medium with 10% fetal calf serum. Epithelial (L929, ATCC CCL-2) and neuronal cells (SHSY5Y, ATCC CRL-2266) were used as host cells. Interaction assay was performed by adding 105 trophozoites to cell monolayers at different times (15, 30 and 45 min). After, cells were exposed to antibodies against *A. castellanii*, secondary antibody -Rhodamine and co-stain with Ph-FITC. The preparations were observed by confocal microscopy. Biotin and biotinylated lectins (AGT and ConA) were used to labell the protein and glycoprotein of trophozoite surface. To determine the proteins that could present affinity to host cells, biotinylated proteins of *A. castellanii* were added to the host cells. The bind proteins were detected by streptavidin-peroxidase. During the infection, there are alterations of cytoskeleton of host cells and trophozoite maintained the actin filaments in acanthopodia. *A. castellanii* show surface proteins with Mr> 180-108kD, Mr> 85-32kD, Mr> 28-17kD. Three proteins with MW > 130, 90 and 54 kD show affinity to epithelial and neuronal cells, some of this molecules are glycoproteins. In summary: i) in model in vitro our study, show that the host cell response to contact *A. castellanii* and show adherence parasite. ii) After, injuries and eventually kills cells and iii) the molecules that show affinity to host cell initiate the adhesion process and cell invasion. We are currently working to identify these molecules and characterize signal mechanism that regulated the invasion.

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The Role of Lipid Raft Aggregation in the Infection of Type II Pneumocytes by *Mycobacterium tuberculosis*.

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Dynamic, cholesterol-dense regions of the plasma membrane, known as lipid rafts (LR), have been observed to develop during and may be directly involved in infection of host cells by various pathogens. This study focuses on LR aggregation induced in alveolar epithelial cells during infection with *Mycobacterium tuberculosis* (*Mtb*) bacilli. We report dose- and time-dependent increases in LR aggregation after infection with *Mtb* bacilli at multiplicities of infection of 1, 10 and 100 from 2-24 hr post infection (hpi). Specific strain-dependent variations in the number of LR aggregates produced were noted among H37Rv, HN878 and CDC1551 during the 24 hour infection period. Treatment of epithelial cells with culture filtrate protein, total lipids and gamma-irradiated whole cells from each strain failed to induce the level of LR aggregation observed during infection with any of the live strains. However, filtered supernatants from infected epithelial cells did produce comparable LR aggregation, a process that was inhibited in the presence of a bacterial protein synthesis inhibitor, suggesting a secreted mycobacterial

protein produced during infection of host cells is responsible for LR aggregation. Disruption of lipid raft formation, with the LR-disruption agent Filipin III, prior to infection indicates that *Mtb* bacilli utilize LR aggregates for internalization and survival in epithelial cells. This study provides evidence for significant mycobacterial-induced changes in the plasma membrane of alveolar epithelial cells and that *Mtb* strains vary in their ability to facilitate aggregation and utilization of LR.

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AHNAK is Required for the Formation of *Salmonella*-Induced Plasma Membrane Ruffles During Invasion of Nonphagocytic Cells.

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Acute gastroenteritis due to nontyphoidal *Salmonella* infection is one of the most common foodborne illnesses in the world, with an estimated 90 million cases and 150,000 deaths occurring annually. A crucial component of *Salmonella* pathogenicity is their ability to actively invade nonphagocytic intestinal epithelial cells. To do this, *Salmonella* use a type III secretion system to translocate a cohort of bacterial effector proteins into the host cell cytosol where they drive actin polymerization at the entry site. Rapid rearrangement of the actin cytoskeleton leads to the localized formation of prominent plasma membrane ruffles that engulf the bacteria in a manner reminiscent of macropinocytosis. Several aspects of *Salmonella* invasion are well understood but we still lack a complete understanding of which host cell proteins are required. We recently demonstrated that the giant phosphoprotein AHNAK is enriched at *Salmonella*-induced plasma membrane ruffles. AHNAK is a membrane-associated protein that has been implicated in vesicle-mediated membrane repair processes, interacts with actin, and potentially acts as a scaffolding protein during membrane signalling events. To examine the role of AHNAK in *Salmonella* invasion, we performed siRNA-mediated knockdown of AHNAK in HeLa cells and consequently observed a ~75% decrease in *Salmonella* invasion. To validate these results, we examined the ability of *Salmonella* to invade mouse embryonic fibroblasts (MEFs) isolated from AHNAK^{+/+} and AHNAK^{-/-} mice and found that *Salmonella* invasion was decreased by ~80.0% in the AHNAK^{-/-} MEFs. These results clearly demonstrate that AHNAK is required for efficient *Salmonella* invasion. Our next objective was to determine the mechanism by which AHNAK contributes to *Salmonella* invasion. Formation of *Salmonella*-induced plasma membrane ruffles is a hallmark of invasion. Therefore, we examined the ability of *Salmonella* to induce plasma membrane ruffles during invasion of AHNAK-depleted cells. Surprisingly, we were unable to identify *Salmonella*-induced plasma membrane ruffles on AHNAK-depleted cells using either immunofluorescence microscopy or scanning electron microscopy. Future experiments will include examination of *Salmonella* invasion by live-cell fluorescence microscopy. This technique will allow us to observe the interaction between the bacteria and host cell in real-time and will allow us to better determine how AHNAK contributes to the formation of the *Salmonella*-induced plasma membrane ruffles.

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The Role of ELMO1 in the Internalization of Enteric Bacteria and Regulation of Inflammatory Responses.

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The World Health Organization estimates that each year as many as 4-6 million people die of enteric bacterial infections. After invading intestinal epithelial cells, bacteria gain access to the subepithelial tissue and the lamina propria where they encounter and infect phagocytes. However, little is known about how phagocytes internalize enteric bacteria reaching the lamina propria in order to clear the infection. Clearance of bacteria by phagocytes plays an essential role in the host response to infection. Bacterial internalization requires the activation of small Rho GTPases such as Rac1 that leads to rearrangement of the actin cytoskeleton and creation of the plasma membrane ruffle. It has been shown previously that the BAI1-ELMO1-Dock180 pathway stimulates Rac1 activation which has a pivotal role in producing the membrane ruffle. ELMO1 was first identified as a protein required for Engulfment and cell Motility and phagocytosis of apoptotic cells. Recently we found that BAI1 (Brain Angiogenesis Inhibitor 1) recognizes Gram-negative bacteria through their lipopolysaccharide (LPS) and therefore acts as a receptor for bacterial attachment. We hypothesized that recognition of Gram-negative bacteria by BAI1 leads to ELMO1-mediated signaling events that regulate bacterial internalization and the subsequent host inflammatory responses in the intestine. Phagocytes in which ELMO1 expression was depleted by shRNA (J774 cells) as well as macrophages isolated from the small intestines of ELMO1 KO or wildtype mice were exposed to Salmonella. Subsequently, bacterial binding, internalization, Rac activation and cytokine responses were assayed. In addition, ELMO1 KO mice were infected and the bacterial burden and inflammatory response assessed in multiple tissues. We found that the down regulation of ELMO1 in macrophages reduced internalization of Salmonella and decreased Rac1 activation following infection. Bacterial internalization was virtually ablated in intestinal macrophages isolated from infected ELMO1 KO mice. Interestingly, inhibition of the ELMO1/Rac function significantly impaired the pro-inflammatory response to infection in cells examined in vitro. Moreover, ELMO1-deficient mice showed attenuated inflammatory responses in the ileum, spleen and cecum. Together these findings suggested a novel role for ELMO1 in Rac1 activation to control bacterial internalization into intestinal macrophages and the subsequent inflammation during enteric diseases.

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Infection of A549 human type II epithelial cells with *Mycobacterium tuberculosis* induces changes in mitochondrial morphology, distribution and mass that are dependent on the early secreted antigen, ESAT-6.

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Pulmonary infection by *Mycobacterium tuberculosis* (*Mtb*) involves the invasion of alveolar epithelial cells, however host-pathogen interactions in these cells are just starting to be characterized. In electron microscopy studies we observed extensive redistribution and remodeling of mitochondria in A549 type II human alveolar epithelial cells infected with the virulent strain *Mtb* Erdman at late stages of infection, specifically 72 and 96 hours post-infection (hpi). In order to further investigate these changes, we used Mitotracker Red® to assess changes in mitochondrial morphology/ distribution and mass from 6-72 hpi by both confocal microscopy and flow cytometry respectively. Early in infection there was no apparent effect on

mitochondrial morphology, however, by 36-48 hpi mitochondria appeared fragmented with small spheres clustered in a perinuclear localization. In addition, the median intensity fluorescence (MFI) in flow cytometry experiments decreased by 30% (average of 3 experiments) at 48 hpi with a further decrease at 72 hpi suggesting a reduction in mitochondrial mass. Mitotracker Red® accumulation is dependent on mitochondrial membrane potential (Ψ_m), so these results could indicate either a reduction in Ψ_m or a direct effect on numbers and structure of mitochondria. Extensive colocalization of Mitotracker Red® with the integral membrane protein COXIV at 48 hpi indicated that the decrease in numbers and changes in distribution/morphology of the mitochondria were due to direct effects on structure. In contrast, mitochondria in cells infected with the non-virulent strain *M. bovis* BCG appeared similar to uninfected control cells. A virulence factor present in *Mtb* Erdman that is lacking in *M. Bovis* BCG is the early secreted antigen, ESAT-6 which has been shown to be important for infection in type II pneumocytes. Therefore, we performed similar experiments using the parental strain *Mtb* Erdman, an ESAT-6 deletion mutant and its complement. MFI decreased in the WT and complemented strains versus uninfected controls by 65% and 45% respectively; no decrease was detected in the deletion mutant. These results indicate an involvement of ESAT-6 in the perturbation of mitochondria by virulent *Mtb*. Current studies are addressing whether this is a direct effect of ESAT-6 on mitochondrial membranes and whether other co-effectors are necessary.

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Bax/Bak Orchestrate Ion Transport, Apoptosis and Inflammatory Response of Host Cells to *Pseudomonas aeruginosa* Quorum-Sensing Molecule Homoserine Lactone.

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Pseudomonas aeruginosa, which accumulate in the lungs of cystic fibrosis (CF) patients, use N-(3-oxododecanoyl)-homoserine lactone (C12) as a quorum-sensing molecule. C12 also affects host cells: activating the IP₃ receptor, stimulating cAMP and CFTR, triggering the ER unfolded protein response and apoptosis and both pro- and anti-inflammatory responses. The present work tested whether Bax and Bak were involved in the effects of C12 on fibroblasts. In wild type mouse embryo fibroblasts (wt MEF's) C12 (50µM) elicited apoptosis-associated blebbing of plasma membranes, condensation of nuclei, release of cytochrome C from mitochondria and activation of caspase 3/7. C12 also caused the ER to release Ca²⁺ and mitochondria to depolarize. C12 caused none of these effects in Bax^{-/-}/Bak^{-/-} (dko) MEF's. C12 caused NF-κB p65 to enter the nucleus from the cytosol, but also inhibited NF-κB-regulated luciferase expression in wt but not in dko MEF's. C12 stimulated expression/transcription but inhibited secretion of proinflammatory cytokines IL6 and KC in untreated and in TNFα-treated wt MEF's. In contrast, C12 did not affect NF-κB or expression or secretion of cytokines in either untreated or in TNFα-treated dko MEF's. Thus, C12-triggered events all required Bax/Bak, indicating the responses were mediated through a common mechanism that shares many characteristics with pattern associated molecular pattern receptors (e.g., TLR's and NODs). It is proposed that C12 regulates Bax/Bak, which causes: (i) depolarization of mitochondria; (ii) release of cytochrome C (leading to activation of caspase 3/7 and apoptosis); (iii) activation of IP₃R (and release of Ca²⁺ from the ER into the cytosol, activation of STIM1 and adenylate cyclase and cAMP/PKA and CFTR); (iv) activation of NF-κB and expression of cytokine genes but inhibition of cytokine production and secretion resulting from effects of C12 to trigger ER stress and inhibit protein synthesis.

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Role of Aquaporin 9 in Leukocyte Activation and Differentiation.A. Holm¹, J. Gardin¹, T. Karlsson¹, V. Loitto¹, J. Wetterö¹, E. Vikström¹, K-E. Magnusson¹;¹Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

The aquaporins (AQPs) are a family of water channels that transports water and other small uncharged solutes over the plasma membrane. They are involved in cellular events like volume and shape regulation. The aquaglyceroporin AQP9 is the main AQP in human leukocytes and has been suggested to play a pivotal role in inflammatory responses like cell migration, differentiation and metabolism. Thus, we chose to investigate the role and regulation of AQP9 expression in human primary immune cells in response to inflammatory stimuli. The AQP9 mRNA expression increased upon inflammatory stimuli both with *Salmonella* lipopolysaccharide (LPS) and with *Pseudomonas aeruginosa* quorum-sensing molecule N-3-oxo-dodecanoyl-homoserine lactone (3O-C12-HSL). The administration of the TNF inhibitor Infliximab seemed to block the increase of AQP9 mRNA expression, further indicating the role of AQP9 in inflammatory responses and suggesting a regulatory pathway of expression through NFκB. Moreover, the human promyelocytic cell line HL-60 showed a significant increase in AQP9 expression when differentiated towards macrophages and neutrophils, suggesting additional importance for AQP9 in inflammatory cells and opening up for AQP9 as a differentiation marker with a role in functional cell maturation.

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Role of The Type Three Secretion System effectors family NleG.M. Lomma¹, G. Frankel¹; ¹Imperial College London, London, UK

Many bacterial pathogens use type III secretion systems (T3SS) to inject 'effector' proteins into the cytosol of the host cell to subvert multiple signalling pathways. T3SS effectors can either be pathotype specific, functional to a particular infection strategy, or common to multiple pathogens to target conserved host cell signalling pathways. Although *Salmonella* and *E. coli* use very different infection strategies, they express homologous effectors named NleGs.

The aim of this study is to elucidate the function of the NleGs and investigate their role during bacterial infection. Effectors belonging to the NleG family have been recently described as a new class of bacterial E3 ubiquitin ligase. Indeed, all NleGs share a conserved C-terminus domain, with a U-box fold and E3 ubiquitin ligase activity, and a more variant N-terminal domain. Importantly, our preliminary data show that different members of this family show different intracellular localisation, suggesting that they may serve very different functions during infection. As example, one of the NleGs expressed by enterohaemorrhagic *E. coli* O157:H7 binds the NA⁺/H⁺ exchanger regulatory factors 1 (NHERF1) and is translocated to the nucleus upon transfection and during infection. Strikingly, NleG from *Salmonella enterica* serovar Typhi and its homologous in *Salmonella bongori* show a completely different localisation pattern as they are found on vesicles positive for the recycling endosomes marker Rab11 and the autophagosomal marker LC3.

The role of the ubiquitin ligases in *E. coli* and *S. Typhi* infection remains by large unknown, however understanding the function of this new class of T3SS effectors may shed a new light on the infectious strategies of enteric bacteria.

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A novel role for lipid droplets in the organismal antibacterial response.

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Histones are fundamental components of eukaryotic chromatin, and are therefore abundant in essentially all animal cells. Histones and histone fragments are antimicrobial in in vitro assays. In principle, then, histones normally present in cells could confer protection against microbes that exploit intracellular niches. However, until now this has seemed unlikely for two reasons. First, histones typically are located in the nucleus, excluding contact with cytosolic bacteria. Second, because unconfined histones cause genomic instability, hypersensitivity to DNA-damaging agents, and lethality, cells minimize excess histones by active mechanisms. Lipid droplets (LDs), the ubiquitous highly dynamic organelles, found in a wide range of organisms store lipids, but much less are known about their other biological functions. Our recent study suggested that LDs could sequester proteins like histones that might otherwise cause cellular distress. Consistent with functional sequestration, we show that histones are tightly bound to droplets and could act as intracellular antibacterial defense system. We show that in a *Drosophila* embryo model system, wild type embryos having lipid droplets bound with histones are extremely resistant to bacterial challenge while mutant embryos having lipid droplets lacking histones are very susceptible. While more work remains to understand the role of histones in adult immunity, our initial data is consistent with the hypothesis that the embryonic system described above may function in adult flies as well. These findings prove that histones on lipid droplets contribute to innate immunity in flies. This intracellular antibacterial defense system may be conserved, since mice respond to a simulated bacterial infection by increasing droplet-bound histones in the liver.

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The role of orphan nuclear receptor NR4A1 in the defense against bacterial pore-forming toxins.

