PAK4 phosphorylates p53 at serine 215 to promote liver cancer metastasis

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Abstract

P21-activated protein kinase 4 (PAK4), which is a member of the Group II PAK protein kinase family, has been shown to play significant roles in cellular signaling pathways including cell migration, cell survival and cellular transformation. Overexpression of PAK4 has been reported in cancers, but the oncogenic properties of PAK4 are not well defined. Here we showed that PAK4 mRNA and protein were overexpressed in clinical hepatocellular carcinoma (HCC) samples. Clinicopathological correlation analysis showed that overexpression of PAK4 significantly associated with aggressive and metastatic tumor phenotypes. Also, a nuclear isoform of PAK4, which lacked the exon two, was isolated during our characterization of PAK4. Using stable PAK4 overexpressing and knockdown clones, we showed that PAK4 was required for HCC cell migration. To understand how PAK4 promotes hepatocarcinogenesis, we observed that PAK4 could directly phosphorylate p53 at serine residue 215. Functionally, the S215 phosphorylation not only attenuated the transactivating activity of p53, but also inhibited the p53-mediated suppression of HCC cell invasion. Taken together, our data showed that PAK4 was frequently overexpressed in HCCs and overexpression of PAK4 promotes HCC metastasis via regulating p53 by phosphorylation.

Introduction

The p21-activated serine/threonine protein kinases (PAKs) family plays a critical role in controlling cytoskeletal dynamics and is involved in several cellular processes including cellular motility, survival and hormonal signaling. PAKs function as downstream effectors of the small Rho GTPases Rac1 and Cdc42 (1). PAK4 belongs to the Group II PAKs. Unlike Group I PAKs, the association of an active, GTP-bound Cdc42 or Rac does not enhance the autophosphorylation as well as the activity of PAK4 (2). However, a recent study showed that PAK4 contains a pseudo-substrate domain, which contributes significantly to its autoregulation (3). For the regulation of cytoskeletal dynamics, it has been demonstrated that PAK4 potentiated the formation of filopodia induced by the constitutively active Cdc42 (2). A constitutively active form of PAK4 (S445N) could induce cell rounding and dissolution of stress fiber (4). Overexpression of PAK4 has been reported to promote ovarian and prostatic cancers progression (5,6). In addition, a recent genome wide study of the miRNA expression profile in HCC has identified miR-199a/b-3p, which targeted PAK4 to promote hepatocarcinogenesis (7). However, the detail molecular mechanism by which PAK4 promotes cancer formation remains largely unknown.

In this study, we evaluated the PAK4 mRNA and protein expression levels in paired clinical HCC samples. With the availability of an exon 2-deleted PAK4 isoform (PAK4β), we delineated a nuclear exporting signal that dictates the localization of PAK4. To understand the mechanism by which PAK4 exerts on carcinogenesis, we demonstrated that p53 was a direct substrate of PAK4 and the phosphorylation of p53 by PAK4 not only inhibits the p53 activity, but also promotes cell migration. Taken together, our data has revealed a novel mechanism by which PAK4 enhances HCC metastasis by phosphorylation of p53.

Materials and Methods

Antibodies and chemicals. Rabbit anti-phospho-p53 (S215) antibody was raised against a synthetic peptide RNTFRHpSVVVPYE (a.a.209-221) (Applied Biological Materials, Richmond, BC, Canada). Cisplatin and etoposide were from Merck KGaA (Darmstadt, Hesse, Germany). All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Cell culture Human HCC cell lines Hep3B, HepG2, HLE, Huh-7 and PLC/PRF/5, cervical adenocarcinoma cell line HeLa and embryonic kidney cell line HEK293T were purchased from the American Type Culture Collection (Manassas, VA, USA). The authentication of these cell lines was assured by the provider by cytogenetic analysis. Human HCC cell lines H2M and H2P were from Guan XY, HKU. MHCC97L cell line was from Liver Cancer Institute, Fudan University, China. SMMC-7721, Bel-7402 and LO2 cell lines were gifts from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All cell lines were regularly authenticated by morphologic observation under microscopy and tested for the absence of mycoplasma contamination. All cells were maintained in DMEM (GIBCO-BRL, Grand Island, NY, USA) with 10% fetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin. Stable overexpressing or knockdown clones were selected by medium with 0.8mg/ml of G418 (Invitrogen, Carlsbad, CA, USA).

Reverse transcription-PCR Quantitative RT-PCR (qRT-PCR) was performed as described previously (8). The sequence of Taqman probe (Applied Biosystems, Foster City, CA, USA) for PAK4 was 5'-GCGGCGCCGAGCCGATGAGTAACCC-3'. For RT² Profiler PCR Array Human Tumor Metastasis, data was analyzed using the software at PCR Array Data Analysis Web portal (Qiagen, Germantown, MD, USA),

according to manufacturer's protocol. PCR amplification for both PAK4 and β-actin was performed at the annealing temperature of 57°C for 25 cycles.

Western Blotting, GST affinity pull-down and luciferase reporter assays The assays were performed as described previously (8). Fractionation was performed using NucBuster Protein Extraction Kit (Novagen, Madison, WI, USA) according to manufacturer's protocol.

Co-immunoprecipitation Procedure was described previously (8). Cells were lysed with NETN buffer with protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Antibodies used for immunoprecipitation and immunoblotting were indicated in the figures.

Colony formation, Transwell migration, Matrigel invasion and wound healing assays The assays were performed as described previously (8). For wound healing assay, cells were pre-treated with 10μg/ml mitomycin C for 3 hours. Three independent experiments were performed for each of the assays.

Confocal microscopy. Procedure was described previously (8). Images were captured by Carl Zeiss LSM 510 microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA) at the Faculty Core Facility, HKU.

Immunohistochemistry (IHC) staining. The IHC staining for PAK4 (1:100 dilution) and phospho-PAK4 (S474) (1:50 dilution) was performed as described previously (8).

Electrophoretic mobility shift assay (EMSA). Nuclear extract was prepared as described previously with some modifications (9). The nuclear pellet was extracted with cold buffer (20mM HEPES, pH 7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT and protease inhibitor cocktail). The nuclear extract was incubated with the ^{32}P-labeled oligonucleotide encoding the p53 consensus DNA binding site (5'-TACAGAACATGTCTAAGCATGCTGGGGACT-3') (Santa Cruz Biotechnology,

Dallas, TX, USA). Competition for p53 DNA binding was performed with unlabeled (X100) or mutant oligonucleotides (5'-TACAGAATCGCTCTAAGCATGCTGGGGACT-3'). The protein-DNA complexes were separated by electrophoresis through a 5% native polyacrylamide gel.

***In vitro* kinase assay.** The assay was performed as described previously (8). The kinase reaction was performed at 30°C for 20 minutes with γ-^{32}P-ATP.

Statistical analyses. Student *t* test and χ^2 or Fisher's exact test analyzed by SPSS 18 (SPSS Inc, Chicago, IL, USA) were used as appropriate. Tests were considered significant when $P < 0.05$.

Results

PAK4 was frequently overexpressed in HCC tumor tissue samples. PAK4 overexpression has been reported in various cancerous cell lines (10,11). However, little is known about the expression status of PAK4 in primary cancer. To examine the expression of PAK4 in HCC, the PAK4 transcript in HCC and its corresponding nontumorous liver tissues was determined by qRT-PCR. After normalization with 18S rRNA, 60.3% (35/59 cases) of the HCC had a higher expression of PAK4 (T/NT > 2) compared with the corresponding nontumorous tissues ($P < 0.0001$, Wilcoxon matched pair test; Fig. 1A). To corroborate the upregulation of PAK4 protein in HCCs, we examined the PAK4 protein expression by Western blotting. Overexpression of PAK4 protein (T/NT > 2) was observed in the HCC cases with PAK4 transcript upregulation, whereas PAK4 transcript that had a similar expression level (Case 211) showed no significant difference in the PAK4 protein level (Fig. 1B). To further confirm the overexpression of PAK4 in tumor cells, IHC staining was performed using total and phospho-specific (serine-474) anti-PAK4 antibodies. Interestingly,

strong cytoplasmic staining was observed for total PAK4 and strong nuclear staining was observed for p-PAK4 (S474) in HCC cells (Fig. 1C), indicating that active form of PAK4 preferentially localizes to the nuclei of HCC cells.