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Pore-forming toxins (PFTs) are the most prevalent cytotoxic bacterial proteins and are generally required for pathogenesis in the large majority of problematic bacterial infections. PFTs function by perforating host cell membranes, mainly to ultimately aid bacteria in immune evasion and spreading to different compartments within the host. Membrane repair mechanisms and several physiological defense pathways have been identified, but fine details on how host cells defend against PFTs remain elusive. We performed genome-wide microarray analysis of human A549 cells exposed to the *Streptococcus pneumoniae* PFT pneumolysin. We found that 113 genes were differentially expressed specifically in response to the pore-forming activity of PLY. Among others, orphan nuclear receptors of the NR4A class were strongly upregulated. This effect extended to HeLa cells exposed to the *Gardenerella vaginalis* PFT vaginolysin. NR4A1 is a known player in metabolism and various immune responses, and depending on its phosphorylation state and associated subcellular localization, NR4A1 can trigger apoptosis (mitochondrial localization) or function as a transcription factor (nuclear localization). Using fluorescence microscopy and Western blotting, we confirmed upregulation of NR4A1 at the protein level, and found that PFT exposure alters the phosphorylation state of NR4A1 while its localization appears to remain predominantly nuclear. These and other, preliminary data are consistent with a role for NR4A1 as a transcription factor in cellular immune defenses against

PFTs. Investigation of the effect of loss or gain of NR4A1 function on PFT defense is ongoing, as are the identification of upstream regulators and downstream effectors.

2466B1448

***Chlamydia* effectors target the host ESCRT system.**

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Chlamydiae are Gram-negative obligate intracellular pathogens that replicate within a membrane-bounded compartment termed inclusion. Throughout their development they use a type III secretion (T3S) system to translocate effector proteins into the inclusion membrane or in the host cell cytoplasm, thereby modifying the eukaryotic environment. We have previously described a new family of T3S effector proteins present in all *Chlamydiae*. It is defined by a common C-terminal domain of unknown function, DUF582, found only in *Chlamydiaceae*. Each genome encodes 4 or 5 proteins of the family, which are expressed at the mid and late phases of the infectious cycle.

A yeast two-hybrid screen performed with the C-terminal domain of two members of the family from *C. trachomatis* suggested the ESCRT system as a target. The ESCRT system drives the sorting towards degradation compartments of ubiquitinated proteins and the formation of multivesicular bodies. We confirmed the two hybrid interaction data by immunofluorescence and immunoprecipitation in cells transfected with DUF582. During infection, one component of the ESCRT machinery gradually disappeared after the mid phase of infection, consistent with the kinetics of DUF582 protein expression. When overexpressed, it was recruited to the inclusion membranes at the late time points. Also, its depletion using different siRNA strongly stimulated *Chlamydia* development resulting in an increased progeny. The quantity of DUF582 proteins was specifically increased in depleted cells (compared to other bacterial markers), providing an independent link between the ESCRT system and this family of proteins.

Altogether, those results suggest that the ESCRT machinery normally limits *Chlamydia* growth and that *Chlamydia trachomatis* is manipulating it during infection. Our current work is focusing on understanding the resulting benefit for the bacteria.

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Luminal release of exosomal vesicles from biliary epithelium contributes to TLR4-mediated mucosal anti-*Cryptosporidium parvum* defense.

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Exosomes are membranous nanovesicles released by most cell types from multivesicular endosomes. They are speculated to transfer molecules to neighboring or distant cells and modulate physiological and pathological procedures, such as angiogenesis, cell proliferation, and tumor-cell invasion. Exosomes released from gastrointestinal epithelium to the basolateral side have been implicated in antigen presentation. Here, we reported that luminal release of exosomal vesicles from biliary epithelium is increased following infection by a protozoan parasite, *Cryptosporidium parvum*. Release of exosomal vesicles involves activation of TLR4/IKK2 signaling through promoting SNAP23-associated vesicular exocytotic process. Downregulation of let-7 family miRNAs by activation of the TLR4 signaling increases SNAP23 expression, coordinating exosome release in response to *C. parvum* infection. Intriguingly, exosomal vesicles carry antimicrobial peptides of epithelial cell origin, including cathelicidin-37

and beta-defensin 2. Activation of TLR4 signaling enhances exosomal shuttle of epithelial antimicrobial peptides. Exposure of *C. parvum* sporozoites to released exosomal vesicles decreases their viability and infectivity both in vitro and ex vivo. Direct binding to *C. parvum* sporozoite surface is required for the anti-*C. parvum* activity of released exosomal vesicles. Our data indicate that TLR4 signaling regulates luminal release of exosomes and exosomal shuttling of antimicrobial peptides from biliary epithelium, revealing a new arm of mucosal immunity mechanisms relevant to biliary antimicrobial defense.

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Investigating dynamics of microbial colonization of the zebrafish gut using light sheet microscopy.

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Microbial colonization of the intestine is an important event in the development of vertebrate animals, playing a role in the maturation of host immunity and the establishment of normal digestive function. Recent advances in genomic technologies have led to a greater understanding of the composition of microbial communities in animals. However, genomic approaches lack the spatial and temporal resolution needed to characterize the dynamics of how complex symbiotic populations arise, and how their constituent members compete and cooperate with one another. Using light sheet microscopy we are able to achieve the speed, resolution, and large field of view necessary to image the initial bacterial colonization of initially germ-free zebrafish intestines. This technique allows us to characterize both bulk population dynamics over the length of the entire organ and the behaviors of individual bacteria in *vivo*.

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In vivo passage changes the protein profile of *Klebsiella pneumoniae* and may associate with bacterial virulence.

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Enterobacterium *Klebsiella pneumoniae* (KP) causes opportunist infections including urinary tract (UTI), wound and pulmonary infection. Emerging evidence indicates that the KP causes liver abscess (LA) in Southeast Asia and the disease is especially severe in diabetic patients. Comparison of 1-D protein profile of Kp3 (a UTI KP strain) and Kp5 (LA KP strain) revealed that TerD/E is unique in Kp5. Using a pair of primers amplify the KP genome of several clinical KP strains, we observed that gene *kpv1* only present in LA-KP strains, but not in UTI-KP strains. Attempt to compare the 2D gel protein profile of Kp3 and Kp5 did not show comprehensive results. As the Kp5 strain has been cultured in vitro for several passage, we wondered if passage in vivo (mice) might reveal different protein profile, we therefore compared the Kp5, HepG2- and mice-passed Kp5 (Kp5m) protein expression profile by 2D gel analysis. Results on MALDI-TOF analysis revealed that Kp5m expressed several changes in proteins, including ATP synthase subunit alpha / epsilon chain, TolB, Rbose-5-phosphate isomerase A, elongation factor Tu (EF-Tu) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Further analysis revealed that *terD/E* and *kpv1* genes are on a large (>200 kb) plasmid. Immune sera obtained from surviving mice infected with Kp5 detected bacterial EF-Tu, GAPDH, outer membrane protein A (OmpA) and fructose-bis-phosphate aldolase (FPA) indicating possible surface location of these proteins. Differential fractionation of Kp3, Kp5 and Kp5m bacterial extract

revealed that EF-Tu, OmpA, FPA and GAPDH were increased in membrane fraction for Kp5 and Kp5m; with higher amount in Kp5m than in Kp5. These results suggest that Kp5 may express EF-Tu, OmpA, FPA and GAPDH on surface detectable by immune serum during infection and may contribute to bacterial virulence in vivo, since antibodies to these proteins protect the infected mice.

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Pleurocidin exerts synergistic antibacterial effect in combination with antibiotics and antibiofilm activity.

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Pleurocidin, a 25-mer antimicrobial peptide, is known to exert bactericidal activity. However, the synergistic effect and its mechanism(s) of pleurocidin in combination with conventional antibiotics, and the antibiofilm activity of the peptide are weakly understood. All combinations of pleurocidin and antibiotics showed synergistic interaction against bacterial strains (fractional inhibitory concentration index (FICI) ≤ 0.5) except for *Enterococcus faecium* treated with a combination of the peptide and ampicillin (FICI = 0.75). In assay using fluorescent dye 3'-(*p*-hydroxyphenyl) fluorescein (HPF), we identified that pleurocidin alone and combinations with antibiotics induced formation of hydroxyl radical. The oxidative stress was caused by a transient NADH depletion and the addition of thiourea prevented bacterial death, especially in case of combined treatment of pleurocidin and ampicillin. Combination of pleurocidin and erythromycin increased permeability of bacterial cytoplasmic membrane. Additionally, the antibiofilm effect of pleurocidin was examined by tissue culture plate method. Pleurocidin exhibited a potent inhibitory effect on preformed biofilm of bacterial organisms. In this study, we suggest that pleurocidin synergizes with antibiotics by means of hydroxyl radical formation or membrane-active mechanism, and exerts to antibiofilm activity.

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Classification of the normal gut microbiome of harvestmen (Order Opiliones, Class Arachnida).

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The spider-like arthropods known as the “harvestmen,” or more commonly “daddy long-legs” (Order Opiliones, Class Arachnida) are familiar though rarely-studied animals. Fossil records of harvestmen indicate they have been in existence for over 300 million years. Although closely related to the true arachnid spiders (Order Araneae), harvestmen neither produce venom nor liquefy prey to ingest enzymatically pre-digested food as true spiders do. Rather, harvestmen are small-bodied predators and scavengers that masticate solid food, suggesting the necessity of a somewhat more complex digestive physiology than that of the true spiders. The normal gut bacterial flora of Order Opiliones has never been classified; we hypothesize that the normal digestive flora found in harvestmen will be more diverse than that found in true spiders. To investigate this hypothesis, we have begun a preliminary survey of aerobic bacterial isolates from the gut and feces samples of two harvestmen species, *Leiobunum* sp. and *Vonones ornata*. Our presumptive microbial identifications have initially been limited to aerobic, non-fastidious isolates using manual morphological and biochemical microbiology methods. We compared isolates from harvestmen to those from a common and representative true spider, *Gasteracantha cancriformes* (the spiny-backed orbweaver). From *V. ornata*, we have identified gram negative *Pseudomonas* spp., *Escherichia coli*, and *Klebsiella* spp., and gram positive *Staphylococcus* spp. From *Leiobunum*, we have identified gram negative *Escherichia coli*, *Enterobacter* spp., and *Salmonella enterica*., and gram positive *Streptococcus* spp.,

Staphylococcus spp. and *Bacillus subtilis*. In contrast, cultures from *G. cancriformes* have thus far only yielded identification of gram positive *Bacillus* spp. and *Lactobacillus* spp. From this preliminary survey of aerobic bacteria, we suggest the normal gut microbial flora of harvestmen do exhibit greater diversity than those of true spiders. Future studies will utilize genetic methods for bacterial identification confirmation, and will expand to include classifications of fastidious and anaerobic normal gut microbiota. Given recent interests in the influence of normal gut microbiome on health and disease, our findings will not only serve to further the understanding of the complex digestive system in this ancient lineage of arachnids, but may lead to a new invertebrate model for gut microbiome research.

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Tackling the *Coxiella burnetii* intracellular cycle by high-throughput screening.

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Bacterial pathogens have developed countless strategies to subvert signalling pathways of eukaryotic cells and invade the host, find a suitable replicative niche and spread the infection. Understanding host-pathogens interactions is therefore fundamental to elaborate effective strategies to counter the key steps of the infectious process. *Coxiella burnetii*, the agent of the zoonosis Q fever, is an extremely infectious emerging pathogen causing large outbreaks with a very high economic and health burden. Its obligate intracellular nature had so far severely hampered its study and we ignore most aspects of its interactions with host cells. However, the recent development of a specific axenic growth medium allows today the genetic manipulation of this microbe, opening the way to its detailed investigation. Our aim is to understand the cell biology of *C. burnetii* infections by a novel approach that brings together high-throughput screening and cellular microbiology. We are generating the first bank of *C. burnetii* mutants by transposon mutagenesis and developing high-content functional assays for the rapid and unbiased identification of the bacterial factors involved in the intracellular replicative cycle of *C. burnetii*. To date we have isolated 2000 mutants, 900 of which have been sequenced and screened. To understand how *C. burnetii* invades the host cell, we have identified 37 mutations that severely affect host cell invasion. Among these, we are further characterising three independent mutations in a gene that codes for a bacterial transmembrane protein. This could lead to the identification of the first bacterial ligand that mediates *C. burnetii* internalization within host cells. To understand how *C. burnetii* secretes bacterial effectors into the host cytoplasm we have characterised the phenotype of 19 mutations in genes involved in the bacterial type 4 secretion system (T4SS) and classified these genes into 3 functional classes depending on the morphological characteristics of their phenotype. Furthermore, we have identified 41 mutations that severely affect intracellular replication of *C. burnetii*. Of these, 27 mutations affect genes coding for proteins of unknown function which we are further characterising. The remaining 14 mutations are found in intergenic regions of the *C. burnetii* genome and may be key to the identification of bacterial non-coding RNAs that play an important role in infection. Additional screens are currently being optimised to identify the bacterial factors that protect *C. burnetii* infected cells from apoptosis and those regulating cell-to-cell spread of bacteria during infections. Importantly, our studies on *C. burnetii* will also serve to standardise novel, robust high-throughput assays for the study of other intracellular bacterial pathogens.

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Characterization of Foodborne Staphylococcus Profiles.J. N. Onyekaba¹, E. M. Arrey-Mbi¹; ¹Natural Sciences, Clayton State University, Morrow, GA

The cellular fatty acid profiles of eight strains of *Bacillus*, *Staphylococcus*, and *Enterobacteriaceae* (*Escherichia coli*, and *Salmonella*) were analyzed by comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GCxGC/TOF MS). A novel template method was developed to standardize the raw GCxGC retention data through the use of a chemical indexing mixture. Analyte retention coordinates were normalized in the primary dimension with respect to a series of n-alkanes (Kovats index) and in the secondary dimension with respect to a series of aromatic hydrocarbons (Lee index). Fatty acid profiles extracted from the templates were compared by multidimensional scaling (MDS) and principal component analysis (PCA). Differences in the profiles of Gram positive and Gram negative bacteria were observed, and a series of heterogeneous mixtures comprised of different fractions (containing one Gram positive and one Gram negative bacterium).

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Identification and characterization of the BI-ARC triple-A ATPase from *Bifidobacterium longum* subsp. *infantis*M. Guzmán-Rodríguez¹, A. Barba de la Rosa¹, L. Santos¹; ¹Molecular biology, Ipcyt, San Luis Potosi, Mexico

Analysis of *Bifidobacterium longum* genome revealed a new gene, *bl-arc*, which codes a 56.1-kDa protein containing one AAA domain. Phylogenetic classification made by CLANS positioned the sequence into the ARC AAA divergent branch of the AAA ATPase family of proteins. N-terminal sequence analysis indicates this protein is closely related to ATPases involved in the binding and recognition of proteins prior to their proteolysis such as *Rhodococcus erythropolis* ARC, *Archaeoglobus fulgidus* PAN and the human proteasomal Rpt1. We have cloned the gene; the full-length recombinant BI-ARC was over expressed in *E. coli*, and purified it as a homohexameric complex. Functional characterization shows that BI-ARC is Mg⁺²-dependent and *N*-ethylmaleimide-sensitive ATPase activity. ATP hydrolysis followed Michaelis-Menten kinetics with a Km for ATP of 327 μM and Vmax at 37°C of 492 pmol of ATP hydrolyzed per μg of BI-ARC per min. Maximum activity was reached at 37°C and pH 5.0, however it remained stable at a wide pH range (1.5-5.0). Gene organization of *B. longum* locates BI-ARC in a region flanked by a cluster of genes that include several pup proteins. These findings point to a possible role as a chaperone in the degradation pathway via pupylation.

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Cholecystokinin inhibits inducible nitric oxide synthase expression in lipopolysaccharide induced peritoneal macrophages.E. C. Carnio¹, R. S. Saia¹, L. G. Branco¹; ¹Physiology, University of Sao Paulo, Ribeirao Preto, Brazil

We investigate the possible modulatory role of cholecystokinin (CCK) on cytokines production and inducible nitric oxide synthase expression (iNOS) in lipopolysaccharide (LPS)-stimulated peritoneal macrophages of rats.

All experimental procedures were approved by the Comitê de Ética em Experimentação Animal - FMRP (protocol nº 152/2009). Male Wistar rats (200-250 g) were used to collection of peritoneal macrophage. Four days before the collection of peritoneal fluid, all rats received an intraperitoneal injection of thioglycollate broth. Cytokines (TNF-α and IL-10) were evaluated in

culture supernatant using ELISA kits, as well as iNOS expression in tissue and cell lysates. Data was compared using one-way analyses of variance (ANOVA) and significant differences were obtained using the Tukey multiple variances post hoc test.

Peritoneal macrophage culture pre-treated with different CCK concentrations (10⁻¹⁰ or 10⁻⁶ M) before LPS (1 µg/mL) reduced nitrite production at 24 and 48h (p<0.01), as well as iNOS protein and RNAm synthesis. The both CCK tested concentrations reduced NF-κβ p65 levels in the nucleus at 30, 60 and 120 minutes (≈ 52.97 - 58.1%), associated with increased p65 and IκBα cytoplasmatic contents in comparison to LPS group. However, only CCK 10⁻¹⁰ M + LPS group increased IL-10 levels in macrophage culture supernatant (1221.90 ± 68.19 vs. 730.25 ± 43.70 pg/mL; p<0.05) at 24h after LPS stimulation. The IL-10 neutralization with specific polyclonal antibody reverted the inhibitory effect of CCK on nitrite and iNOS synthesis (p<0.05).

CCK may regulate negatively nitrite production and iNOS expression in LPS-stimulated peritoneal macrophage culture, through decreased NF-κβ activation, IκBα degradation and an auto-regulatory IL-10 mechanism.

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SDF-1/CXCL12 induces migration of lymphocytes by a mechanism pannexin1 hemichannel dependent.

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In the last few years the role that pannexin hemichannels play in immune cells has received extensive attention. Our previous work showed that HIV infection induced a biphasic opening of these channels, an early opening between 5-30 min and a late opening between 48-120 h after HIV exposure. The events of viral attachment and infection involves interaction of the virus with the cellular receptors, CD4 and CCR5 or CXCR4, depending on the HIV isolate used. Thus, we proposed that activation of specific chemokine receptors results in the physiological opening of pannexin-1 hemichannels in T lymphocytes.

We determined that treatment of T lymphocytes with SDF-1/CXCL12, a key chemokine in lymphocyte migration and HIV infection, induces a transient opening of pannexin-1 hemichannels, but not connexin43 hemichannels. Blocking pannexin-1 hemichannels blocked the lymphocyte migration induced by SDF-1/CXCL12, suggesting that opening of pannexin-1 hemichannels is essential for lymphocyte migration. Our studies of localization indicate that pannexin hemichannels are mainly localized at the leading edge of the cell during migration. Thus, our findings demonstrate that Panx1 hemichannels play an essential role in HIV infection and in lymphocyte migration.

2478

A Trojan host story: the role of MMP-7 in Giardia SM-induced host pathogenesis

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The intestinal barrier constitutes a single layer of intestinal epithelial cells that are linked by paracellular cadherin and tight junctions. The breach of epithelial barrier may lead to influx of antigen products and microorganisms located in the intestinal lumen. Giardiasis is one of the most commonly identified waterborne intestinal diarrheal diseases. The Giardia parasitic products enhance monolayer permeability and decrease of transepithelial electrical resistance were documented. However, the downstream signaling that cause intestinal epithelial barrier defects is unclear.

The downstream signaling or activation pathways responsible for Giardia duodenalis -induced epithelial barrier defects associated with cleavage of cadherin and tight junctions are unknown. One of the possible hypothesis is that the expression of MMP-7 could lead to cadherin junction destruction during the Giardia sonicate exposure. Interestingly, the significant increasing level of expression of MMP-7 was observed by immunofluorescence staining and western blot analysis. The destruction of intestinal mucosa microvillus correlated to Giardia duodenalis induced MMP-7 expression activities. The shedding and priming of microvillus cause the reduction of the thickness intestinal mucosa layer. The Giardia duodenalis GSM surface binding of MMP-7 were observed in the area where the active intestinal mucosa microvillus shedding and priming during the in vivo Giardia duodenalis GSM challenges and the Giardia surface binding of host MMP-7 could facilitate its invasiveness.

In order to investigate MMP-7 if the facultative protease responsible for intestinal epithelial junction destruction, the MMP-7 knock-out mice and wild type both were performed the intestinal obstruction procedure then the HRP flux was measured in the Ussing chamber assay for determining by the level of mucosal-to-serosal flux of horseradish peroxidase (HRP type II, MW = 44 kD, Sigma). The results demonstrates that the dramatic HRP fluxes observed after 30 minutes measuring in wild type mice, however MMP-7 OK demonstrated the intact epithelial junction barrier and no obvious HRP fluxes observed and the junctional destruction induced bacteria ntranslocation were determined by enteric bacteria translocation assay. The liver and spleen tissue homogenates from challenged mice at different time courses were inoculated into the fresh blood agar and the bacteria colonies were counted. The results demonstrate that the obvious reduction of colonies growth in liver and kidney of the challenged MMP-7 KO group compared to the wild type mouse

Imaging Technologies, Single Molecule Imaging, and Super-Resolution II

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Quantitative Intravital Microscopy of Liver Transport.

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Because of its unique ability to collect fluorescence images from deep in biological tissues, intravital multiphoton microscopy has become a valuable tool in several areas of biological research, including neurobiology, cancer biology and immunology. Here we describe methods of quantitative intravital microscopy that we have developed to characterize cholestatic liver injury. Special methods of tissue immobilization, multiphoton microscopy and digital image analysis were developed to support dynamic measurements of the kinetics of transport from the sinusoids into the cytosol and from the cytosol into the bile canaliculi in individual hepatocytes *in vivo*. Using a combination of different fluorescent probes, we have combined transport assays with measures of microvascular function, inflammation and cell viability to provide integrated measures of liver injury. The sensitivity of this approach is demonstrated in quantitative analyses of the acute effects of cholestatic drugs and the effects of chronic kidney disease.

2480

Single-shot superresolution fluorescence image by modified spinning disk confocal microscope.

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Most of the recently developed superresolution fluorescence microscopy techniques sacrifice their temporal resolutions to break the diffraction barrier of spatial resolution. A single superresolution image is reconstructed from tens to tens of thousands of time-sequential signals, which often takes a few seconds or longer. The movement in the sample during this acquisition period makes motion blur, which challenges the application of superresolution techniques to live specimens. Here, we report a novel superresolution technique based on the spinning disk confocal microscopy. Theoretically an ideal confocal microscope with infinitesimally small aperture yields an image that contains the information of higher spatial frequency structures up to twice better than the diffraction limit. But its image is too weak for the application to the biological samples. Conventional confocal microscopes, therefore, usually use the aperture size close to the size of the Airy disk, which increases the signal with higher photon correction efficiency at the expense of spatial resolution close to the diffraction limit. We have modified the spinning disk to balance the resolution and the signal intensity. The apertures of the confocal slit were narrowed to increase the resolution. Their intervals were adjusted to achieve acceptable photon correction efficiency with minimal crosstalk between the adjacent slits. A single shot image taken with this modified spinning disk was further processed with a spatial frequency filter to enhance the higher spatial frequency components. This technique, thus, enables the high temporal resolution superresolution imaging with twice better resolution than the diffraction limit. It has no requirements on the fluorescent dyes, so that multi-color superresolution images were obtained with conventional fluorescent dyes or fluorescent proteins. This technique will be, thus, suitable for many cell biology applications.

2481

Live-cell molecular imaging in the oxidative environment using cysteine-free fluorescent proteins.T. Suzuki¹, S. Arai¹, I. Wada¹; ¹Cell Science, Fukushima Medical University, Fukushima, Japan

Direct visualization of target molecules using fluorescent proteins (FP) is a powerful approach to analyze molecular mechanisms in living cells. Extensive efforts have been devoted to improve the tag property. However, molecular imaging in the extracellular environment, including the lumen of the secretory pathway, has been limited due to cotranslational oxidation of cysteine residues in FP. Recently, accelerated folding of GFP was shown to reduce the chance of disulfide-bond formation (Traffic (2011) 12:543). Here, we report that each cysteine residue could be substituted with a specific amino acid without loss of molecular brightness (PLoS ONE (2012)7(5)e37551). Although a single molecule photobleaching experiment revealed that only the GFP monomer emitted fluorescence, the presence of cysteine in the fluorescent FP monomer largely affected both motion and folding of the fused protein in the endoplasmic reticulum (ER). An extreme case was a prion fusion protein whose fusion with the conventional FP caused the formation of a SDS-resistant aberrant structure and the retention in the early secretory pathway. Its tagging with cysteine-free (cf)-FP allowed rapid transport to the cell surface where transient raft-association of the single molecule was observed. We used these new tags to determine how many misfolded soluble cargo were trapped in a chaperone complex in the ER lumen. The photon counting histogram of the cf-FP fusion of permanently misfolded antitrypsin mutant, null(HongKong), indicated that only a monomer was found in a single complex and the inhibition of glucose processing did not alter the stoichiometry. This may explain the highly mobile nature of the misfolded cargo, which must be required for efficient quality control. Development of various types of cf-FP should help understanding the molecular mechanisms in secretion as well as various molecular events in the extracellular space.

2482

Engineering a bright monomeric green fluorescent protein derived from *Branchiostoma lanceolatum*.N. C. Shaner¹, G. G. Lambert¹, A. Chamma², Y. Ni², P. J. Cranfill³, M. A. Baird³, B. R. Sell³, R. N. Day⁴, M. W. Davidson³, J. Wang²; ¹The Scintillon Institute, San Diego, CA, ²Allele Biotechnology and Pharmaceuticals, Inc., San Diego, CA, ³The Florida State University, Tallahassee, FL, ⁴Indiana University School of Medicine, Indianapolis, IN

Here we report mNeonGreen, a novel monomeric yellow-green fluorescent protein derived from LanYFP. Most newly cloned green and yellow fluorescent proteins are not subjected to further engineering simply due to their lack of improved properties relative to existing GFPs. LanYFP, a tetrameric yellow fluorescent protein from the cephalochordate *Branchiostoma lanceolatum*, exhibits an unusually high quantum yield (~0.95) and extinction coefficient (~150,000 M⁻¹cm⁻¹), making it a very attractive candidate for further development. Cephalochordate fluorescent proteins are evolutionarily distant from all other fluorescent protein lineages, and LanYFP has no close homologs whose structures have been solved. However, structure prediction and docking algorithms allowed us to successfully identify the initial target side chains leading to monomerization, and to apply a traditional directed evolution approach to generate a monomeric variant. The final variant, mNeonGreen, is among the brightest monomeric green fluorescent proteins yet described, performs exceptionally well as a fusion tag, and shows great promise as a FRET acceptor for the newest generation of cyan fluorescent proteins.

2483

An optimized FRET trio for live-cell imaging of multiple protein-protein interactions.*B. L. Scott¹, A. D. Hoppe¹; ¹Chemistry and Biochemistry, South Dakota State University, Brookings, SD*

Fluorescence resonance energy transfer (FRET) microscopy is a powerful tool to quantify subresolution protein-protein interactions inside living cells. Recent efforts in fluorescent protein engineering, image acquisition and data analysis have made it possible to measure simultaneously, multiple protein-protein interactions inside living cells by FRET. Here, we characterized the optimal FRET trio to improve the spatiotemporal resolution of these measurements. We focused on readily available cyan, yellow, and red (C-Y-R) proteins that have high quantum yields, large extinction coefficients and exhibit the best possible photostability. Based on published data, we selected the following C-Y-R proteins: CyPet/TFP1, mCitrine/YPet, and TagRFP/mCherry. We generated structurally-equivalent constructs of linked donor/acceptor pairs and measured their FRET efficiency by acceptor photobleaching. Based on these FRET efficiencies and photostability, we determined that TFP, YPet and mCherry were the optimal FRET trio. This conclusion was confirmed by linked-trio constructs created with varied ordering and various fluorophores. Our optimal construct was a TFP-YPet-Cherry which exhibited the highest overall FRET efficiencies: $E_{TFP-YPet} = 0.47$, $E_{TFP-Che} = 0.15$ and $E_{YPet-Che} = 0.37$. We believe this construct will be a useful tool for calibrating microscopes for three-color FRET experiments leading to improved monitoring of multiple protein-protein interactions in live cells.

2484

New single-chain Rac1 biosensor shows GTPase function in invadopod dynamics.*Y. Moshfegh¹, J. Bravo-Cordero¹, J. Condeelis¹, L. Hodgson¹; ¹Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY*

Members of the Rho family of small GTPases are heavily involved in cell motility and migration, and are thought to participate in the regulation of cancer metastasis. To study the function of Rac1 in highly invasive tumor cell migration, we developed a novel, genetically-encoded Rac1 FRET biosensor based on an intra-molecular design. The new biosensor is a substantial improvement over the previous-generation Rac1 biosensor because of its single-chain arrangement, ensuring an equimolar distribution of FRET donor and acceptor, and producing a more accurate readout. Additionally, our design maintains the correct C-terminal lipid modification of full-length Rac1, enabling proper interaction with upstream regulators (GEFs, GAPs, and GDIs).

We used this new biosensor to explore the molecular basis for the involvement of Rac1 in the turnover dynamics of invadopodia, which are F-actin-rich protrusions with proteolytic activity that are exclusive to invasive tumor cells and are thought to be crucial for cell invasion and metastatic phenotypes. The biosensor has shown that there is an exclusion of Rac1 activity from the core of the invadopodial structures of MTLn3 rat carcinoma cells. This exclusion dissipates and Rac1 activity is elevated when stable invadopods disappear, suggesting that a lack of Rac1 activity is necessary for invadopod maintenance, and activation of the GTPase could possibly be involved in disassembly. PAK1, one of the major downstream effectors of Rac1, phosphorylates cortactin, which is a key protein in these structures, and upon this kinase activity, PAK1 releases cortactin. Taken together, we have developed a model for the mechanism of invadopod disassembly, where Rac1 activation in the core of an invadopodium will induce kinase activity of PAK1, which will phosphorylate cortactin, causing a destabilization of the invadopod's principal structural molecule and therefore leading to dissolution. This mechanism may be critical for the proper turnover of the invasive structures during migration

and invasion of tumor cells *in vivo*, where a balance of invasive invadopodial matrix-degradation activity and the protrusive force of leading edge projections must be carefully coordinated to achieve a maximally invasive phenotype.

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Internalization of near infrared labeled transferrin into breast cancer cells using FRET tomography imaging.

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Transferrin (Tfn) is an iron-binding protein used to transport iron via cell surface Tfn receptors (TFR) into the cytoplasm. The dimeric nature of TFR allows the use of Förster Resonance Energy Transfer (FRET) based detection methods to identify receptor-bound, fluorophore labeled Tfn donor and acceptor pairs from unbound Tfn. We report the use of near infrared (NIR)-FRET tomographic imaging to detect internalization of Tfn based on the reduction of donor fluorophore lifetime using a multispectral time resolved fluorescence molecular tomography (FMT) imaging system. Our main goal is to distinguish breast cancer cells (T47D) from normal cells (HMEC) via quantification of cellular Tfn uptake within live mice, due to the wide use of Tfn as a carrier for anti-cancer drug delivery systems. T47D and other cancer cells show higher TFR expression and more rapidly internalize Tfn compared to HMECs in which Tfn is co-localized to early endosome 1 (EEA1) positive markers, thus validating specific cellular internalization of Tfn. In support, we demonstrate increased % FRET efficiency (E%) at higher intensity thresholds of acceptor molecules in breast cancer cells compared to controls using confocal FRET analysis. To establish *in vivo* conditions, we tested temporal lifetime measurements of several donor NIR fluorophore candidates using our time-resolved imaging platform with data fitted into a bi-exponential decay function to estimate non-FRETing donor lifetimes. NIR FRET donor-acceptor AF700-Tfn and AF750-Tfn pair was selected as the optimal FRET pair under our imaging conditions due to the longer donor lifetime and higher quantum yield of AF700. Alexafluor NIR fluorophores AF700 and AF750 conjugated to Tfn molecules (AF700-Tfn/AF750-Tfn) were used for uptake studies into T47D and HMECs. Cells were imaged using a time-resolved wide-field illumination strategy, with whole-body functional and fluorescence tomographic data sets at high-spatial and high-spectral densities (690nm-1020nm spectral range), and acquired using a 300ps time gate and a 40ps time delay to obtain time point spread function (TPSF) over a 4.6ns time window (116 gates). Co-internalization of AF700-Tfn and AF750-Tfn into T47D cells show a positive increase in fractional amplitude of FRETing donor Tfn molecules as A:D ratios increased from 1:4 to 4:1, as expected using fluorescence lifetime based FRET tomographic imaging. In support, mean photon counts show FRET-induced quenching at 4:1 compared to the 1:4 A:D ratio. We demonstrate a detection of Tfn internalization based on FRET events in cancer cells at a threshold of approximately 1×10^4 cells, a number well below current clinical detection capabilities. We thus show proof-of-principle detection of NIR FRET in breast cancer cells in which these signals could be a powerful and non-invasive tool to identify the presence of tumors *in vivo* and to optimize targeted delivery systems based on Tfn-TFR mediated uptake into cancer cells.

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Multispectral/Multimodal 3D Image Reconstruction without Dyes.

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Multimodality and multispectral imaging, using 3D optical sectioning and 3D image reconstruction, are proposed as an approach to producing contrast with live samples without using exogenous contrast media. Contrast agents, such as fluorescent or absorbing dyes, can be toxic to live samples or alter cell behavior. Current modes of producing 3D image sets from a light microscope, such as 3D deconvolution algorithms and confocal microscopy, often require dyes. Phase contrast, brightfield and darkfield microscopy, with proper microscope adjustments, can generate contrast without using dyes. The image reconstruction algorithm is based on an underlying physical model that includes 3D distributions of scattering potential, which models the absorption and phase character of the sample. The algorithm is based upon optimizing/solving an objective function, called the I-divergence, while solving for the 3D scattering potential that provides this optimization. Unlike deconvolution algorithms, the method produces two output image sets instead of one. Preliminary images will be presented.

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Quantitative Imaging of Protein Complexes.