Overexpression of PAK4 transcript was associated with more aggressive tumor behavior. To understand how PAK4 expression is related to HCC, clinicopathological association analysis was performed. Overexpression of PAK4 was significantly correlated with the presence of venous invasion, liver invasion, poorer tumor cell differentiation (classified according to Edmondson grading) and the late pTMN stage, suggesting a more aggressive tumor behavior and a higher incidence of metastasis (Table 1). However, no significant correlation was found between PAK4 overexpression with patient survival rates by the log-rank test (data not shown).

Overexpression of PAK4 promoted HCC cell migration. To study the functional role of PAK4 in HCC, two stable PAK4 overexpressing clones (PAK4-C1 and -C2) and two vector control clones (GFP-C1 and -C2) were established in the HCC cell line SMMC-7721, which has a low endogenous level of PAK4 (Fig. 5D). After confirming the stable expression of PAK4 (Fig. 2A, top panel), the cell proliferation rate of stable PAK4 overexpressing clones were compared with vector controls, but no significant difference was observed (Supp. Fig. 1B). Interestingly, we observed that stable expression of PAK4 induced a morphological change, resulting in elongated and accumulated spiky structures at the cell peripheries (Fig. 2A). To address whether the stable PAK4 overexpressing clones have an increased motility, Transwell migration and wound healing assays were performed. In the Transwell migration assay, the number of migrated cells in stable PAK4 overexpressing clones was remarkably increased compared with the control clones (Fig. 2B). Consistently, in the wound healing assay, wound closure of the stable PAK4 overexpressing clones

was significantly faster than that of the control clones (Fig. 2C). To assess the invasiveness of stable PAK4 overexpressing clones, Matrigel invasion assay was performed. As shown in Fig. 2D, the number of invaded cells in stable PAK4 overexpressing clones was significantly higher than that of the control clones. Overall, these results indicated that PAK4 not only promotes HCC cell motility, but also enhances the invasiveness of HCC cells.

Knockdown of PAK4 inhibited HCC cell migration. To further confirm the effect of PAK4 on HCC cell motility, we adopted a loss-of function approach to specifically knockdown PAK4 using the vector-based short hairpin RNA (shRNA). The HCC cell line, HLE, was chosen for the establishment of stable PAK4 knockdown clones due to its high endogenous PAK4 protein level (Fig. 5D). Consistently, the migration rate measured by Transwell migration assay was significantly decreased in the stable PAK4 knockdown clones (Fig. 2E) while no significant differences were observed for the proliferation rate (data not shown).

Exon 2 conferred cytoplasmic localization of PAK4. A recent study has shown that another Group II PAK family member PAK5 contains a nuclear exporting signal (NES), which may have an important role in the anti-apoptotic activity of PAK5 (12). In the IHC staining, predominant nuclear staining of phospho-PAK4 was observed in the HCC cells. Moreover, during the cloning of PAK4, we obtained from Japan Kazusa DNA Research Institute an isoform of PAK4, in which the complete exon 2 (a.a.69-221) was missing. Hence, we designated this form as PAK4β and the previous full-length form of PAK4 as PAK4α in the remaining discussion. To detect the existence of PAK4β, RT-PCR were performed on an immortalized hepatocyte cell line (LO2) and a panel of HCC cell lines using a pair of PCR primers derived from exon 1 and 3 sequences. The result showed that the transcripts of both PAK4α and β

were amplified in all the HCC cell lines examined (Supp. Fig. 1A). PAK4β protein was also detectable in all the HCC cell lines tested except LO2 and H2M by Western blotting (Fig. 5D), confirming the presence of PAK4β in HCC cells. When we studied the subcellular localization of PAK4, GFP-tagged PAK4α was found mainly localized in the cytoplasm, whereas PAK4β was localized in the nuclei (Fig. 3A). Treatment of cells with leptomycin B (LMB), a specific inhibitor for the nuclear export machinery, caused the cytoplasmic PAK4α to translocate into nuclei (Fig. 3A). Such effect was not cell-line specific because similar translocation pattern was also observed in other HCC cells, HLE and SMMC-7721 (data not shown). To further confirm the subcellular localization of endogenous PAK4, we performed cellular fractionation assay. PAK4α was mainly found in the cytoplasmic fraction while the nuclear fraction contained a shorter polypeptide, corresponding to the size of PAK4β, which was also recognized by the polyclonal anti-PAK4 antibody targeting the N-terminal of PAK4 (Supp. Fig. 2A). In addition, the constitutively active form of PAK4α (S445N) also translocated to the nuclei in the presence of LMB (Fig. 3B), indicating that the NES signal operated effectively in activated PAK4. To identify the NES sequence, we subjected the amino acid sequence of PAK4α to the NES search engine, PATTINPROT. Six potential NES sequences were identified, but only one locates in the exon 2, corresponding to the a.a.82-94 of PAK4α (Supp. Fig. 2B). To consolidate the autonomy of NES sequence, we fused the NES sequence to the C-terminal of GFP protein. The fusion protein was observed to solely localize in the cytoplasm and treatment with LMB induced re-localization of the fusion protein throughout the whole cell (Supp. Fig. 2C), suggesting that a.a.82-94 is a functional NES sequence. The critical residues for the NES signal were further characterized by mutating the conserved residues lysine 82 (L82) and valine 94 (V94) to alanine. The result showed

that single substitution of L82A or V94A had a limited effect on the re-localization of the mutant proteins to the nuclei (Fig. 3C). However, substitutions of both L82 and V94 to alanine (PAK4 LVAA) completely abolished the cytoplasmic localization pattern (Fig. 3C). Furthermore, the fractionation assay confirmed that the PAK4 LVAA was found in the nucleus, whereas the PAK4α WT was mainly localized in the cytoplasm (Fig. 3D).

PAK4 interacted with p53 and phosphorylated p53 at serine 215. A recent study using PAK chemical inhibitor PF-3758309 demonstrated that PAK4 may act upstream of p53 signaling, but the detailed mechanism remains elusive (13). To investigate whether p53 is a substrate of PAK4, *in vitro* PAK4 kinase assay was performed. As shown in Fig. 4A, both WT and a serine-15-to-alanine (S15A) mutant of GST-fused p53 (GST-p53) were phosphorylated to a similar extent by the bacterially purified His-tagged PAK4α (His-PAK4), indicating that p53 is a direct phosphorylation substrate of PAK4, but PAK4 phosphorylation of p53 did not occur at S15, which is important for the regulation of p53 protein stability (14). The specificity of PAK4 kinase activity was demonstrated by the phosphorylation of a specific substrate control, GST-PAK4tide (Fig. 4A) (8). To map the PAK4 phosphorylation site on p53, we generated three truncation mutants of p53, namely M1, M2 and M3, according to the transactivation (TAD), DNA binding (DBD) and C-terminal regulatory (CTD) domains of p53 (Fig. 4B). Although the construct of truncation mutant carrying a.a.1-92 had been generated, the expressed protein was completely insoluble during purification. Hence, the three purified truncation mutants were used in the PAK4 kinase assay. As shown in Fig. 4C, His-PAK4 phosphorylated significantly the mutants M1 and M2, but not M3, suggesting that the PAK4 phosphorylation site(s) may be located in the p53 DBD. Using GST affinity pull-down assay, we showed that

M2, but not M3, directly associated with PAK4 with a high affinity (Fig. 4D). To answer whether PAK4 and p53 interact intracellularly, co-immunoprecipitation was performed. As expected, p53 WT and M2 were detected in the PAK4α immunoprecipitate (Fig. 4E). Interestingly, we observed that the PAK4 LVAA revealed a stronger interaction with p53 WT and M2 (Fig. 4E, lanes 15 and 16, respectively) than PAK4α. Similar results were also obtained in the reciprocal co-immunoprecipitation (Fig. 4E, lanes 24 to 27). Based on the PAK4 phosphorylation site consensus sequence: R-X-S-W/I/V/Y-A/Y-S (R: arginine; X: any; S: serine; W/I/V/Y: tryptophan/isoleucine/valine/tyrosine; A: alanine) (15), a potential PAK4 phosphorylation site was identified at the serine residue 215 (S215) within the DBD, which is also a phosphorylation site for Aurora kinase A (16). To verify the S215 phosphorylation by PAK4, we generated a non-phosphorylatable mutant by substituting S215 with an alanine residue (S215A) and performed the *in vitro* kinase assay. While the p53 WT was significantly phosphorylated by PAK4α and Aurora kinase A, mutation of S215 completely abolished the phosphorylation (Fig. 4F), strongly indicating that the S215 is a putative PAK4 phosphorylation site.