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The dynamic composition of protein complexes can have a profound impact on their function. However, conventional pull-down assays cannot provide dynamic information, and sample preparation may affect complex stability. Here, we combine total internal reflection (TIRF) imaging with quantitative image and data analysis to examine the stoichiometry of protein complexes as well as the dynamics of protein associations. Fluorescently labeled proteins are immobilized on glass coverslips, and perfused with other fluorescently tagged proteins or small molecules. Complexes are imaged on a TIRF microscope to selectively illuminate bound proteins. We determine copy number and relative positions of fluorescent proteins inside complexes by counting photobleaching events, using computational super-resolution and mixture-model fitting algorithms for spot detection. We then statistically correct the results for experimental artefacts, such as expression levels and pre-bleaching of fluorophores, which can reach levels of 20%. We further determine binding kinetics by measuring dwell time of fluorescently labeled proteins on immobilized substrates using the same computational approach. Together these methods have allowed us to determine that CENP-A exists in octameric nucleosomes throughout the cell cycle, that myosin adopts multiple conformations on the cortex of the *C. elegans* zygote that are differentially sensitive to perturbations, and that the interaction of KNL2 with CENP-A depends on its phosphorylation and its Myb-domain structure.

2488

An image-based high content screening for chemotherapy-induced neurotoxicity.

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Central and peripheral neurotoxicity are common complications associated with chemotherapy. Major pitfalls including inappropriate cell model, insufficient analytic parameters, and image selection bias are noticed in the most in vitro neurotoxicity studies. To differentiate the cellular and subcellular parameters associated with neuron morphology, we developed an image-based high content screening of neurotoxicity. We chose the primary culture of cortical neurons instead of immortalized neuronal-like cells or human stem cell-derived neural cultures. Three categories of parameters, including neuron viability, neurite outgrowth, and synapse formation, were analyzed in our image-based high content screening. Microtubule-associated protein 2 and synaptophysin I were utilized to stain neuronal dendrite and synaptic puncta, respectively. Automated image acquisition by high throughput fluorescent microscope enabled to take quantitative images for massive image information. Appropriate image analysis algorithms were designed to define neuron numbers in a heterogeneous cell population of primary neuron culture. Eleven chemotherapeutic agents were used to test their neurotoxic effects. Paclitaxel and vincristine, which disrupt microtubule dynamics, differentially damaged neuron number, neurite outgrowth, and synaptogenesis within their therapeutic concentrations. For platinum-containing drugs, cisplatin significantly affected the three categories of parameters compared to carboplatin in the therapeutic ranges. In terms of ifosfamide and its catabolite chloroacetaldehyde, chloroacetaldehyde severely affected neurite outgrowth and synaptogenesis. Topoisomerase inhibitors, including doxorubicin and its liposomal formulation lipo-Dox®, etoposide, and topotecan, as well as nucleoside analogue gemcitabine showed less neurotoxicity. Collectively, our data demonstrate that automated image acquisition and analysis can be used to effectively differentiate the effects of chemotherapeutic agents on cellular neurotoxicity. This high-content screening assay provides a useful platform to filter out neurotoxic chemicals and further discover neuroprotective agents.

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An in-focus 3-well glass-bottom plate suitable for simultaneous observation of untreated cells and cells treated with two different drugs and application to cell-lineage analysis of mouse m5S cells visualized with mCherry-histoneH3.

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Focusing on living cells in multi-well glass-bottom plates is often tedious and frustrating, especially when performing multi-point time-lapse imaging at higher magnification over a long period. We designed an in-focus 3-well glass-bottom plate suitable for multiple-point long-term live-cell imaging under a microscope equipped with a 40x objective. The surface of the glass-bottom plate was made almost flat in order to capture time-lapse images of three wells in one experiment without an expensive focusing system such as Perfect Focus. We further constructed m5S mouse embryonic fibroblast cell lines whose nuclei have been visualized with mCherry-histone H3. The doubling time of one cell line, grown in each well for 30 h, was almost constant, indicating a nearly uniform culture condition in each well, including temperature and humidity. We further confirmed the effectiveness of this novel plate on multi-point time-lapse

imaging through the simultaneous observation of untreated cells and cells treated with taxol or thapsigargin. Drug-induced mitotic arrest or apoptosis was compared with the normal growth of untreated controls over the same time scale in individual experiments.

2490

Single cell visualization of RNA and DNA using oligonucleotide libraries and molecular beacons.

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Fluorescence microscopy is one of the best methods for following biological events at the single cell level, limited only by the availability of specific labeling reagents for the cellular components of interest. We have generated fluorescence probes to visualize DNA and RNA in single cells to further our understanding of the extent of cellular heterogeneity in populations of cells.

Fluorescence in situ hybridization (FISH) is a powerful technique for studying the structure, organization, and localization of nucleic acids within individual fixed cells. We have combined our ability to select in silico the highest performance metrics for FISH probe design with de novo chemical synthesis of DNA in massively parallel reactions to produce high resolution fluorescent paints for DNA and RNA. Using these oligonucleotide library-derived probes on DNA, human genomic regions as small as 1.8 kb and as large as whole chromosomes were visualized in both metaphase and interphase cells with the same simple assay protocol. RNA-specific probes were also generated that detect the localization of a variety of coding and non-coding RNAs with both conventional wide-field fluorescence and structured illumination microscopy. Using probes designed specifically to transcribed vs. non-transcribed regions, we have simultaneously detected DNA and RNA from the same locus in the same single cell. Our oligoFISH methods are also compatible with co-detection of cellular proteins by immunocytochemistry. The ability to generate high performance FISH probes using chemically synthesized oligo libraries that works flexibly with co-detection of other molecules yields a valuable tool for studies of how localization of specific nucleic acids impacts biological function.

Despite its advantages and applications, FISH is performed on fixed cells and is an inherently static measurement technique, whereas cellular processes are very dynamic. In order to better understand how RNA expression and localization change in living cells, we have employed molecular beacons to label mRNA and monitor the signal over time. Molecular beacons designed against nestin mRNA were validated in solution in a plate reader and ultimately tested in undifferentiated P19 cells, expressing high levels of nestin. FISH probes and molecular beacons are powerful and complementary tools for visualizing the heterogeneity in expression and localization of nucleic acids within populations of cells.

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Fluorescence imaging of protein dynamics on long single-stranded DNA.

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Recent advances in single-molecule fluorescence imaging techniques have allowed the direct observation of protein dynamics on DNA, but the progress has been largely limited to double-stranded DNA (dsDNA) or short single-stranded DNA (ssDNA). Here, we present a single molecule imaging approach for observing dynamics of proteins on long ssDNA of thousands of nucleotides in length. Using a hybrid instrument combining single-molecule fluorescence and

force spectroscopy, we could visualize the dynamics of proteins interacting with a long ssDNA similar in length to what's generated during DNA metabolic processes. Our ultimate goal is to study many different proteins binding to the same long ssDNA and performing their function in coordination in each other. As a step toward the goal, here, we demonstrate the applicability of our methods to the dynamics of two different proteins: (1) the unidirectional motion of *Escherichia coli* (*E. coli*) UvrD helicase and (2) the diffusion of *E. coli* single-stranded DNA binding protein (SSB). With the multidimensional data obtained with our platform, we could capture the entire sequence of binding, translocation, unwinding initiation of UvrD helicase with single molecule resolution. With SSB, we found that the diffusion coefficient is three orders of magnitude higher than what was determined from SSB diffusion on short ssDNA suggesting that on long ssDNA that mimics physiological setting, SSB can migrate via a long range inter-segmental transfer. Force dependence of diffusion further supports the interpretation.

2492

A robust and convenient tool for image segmentation.

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Fluorescence microscopy is a key method for unraveling spatial and temporal processes in cell biology. The advent of automated microscopy, as well as the desire to characterize ever more subtle phenotypes have increased the need for automated quantification of fluorescent image data.

Image segmentation is the first step of image quantification, where signal of interest is distinguished from the background. It is critical that segmentation is sensitive, specific and robust. Balancing sensitivity, specificity, and robustness is a challenge: Higher sensitivity can be achieved by employing more prior knowledge about the signal of interest, but the more prior knowledge is employed, the less robust the analysis becomes. In addition, image segmentation faces the challenge that employing more prior knowledge often requires optimizing multiple parameters for optimal performance, and that pre-processing of the image renders the determination of the threshold for classifying pixels into signal or noise highly unintuitive.

Here, we present a novel tool for image segmentation that is sensitive, specific, and robust, usually requires adjusting one intuitive parameter only, and that decides between signal and background on statistical grounds. Since our algorithm makes very limited assumptions about the fluorescent image, it is applicable to a wide range of signal sizes and shapes, from spots via chromosomes to cells.

Our segmentation algorithm relies on two assumptions about fluorescent images: that the signal is brighter than the background, and that noise is spatially uncorrelated. For each neighborhood in the image, the algorithm counts how many pixels are brighter than, say, two noise standard deviations. If there are significantly more bright pixels than what would be expected from a signal-free image containing only noise, the center of the neighborhood is considered to be a signal pixel. The significance level is set by deciding on the number of expected false positive pixels in the image, which directly allows controlling specificity. Even though the algorithm identifies signal through its spatial correlation, it compares favorably with state-of-the-art spot detection algorithms, such as H-Dome or MSVST, and it performs excellently on published benchmarks.

We demonstrate the power and convenience of using our segmentation tool on the example of the mechanism of P-body assembly. P-bodies, or mRNA processing bodies, are agglomerates of mRNA with factors involved in mRNA degradation and translation repression. Current understanding of P-bodies, especially the mechanisms of assembly, remains limited. Cellular perturbations affecting P-bodies are frequently reported to increase P-body size and number,

suggesting P-bodies assemble via passive precipitation. Here, we show that P-body nucleation and growth are independently regulated in mammalian cells. We use our segmentation tool to statistically identify P-bodies significant signal clusters in immunolabeled images, and we run the algorithm on the background fluorescence of the same staining to detect the cells. We report that phosphorylation of the eukaryotic initiation factor 4E-T by the kinase JNK is required for P-body growth, but not P-body nucleation.

In sum, we present an image segmentation tool that will make it possible for all cell biologists to conveniently and correctly segment fluorescent images, and thus allow accurate quantification of fluorescent image data.

2493

ImageJ plugins for automated intelligent confocal microscopy and their application to systems biology questions.

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In order to obtain statistically relevant information in microscopy experiments one often needs to image a large number of specimen. Recently, we presented "Micropilot" (Conrad et al., 2011, Nat. Methods), a software that communicates with microscopes, automatically detects relevant objects within an image (e.g. mitotic cells) and subsequently launches complex microscopy protocols in order to interrogate the object of interest in more detail. Micropilot is written in LabVIEW and uses supervised learning algorithms for object identification.

To enhance the flexibility and openness of our automated intelligent imaging efforts, we now present a series of ImageJ Plugins that retrieve the current image from a microscope, facilitate object detection, and signal whether a specific imaging protocol should be launched on the detected object. ImageJ is free, open-source, widely used in the biological community and contains a large number of image processing operations. Through "Macro Recording" one can readily set up an automated microscopy workflow, combining our Plugins with all ImageJ commands. This provides a wide range of (3-D) object detection methods, ranging from simple thresholding to pixel-based supervised classification.

To demonstrate the potential of the technology in systems biology applications we conduct automated fluorescence recovery after photobleaching (FRAP) experiments on a Zeiss LSM780 confocal microscope in order to measure the kinetics of vesicular coat complex II (COPII) components turnover at ER exit sites in cells treated with siRNAs targeting genes that have been proposed to play a role in ER to Golgi transport (Simpson et al., 2012, Nat. Cell Biol.).

2494

The Open Microscopy Environment: Open Image Informatics for the Biological Sciences.

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Despite significant advances in cell and tissue imaging instrumentation and analysis algorithms, major informatics challenges remain unsolved: file formats are proprietary, facilities to store, analyze and query numerical data or analysis results are not routinely available, integration of new algorithms into proprietary packages is difficult at best, and standards for sharing image data and results are lacking. We have developed an open-source software framework to address these limitations called the Open Microscopy Environment (<http://openmicroscopy.org>). OME has three components—an open data model for biological imaging, standardised file

formats and software libraries for data file conversion and software tools for image data management and analysis.

The OME Data Model (<http://ome-xml.org>) provides a common specification for scientific image data and has recently been updated to more fully support fluorescence filter sets, the requirement for unique identifiers, screening experiments using multi-well plates. The OME-TIFF file format (<http://www.loci.wisc.edu/ome/ome-tiff.html>) and the Bio-Formats file format library (<http://www.loci.wisc.edu/ome/formats.html>) provide an easy-to-use set of tools for converting data from proprietary file formats. These resources enable access to data by different processing and visualization applications, sharing of data between scientific collaborators and interoperability in third party tools like Fiji/ImageJ.

The Java-based OMERO platform includes server and client applications that combine an image metadata database, a binary image data repository and high performance visualization and analysis. The current release of OMERO (4.4.3; <http://openmicroscopy.org/site/support/omero4/downloads>) includes a single mechanism for accessing image data of all types—regardless of original file format—via Java, C/C++ and Python and a variety of applications and environments (e.g., ImageJ, Matlab and CellProfiler). Support for large images from digital pathology is included. This version of OMERO includes a number of new functions, including SSL-based secure access, distributed compute facility, filesystem access for OMERO clients, and a scripting facility for image processing. Demos of OMERO are available at <http://openmicroscopy.org/site/products/feature-list>.

Proteomics and Genomic Methods

2495

PSI Structural Biology Knowledgebase: New Ways to Enable Your Research.

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The Protein Structure Initiative Structural Biology Knowledgebase (SBKB, <http://sbkb.org>) is the scientific web portal that integrates biological, experimental, and structural data about proteins. SBKB delivers comprehensive information, including 3D structures from the Protein Data Bank, annotations from 150+ open biological resources, target history and protocols from TargetTrack, theoretical models in Protein Model Portal, technology reports from the PSI Technology Portal, PSI articles from the PSI Publications Portal, and research and technical highlights from Nature Publishing Group. We will present several examples on how structural data found in the SBKB and its portals can enable biological research.

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2496

The PSI: Biology-Materials Repository: A Resource for Protein Expression Plasmids.

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The Protein Structure Initiative: Biology-Materials Repository (MR; <http://psimr.asu.edu>) sequence-verifies, annotates, stores, and distributes protein expression plasmids and vectors created by the Protein Structure Initiative (PSI). Researchers can find and request PSI plasmids in a freely searchable database called DNASU (<http://dnasu.asu.edu>), which in addition to the PSI collection, distributes over 162,000 plasmids created by our laboratory, by other consortia, and by individual laboratories. Each plasmid links to detailed annotations, including the full-length sequence, vector information, associated publications, and resources such as the PSI Structural Biology Knowledgebase (<http://sbkb.org>), which facilitates cross-referencing of a particular plasmid to additional protein annotations and experimental data. As of September 2012, over 60,000 protein expression plasmids and 97 empty vectors from the PSI are available upon request from DNASU. In addition to providing these materials to the research community, the MR strives to expedite and simplify plasmid requests through its expedited material transfer agreement (EP-MTA) network, where researchers from network institutions can order and receive PSI plasmids without institutional delays. With the added biological focus in PSI: Biology, the distribution of these materials will help researchers expand the knowledge of the role of proteins both in normal biological processes and in disease.

2497

A genomic resource to study kinesin and myosin motors in mammalian cells.

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Microtubules and actin filaments define orthogonal, polarized networks required for intracellular trafficking, cell motility and mitosis. An important class of proteins that works with these polymer systems is the P-loop cytoskeletal motor superfamily consisting of myosin and kinesin motor proteins. However, there is no comprehensive resource to study motor protein function in any given cell type and few of the 39 myosin and 43 kinesin genes in the human genome have been characterized to date. Bacterial artificial chromosome (BAC) transgenes are ideal vectors to investigate gene function because they contain native cis-regulatory sequences for proper control of expression and splicing. Here, we report a collection of 243 recombinant BAC transgenes and 227 stable HeLa BAC lines to investigate the function of 71 (of 82) kinesins and myosins. BACs derived from mouse or human BACs contain cleavable, universal N- or C-terminal tags containing GFP for live-cell imaging and systematic immunoaffinity capture. Motors endogenous to HeLa are expressed as full-length fusion proteins and are generally expressed at native levels. Moreover, the changes in protein levels through the cell cycle are similar for human kinesins and their mouse orthologs. Imaging of the HeLa BAC lines revealed that 21 (of 23 reported) motors were properly targeted as N- or C-terminal fusion proteins. A subset of motor transgenes was also introduced into mouse embryonic stem cells and displayed localization similar to HeLa, indicating the BAC transgene collection is a portable system to investigate motor protein function across multiple cell lines. The BAC and HeLa collection is available to the broader cell biology community with resource access described at this poster.

2498

Investigating natural genetic variation in lethal heat and salt shock survival in yeast.

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When exposed to a mild stress, yeast activate the environmental stress response, a transcriptional response involving hundreds of genes, thereby increasing their resistance to future stresses. This resistance to future lethal stress occurs even when the initial and future lethal stresses are different. Most studies that have identified genes underlying induced stress resistance traits use deletion strains or mutation screens in a single genetic background, commonly a laboratory strain. Here we seek to discover natural genetic variation between yeast strains that has a functional effect on induced stress resistance, using quantitative trait locus (QTL) mapping. QTL mapping is a method for identifying genomic positions where genetic variation underlies phenotypic variation. We use extremely large pools of yeast segregants and bulk selection, extreme QTL mapping (X-QTL), to map QTLs for survival of severe heat and salt shocks with or without pretreatment from either shock. Two parent strains, a lab strain and a clinical isolate, showed heritable differences in stress survival and were used for X-QTL. Using these techniques we have identified two QTL for survival of extreme salt shock, both favoring the allele from the clinical isolate. The mapping will be repeated for more conditions and the genes underlying the detected QTL will be identified by allele replacement.

2499

Bridging the gap between tissue structural properties and gene expression.

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The structure/function relationship is fundamental to our understanding of biological systems at all levels, and drives most, if not all, techniques for detecting, diagnosing, and treating disease. The predominant means of collecting structure/function data in biomedicine is reductionist and has thus led to a proliferation of complex data (e.g., gene expression arrays, digital images) that capture only a fraction of this relationship because they are limited to one organizational scale each. Current systems-based analysis approaches are hindered by the lack of integration between different forms of reductionist data. Our goal is to overcome this data impedance barrier between molecular, cellular, and tissue-level scales, and vertically integrate them into a uniform feature space that facilitates development and testing of multiscale, mechanistic hypotheses. As proof of concept, we collected two different forms of data on human breast tissue: (1) structural features computed from graph theory-based models ("cell graphs") of histopathology images and (2) publicly available gene data sets (GDS) from the NCBI Gene Expression Omnibus (GEO) database. We queried three classes of expression data, which tightly correspond to similar classes for the structural features from histopathological image data, namely low (normal breast tissue/expression), medium (ductal carcinoma) and high (malignant) levels of cancer. We applied singular value decomposition on the matrices L, M, and H, to extract the top eigen-genes (linear combinations of all 14718 genes) that capture 75% of the total variance. Next we added up the contribution/coefficient of each original gene in each of the chosen eigen-genes scaled by the corresponding singular value, and finally selected the top k=100 genes for each class. These genes contribute the most to the eigen-genes, and thus may be considered to be the most informative in terms of variance. Ultimately, this results in a 207 x 3 matrix E of top genes, and their average normalized expression value in each of the three

classes. Next, we linked the expression data with a 35 x 3 structural feature matrix S comprising average cell-graph feature values from the histopathological images from the same three classes. We linked them by creating a 207 x 35 molecular x structural feature kernel matrix K obtained as a dot-product of each row of E and S. Finally, we identified the top genes that correlate well with the structural features. The results of this approach reveal correlations between cell graph features and specific genes that reflect the different phenotypes of the samples.

2500

On-chip activity assay of tissue transglutaminase and blood coagulation factor XIII in differentiated monocytic cells.

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Transglutaminases (TGs) are a family of enzymes consisting of nine isozymes, including TG1-TG7, which are involved in Ca²⁺- and thiol-dependent post-translational modifications of proteins, and blood coagulation factor XIII (FXIII). Among the isozymes, FXIII and TG2 are transglutaminase family members that are involved in various physiological functions, including vascular pathophysiology, bone development, and cancer progression. However, investigation of their differential functions is limited by the lack of high-throughput and isozyme-specific activity assays. Therefore, we developed an on-chip activity assay system to simultaneously determine the transamidating activities of blood coagulation factor XIII (FXIII) and transglutaminase 2 (TG2) by use of fibrinogen arrays. For the on-chip activity assay, we fabricated protein arrays by immobilizing fibrinogen onto the 3-aminopropyltrimethoxysilane surface of well-type arrays, and we determined transamidating activity by probing biotinylated fibrinogen with Cy3-conjugated streptavidin on arrays. We optimized assay conditions, such as buffer pH, concentrations of dithiothreitol and 5-(biotinamido)pentylamine, and incubation time, and we created equations to determine specific FXIII and TG2 activities in samples. We successfully applied this assay system to monitor changes in FXIII and TG2 activities in THP-1 monocytic cells differentiated with phorbol 12-myristate 13-acetate and interleukin-4. This activity assay is sensitive and suitable for high-throughput determination of FXIII and TG2 activities and thus has a strong potential for investigating the differential functions of these isozymes in cell signaling and cardiovascular pathophysiology research.

2501

AdHTS: A high-throughput system for generating recombinant adenoviruses.

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The need for efficient high-throughput gene delivery system for mammalian cells is rapidly increasing with the growing request for functional genomics studies and drug discoveries in various physiologically relevant systems. However, plasmid-based gene delivery has limitations in transfection efficiency and available cell types. Viral vectors have great advantages over plasmid-based vectors, but construction of recombinant viruses remains to be a big hurdle for high-throughput applications. Here we demonstrate a rapid and simple high-throughput system for constructing recombinant adenoviruses which have been used as efficient gene delivery tools in mammalian systems in vitro and in vivo. By combining Gateway-based site-specific recombination with Terminal protein-coupled adenovirus vector, the adenovirus high-throughput

system (AdHTS) generates multiple recombinant adenoviruses in 96-well plates simultaneously without the need for additional cloning or recombination in bacteria or mammalian cells. The AdHTS allows rapid and robust cloning and expression of genes in mammalian cells by removing shuttle vector construction, bacterial transformation, or selection and by minimizing effort in plaque isolation. By shortening the time required to convert whole cDNA library into desired viral vector constructs, the AdHTS would greatly facilitate functional genomics and proteomics studies in various mammalian systems.

2502

Systemically identify oxidized methionine peptides under different Photofrin location in human A431 cells by quantitative proteomics approach.

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Photodynamic therapy (PDT) is a minimally invasive therapeutic clinical treatment for some cancer and non-tumor disorders. This process requires a photosensitizer which can absorb light and produce reactive oxygen species to damage biomolecules in target cells. Photofrin is the most widely used photosensitizer. Our previous studies showed that different distribution of Photofrin in cells results in distinct cell death after PDT. Using human epidermoid carcinoma A431 cells as model, we herein applied SILAC-based quantitative proteome approach and a unique methionine peptides enrichment protocol to systemically analyze the relationship between the subcellular location of Photofrin and the Photofrin-PDT-mediated protein oxidation (on Met residues). Our data showed that when Photofrin mainly localized to plasma membrane (condition I), intracellular organelles (condition II) and whole cell (condition III), the percentage of highly oxidized (i) membrane proteins is significantly higher in condition I (I: 13.33%, II: 3.03%, III: 0.76%) ; (ii) Golgi proteins in condition II (I: 3.33%, II: 18.18%, III: 9.09%); and (iii) mitochondrial proteins in condition III (I: 4.44%, II: 15.66%, III: 30.3%). The results indicate that PDT with Photofrin targeted to distinct subcellular localizations can affect the redox proteome in a site-specific manner in living cells. Among those highly oxidized proteins detected post Photofrin-PDT, we select cathepsin D and EGFR for further study. Mapping the observed oxidized Met residues into the 3D structure of cathepsin D and EGFR showed that these oxidized Met residues mainly distribute on the surface of the protein. We also provided evidence to show that Photofrin-PDT can directly inhibit the activity of cathepsin D and influence EGFR phosphorylation in vitro. Collectively, our data demonstrate for the first time that the relationship between protein Met oxidation and PDT with site-specific location of Photofrin in living cells.

2503

Nested PatchPCR for targeted RNA-seq.

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Using a next-generation sequencing approach to gene expression profiling (RNA-seq) offers significant advantages over microarrays. In addition to detecting gene expression ranges at greater sensitivity than microarray technology, RNA sequencing can be used to detect sequence variants (mutations and SNPs), provides allele specific gene expression information, and enables the discovery of splice junctions and fusion transcripts. While whole transcriptome profiling using next-generation sequencing is valuable, its use is limited by sample preparation techniques, low sample throughput and a requirement for ultra deep sequencing to obtain sufficient coverage of low and medium expressed transcripts in the presence of extremely high housekeeping gene expression. This results in high costs for sequencing and analysis. To

increase the utility of RNA sequencing in biomedical research, we have developed a low cost, high-throughput, targeted RNA sequencing method.

We describe the use of Nested PatchPCR, a sensitive, cost-effective and highly scalable target enrichment technology for the analysis of gene expression changes, detection of SNPs and fusion transcripts. Nested PatchPCR is a limited cycle PCR and selective ligation-based single-tube procedure allowing for simultaneous amplification and selection of tens to thousands of targeted regions. Platform-specific sequencing primers incorporated during the Nested PatchPCR reaction allow direct sequencing of products on Illumina or Ion Torrent next-generation sequencing platforms without time-consuming library construction. Here we utilized oligo-dT primed cDNA of K562, MCF7 and Jurkat cancer cell lines to target transcripts from 13 cancer-related genes including BRAF, EGFR, PTEN, TP53 and BCR-ABL1. The targeted RNA-Seq libraries were multiplexed for sequencing on an Illumina GAIIX resulting in 1-2 million read coverage per sample. We demonstrate potential utility of our target enrichment method as a cost-efficient RNA sequencing solution to conducting comparative gene expression studies, identification of variants and for detection of minimally expressed and fusion transcripts.

2504

Serum IgG Autoantibodies: Possible Role in the Clearance of Tissue Debris Under Normal and Diseased Conditions.

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The presence of self-reactive IgG autoantibodies in human sera is largely thought to represent a break-down in central tolerance and is regarded as a harbinger of autoimmune pathology. In the present study, immune-response profiling of human serum from 166 individuals of different age, gender and health status via human protein microarrays demonstrates that IgG autoantibodies are abundant in all human sera, usually numbering in the thousands. These autoantibodies recognize common as well as organ-specific proteins present both on cell surfaces and intracellularly. Furthermore, we found that serum IgG autoantibody profiles are remarkably stable over time, and that age, gender and the presence of specific diseases strongly influence individual autoantibody profiles. Similarly, both complex and stable autoantibody profiles also exist in rat and swine, suggesting evolutionary conservation of this immunological feature among mammals. We propose that expression of IgG autoantibodies has evolved as an adaptive mechanism for the clearance of cell and tissue debris generated day-to-day by the body. In accord with this hypothesis, we have identified specific alterations in autoantibody profiles associated with Alzheimer's and Parkinson's disease pathology. Using these alterations in expression of autoantibodies as biomarkers enables the diagnosis of Alzheimer's and Parkinson's diseases with over 90% accuracy. We anticipate successful identification of other serum autoantibodies as diagnostic biomarkers for a large number of other diseases which involve tissue damage in affected organs. Further investigations are underway to clarify the function of these autoantibodies and elucidate mechanisms underlying their production and abundant presence in human sera. Supported by the Osteopathic Heritage Foundation.

2505

Identification of serological biomarkers for the diagnosis of liver disease using tagged-internal standard assay-based normalization.

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For minimizing systemic experimental variation in the analysis of antibody array data, we developed a novel median-centered/IgM-tagged-internal standard (TIS) assay normalization using median-centering and TIS assay-based determination of serum IgM concentrations. We evaluated five normalization methods by analyzing correlation coefficients and coefficients of variation for six serum proteins using human serum samples from normal controls (n = 25) and patients with liver cirrhosis (n = 25) or hepatocellular carcinoma (HCC; n = 29). Median-centered normalization improved correlation coefficients, while IgM-based normalizations improved coefficients of variation. The TIS assay was more efficient, economical, and reproducible for determining IgM concentrations than enzyme-linked immunosorbent assay. Additionally, we normalized antibody array data for six serum proteins using the median-centered/IgM-TIS assay, and evaluated serum biomarkers through distribution analysis of normalized fluorescence intensities and receiver operating characteristic analyses for the diagnosis of liver cirrhosis and HCC. Apolipoprotein A-1 and a combination of alpha-fetoprotein and C-reactive protein were determined to be potential serological biomarkers for liver cirrhosis and HCC, respectively. Thus, median-centered/IgM-TIS assay normalization is a useful approach for analyzing antibody array data and evaluating serological biomarkers for the diagnosis of liver disease or cancers.

2506

Development of peptidomics assays for mapping the peptides epitopes derived from collagen I and II processing by different metalloproteases and cathepsins.

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In recent years several lines of research indicate the relevance of extracellular matrix remodeling in tissue homeostasis. Several enzymes, including matrix metalloproteinases (MMPs), have been described as modulators of tissue microenvironment through degradation of the extracellular matrix (in particular collagen metabolism), processing of cytokines and growth factors and processing of cellular surface receptors. The MMPs are the primary enzymes involved in collagenolysis. Peptide fragments generated by collagen processing could be presented to the immune system for generation of either tolerance or immunity. In order to reveal new potential MHC class II restricted epitopes processed by MMPs that are distinct and additional to peptides processed by endosomal proteases, we developed peptidomics assays in which the antigen processing was coupled with nano-LC ESI MS/MS sequencing of the peptides epitopes. New peptides derived from collagen-I and -II processing by the recombinant cathepsins S, B, D and L and by the late endosomal fraction isolated from dendritic cells (DCs) were sequenced and compared with the epitopes derived from the activity of pure recombinant MMPs (such as MMPs 1, 2, 3, 7, 9, 13, 14)) and from the enzymatic activity of the plasma membrane fraction isolated from the human primary DCs, leading to the generation of new peptides epitopes (with molecular weights of 800-6,000 Da). The new discovered epitopes suggest new mechanisms for the extracellular antigen processing distinct from those present in the late endosomal compartment.

2507

Novel Chemifluorescent Substrate for Highly Sensitive Protein Detection.*V. Boveia¹, K. Kundu¹, W. M. Volcheck¹, T. Urlacher¹, N. Padhye¹; ¹LI-COR, Inc., Lincoln, NE*

Detection of proteins in a complex biological sample with high sensitivity is playing an increasingly important role in understanding physiological and pathological processes. The low relative abundance of many proteins within a biological sample and the limited quantity of precious sample make sensitive detection of target proteins highly desirable in protein analysis. Chemiluminescence based detection has been the method of choice for the Western blot and ELISA, but the sensitivity of the analysis is compromised as the accumulation of signal intensity is not possible in chemiluminescence. Here, we present a novel near infrared chemifluorescent substrate, where the fluorescence signal will accumulate with each turnover by the HRP enzyme and thus lead to unprecedented sensitivity. The chemifluorescent substrate exhibits 32-fold sensitivity compared to chemiluminescent detection, and 16-fold sensitivity compared to fluorescent dye-conjugated secondary antibody detection in Western blots. The basic workflow remains unchanged and therefore, the chemifluorescent substrate based analysis is amenable to other methods of protein analysis, such as ELISAs and protein arrays.

2508

Streamlined analysis of heterogeneity in cell populations using single-cell gene expression profiling.*A. P. May¹, R. Lebofsky¹, A. Leyrat¹, B. Fowler¹, J. Shuga¹, P. Chen¹, J. Wang¹, D. Toppani¹, M. Thu¹, M. Wang¹, J. West¹, S. Weaver¹, B. Jones¹, D. Kemp¹, M. Norris¹, M. Unger¹, T-H. Charn¹, B. Jones¹; ¹Research & Development, Fluidigm Corporation, South San Francisco, CA*

Single-cell gene expression profiling has recently been used to characterize emergent properties in cell populations that drive lineage choice and specificity in reprogrammed cells; resolution of cell fate decisions in very early embryonic development, and for identification of cancer stem cell biomarkers in tumor biopsies. We have developed a simple, modular workflow for streamlined analysis of cell populations down to the single-cell level. The workflow is centered on two key components: the C₁[™] Single Cell AutoPrep System for automated cell isolation and cDNA preparation, and the Biomark HD[™] system for highly parallel gene expression analysis. Starting from samples containing only a few hundred cells, the preparation system isolates individual cells into discrete compartments within a microfluidic device. Cells can be inspected after isolation using either brightfield microscopy or fluorescent markers to verify cell number and type. Following cell inspection and verification, the selected individual cells are automatically processed to prepare and output high-quality targeted cDNA for gene expression analysis using quantitative or real-time PCR. Targeted cDNA samples are loaded and analyzed in parallel with up to 96 gene expression assays per cell on Dynamic Array[™] IFCs using the Biomark HD system. Data can be analyzed in R, using a custom set of scripts to enable clustering and visualization for identifying interesting expression profiles. This workflow has been used to analyze underlying heterogeneity in a variety of cell types (e.g. K562, HL60, primary keratinocytes, primary fibroblasts), revealing a broad spectrum of previously unappreciated transcriptional variation that underpins function in these diverse cellular systems. This approach provides a powerful, easy to use and high resolution path to generate highly detailed descriptions of heterogeneity and stochastic events within cellular populations.

2509

The Heterogeneity of Cellular Response is Revealed by Gene Profiling at Single Cell Resolution.