PAK4 phosphorylated the S215 site of p53 within the cells. To elucidate whether the S215 phosphorylation occurs endogenously, we co-transfected the expression constructs of Myc-p53 WT or S215A mutant together with PAK4α WT or LVAA mutant into HCC cells. After immunoprecipitation, their phosphorylation status was examined using an anti-phospho-serine antibody. Compared with the vector control, ectopic expression of PAK4α WT and LVAA could significantly increase the phosphorylation of p53 by 3.0 and 16.3 folds, respectively (Fig. 5A), whereas the signal was completely abolished on the p53 S215A mutant even in the presence of PAK4α WT or LVAA, confirming that S215 is an intracellular PAK4

phosphorylation site. To specifically detect the phosphorylation of S215, a phospho-specific antibody was raised. The specificity of the anti-p-p53 (S215) antibody was verified by *in vitro* PAK4 kinase assay. As shown in Fig. 5B, WT but not the S215A mutant of p53 was recognized by the anti-p-p53 (S215) antibody incubated with His-PAK4α (lanes 3 and 5, respectively) or His-Aurora kinase A (lanes 7 and 8, respectively). Next, we sought to detect the endogenous phosphorylation of p53 by transfecting SMMC-7721 cells with constructs expressing p53 WT or S215A together with PAK4α. The anti-p-p53 (S215) antibody only detected the signals in cells co-expressing PAK4α and p53 WT, but not in cells co-expressing the S215A mutant (Fig. 5C). Using anti-p-p53 (S215) antibody, we detected strong S215 phosphorylation signal in HCC cell lines with high endogenous PAK4 expression, including HepG2, HLE, H2P and MHCC97L (Fig. 5D). However, silencing of PAK4 expression by stably shRNA or transiently siRNA in knockdown HLE cells remarkably lowered the S215 phosphorylation signal (Fig. 5E and Supp. Fig. 3, respectively), strongly suggesting that the S215 of p53 is an endogenous PAK4 phosphorylation site.

PAK4 phosphorylation attenuated p53 tumor suppressive, transactivating and DNA binding activities. To evaluate whether PAK4 phosphorylation on p53 affects cell growth, colony formation assay was performed. As shown in Fig. 6A, a significant increase in the number of colonies was found in cells transfected with the S215D mutant compared with WT or S215A mutant, suggesting that phosphorylation of S215 attenuates p53 growth suppressor activity. To examine if PAK4 phosphorylation has an impact on the transactivating activity of p53, p53-responsive luciferase reporter assays were performed. While co-transfecting with PAK4α significantly inhibited the transactivating activity of p53 WT in a dose-dependent

manner, similar inhibition was not observed in the S215A and S215D mutants, and the S215D mutation led to a significant reduction in p53 transactivating activity (Fig. 6B). To further evaluate if nuclear localization of PAK4 is essential for the inhibition of p53 activity, PAK4 LVAA was co-transfected with p53-responsive luciferase reporter into HCC cells. The PAK4 LVAA exhibited a much potent inhibitory effect on p53 transactivating activity compared with WT (Fig. 6C), suggesting that the nuclear localization of PAK4 augmented the inhibition of p53 activity. Then, we wondered if the phosphorylation of S215, which located within the p53 DBD domain, would affect the DNA binding activity of p53. Using the EMSA, we compared the DNA binding affinity of p53 isolated from the PAK4/p53 and PAK4 LVAA/p53 co-transfected lysates. Co-transfection of PAK4 and PAK4 LVAA significantly decreased the ability of p53 to form a complex with the p53 oligonucleotides by about 10% and more than 30%, respectively (Fig. 6D, lane 5 and 6), implicating that PAK4 phosphorylation lowered the ability of p53 to bind DNA. Treatment of SMMC-7721 cells with two chemotherapeutic agents, cisplatin and etoposide, significantly suppressed the colony formation and upregulated $p21^{CIP1/Waf1}$ expression (Fig. 6E). However, both of these effects were alleviated by the ectopic expression of PAK4, and were completely abolished by the expression of PAK4 LVAA, without significantly affecting the p53 protein level. Since our clinicopathological features analysis indicated that overexpression of PAK4 was associated with a more metastatic phenotype, we sought to examine the impact of p53 phosphorylation on cancer metastasis. Stable p53 WT, S215A and S215D mutants overexpressing clones were established with SMMC-7721 cells, and were confirmed by Western blot (Fig. 6F). Using Transwell migration (Fig. 6F) and Matrigel invasion (Fig. 6G) assays, we demonstrated that overexpression of WT and S215A significantly reduced the number

of migratory and invasive cells, whereas the S215D mutant had negligible effects. However, transient transfection of PAK4α into the stable p53 WT clone could partially rescue the migration suppressive effect of p53 (Fig. 6H), whereas the S215A mutant could not be reversed by PAK4α, indicating that p53 could suppress HCC cell invasion but was abolished by PAK4 phosphorylation. Similar phenomenon was also observed in PAK4 knockdown HLE cells. Transiently expression of the S215A mutant, but not WT, significantly suppressed the vector clone migration, whereas expression of WT and S215A showed no significant difference on cell migration in stable PAK4 knockdown clones, suggesting that the p53-mediated inhibition of cell migration is PAK4 and S215 phosphorylation dependent (Suppl. Fig. 4A).

To search the genes responsible for the attenuation of cell migration and invasion exerted by p53, a qRT-PCR array was performed using the RNA extracted from the stable p53 overexpressing cells. Among the 84 metastasis-related genes screened, the transcript level of three metastasis suppressors, cadherin 6 (CDH6), cyclin-dependent kinase inhibitor 2A (CDKN2A) and kisspeptin-1 receptor (KISS1R), was significantly downregulated in the S215D mutant compared with WT and S215A mutant cells, whereas two metastasis enhancers, fibroblast growth factor receptor 4 (FGFR4) and vascular endothelial growth factor A (VEGFA), were significantly upregulated in the S215D mutant cells (Fig. 6I). Consistently, the protein levels of the three metastasis suppressors were downregulated in the stable S215D mutant compared with WT and S215A mutant cells (Fig. 6J), indicating that p53 may suppress cancer metastasis via regulating specific metastasis-related genes. In the high endogenous PAK4 expressing HCC cell lines, at least one, if not all, of these metastatic suppressors were observed to be differentially expressed, supporting the

notion that the regulation of p53 by PAK4 is relevant to cancer metastasis (Supp. Fig. 4B).

Discussion

Overexpression of PAK4 has been demonstrated in various cancer cell lines and clinical samples, such as ovarian and gastric cancers (6,10,17). Although evidences have shown that PAK4 is involved in the hepatocyte growth factor (HGF), c-Src/epidermal growth factor (EGF) and Smad2/3 signaling pathways, the molecular mechanism by which PAK4 promotes cancer formation is still far from clear (6,17,18). In this study, we demonstrated that PAK4 transcript and protein are frequently overexpressed in HCCs (Fig. 1). Clinicopathological analysis revealed that overexpression of PAK4 mRNA is significantly associated with poorer prognosis with high incidence of metastasis (Table 1). Consistently, we observed that stable PAK4 overexpression in HCC cells induces morphological changes that are likely to be related to the aberrant regulation of actin cytoskeletal structure, as illustrated by the spiky structures (Fig. 2A, bottom panel). This observation complements and is consistent with the findings reported by Qu *et al.*, who demonstrated that expression of a constitutively active PAK4 induces cell rounding and poor adhesion of NIH3T3 cells (19). The effect of PAK4 on HCC cell migration and invasiveness was further confirmed by Transwell migration, wound healing and invasion assays (Fig. 2B-D). Complementary to the overexpression, stable knockdown of PAK4 resulted in a decrease in the cell mobility (Fig. 2E).