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Many biologically important and clinically relevant cells are not only present in rare quantities, but are often found in mixtures with other cell-types. Measuring and modeling the gene expression and regulatory networks that are altered during signaling changes at single-cell resolution is technically and computationally difficult. Such challenges are particularly pertinent to the field of stem cell biology and cellular differentiation. Here, we describe the use of an integrated single-cell isolation and gene expression profiling platform to address the heterogeneity of cellular identities and responses to changes. This platform utilizes a C1 single cell capture system (Fluidigm, CA) coupled with a BioMark HD system (Fluidigm, CA), a high-throughput quantitative PCR platform. We have developed experimental and computational protocols to determine the sensitivity, reproducibility and accuracy of the platform. We have developed a probabilistic framework to incorporate notions of variability in analyzing gene expression in single cells. We have applied this Fluidigm system and our framework to analyzing hundreds of human embryonic stem cells (ESCs), patient induced pluripotent stem cells (iPSCs) and somatic cells at the single-cell level, identifying previously unidentified gene expression patterns that distinguish the pluripotent from differentiated states. Our study also revealed a dynamic and progressive response within each cell population when ESCs or iPSCs were subjected to stimuli or differentiation signals. In conclusion, multi-parallel gene profiling studies at single cell resolution will be fundamentally important and necessary to dissect cellular heterogeneity in biological processes.

Physical, Chemical, and Synthetic Cell Biology

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Molecular basis of Morphogenesis in Multicellular Systems via High Content Screening.

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Cells in 3D culture have been shown to grow, interact, and behave more like cells in a living organism than those that are grown in a monolayer culture. This is in part due to cellular organization and cell-cell contact and unique cell-extracellular matrix (ECM) adhesions and motility phenotypes. 3D cell culture models offer certain advantages over the 2D models. In the former, each cell forms an ellipsoid with dimensions of 10-30 μm , while in the latter, each cell remains flat with a thickness of a few micrometers. In the former, the entire cell surface is exposed to other cells or the ECM, but in the latter, a very small percentage of the cell surface area is exposed to other cells. These differences in shape and surface are especially significant, given that approximately 30% of the genome is thought to encode membrane-bound macromolecules. Studies have shown that these simple morphological organizations have a profound impact on cellular functions such as morphogenesis, differentiation, developmental, tumor progression, response to treatment, and patterns of gene.

A major barrier for morphometric analyses of multicellular systems has been accurate cell-by-cell analysis of each colony that has been imaged through confocal microscopy in 3D. We present a validated computational assay for characterizing morphometric indices on a cell-by-

cell basis in the context of the colony organization. The computational pipeline has been applied to four different cell lines of an epithelial origin, with known genetic aberrations, to characterize colony growth as a function of individual cellular morphology. The pipeline provides an integrated computational, storage, and visualization for morphometric and bioinformatics analysis of complex multicellular systems that are imaged in 3D. As a result, specific morphometric indices can be identified that correlate with colony morphogenesis. Finally, molecular data are also associated with a subset of morphometric indices to predict molecular aberration.

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High throughput quantification of collective cells migration: Studying the effects of HGF/SF-Met and Glucose metabolism.

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Collective cell migration plays a major role in many essential biological processes. Understanding the molecular and cellular mechanisms of this unique migration mode is limited despite decades of extensive investigations. Numerous computational methods, measures, and software tools exist for microscopic single-cells dynamics analysis, but the collective-migration setting has been neglected. The current explosive growth in the volume of microscopic data requires high throughput analyses to enable applications such as understanding molecular and cellular metastasis mechanisms and drug-screening.

We present a set of quantitative measures, based on live cell imaging of combined bright field and fluorescence confocal microscopy to demonstrate enhanced analyses and better understanding of collective cell motility in a high throughput manner. The toolbox includes multi-cellular segmentation, motion estimation and tracking, motion in clusters and a measure for cells' individuality, measures for indirect morphological features, and spatial-temporal motility measures and visualization.

We focus on the induction of collective tumor cell migration by Hepatocyte Growth Factor / Scatter Factor (HGF/SF) – Met-signaling, master regulators of cell motility in normal and malignant processes. It is demonstrated that HGF/SF-Met-signaling dramatically alters the morpho-kinetic dynamics of collective migration of breast cancer tumor cells: a "wave" of increasing velocities propagates back from the leading edge during in vitro wound healing assay. Met-inhibition reduces this wave.

An open-source, freely available software for high-throughput analysis of collective cell migration, currently developed in our laboratory, is applied to study the cross talk between glucose metabolism of cancer cells and Met tyrosine kinase growth factor receptor signaling. Preliminary results indicate that Met-activation by HGF/SF is more prominent than glucose metabolism in inducing collective migration of tumor cells.

Low concentrations of exogenous expressions of YFP- WT-Met, activating and inhibitory forms were expressed in a monolayer of tumor cells to simulate the phenomenon of cells that maintain amoeboid-like motility while traversing in a tissue consisting of epithelial cells. Individuality of cells is a fully-automated quantitative measure that examines the motility of transfected cells in relation to their vicinity of untreated cells. Activating Met-variants are generally characterized by

lower individuality derived from cells' amoeboid-like motility; Met-inhibition induces epithelial motility also characterized by lower individuality, while HGF/SF-induces increased individuality. It is concluded that the activation of Met-signaling by HGF/SF is the prominent component in individuality of cells.

Our findings may shed light on the molecular and cellular alterations that enable metastasis formation in breast cancer and aid in identifying new targets for therapy.

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Automated Bayesian inference approaches to characterize the nature and onset of particle motion in live cells and embryos.

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Quantitative analysis of particle motion from particle tracking datasets—such as cell trajectories during embryonic development, receptor dynamics in cell membranes, and chromosome and kinetochore motions during spindle assembly—is a powerful approach to understanding the mechanism of transport and onset of directed motion in biological systems. However, inferring motion models from single-particle trajectories (SPTs) is non-trivial due to noise from both sampling limitations and heterogeneity in biological samples. We present two complementary approaches based on Bayesian inference to perform objective and automated analysis of SPTs. The first is a multiple hypothesis testing approach to determine the most likely mode of motion from mean-square displacement (MSD) curves derived from particle trajectories. This approach handles a large set of competing motion models—including diffusion, anomalous diffusion, confined diffusion, and directed motion—and determines which model is most justified by the evidence present in the available MSD curves. The second approach fits raw particle trajectories with a Hidden Markov Model (HMM) to determine the most likely diffusion coefficient and velocity at each step along a trajectory, enabling the identification of transient motion states and dynamic transitions between motion models. These methods avoid overfitting by using an objective Bayesian framework to penalize model complexity and account for noise. We demonstrate the utility of these procedures by applying them to analyze cell dynamics during gastrulation in developing *Drosophila* embryos. We find that cells and cell junctions in embryos undergoing ventral furrow formation initially exhibit random motion that transitions to directed motion as the furrow begins to invaginate. The timing of the initiation of directed motion varies spatially across the embryo surface, providing insight into the mechanism of symmetry-breaking during this important furrowing event. These studies pave the way for use of these automated approaches in higher-throughput screens of particle trajectories in living systems.

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A Genetic Algorithm-based Method to Automatically Characterize and Classify Neurons.

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It has been well documented that neurons exhibit a large variety of morphologies, ranging from the highly complex Purkinje neurons to the simple bipolar retinal neurons. It has also been

shown that the morphology of neurons is affected under pathological conditions. Historically, the morphology of neurons was manually characterized by axonal projection, dendritic branching pattern, soma morphology, and dendritic spine density. As a result, the human characterization is an extremely time consuming and laborious task. This study proposes an automatic classification method by identifying a set of morphological features to characterize and classify neurons based on an inheritable bi-objective combinatorial genetic algorithm (IBCGA).

To evaluate the classification method in classifying neurons with different neuronal morphologies, we used shRNA-mediated gene silencing to suppress a subset of the microtubule-based motors (Kif2a, Kif3c, Kif5b, Kif11, Kif18a, Kif21b, Kif22, Kif23, and Dync1h1) in P19-derived neurons. Microtubule-based motors are responsible for intracellular transport and force generation in neurons; hence, they are essential for neuronal morphogenesis. When these motors are individually suppressed by shRNA, the motor-suppressed neurons each possess a specific morphological phenotype. High-throughput fluorescence microscopy was utilized to acquire these neuronal images. Automated image analysis and IBCGA were then used to quantify and distinguish these 10 classes of morphologically distinct neurons (1 un-suppressed control and 9 motors suppressed). During the training process, 17 out of 703 bioimage features were selected by IBCGA and the classifier of using support vector machine with the 17 features has a prediction accuracy of 92% in distinguishing these 10 classes of morphologically different neurons. To test whether our proposed method can use phenotypic output to predict genotypic perturbation, we classified 3 additional sets of neuronal images (Kif5c-, Dctn2-, and Dync1h1-suppressed) into previously characterized 10 classes. These 3 sets of motor-suppressed neurons were correctly assigned into their respective classes that were consistent with their reported functions. Taken together, the proposed classification method provides an unbiased and efficient way to characterize and classify neurons based on the identified 17 morphological features. This opens up the possibility of using phenotypic output to predict the genotypic perturbation.

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Wrangling phosphoproteomic data to elucidate cancer signaling pathways.

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Analysis of biological data sets is essential for generating hypotheses that guide research, yet the rate of data generation has exceeded our capacity to evaluate the data. The challenge is to discern meaningful patterns in complex data sets, and then convey results in a way that can be easily appreciated. Proteomic data is especially challenging because mass spectrometry detectors often miss peptides in complex samples, resulting in sparsely populated data sets. Using the R programming language and techniques from the field of pattern recognition, we have devised methods to resolve and evaluate clusters of proteins related by their pattern of expression in different samples in proteomic data sets. We examined tyrosine phosphoproteomic data from lung cancer and samples and neuroblastoma cell lines. We calculated dissimilarities between the proteins based on Pearson or Spearman correlations and on Euclidean distances, whilst dealing with large amounts of missing data. The dissimilarities were then used as feature vectors in clustering and visualization algorithms. The quality of the clusterings and visualizations were evaluated internally based on the primary data and externally based on gene ontology and protein interaction networks. The results show that t-distributed stochastic neighbor embedding (t-SNE) followed by minimum spanning tree methods groups sparse proteomic data into meaningful clusters more effectively than other methods such as k-means and classical multidimensional scaling. Furthermore, our results show that

using a combination of Spearman correlation and Euclidean distance as a dissimilarity representation increases the resolution of clusters. Our analyses show that many clusters contain one or more tyrosine kinases and include known effectors as well as proteins with no known interactions. Visualizing these clusters as networks elucidates previously unknown tyrosine kinase signal transduction pathways that drive cancer. Our approach can be applied to other data types, and can be easily adopted because open source software packages are employed.

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Genome-wide, high-content screening reveals novel gene regulators of interphase microtubules and cellular shape.

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Microtubules are a highly conserved, crucial element for most vital processes in eukaryotes including global cell shape, intracellular transport, organelle positioning, and cell division. Therefore, elucidating the mechanisms involved in precise microtubule organization is essential to understanding many fundamental aspects of all eukaryotic life. Furthermore, it may provide critical insights into microtubule-associated diseases, including cancer and Parkinson's disease.

We have developed a high-throughput/high-content microscopy pipeline to screen a genome-wide collection of fission yeast (*Schizosaccharomyces pombe*) knockout mutant strains for knockouts deregulating interphase microtubules. Using a custom-made automated image processing and data analysis pipeline that segments cells in 2D and microtubules in 3D, we have quantitatively analyzed over half a million images of GFP-tubulin expressing cells and extracted over one hundred individual feature measurements from close to two million cells representing more than 3000 different gene knockouts. We used microtubule and cell shape features to quantitatively characterize the mutants based on statistical and geometric features, and applied statistics and data mining techniques to classify strains based upon their microtubule patterns, cell cycle stage and cell shape phenotype. In parallel, visual screening was carried out for comparison and quality control.

Tens of genes known to regulate cellular shape and microtubules, such as those encoding the prefoldin complex of chaperones, were successfully identified as well as hundreds of novel candidate gene regulators that we are in the course of testing and validating. Here, we describe the development and current status of the screen.

2516

Development of New Near IR Dye for In Vivo Imaging Applications.

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Near infra red (NIR) fluorophores are commonly used in cell-based assays or *in vivo* imaging applications. They can be useful in specific imaging or assay application based on the dye's characteristic excitation and emission spectra properties, or relative Hydrophyllicity/hydrophobicity attributes. In this study we have evaluated a new cyanine-based

dye with excitation /emission of 684 / 706 nm, and features that demonstrate excellent characteristics for *in vivo* and *ex vivo* imaging. In general, the dyes that contain a greater number of negative charges display high water solubility and clearance, while more hydrophobic dyes often clear via a hepatic pathway. This dye was conjugated to primary and secondary antibodies which were then used for fluorescence immunostaining in various cell types. In addition, the dye was tested in *in vivo* for optical biodistribution and clearance in nude mice. For *in vivo* studies, the mice received an IV injection of dye via the retro orbital plexus, and were imaged on a Carestream MSFX imager (690nm excitation / 750nm emission) before injection and at 0h, 3h, 6h, 12h, and 24h post injection. After the final time point, animals were sacrificed and organs were collected for *ex vivo* imaging. Toxicity of the dye was evaluated by histological analysis of the dissected tissue section.

The data showed that when this dye was conjugated to an antibody at low molar excess, it had high labeling efficiency that resulted in high fluorescence intensity, good specificity, high signal-to-background ratio, and photostability in cellular imaging applications. The biodistribution and clearance of this dye showed rapid clearance through kidney and/or gastro-intestinal tract. This new NIR dye is an excellent tool for imaging through tissues to circumvent endogenous fluorescent biomolecule interference or quenching.

2517

Identifying transience: considerations in dynamic monitoring of biological events in *Arabidopsis thaliana* biosensors.

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One of the major advantages of Fluorescent Proteins (FPs) is their robust and easy detection; this is in addition to their ability to be fused to other protein domains that can confer additional properties such as organelle compartmentalization or altered stability levels. These properties have practical ramifications when designing a biosensor. Specifically, we consider different aspects of both reporter-protein and promoter dynamics in relation to characteristics (e.g. kinetics, "calibration", and baseline measurements) that render our FP-based biosensor suitable as a tool for real-time measurement and analysis. Our work is contextualized in a proof-of-concept system for automated monitoring of plant temperature stress, through interfacing cellular heat shock responses with a computer-linked fluorescence meter. Overall we demonstrate the value of particular biosensor characteristics and the feasibility of using a cell's genetic response to stimuli for achieving practical goals in the monitoring or control of biological systems. This is further aided by our preliminary work towards developing new FP-reporter constructs for more dynamic visualization of promoter activity in plants.

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Direct labeling of 19F-perfluorocarbon onto engineered multilayered cell sheet for MRI-based non-invasive cell tracking.

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Background: Regenerative medicine with the use of stem cells has appeared as a potential therapeutic alternative for many diseases. Numerous questions remain regarding the viability, biology and the fate of grafted cells. Recent technological advances in chemistry and imaging have allowed monitoring the injected cells in a non-invasive way. However, only individualized group of cells were labeled and tracked *in vivo* so far, since the cell labeling techniques chiefly relies on the inherent cellular uptake mechanism upon foreign labeling agents. In order to

investigate the possible cell migration from the grafted cell sheet to other vital organs, the present study undergoes cell sheet labeling optimization. For the first time, we propose a direct labeling of an organized multilayered cell sheet, for MRI-based in vivo cell tracking post grafting. A perfluorocarbon tracer will be employed to enable specific, quantitative evaluation of the graft by 19F MRI. Using a new temperature-responsive culture surface (UpCell®, CellSeed Inc, Japan), we engineered a multilayered cell sheet, which will be labeled with CS-1000 perfluorocarbon (Celsense, Inc., Pittsburgh, PA). Experiment: Oral mucosal epithelial cells were isolated from rabbit oral mucosal epithelium and were cultivated in the unique culture surfaces to engineer multilayered cell sheets. A dose response study was performed to determine the optimal concentration of the reagent CS-1000 19F-perfluorocarbon. A multiple labeling approach was also used with optimal concentration to successfully label the cellsheet. The labeled cell sheets were harvested, and ten thousands cells were sampled and re-isolated for colony forming assays (CFAs) to validate the clonogenicity of the cells. The remaining part of cell sheets was subjected to 19F NMR spectral analysis at 7T to determine the labeling efficiency. Results: Labeled cells incubated twice with 5 mg/ml of CS-1000 showed a 3.7-time higher labeling efficiency than those labeled only 1 time at the same concentration. The efficiency climbed with increasing the concentration, reached the plateau at 10 mg/ml, and kept steady until the concentration of 20 mg/ml (10 mg/ml was the maximum efficient labeling concentration). Therefore, labeling at 10 mg/ml of CS-1000 2 times during the culture of a cell sheet was found to be an optimal condition. H&E staining of the labeled cell sheet showed that the labeling did not change the cell morphology. As well, no apparent negative effects of the CS-1000 on the clonogenicity of cell sheet were observed. In addition CS-1000 labeling didn't change the p63 expression and the proliferative potential of the engineered cell sheets and no significant effect was observed on the number of cells and the cell viability. Conclusion: We have succeeded in labeling an organized multilayer cell sheet with 19F CS-1000 perfluorocarbon, without apparent toxic effects. This new approach opens new perspective to monitor the migration of cells, coming from an organized engineered tissue.

2519

Stretching Von Willebrand Factor Multimers and Dimers with the Optical Trap.

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Von Willebrand Factor (VWF) is a multimeric plasma protein that is critical for both hemostasis and thrombosis. VWF size in blood is mediated by ADAMTS13, a metalloprotease that cleaves the Tyr1605-Met1606 bond in the A2 domain of VWF. This process is regulated by many factors including denaturing agents like urea, shear stress in blood, and other VWF domains like A1. It has been found that single A2 domains, either in its native or recombinant form, can be unfolded by an increasing pulling force and the unfolding force depends on the force loading rate. A2 unfolding facilitates its cleavage by ADAMTS13. In this work, we stretched both native VWF multimers and recombinant VWF dimers with the optical trap. The VWF molecules were immobilized on plastic beads with antibodies. We found that some A2 domains (about 16%) of native VWF multimers showed an intermediate state before its fully-unfolded conformation. The unfolded length of this intermediate state was about 35 nm. We also found that the A2 domains of recombinant VWF dimers were unfolded at smaller forces, compared with native VWF multimers. With deletion of the A1 domain or deglycosylation, the unfolding forces became even smaller. Three mutations in VWF dimers (G1609R, G1629E, and G1631D) rendered them devoid of any unfolding behavior, indicating that the A2 domains in them became so weak that their unfolding forces were not measurable. From stretching VWF dimers, we calculated an extensional stiffness of about 0.5 pN/nm for VWF monomers before any domain unfolding.

These data show clearly how VWF molecules are extended by force and how their cleavage can be tuned via deglycosylation, domain deletion, and point mutation.

2520

Small Angle Neutron Scattering Study of Green Fluorescent Protein under Macromolecular Crowding: A Tale of Two Dimers.

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Proteins typically function in the crowded cellular environment where they interact with a dense mixture of other biological macromolecules. Concentrations can reach up to 400 mg/ml in the cytosol of some *E. coli*. Such conditions, often referred to as 'crowded' are in stark contrast to the very dilute solutions used in most biochemical and biophysical studies. Here, small-angle neutron scattering (SANS) was employed to study the structure and self-association of green fluorescent protein (GFP) in solutions containing human serum albumin (HSA) at concentrations from dilute (5 mg/ml) to crowded (200 mg/ml). HSA, a common protein in blood serum, affords a more realistic mimic of the biological environment than the more commonly used crowding agents, such as polyethylene glycol or Ficoll. By using perdeuterated GFP and hydrogenated HSA, it was possible to directly probe GFP in the solutions by matching the scattering length density of HSA with an appropriate D₂O/H₂O buffer mixture, thereby eliminating its (SANS) signal. At low HSA concentrations, GFP exists in a side-by-side dimeric conformation that is found when GFP is free in solution. As the concentration of HSA increases past 100 mg/mL, GFP transitions to an ensemble of states that is an equilibrium between a monomeric state and end-to-end dimeric configuration. This experiment is a good demonstration that SANS can be used to probe individual component within complex biological systems, assemblies and composites by exploiting the natural contrast difference between biomolecules. Furthermore, the high penetration power of neutrons and the lack of radiation damage make it useful to study biomolecules even in the cell.

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Macromolecular crowding effects on cell-free protein synthesis are dependent on the concentration of free magnesium.

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Cell-free synthetic biology is a promising approach for understanding, harnessing, and expanding the functions of natural biological systems without using living cells [1]. One of the most prominent example of cell-free biology is the cell-free protein synthesis (CFPS) which provides an open, versatile and rapid platform for protein synthesis [1,2]. However, CFPS is routinely carried out in relatively dilute solutions, where a common intracellular feature, macromolecular crowding is ignored [3]. We earlier investigated the CFPS under macromolecular crowding environments emulated with three different crowding agents, and found that these crowding agents substantially enhanced the transcription, but generally inhibited the translation.[3]. It has been proposed that such different responses might be attributed to the difference in other environmental factors, such as temperature and salt concentrations [3]. In this study, we further examined the macromolecular crowding effects on *in vitro* transcription, translation and coupled transcript/translation reaction at different concentrations of free magnesium ion (Mg⁺⁺). We found that macromolecular crowding effects enhanced these *in vitro* reactions at low concentrations of free Mg⁺⁺, but inhibited the reactions at high

concentrations of free Mg⁺⁺. The results from this research may allow us to better mimic the biological process in an *in vitro* setting.

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2522

Microenvironment of microtubules probed with fluorescence correlation spectroscopy.

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One aspect not completely understood regarding microtubule based transport is how motor proteins are able to move cargo through the cytoplasm when only a few are available to move each cargo and the force generated by each motor protein is small, 7 pN or less. This is surprising considering the high concentration of macromolecules and other proteins present in the cytoplasm through which cargo must move. To study the forces required to move organelle-sized particles through the cytoplasm, we have performed a series of measurements using magnetic tweezers. In these experiments, forces greater than 100 pN were required to move 0.3 μm magnetic beads in the cytoplasm. However, these experiments were performed on particles that had been injected into the cytoplasm and were not moving along microtubules. Transport of organelles at lower forces could be explained by spatial variation in the physical properties of the cytoplasm near microtubules. We have used fluorescent correlation spectroscopy (FCS) to compare the physical properties of the cytoplasm surrounding microtubules and elsewhere in the cell as a comparison. In PtK₂ cells expressing GFP-tubulin, fluorescently labeled 10 kD dextran was observed to have diffusion coefficients up to eight times higher near microtubules than elsewhere in the cell, demonstrating that microtubules are surrounded by a “high diffusion” zone. This spatial variation in the physical properties of the cytoplasm may facilitate transport of large cargo along microtubules.

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DNA target sequence identification mechanism for dimer-active protein complexes.

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Protein-DNA interactions are essential to cellular processes, many of which require proteins to recognize a specific DNA target-site. This search process is well-documented for monomeric proteins, but not as well understood for systems that require dimerization or oligomerization at the target site for activity. We present a single-molecule study of the target-search mechanism of Protelomerase TelK, a recombinase-like protein that is only active as a dimer. Interestingly, we observe that TelK undergoes 1D diffusion on non-target DNA as a monomer, as expected, but becomes immobile on DNA as a dimer or oligomer despite the absence of its target site. We further show that TelK condenses non-target DNA upon dimerization, forming a tightly bound

nucleo-protein complex. Together with simulations of dimer-active protein search, our results suggest a search model whereby monomers diffuse along DNA, and subsequently dimerize to form an active complex on target DNA. These results show that target-finding occurs faster than nonspecific dimerization at biologically relevant protein concentrations. This model may provide insights into the search mechanisms of proteins that are active as multimeric complexes for a more accurate and comprehensive model for the target-search process by sequence specific proteins.

2524

Using Sequencing to Measure DNA Looping Probability in E. Coli.

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Many of the processes that make it possible to copy, control, access and rearrange the information contained in genomes require physical deformations of the underlying DNA substrate. However, the question of how such deformations in vivo depend upon the specific nucleotide sequences of the DNA remains largely unanswered. In this work, we harness the high-throughput potential of in vivo studies to explore the connection between sequence and deformation through the specific example of DNA looping in transcriptional regulation. The looping probability of randomly generated looping sequences will be reported via yellow fluorescent protein output from the gene subject to repression by looping. Sequence information will be gleaned from these cells using flow cytometry to sort cells into bins based upon their fluorescent output, which reflects looping probability. Sequencing will be performed upon cells displaying high and low looping probabilities, and mutual information will be used to find correlations between sequence and looping probability, and will serve as a springboard for studying the connection between sequence and physical deformations in other contexts.

2525

Quantitative dissection of gene regulation through DNA loop formation.

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The study of genetic circuits has reached a state of maturity that permits direct and stringent comparison between theories of transcriptional regulation and the response of living cells. We have developed and tested such models of gene expression based on equilibrium statistical mechanics. The models require only a few input parameters: binding energies of proteins, copy numbers of molecular components, and the mechanical properties of DNA. These parameters are measured in simple control experiments and used to predict gene expression levels for genetic circuits involving different combinations of these parameters. Such first principles biological models require no fitting parameters and make quantitative and precise predictions about gene regulation over a wide range of parameter space.

Equations derived from these models define the input-output function of each genetic circuit, and predict how each system component modulates gene expression. We independently tuned each parameter of the model and experimentally tested whether the gene circuit responded as predicted. Model predictions were tested by creating these genetic circuits in E. coli, and using fluorescent reporters to quantify the level of gene expression for each circuit. We tuned

transcription factor copy number over two orders of magnitude and varied the operator binding energies resulting in several orders of magnitude change in dissociation constants.

Next we implemented the model to examine the role of DNA mechanical properties in the context of transcription factor-mediated loop formation. The tetrameric transcription factor Lac repressor is able to increase the level of gene repression by binding to two operators and looping the intervening DNA. We use this system to ask whether the sequence-dependent flexibility of the DNA in the loop influences gene expression. By incorporating DNA sequences with known sequence-dependent flexibilities into the looping region, we found that the intrinsic flexibility of the looping DNA does not influence repression. DNA sequences shown to be flexible *in vitro*, including in our own tethered particle motion assays, do not lead to increased repression. We resolve the lack of *in vivo* sequence dependence by theoretically and experimentally exploring the role of the DNA-bending protein HU in loop formation. We found that after knocking out HU, sequence-dependent loop formation is restored *in vivo*, and use the models to suggest how the presence of DNA-bending proteins buffers away the influence of sequence-dependent mechanics on gene regulation.

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Bridging Length Scales of Chromatin Biophysics.

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Eukaryotic cells must overcome the daunting challenge of packaging meters of DNA into a micrometer sized nucleus while retaining accessibility of the genome to transcriptional machinery. They do so by employing a hierarchical, dynamic chromatin structure which harnesses the physical properties of DNA and interacting proteins on a wide range of length scales. We use multi-scale modelling to address physical aspects of chromatin at different levels of the packaging hierarchy.

At the smallest scale of chromatin packing, DNA is wrapped around histone proteins to form nucleosomes. We generate a coarse-grained mechanical model for nucleosomal fluctuations during transcription by RNA polymerase. We show that unwrapping of nucleosomal DNA generates rapidly fluctuating forces on the polymerase and that the time scale and magnitude of these fluctuations dictate key features of the transcription kinetics. In addition to elucidating mechanical aspects of nucleosome structure and dynamics, our results imply a general biophysical principle. Namely, we highlight the important role of force fluctuations in determining biomolecular kinetics in the dynamic environment of a living cell.

On a large scale, arrays of nucleosomes are believed to condense into regular helical fibers under physiologic conditions.

We study how the elastic properties of DNA and nucleosome geometry impact the formation of such compact chromatin fibers. We demonstrate a wide variety of energetically accessible fiber structures, indicating that a multiplicity of different structures are likely present *in vivo*.

Zooming out to the scale of an entire genome presents particular modelling challenges, as gigabase lengths of DNA cannot be handled by the same techniques that capture individual nucleosomes. This disparity of length scales necessitates a systematic process for generating coarse-grained models to address longer length phenomena. We develop a generalizable procedure for coarse-graining of polymer models by mapping onto an effective shearable worm-like chain that maintains the correct statistics at long length scales.

We apply this procedure to study the thermodynamics of DNA with periodic kinks generated by bound proteins. We consider looping of DNA-nucleosome arrays on different length scales, demonstrating the crucial impact of local geometry on the distribution of loops. These calculations provide a jumping-off point for investigating the condensation of nucleosome arrays into dense chromatin structures.

These multi-scale studies further our understanding of how the complex hierarchy of chromatin packing and processing arises from the basic physical properties of DNA and interacting proteins.

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Measuring enzyme copy numbers and single-molecule reaction rates in living cells.

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To gain direct insight into enzyme function and organization of reaction pathways, we adapted PALM super-resolution fluorescence microscopy as a general technique to quantify enzyme copy numbers, reaction rates, and their spatial distribution and movement at single-molecule resolution in living cells. Here, we discuss the relative merits and downsides of this technique compared to slimfield excitation microscopy at millisecond temporal resolution and conventional fluorescence imaging by means of two example applications.

(1) We investigated general organization principles of DNA repair pathways. Albeit essential for cell survival, DNA repair itself poses a threat to chromosome stability, generally involving toxic gapped or nicked DNA intermediates. Reaction rates of subsequent repair pathway steps hence have to be well balanced, depending on the copy number distributions, damage search mechanism, and the spatial distribution of repair sites and enzymes. Here, we provide a comprehensive quantitative account of the ubiquitous DNA synthesis and ligation steps by directly visualizing individual DNA Polymerase I (Pol) and DNA Ligase (Lig) enzymes searching and repairing DNA damage in live E.coli. Consistent with early reports, we measure ~400 and ~200 copies of Pol and Lig per cell. Upon DNA damage by mutagen methyl-methanesulfonate (MMS), cells showed a 4-5 fold increase in stationary Pol and Lig molecules within minutes of MMS exposure; individual tracks displayed binding and dissociation events. We quantified repair activity at the single-cell level over time and for varying MMS concentration and found that Pol and Lig exhibit balanced enzymatic activities in excess copy numbers to saturate substrates. This ensures a minimum presence of unbound DNA breaks and high repair capacity to prevent accumulation of toxic DNA repair intermediates.

(2) Combination of our general method with multi-color fluorescence microscopy further provided insight into the structure and function of MukBEF, Structural Maintenance of Chromosomes (SMC) complexes with a central role in E.coli chromosome separation. By distinguishing chromosome bound and diffusing molecules, we establish that MukBEF accumulates in 1-3 immobile clusters per cell that are further organized into sub-clusters with a size < 40 nm. Diffusion of the individual components MukB, E, and F outside clusters was similar despite differences in the individual molecular weights, suggesting that stoichiometric MukBEF complexes assemble in solution.

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Hematopoietic stem and progenitor cells contain functional subsets different in their NF- κ B regulated cytokine producing capacity.

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The ability of hematopoietic stem and progenitor cells (HSPCs) to replenish the hematopoietic repertoire and to maintain the proper numbers of various hematopoietic cells has long been considered their sole function. Little is known about direct immune effector function of HSPCs during an infection. In fact, whether HSPCs can directly sense an infection remains to be substantiated with direct evidence. In this study, we have combined both transgenic and genetic knockout mouse models with a novel single cell barcode proteomics microchip technology to tackle these questions (Ma, C. et al. *Nature Medicine*, 2011, 17, 738-743). We show that although long-term HSCs (as defined by L-K+S+CD150+CD48-) do not secrete cytokines upon TLR ligand stimulation, short-term HSCs and multipotent progenitor cells (MPPs) (as defined by L-K+S+) can produce copious amounts of cytokines upon direct Toll-like receptor (TLR)-4 and TLR-2 stimulation, indicating that L-K+S+ cells can directly participate in an immune response by producing a myriad of cytokines, upon bacterial infection. Within the population of L-K+S+ cells we detect multiple functional subsets of cells, specialized in either myeloid or lymphoid-like functions. Moreover, we show that the cytokine production by L-K+S+ cells is regulated by the NF- κ B activity, as p50-deficient L-K+S+ cells show reduced cytokine production while microRNA (miR)-146a-deficient L-K+S+ cells show significantly increased cytokine production, which is rescued by the double knockout mouse model. The cytokine production in knockout models is less regulated and proteins have lower correlation than those in the wild type model. miR-146a deletion skews L-K+S+ cells toward a myeloid phenotype as well. This study has provided the first evidence of an immune effector function of HSPCs upon TLR stimulation that is regulated by the TLR-NF- κ B signaling axis and suggested the existence of multiple functional subsets within HSPCs.

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Elucidating the Mechanism behind Stem-Cell Derived Hepatocytes using Transcriptome Analysis.

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The capability of human embryonic stem cells to differentiate into cell types of hepatic lineage holds great promise in hepatocyte transplantation for regenerative therapy of liver failure and in hepatocyte based drug toxicity testing. We have previously developed a four-step protocol mimicking the signaling in embryonic liver development using a combination of growth factors and cytokines. In addition to expression of hepatic-specific genes, mature hepatocyte function was demonstrated in albumin secretion, cytochrome p450 activity, as well as urea metabolism. We further demonstrated that these hepatocyte-like cells, when cultured as three dimensional spheroids, had increased expression and/or activities of liver specific genes as well as a larger subpopulation of cells exhibiting differentiated markers such as PEPCK and ASGPR. We also explored the possibility of expanding cells along the path of hepatic differentiation. At least in two stages, cells isolated from differentiation culture were successfully expanded by replating in sub-confluent conditions to retain the capability for further differentiation into the hepatic-like cells.

To evaluate the progress of hepatic differentiation, transcriptome analysis was performed on various cell populations isolated along the course of the protocol and compared to

transcriptome data from other published hepatic differentiation from primary human hepatocytes as well as fetal and adult livers. The transcript levels of a number of liver specific genes, such as albumin approached that of primary hepatocytes. However, these cells did not demonstrate a complete switch in expression of key metabolic isozymes, such as AldoA and AldoB, from the ES state to the hepatocyte state indicating incomplete differentiation. Gene Set Enrichment Analysis (GSEA), was employed to identify key functional classes of genes that are highly dynamic during differentiation. Results of the transcriptome analysis will then be used as a guide in our future optimization of embryonic stem cells differentiation to hepatic lineage.