During the cloning of PAK4, we characterized an exon 2-spliced isoform of PAK4 (PAK4β), which exhibits nuclear localization, in contrast to the cytoplasmic localization of wildtype PAK4 (PAK4α) (Fig. 3A). The transcript and protein of

PAK4β can be observed in a number of HCC cell lines (Supp. Fig. 1A and 5D, respectively). Using site-directed mutagenesis, we validated a NES signal located at exon 2 and that is important for the nucleo-cytoplasmic shuttling of PAK4α (Fig. 3C). Similar finding was reported in a recent study showing that PAK4 is a nucleo-cytoplasmic shuttling protein (20). Surprisingly, we noticed that although the IHC staining for total PAK4 was mainly localized in cytoplasm, staining for phospho-PAK4 was mainly in nuclei (Fig. 1C). This result indicated that a subset of phosphorylated and potentially activated PAK4 proteins are preferentially translocated to the nuclei of HCC cells, but the reason for that remains unknown. It is unlikely that the phospho-PAK4 antibody cross-reacted with other Group II PAK family members, i.e. PAK5 and PAK6, because both PAK5 and PAK6 expression levels were relatively low in the HCC cases examined. Since the S445 site is conserved in PAK4β form, we cannot really distinguish whether the nuclear signal of phospho-PAK4 is specifically due to the presence of PAK4β form in HCC cells. Currently, it still remains unclear whether the PAK4β isoform is exclusively expressed in HCC or the nuclear phospho-PAK4 represents a pathological species of PAK4 with specific modification. Since the constitutively active mutant of PAK4 (S445N) did not confer a nuclear localization pattern of PAK4 protein, the possibility that the observed phospho-PAK4 staining in HCC tissue represents a form that is recruited by other protein partners or altered by other modifications cannot be completely ruled out. Such translocation upon activation has been clearly demonstrated by another member of the Group II PAKs PAK6 (21). While the importance of nuclear localization of PAK4 is still under speculation, the significance of nuclear localization of PAK1 has previously been implicated in breast tumor

formation during the transition from ductal hyperplasia to ductal carcinoma *in situ* and then to adenocarcinoma in transgenic mice (22).

Despite mounting evidence pointing to a role of PAK4 in tumorigenesis, it remains unclear how PAK4 overexpression causes carcinogenesis. Recent evidence implicates a link between PAK4 and p53 signaling (13), but the detail mechanism for the regulation of p53 by PAK4 is largely unknown. Here we provided evidence to support that PAK4 phosphorylates and inactivates p53 directly to promote HCC metastasis. By means of *in vitro* kinase, GST affinity pull-down, co-immunoprecipitation and phospho-specific antibody detection assays (Fig. 4C-F and Fig. 5), we demonstrated that PAK4 phosphorylates p53 at serine 215 intracellularly. Using wildtype and a nuclear form of PAK4 (PAK4 LVAA), we further demonstrated that S215 phosphorylation of p53 significantly diminishes the DNA binding, transactivating and tumor suppressive activities of p53 (Fig. 6A-D), suggesting that PAK4 is an inhibitory kinase of p53. Given that PAK4 overexpression is associated with more metastatic phenotype in clinicopathological analysis, we hypothesized that PAK4 phosphorylation of p53 may also play a role in cancer metastasis. Using the stable PAK4-phosphorylation defective (S215A) and mimetic (S215D) mutants of p53 overexpressing clones, we demonstrated that p53 is able to suppress HCC cell migration and invasion, but more surprisingly the S215 phosphorylation of p53 completely reverts the effect on the suppression of HCC cell migration and invasion (Fig. 6F and G). These effects have been observed in at least two HCC cell lines, i.e. SMMC-7721 and HLE (Supp. Fig. 4A), indicating that the regulation of p53 by PAK4 is not cell-line specific. Subsequent analysis using a qRT-PCR array of 84 known cancer metastasis-related genes showed that p53 S215D, compared with WT, specifically enhances the regulation of several previously uncharacterized p53-

regulated metastasis genes, including CDH6, CDKN2A, KISS1R, FGFR4 and VEGFA, indicating a gain-of-function effect of p53 by PAK4 phosphorylation. It has been reported that the S215 site is targeted by Aurora kinase A to inactivate p53 (16). A recent study also showed that Aurora kinase B targets the same site to enhance the degradation of p53 (23). Currently, it is unclear if PAK4 and Aurora kinases will compete with each other for the same site to regulate p53. However, our existing data revealed a novel role of p53 in suppressing HCC cell migration and that PAK4 can moderate the suppressive effect of p53 through S215 phosphorylation. We believe that it will be of interest to explore the interplay between different p53 mutations and S215 phosphorylation on the ability to suppress HCC cell migration. Further investigation on whether S215 phosphorylation coordinates the cell cycle progression and cell migration is currently undergoing.

We observed that forced expression of PAK4α and PAK4 LVAA protects HCC cells from DNA damaging drug-induced cell death (Fig. 6E). Although the detailed mechanism for such effect remains to be determined, the fact that PAK4 acts as a negative regulator of p53 may present an exciting opportunity to enhance the chemo-sensitivity of HCC cells using PAK4 inhibitor in single or combined chemo-treatment. In summary, we demonstrated the oncogenic properties of PAK4 in HCC and provided the first evidence to support an important role of p53 phosphorylation in PAK4-mediated cancer metastasis.

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Figure legends

Figure 1. Overexpression of PAK4 in HCCs. (A) qRT-PCR results of HCC (T) and corresponding nontumorous (NT) liver tissues (59 pairs, $P < 0.0001$, Wilcoxon matched pair test). Horizontal lines represent medians. (B) Representative results of Western blotting analysis. Numbers indicate the fold of relative expression (T/NT). (C) Representative cases of immunohistochemical staining for PAK4 and phospho-PAK4 (S474) in HCC (T) and corresponding nontumorous (NT) liver tissues.

Figure 2. Promotion of HCC cell migration and invasion by PAK4. (A) PAK4 expression in stable PAK4 overexpressing (PAK4-C1 and -C2) and GFP control (GFP-C1 and -C2) SMMC-7721 cells was detected by immunoblotting. (B) Stable PAK4 and GFP clones were used for Transwell migration, (C) wounding healing and (D) invasion assays. In (B) and (D), $*P < 0.05$, $\#P < 0.05$. In (C), $*P < 0.04$, $\#P < 0.04$. (E) Stable PAK4 knockdown (Sh-C1 and -C2) and vector control (V-C1 and -C2) cells were established in HLE cells and used for Transwell migration assay. Insets: Western blotting analysis. $*P < 0.02$; $\#P < 0.05$. For (B) to (E), one of three independent experiments was shown. Scale bar = 100μm. Error bars, mean ± SD of samples. *t* test compared with GFP-C1/V-C1(*) or GFP-C2/V-C2 (#).

Figure 3. Nuclear localization of PAK4. (A) HeLa cells transfected with constructs expressing GFP-PAK4α or GFP-PAK4β were treated with vehicle (Veh) or leptomycin B (LMB; 15nM) for 5 hours. Localization of PAK4α or PAK4β was identified by the presence of GFP signal (green). Nuclei (blue) were stained with DAPI. Scale bar = 20μm. (B) Similar to (A), but transfected with constructs expressing GFP-PAK4α or S445N mutant. (C) Similar to (A), but transfected with constructs expressing GFP-PAK4α mutants of L82A, V94A or LVAA. (D) HeLa cells were transfected with constructs expressing PAK4α or PAK4 LVAA. The

cytoplasmic (Cyto) and nuclear (Nu) fractions were extracted for Western blotting analysis.

Figure 4. Phosphorylation of p53 by PAK4. (A) GST-p53 WT and S215A were phosphorylated by His-PAK4α in *in vitro* kinase assay. GST and GST-PAK4tide were used as negative and positive controls, respectively. Autoradiography and Coomassie blue staining gel (for protein loading) were shown. (B) Schematic diagram of p53 and its truncation mutants. TAD: transactivation domain; DBD, DNA-binding domain; 4D, tetramerization domain; CTD, C-terminal regulatory domain. (C) Similar to (A), WT or mutants of GST-p53 were phosphorylated by His-PAK4α. Autoradiography and Coomassie blue staining gel were shown. Asterisks indicate corresponding positions of proteins. (D) WT or mutants of GST-p53 and His-PAK4α were used for GST affinity pull-down assay. The proteins were detected by immunoblotting. (E) HEK293T cells co-transfected with constructs expressing PAK4α or LVAA mutant and WT or mutant of p53 were harvested for immunoprecipitation with anti-c-Myc agarose conjugate or anti-GFP protein-G Sepharose, and immunoblotting. ms, mouse. rb, rabbit. (F) GST-p53 WT or S215A was phosphorylated by His-Aurora A or His-PAK4α in the *in vitro* kinase assay. Autoradiography and Coomassie blue staining gel were shown.