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Hot spots for allosteric regulation on protein surfaces.

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Co-evolution analysis indicates a general architecture for natural proteins in which sparse networks of amino acids underlie basic aspects of structure and function. These networks, termed sectors, are spatially organized such that active sites are physically linked to particular surface sites distributed throughout the protein structure. This suggests that sectors represent an evolutionarily conserved "wiring" mechanism that effectively functionalize certain sites on the protein surface for the gain of allosteric regulation. Using the metabolic enzyme dihydrofolate reductase as a model system, we show that: (1) the sector is strongly correlated to a distributed network of residues undergoing millisecond conformational fluctuations associated with enzyme catalysis, and (2) sector-connected surface sites are statistically preferred locations for the emergence of allosteric control in vivo. Thus, perturbations at specific sector connected surface positions are able to rapidly initiate conformational control over protein function. These findings suggest that the heterogeneous sector architecture of proteins might enable the evolution of intermolecular communication and regulation.

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Saccharomyces cerevisiae strains evolved for increased medium-chain alcohol tolerance.

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Biofuels are a promising alternative to traditional fuel sources that are fraught with negative environmental impacts and rising costs. Medium-chain alcohols such as *n*-butanol and *n*-hexanol have higher energy densities and less corrosivity than ethanol, making them promising biofuel molecules¹. However, these solvents are toxic and are thus produced at low levels². A solution is to engineer or evolve production organisms that are tolerant to industrially relevant solvent concentrations. This will allow the cell to survive higher solvent concentrations and thus produce more biofuels. *Saccharomyces cerevisiae* is an attractive biofuel production host because it is genetically tractable, resistant to phage infection, and industrial fermentations are well established.

Toward the goal of highly tolerant production hosts, we have evolved a laboratory strain of *S. cerevisiae* to be more tolerant toward the medium-chain alcohol *n*-hexanol. These evolved strains display log-phase growth rates more than double that of the wild-type strain in the presence of toxic levels of *n*-hexanol. The phenotype is stable in the absence of solvent pressure, and is dominant: when mated with a non-tolerant yeast, the diploids have tolerance levels matching those of the tolerant haploid strains. Sporulation and tetrad analysis indicates that multiple genes are involved. The tolerance extends to other medium-length alcohols such

as *n*-pentanol and *n*-heptanol, but not *n*-butanol. Efforts to identify the genes responsible are underway, and will be useful for engineering effective biofuel production hosts.

1) Steen et al., *Microb Cell Fact*, 2008. 2) Dellamonoco et al., *Nature*, 2011.

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Novel positive allosteric modulators of the human $\alpha 7$ nicotinic receptor modulate the antidepressant activity elicited by nicotine.

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Major Depressive Disorder is extremely prevalent in the U.S. population (~20%) and one of the leading causes of disability and morbidity in the world. The aim of this study is to characterize the structural and functional interactions of novel positive allosteric modulators (PAMs) with different nicotinic acetylcholine receptors (AChRs), and to determine its modulatory activity on nicotine induced antidepressant effects in mutant mice. Ca²⁺ influx results indicate that these compounds are not agonists of the human (h) $\alpha 4\beta 2$, $\alpha 3\beta 4$, $\alpha 7$, and $\alpha 1\beta 1\gamma\delta$ AChRs. Compounds 2-4 are specific PAMs of h $\alpha 7$ AChRs, whereas compounds 1-4, 7, and 12 are noncompetitive antagonists of the other AChRs. Radioligand binding results show that these PAMs enhance [³H]epibatidine binding to h $\alpha 7$ AChRs, indicating that these compounds do not directly, but allosterically, interact with the h $\alpha 7$ agonist sites. Molecular dynamics and docking results suggest that the binding site for PAMs 2-4 is mainly located in the inner β -sheet of the $\alpha 7$ - $\alpha 7$ interface, 12Å from the agonist locus. Two hydrogen bond interactions bridging both (+) and (-) subunit faces are found to be critical for the PAM activity at the h $\alpha 7$ AChR. Based on the forced swimming tests on mutant ($\beta 4^{-/-}$) and wild-type ($\beta 4^{+/+}$) mice, male vs female, we determined that: (1) the $\beta 4$ subunit plays an important role in the antidepressant effect elicited by nicotine. The results indicated a statistically significant increase of the antidepressant effect of nicotine in $\beta 4^{+/+}$ mice compared to $\beta 4^{-/-}$ mice, and (2) PAM-2 modulates this activity in a gender distinct manner. The results indicated that PAM-2 enhances the antidepressant effect of nicotine in female $\beta 4^{+/+}$ mice but diminishes it in male $\beta 4^{+/+}$ mice. In $\beta 4^{-/-}$ mice, there is a statistically significant increase of the antidepressant effect (chronic) of nicotine in PAM-2 treated mice compared to nicotine (only) treated mice. The receptor specificity and the modulatory action on nicotine-induced antidepressant activity elicited by PAMs 2-4 might be therapeutically important for the treatment of depressed patients with Alzheimer's disease.

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Integrin binding activity of Aaa-Gly-Xaa or Xaa-Gly-Aaa motif containing laminin peptides.

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Laminin α chain ($\alpha 1$ - $\alpha 5$ chains) has chain-specific diverse biological functions. The C-terminal globular domain of α chain consists of five laminin-like globular modules (LG1-5) and plays a critical role in the biological activities. The LG modules of laminin α chains consist of a 14-stranded β -sheet (A-N) sandwich structure. Previously, we designed 25 homologous peptides (G1EF1-G5EF5) derived from the loop regions between the E and F strands in the LG modules and identified their biological activities. The results suggested that the peptide containing

(acidic amino acid; Aaa)-Gly-(basic amino acid; Baa) motif in the middle of the sequence interacted with integrins, and the peptides containing Baa-Gly-Baa motif in the middle of the sequence interacted with syndecans. Previously, we pointed out the importance of Aaa residue to interact with integrins. Here, we extracted sequences including Aaa-Gly-Xaa or Xaa-Gly-Aaa motif in the LG modules of laminin α chains except the E-F loop regions. We designed 55 peptides (40 of Aaa-Gly-Xaa; 15 of Xaa-Gly-Aaa) with 19 amino acid length and prepared by solid-phase peptide synthesis. We evaluated cell attachment activity of these peptides using human dermal fibroblasts (HDFs), and identified their integrins binding activity using EDTA or anti-integrin antibodies. Fourteen peptides (A1GG2320, A1GG2824, A1GG2916, A2GG2416, A2GG2841, A2GG2855, A4GG833, A4GG1049, A4GG1251, A4GG1254, A4GG1483, A4GG1544, A5GG3133 and A5GG3441) promoted HDF attachment and eleven of them (A1GG2320, A1GG2824, A2GG2416, A2GG2841, A4GG833, A4GG1049, A4GG1251, A4GG1254, A4GG1483, A4GG1544, and A5GG3441) inhibited by EDTA. Furthermore, the HDF attachments of eight peptides (A1GG2320, A2GG2416, A2GG2841, A4GG1251, A4GG1254, A4GG1483, A4GG1544, and A5GG3441) were inhibited by anti-integrin β 1 antibody and seven peptides had Aaa-Gly-Xaa motif (A1GG2320, A2GG2416, A2GG2841, A4GG1251, A4GG1254, A4GG1544, and A5GG3441). These results suggest that the Aaa-Gly-Xaa motif is critical for integrin binding.

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Imiquimod induces cancer cell apoptosis by damaging mitochondria through a TLR-independent pathway.

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Cancer cells differ from their normal counterparts in many ways, so we should be able to kill them selectively. To reveal targetable differences between cancer and normal cells, we used a chemical genetics approach, starting with a proven therapeutic drug imiquimod, that specifically kills virus-infected and cancerous skin cells while nearby normal cell are undamaged.

The current understanding of imiquimod's mechanism of action (MOA) is that imiquimod acts as a TLR7 agonist to induce cytokine releases in macrophages and maturation of dendritic cells. However, a more potent TLR7 agonist resiquimod has failed the phase III clinical trial due to lack of efficacy, indicating that TLR7 agonism is not imiquimod's only MOA, if at all. In addition, imiquimod was reported to be more potent at direct killing of cultured basal cell carcinoma and squamous cell carcinoma than resiquimod, suggesting an additional target in cancer cells that is more strongly affected by imiquimod.

Our preliminary study revealed that imiquimod treated cancer cells are more dependent on glucose for survival suggesting impaired mitochondria functions. Indeed, using fluorescence microscopy we observed that imiquimod, but not resiquimod causes mitochondria to swell. Characterization of the swollen mitochondria using electron microscopy showed degenerated cristae. To test whether the mitochondria damaging effect depends on membrane receptors, we tested imiquimod in an xenopus egg extract system. Without any plasma membrane components, imiquimod impaired mitochondria oxygen consumption, indicating that its target may reside in mitochondria. Our ongoing research will hopefully elude this target that is crucial to cancer cells, but dispensable to normal cells.

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(+)-Negamycin derivatives promoting premature termination codon-readthrough.

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(+)-Negamycin **1** is a dipeptidic antibiotic containing a hydrazide structure. Although (+)-**1** was not clinically developed due to some toxicity, it was recently reported that (+)-**1** restored dystrophin expression in the muscles of *mdx* mice, an animal model of Duchenne muscular dystrophy (DMD) (Arakawa, M. *et al.*, *J. Biochem.*, 2003). DMD is the most severe form of dystrophinopathy caused by a mutation in the dystrophin gene, which codes an important structural protein dystrophin within muscle tissue. About 10-20% of this congenital disease is caused by nonsense mutations possessing the premature termination codons (PTCs). Hence, a promising approach for the treatment of DMD is the use of drugs to force PTC readthrough, and the potent derivatives of (+)-**1** would be a promising therapeutic candidate for diseases caused by nonsense mutations. Based on our own efficient total synthetic method of (+)-**1** (Hayashi, Y. *et al.*, *Chem. Commun.*, 2008; Nishiguchi, S. *et al.*, *Tetrahedron*, 2010), structure-activity relationship (SAR) study was performed to discover derivatives with a potent readthrough-promoting activity. The biological evaluation was conducted by using the double reporter plasmid possessing PTC between beta-galactosidase and luciferase genes. We found a derivative, (5*R*)-5-hydroxy-6-aminohexanoyl-glycine exhibited not antimicrobial activity but a similar readthrough activity to (+)-**1**, suggesting that the PTC readthrough mechanism can be distinguished from the antimicrobial mechanism (Taguchi, A., Hayashi, Y. *et al.*, *ACS Med. Chem. Lett.*, 2012). Moreover, we synthesized 5-*epi*-negamycin and found that this analog exhibited a similar activity to (+)-**1** in *in vitro* readthrough assay. This result hence prompted us to synthesize a 5-dehydro-derivative, e.g., 5-dehydro-3-*epi*-negamycin **2**, which is a natural product with little antimicrobial activity. Surprisingly, we found that **2** showed a higher *in vitro* readthrough-promoting activity than (+)-**1**. This result suggests that Mother Nature independently evolved readthrough-promoting products like suppressor tRNA, in distinction from aminoglycosides, which show both antimicrobial and readthrough-promoting activities.

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Assessing cytotoxic effect of phthalates on mesenchymal stem cells through analysis of impedance fluctuations.

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Phthalates (PAEs) are a family of compounds widely used in the manufacturing industry and in the production of plastics. The human exposure to these compounds can be through ingestion, inhalation, or even directly by certain medical procedures. Due to the intensive and long term use of PAEs in plastic industry and medication, studies on the connection between the PAEs exposure of pregnant women and its toxic influence on the fetus are urgently needed since data are limited to date. The use of mesenchymal stem cells (MSCs) as *in vitro* model systems has great potential to help develop a biologically based risk assessment of toxic chemical exposure to human beings. In this study we have applied electric cell-substrate impedance sensing to investigate the cytotoxic effect of diethyl phthalate (DEP), dibutyl phthalate (DBP) and their metabolites (MEP and MBP) on MSCs isolated from the human umbilical cord. The basic principle of electric cell-substrate impedance sensing is to monitor the changes in electrical impedance of adherent cells grown on gold-film electrodes. To detect the changes of cell micromotion in response to the challenge of different concentrations of PAEs, the time-series

impedances of cell-covered electrodes were monitored and the values of variance and Hurst coefficient were calculated to verify the difference. These measures describe the motile and persistent behavior of these cells in culture. At high concentrations of PAEs such as 3000 ppm and metabolites such as 80 ppm, a drastic drop of resistance due to the increase of intercellular space was observed almost immediately following the addition. Micromotion measurements of MSC-covered electrodes were taken 20 h after exposure to different concentrations of PAEs. Variance and standard deviation of the increments were used to characterize the level of fluctuations. While a dose-dependent relationship was generally observed from the overall resistance of the MSC monolayer, the analysis of impedance fluctuations distinguished DEP levels as low as 100 ppm and metabolite levels as low as 40 ppm. Our results suggest that 3000 ppm of PAEs and 50 ppm of metabolite can cause a decrease of junctional resistance between cells and the reduction in micromotion.

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Dose-Dependent p21(Cip1/Waf1) DNA Damage Response.

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p21(Cip1/Waf1) is a member of the Cyclin-dependent kinase inhibitor (CKI) class of proteins involved in arresting the cell cycle in response to DNA damage. Previous studies have shown that the N-terminal domain of p21 is involved in binding to and inhibiting the activity of Cyclin-CDK complexes, and the C-terminal domain of p21 binds to and inhibits proliferating cell nuclear antigen (PCNA), a DNA polymerase auxiliary factor involved in DNA synthesis and repair.

Our lab has developed genetic tools and methods to evaluate cellular responses over varying levels of gene expression. Recently, we have developed a library of expression vectors that control the level of protein translation. We have used this vector library to express exogenous p21 levels in an Hct116 human colon cancer cell line in which the endogenous p21 has been inactivated by gene-targeting. We were able to generate a full range of p21 expression levels, including those greater than the p21 levels induced by ionizing radiation and those below levels observed in undamaged wild-type Hct116 cells. This wide range of expression allows us to monitor the behavior of cells exposed to varying amounts of p21, including levels that are physiologically relevant.

Our results show that cells expressing exogenous wild-type p21 are not actively cycling through S phase. To explore the underlying mechanism of p21 expression strength affecting cell cycle arrest, we made two mutant versions of the p21 gene—one that does not bind to Cyclin/CDK complexes (p21 CDK-/PCNA+) and one that does not bind to PCNA (p21 CDK+/PCNA-). While the CDK+/PCNA- mutant significantly inhibited the cell cycle at higher levels of expression, the CDK-/PCNA+ mutant inhibited cell cycle progression only at low levels. Additionally, we have found that wild-type Hct116 cells exposed to ultraviolet (UV) irradiation undergo a UV dose-dependent decrease in p21 levels along with an increase in levels of ubiquitinated PCNA—a marker of trans-lesion synthesis (TLS) DNA repair—at one hour post-UV exposure. This trend is reversed at 24 hours, with high levels of p21 and low levels of ubiquitinated PCNA present at high UV doses, likely indicating that repair mechanisms are no longer attempting to fix the DNA damage. Cells exposed to higher UV doses also exhibited a decrease in pro-caspase 3 levels at 24 hours post-UV exposure, suggesting that the apoptosis pathway may be activated.

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The Structural Basis for Redox-Dependent Conformational Switching in the INAD PDZ5 Domain.*K. I. White¹, R. Ranganathan¹; ¹Green Center for Systems Biology, UT Southwestern Medical Center, Dallas, TX*

General biophysical principles guiding the emergence of function in natural proteins remain elusive. Here, we seek to establish the rules for specifying a complex allosteric activity in the INAD scaffolding protein involved in *Drosophila melanogaster* phototransduction. Light stimulation drives a redox-based conformational switch within the fifth PDZ domain (PDZ5) of INAD, and this activity plays a critical role in mediating rapid signal transduction *in vivo* through the modulation of binding to downstream targets [1]. How does the domain trigger the conformational switch in response to environmental signals, and what is the minimal set of residues in PDZ5 needed to encode this behavior? One approach to this problem is to attempt transplantation of redox switching through stepwise mutation of an unrelated, redox-insensitive PDZ domain. Structural and biophysical data show that simply introducing a pair of cysteines is insufficient to recapitulate redox switching over biologically relevant regimes, suggesting that a more distributed group of residues underlies this process. In this regard, the statistical coupling analysis (SCA) [2, 3] reveals a set of collectively co-evolving residues in the PDZ family that may represent the unit of redox sensitivity and conformational switching. Starting with an unrelated, redox-insensitive PDZ domain (PSD95 PDZ3), I have generated a series of chimeric constructs that represent a gradual transplantation of INAD PDZ5 residues at SCA-identified positions, and preliminary data demonstrate the emergence of PDZ5-like redox properties with only a handful of mutations in addition to the requisite cysteines. The identification of the minimal set of these positions needed and their sufficiency for redox-dependent conformational switching remains as an open question for further study.

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