Figure 5. Phosphorylation of p53 at the S215 site by PAK4. (A) SMMC-7721 cells transfected with constructs expressing PAK4α or LVAA mutant together with p53 WT or S215A mutant were harvested for immunoprecipitation and Western blotting analysis using the anti-phospho-serine and anti-Myc antibodies. Band intensity: p-serine/total immunoprecipitated p53. Input lysates were analyzed by immunoblotting. ms, mouse. rb, rabbit. (B) GST-p53 WT or its mutant carrying serine-to-alanine substitution at S215 was phosphorylated by His-Aurora A or His-PAK4α in the *in*

vitro kinase assay. Western blotting analysis was performed. (C) SMMC-7721 cells transfected with constructs expressing PAK4α together with p53 WT or S215A mutant were harvested for Western blotting with anti-phospho-p53 (S215) and other antibodies. The asterisk indicates the p-p53 (S215) protein. Band intensity: p-p53/total GFP-p53. Intensity in lane 1 was set as 1. (D) Lysates from HCC, LO2 and 293T cells were used for Western blotting analysis with indicated antibodies. The graph shows the relative level of PAK4α normalized with β-actin. Level in LO2 was set as 1. Lysates from 293T cells transfected with constructs expressing Myc-PAK4α or Myc-PAKβ were used as positive controls. (E) Stable PAK4 knockdown (Sh-C1 and -C2) and vector control (V-C2) HLE cells were harvested for Western blotting analysis. Band intensity: p-p53/total p53. Intensity in V-C2 was set as 1.

Figure 6. Regulation of p53 activity by PAK4 phosphorylation. (A) SMMC-7721 cells were transfected with constructs expressing p53 WT or mutants for colony formation assay. Insets: Western blotting analysis. Vector [pcDNA3.1 (+)] and mock transfection (MT) controls were indicated. $*P < 0.02$ (*t* test) compared with vector control. (B) SMMC-7721 cells were co-transfected with p53-responsive luciferase reporter and constructs expressing p53 WT, S215A or S215D mutant together with an increasing amount of PAK4α constructs for luciferase reporter assay. Vector and p53 only were used as negative and positive controls, respectively. Insets: Western blotting analysis. $*P < 0.04$, $**P < 0.003$, $***P < 0.002$ (*t* test) compared with p53 only control. (C) SMMC-7721 cells were transfected with p53-responsive luciferase reporter together with an increasing amount of PAK4α or LVAA mutant constructs for luciferase reporter assay. Vector (pEGFP-C1) was used as negative control. Insets: Western blotting analysis for protein expression. $*P < 0.05$, $**P < 0.02$ (*t* test) compared with vector control. (D) Top: HEK293T cells were transfected with

constructs of p53 and PAK4α or LVAA mutant. Nuclear lysates were extracted for EMSA. Band intensity: p53/oligo complex in lanes 5 and 6 was separately compared with lane 4, which the intensity was set as 1. Bottom: Western blotting analysis. (E) SMMC-7721 cells were transfected with constructs expressing PAK4α or LVAA mutant, and treated with vehicle control (DMSO), cisplatin (CIS; 2μg/ml) or etoposide (ETO; 120μM/ml) for 16 hours. A portion of cells was used for colony formation assay. Insets: Western blotting analysis with remaining portion. Vector (pEGFP-C1) and mock transfection (MT) controls were included. *$P < 0.02$, **$P < 0.002$ (t test) compared with vector control under DMSO treatment. (F) p53 expression in stable p53 WT, S215A and S215D overexpressing, and GFP control SMMC-7721 cells was detected by Western blotting analysis. Stable p53 WT, S215A and S215D overexpressing, and GFP control clones were used for Transwell migration assay, and (G) invasion assay. *$P < 0.0005$ (t test) compared with GFP; #$P < 0.003$ with WT or S215A. (H) Stable p53 WT and S215A overexpressing, and GFP control clones transiently transfected with constructs expressing PAK4α were used for Transwell migration assay. Insets: Western blotting analysis. *$P < 0.0002$ (t test) compared with GFP. Scale bar = 100μm. Error bars, mean ± SD of samples. For (A) to (C) and (E) to (H), one of three independent experiments was shown. (I) PAK4 binds with p53 and phosphorylates p53 at serine 215, which enhances cancer metastasis. In the qRT-PCR array, down-regulation (level < 0.5) and up-regulation (level > 2) of transcript level of several metastasis-related genes were detected in stable p53 S215D overexpressing cells compared with stable p53 WT cells. The genes, from top to bottom, are arranged in a descending order of transcript level. *Similar level in both stable cell lines of p53 WT and S215A mutant. (J) Lysates from stable p53 WT, S215A and S215D mutant, and GFP control SMMC-7721 cells were used

for Western blotting analysis. Band intensity: CDH6/β-actin, p14ARF/β-actin or KISS1R/β-actin. Intensity in stable GFP control cells was set as 1.

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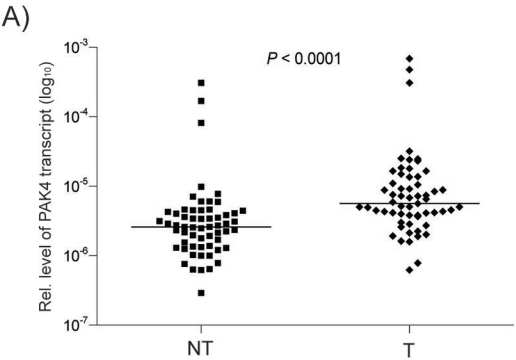
Fig. 1


A)
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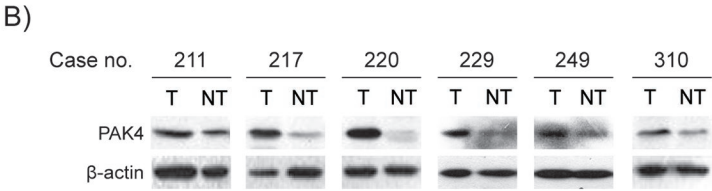
Rel. level of PAK4 transcript (log10)
10-3
10-4
10-5
10-6
10-7
NT
T


B)
Case no. 211 217 220 229 249 310
T NT T NT T NT T NT T NT T NT
PAK4
β-actin
T/NT (protein) 1.14 4.76 6.39 2.47 2.74 2.03
T/NT (mRNA) 1.14 2.15 8.92 5.13 5.40 3.58

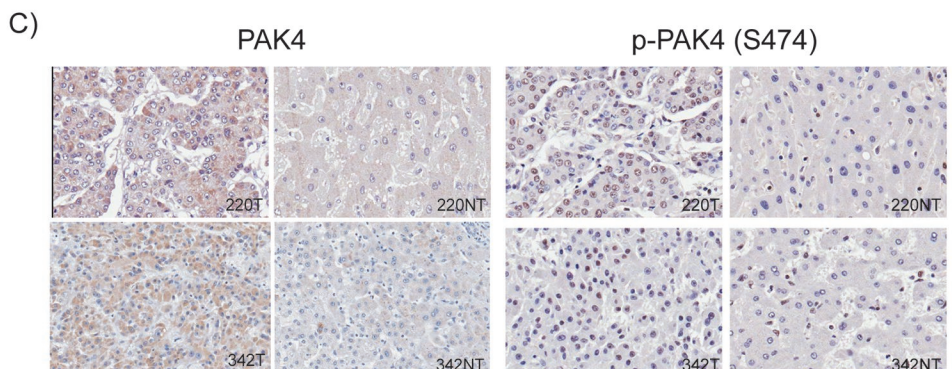

C)
PAK4
p-PAK4 (S474)
220T
220NT
220T
220NT
342T
342NT
342T
342NT

Fig. 2

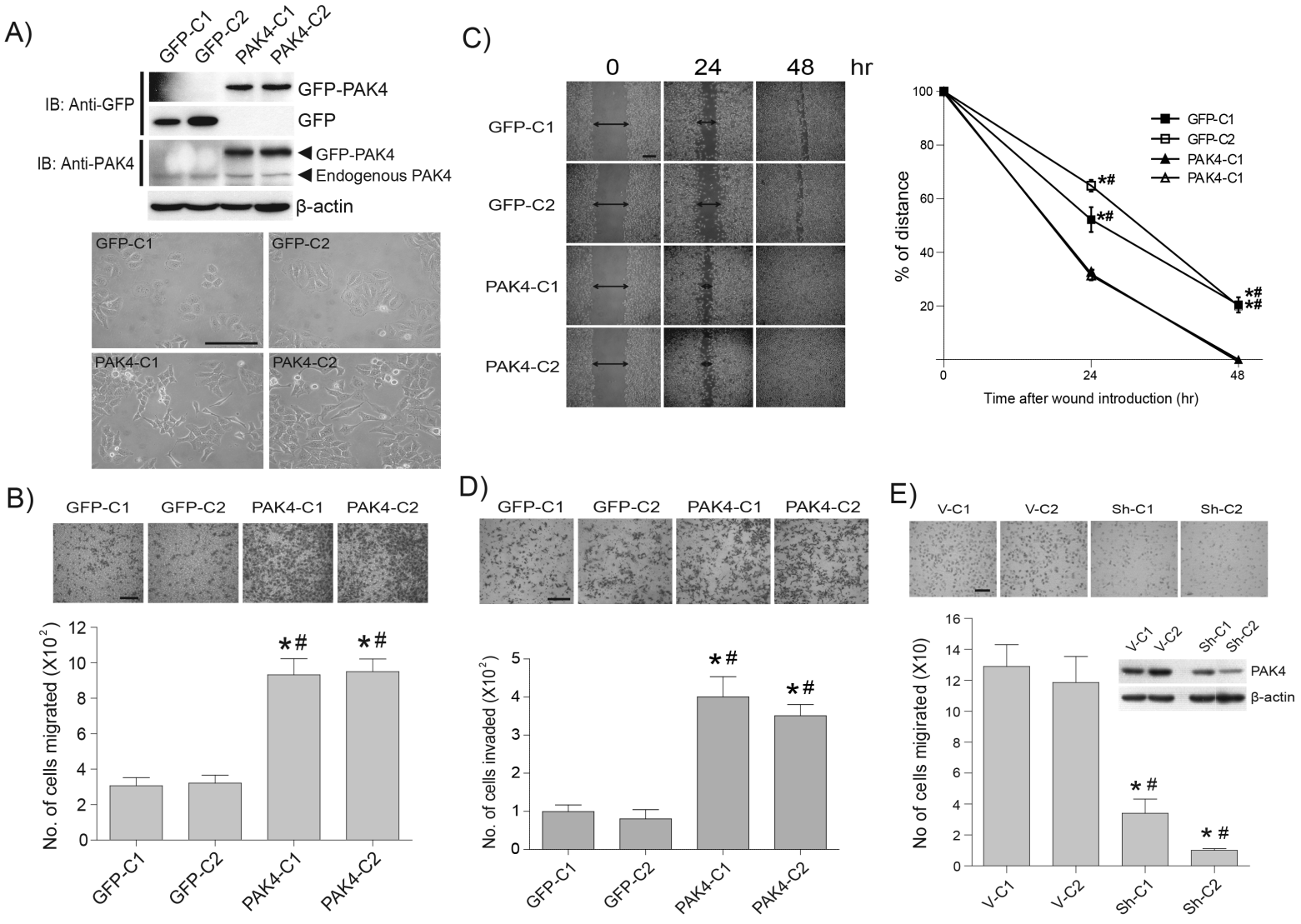

A)
GFP-C1
GFP-C2
PAK4-C1
PAK4-C2
IB: Anti-GFP
GFP-PAK4
GFP
IB: Anti-PAK4
GFP-PAK4
Endogenous PAK4
β-actin
C)
0
24
48
hr
GFP-C1
GFP-C2
PAK4-C1
PAK4-C2
% of distance
Time after wound introduction (hr)
B)
No. of cells migrated (X10²)
D)
No. of cells invaded (X10²)
E)
V-C1
V-C2
Sh-C1
Sh-C2
PAK4
β-actin
No of cells migirated (X10)

Fig. 3

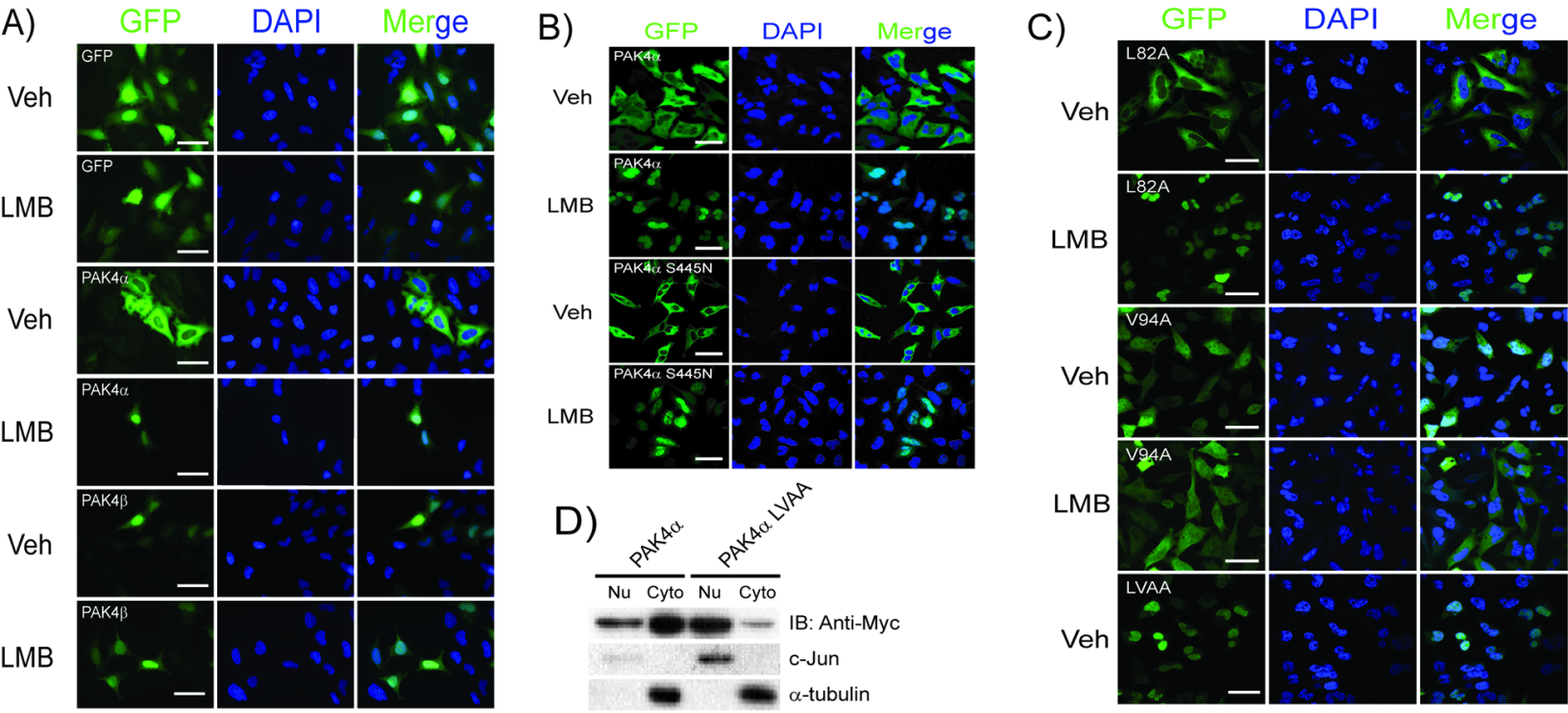

A)
GFP
DAPI
Merge
Veh
LMB
Veh
LMB
Veh
LMB
GFP
GFP
PAK4α
PAK4α
PAK4β
PAK4β
B)
GFP
DAPI
Merge
Veh
LMB
Veh
LMB
PAK4α
PAK4α
PAK4α S445N
PAK4α S445N
C)
GFP
DAPI
Merge
Veh
LMB
Veh
LMB
Veh
L82A
L82A
V94A
V94A
LVAA
D)
PAK4α
PAK4α LVAA
Nu
Cyto
Nu
Cyto
IB: Anti-Myc
c-Jun
α-tubulin

Fig. 4

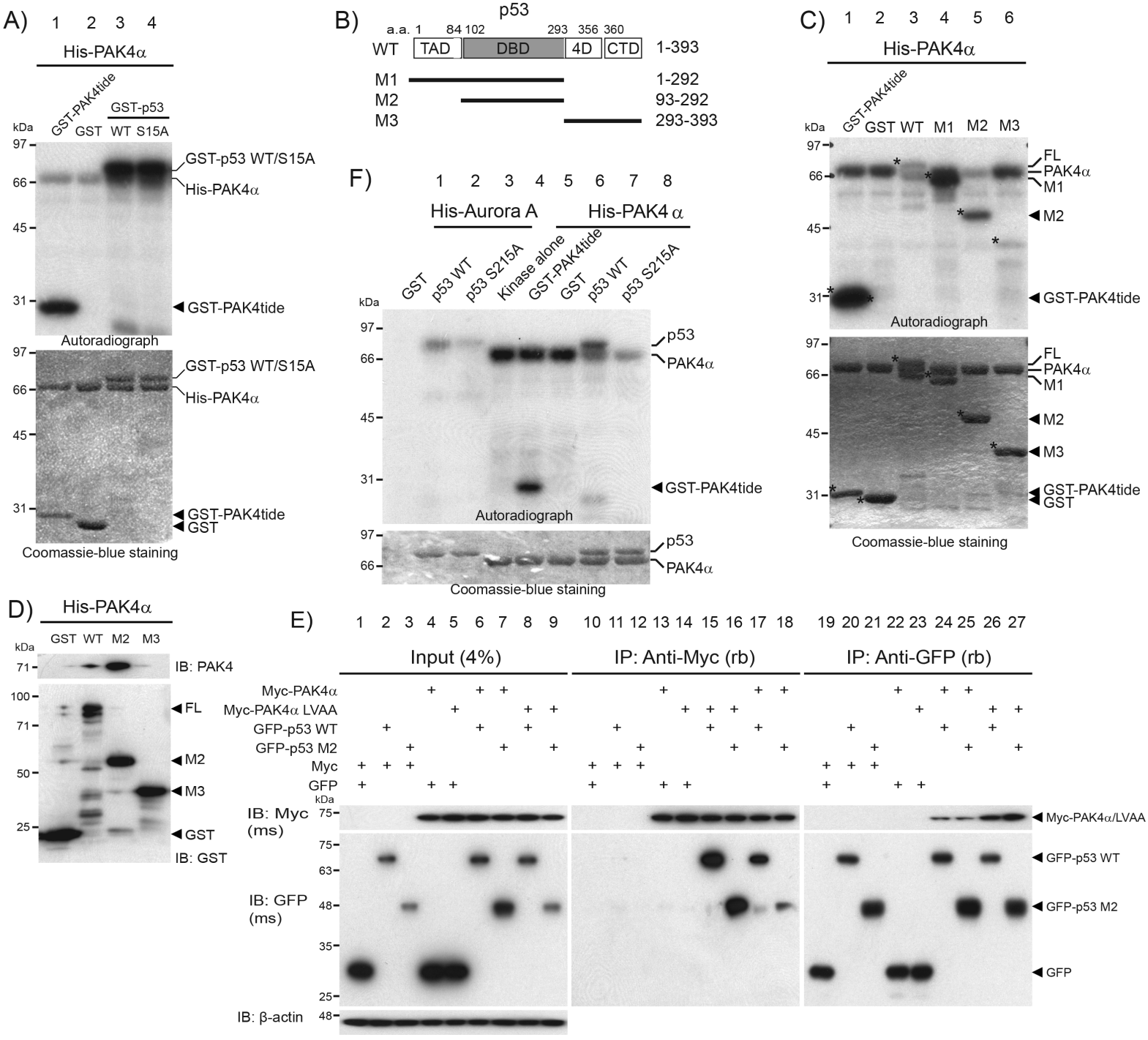

A)
1 2 3 4
His-PAK4α
GST-PAK4tide
GST-p53
kDa
GST WT S15A
97
66
45
31
GST-p53 WT/S15A
His-PAK4α
GST-PAK4tide
Autoradiograph
GST-p53 WT/S15A
His-PAK4α
GST-PAK4tide
GST
Coomassie-blue staining
B)
p53
a.a. 1 84 102 293 356 360
WT TAD DBD 4D CTD 1-393
M1 1-292
M2 93-292
M3 293-393
C)
1 2 3 4 5 6
His-PAK4α
GST-PAK4tide
kDa
GST WT M1 M2 M3
97
66
45
31
FL
PAK4α
M1
M2
GST-PAK4tide
Autoradiograph
M3
GST
Coomassie-blue staining
D)
His-PAK4α
GST WT M2 M3
kDa
71
IB: PAK4
100
71
50
25
FL
M2
M3
GST
IB: GST
E)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Input (4%)
IP: Anti-Myc (rb)
IP: Anti-GFP (rb)
Myc-PAK4α
Myc-PAK4α LVAA
GFP-p53 WT
GFP-p53 M2
Myc
GFP
kDa
IB: Myc (ms)
75
Myc-PAK4α/LVAA
IB: GFP (ms)
75
63
48
35
25
GFP-p53 WT
GFP-p53 M2
GFP
IB: β-actin
48
F)
1 2 3 4 5 6 7 8
His-Aurora A
His-PAK4 α
GST
p53 WT
p53 S215A
Kinase alone
GST-PAK4tide
GST
p53 WT
p53 S215A
kDa
97
66
45
31
p53
PAK4α
GST-PAK4tide
Autoradiograph
p53
PAK4α
Coomassie-blue staining

Fig. 5

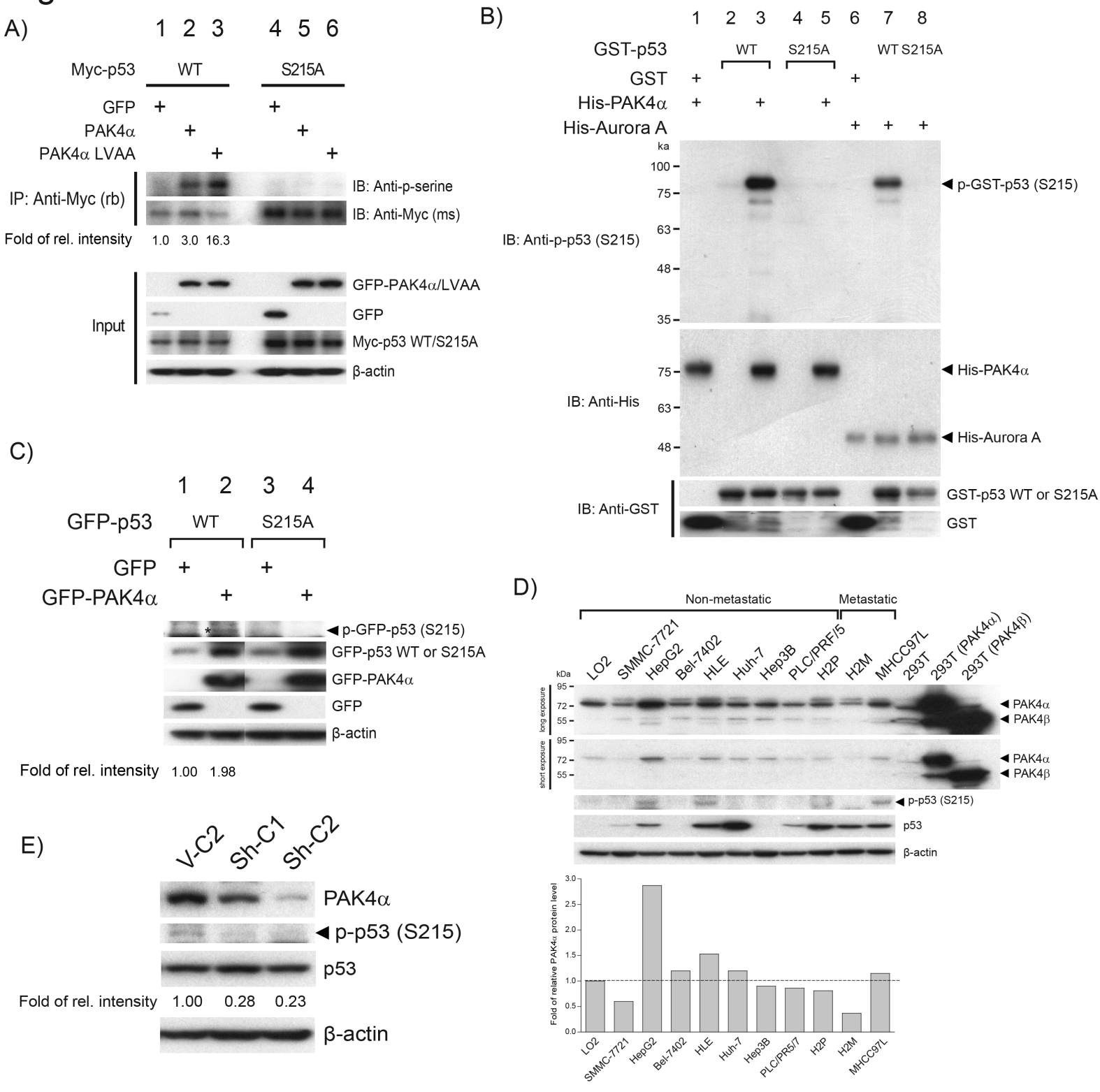

A)
1 2 3 4 5 6
Myc-p53
WT
S215A
GFP
PAK4α
PAK4α LVAA
IP: Anti-Myc (rb)
IB: Anti-p-serine
IB: Anti-Myc (ms)
Fold of rel. intensity
1.0 3.0 16.3
Input
GFP-PAK4α/LVAA
GFP
Myc-p53 WT/S215A
β-actin
B)
1 2 3 4 5 6 7 8
GST-p53
WT
S215A
WT S215A
GST
His-PAK4α
His-Aurora A
ka
100
75
63
48
35
IB: Anti-p-p53 (S215)
◀ p-GST-p53 (S215)
IB: Anti-His
◀ His-PAK4α
◀ His-Aurora A
IB: Anti-GST
GST-p53 WT or S215A
GST
C)
1 2 3 4
GFP-p53
WT
S215A
GFP
GFP-PAK4α
◀ p-GFP-p53 (S215)
GFP-p53 WT or S215A
GFP-PAK4α
GFP
β-actin
Fold of rel. intensity
1.00 1.98
D)
Non-metastatic
Metastatic
LO2
SMMC-7721
HepG2
Bel-7402
HLE
Huh-7
Hep3B
PLC/PRF/5
H2P
H2M
MHCC97L
293T
293T (PAK4α)
293T (PAK4β)
kDa
long exposure
short exposure
95
72
55
◀ PAK4α
◀ PAK4β
◀ p-p53 (S215)
p53
β-actin
Fold of relative PAK4α protein level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
E)
V-C2
Sh-C1
Sh-C2
PAK4α
◀ p-p53 (S215)
p53
Fold of rel. intensity
1.00 0.28 0.23
β-actin

Fig. 6

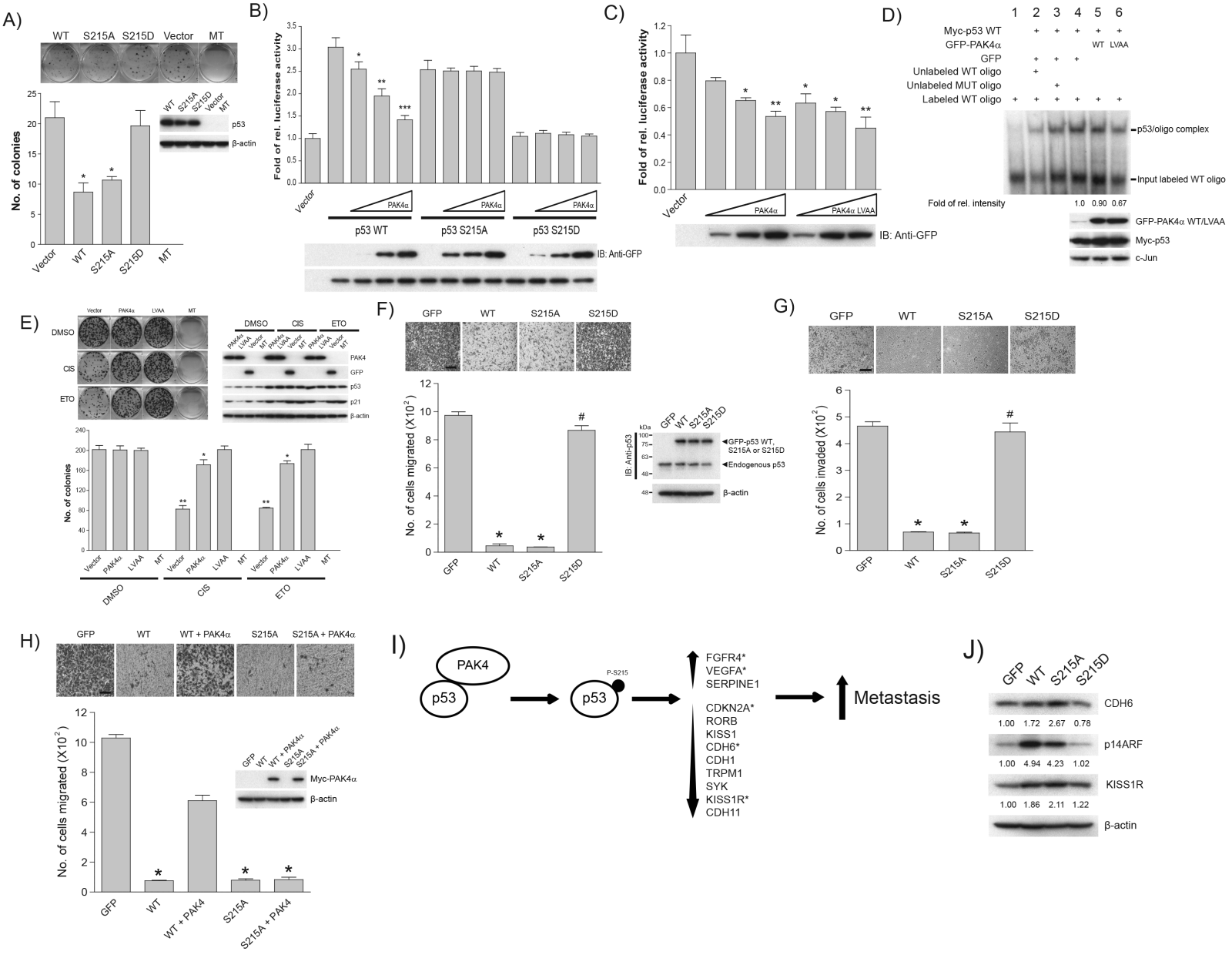

A)
WT
S215A
S215D
Vector
MT
No. of colonies
p53
β-actin
B)
Fold of rel. luciferase activity
Vector
PAK4α
p53 WT
p53 S215A
p53 S215D
IB: Anti-GFP
C)
Fold of rel. luciferase activity
PAK4α LVAA
IB: Anti-GFP
D)
Myc-p53 WT
GFP-PAK4α
GFP
Unlabeled WT oligo
Unlabeled MUT oligo
Labeled WT oligo
p53/oligo complex
Input labeled WT oligo
Fold of rel. intensity
1.0 0.90 0.67
GFP-PAK4α WT/LVAA
Myc-p53
c-Jun
E)
DMSO
CIS
ETO
PAK4
GFP
p53
p21
β-actin
No. of colonies
F)
GFP
WT
S215A
S215D
No. of cells migrated (X10²)
IB: Anti-p53
GFP-p53 WT, S215A or S215D
Endogenous p53
β-actin
G)
No. of cells invaded (X10²)
H)
WT + PAK4α
S215A + PAK4α
Myc-PAK4α
β-actin
No. of cells migrated (X10²)
I)
PAK4
p53
P-S215
FGFR4*
VEGFA*
SERPINE1
CDKN2A*
RORB
KISS1
CDH6*
CDH1
TRPM1
SYK
KISS1R*
CDH11
Metastasis
J)
CDH6
1.00 1.72 2.67 0.78
p14ARF
1.00 4.94 4.23 1.02
KISS1R
1.00 1.86 2.11 1.22
β-actin

Table 1. Clinicopathological correlation of PAK4 overexpression in human HCCs

Clinicopathological features	PAK4		*P*
	Overexpression (T/NT > 2)	No overexpression (T/NT ≤ 2)	
Sex			0.521
Male	29	18	
Female	6	6	
Venous invasion			***0.008***[#]
Absent	11	16	
Present	24	8	
Tumor microsatellites			0.072
Absent	15	16	
Present	20	8	
Direct liver invasion*^			***0.028***[#]
Absent	19	16	
Present	16	3	
Cellular differentiation (Edmondson grading)^			***0.015***[#]
I–II	10	14	
III–IV	25	9	
HBsAg^			0.322
Absent	5	6	
Present	29	17	
Tumor encapsulation^			0.050
Absent	25	10	
Present	10	12	
Tumor size (cm)			0.525
≤5	13	7	
>5	22	17	
No. of tumor nodules			0.649
Single	28	18	
Multiple	7	6	
Background liver disease			0.746
Normal and chronic hepatitis	19	12	
Cirrhosis	16	12	
Tumor pTNM stage^			***0.027***[#]
I-II	10	13	
III-IV	24	9	

*Invasion of tumor to adjacent liver parenchyma

^With cases having unavailable data

[#]*P < 0.05*