

**Differential subcellular localization and activity of kelch repeat proteins
KLHDC1 and KLHDC2**

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Abstract

We have previously identified and characterized human KLHDC2/HCLP-1, a kelch repeat protein that interacts with and inhibits transcription factor LZIP. In this study we identified and characterized a paralog of KLHDC2 called KLHDC1. KLHDC1 and KLHDC2 share about 50% identity at the level of amino acid sequence and both gene loci localize to human chromosome 14q21.3. This cluster of *KLHDC1* and *KLHDC2* genes is highly conserved in vertebrates ranging from pufferfish to human. Both genes are expressed highly in skeletal muscle, but weakly in various other tissues. While KLHDC2 was predominantly found in the nucleus, KLHDC1 is a cytoplasmic protein. Neither KLHDC1 nor KLHDC2 binds to actin. In addition, KLHDC1 was unable to inhibit LZIP/CREB3-mediated transcriptional activation. Thus, KLHDC1 and KLHDC2 have differential localization and activity in cultured mammalian cells.

Key words: kelch repeat protein, *KLHDC1*, *KLHDC2*, transcription factor, LZIP

Abbreviation: BD, Gal4 DNA binding domain; HCA33, hepatocellular carcinoma-associated antigen 33; HCF, host cell factor 1; HCLP, HCF-like protein; HUGO, The Human Genome Organization; KLHDC, kelch domain containing protein

Introduction

The kelch motif of about 50 amino acids in length was first identified in fruit fly kelch ORF1 protein [1] and has subsequently been found in many different polypeptide contexts [2]. It commonly occurs in multiple repeats to form a tertiary structure known as β -propeller, each blade of which has four-stranded antiparallel β sheets radiating outward from a central axis [3]. The kelch repeats are thought to be an evolutionarily conserved module that mediates protein-protein interaction [2]. Many kelch repeat proteins are critically involved in cytoskeletal organization through their association with actin and intermediate filaments [4]. Other kelch repeat proteins participate in diverse physiological processes including cell signaling, protein trafficking and transcriptional regulation [5].

Host cell factor 1 (HCF1) is the prototype of a group of multifunctional transcriptional regulators that have six kelch repeats at the N-terminus [6, 7]. HCF1 coordinates the orderly assembly of enhancer complexes on viral and cellular promoters through the interaction between its kelch domain and various transactivators such as LZIP, VP16, GA binding protein and Oct-1 [8-11]. The kelch repeats of HCF1 have also been found to associate with Set1/Ash2 methyltransferase [12]. They are thought to tether HCF1 to the chromatin and they also play an important role in the regulation of cell cycle progression and cell proliferation [13, 14]. We have previously identified and characterized a novel protein designated HCF-like protein 1 (HCLP-1). HCLP-1 shares significant homology with HCF1 and HCF2. However, unlike HCF that has multiple functional domains, HCLP-1 is composed entirely of six kelch repeats [15]. When HCLP-1 was identified, the only other kelch repeat protein known to consist entirely of

kelch repeats is p40, which binds to Rab9-GTP and is required for endosome-to-trans-Golgi network transport [16]. HCLP-1 specifically interacts with bZIP transcription factor LZIP but not with VP16. This interaction targets the bZIP domain and leads to an inhibition of LZIP-dependent transcription [15]. Because LZIP and other bZIP transcription factors in the CREB3 subfamily have tumor suppressive activity [17, 18], expression of HCLP-1 may influence cell proliferation through the inhibition of LZIP. Indeed, HCLP-1 has independently been identified as hepatocellular carcinoma-associated antigen 33 (HCA33; GenBank AF244137), which is abundantly found in hepatoma tissues. Because HUGO Gene Nomenclature Committee has subsequently named HCLP-1/HCA33 as kelch domain containing protein 2 (KLHDC2), thereafter in this paper we will use this official name.

In this study, we identified and characterized a novel kelch repeat protein KLHDC1 paralogous to KLHDC2. Interestingly, both *KLHDC1* and *KLHDC2* mapped to human chromosome 14q21.3. The two gene loci are found in head-to-tail orientation, forming a small gene cluster. We compared the amino acid sequences of the two proteins and the genomic organization of the two genes. In addition, we also examined the expression and localization patterns as well as the activity profile of KLHDC1 and KLHDC2. Our findings have important implications on the evolution and functional diversification of vertebrate kelch repeat proteins.

Experimental procedures

Plasmids

Reporter plasmid pGal-Luc and expression plasmids for V5-tagged KLHDC2, Gal4 DNA binding domain (BD) and BD-LZIP fusion protein have previously been described [15]. Expression plasmid for V5-tagged KLHDC1 was derived from pcDNA3.1/V5/HisB (Invitrogen). Expression plasmids for BD-VP16-KLHDC1 and BD cJun-KLHDC1 were derived from pM (CLONTECH).

KLHDC1 cDNA (IMAGE clone 5283778; GenBank BC031270) was obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (Berlin, Germany). Nucleotide A at position 560 found to be different from human/chimpanzee genomic sequence and from other KLHDC1 cDNA clones was corrected to G using site-directed mutagenesis and confirmed by DNA sequencing. Full-length cJun cDNA has been described elsewhere [15]. VP16 cDNA [19] was kindly provided by Dr. Gary Hayward (Johns Hopkins University at Baltimore, USA).

Details of KLHDC1/KLHDC2 expression plasmids and oligonucleotide primers used in subcloning are available upon request.

Sequence analysis

Multiple alignment of KLHDC1-related protein sequences was produced with the PILEUP program in the Wisconsin software package (Version 10.2; Genetics Computer Group, Inc., Madison, Wisconsin, USA) and the BOXSHADE server (Version 3.21; URL:

<http://www.ch.embnet.org>). Phylogeny of protein sequences was inferred using the PHYLIP software package (Version 3.63; Joseph Felsenstein, The University of Washington) as previously described [20].

Northern blot analysis

Human multiple tissue Northern blots were purchased from CLONTECH. Northern blot analysis was performed using the protocol recommended by the manufacturer. The probes for Northern blotting were full-length cDNAs of human *KLHDC1* (2.7 kb) and *KLHDC2* (1.7 kb), while human β -actin cDNA (2 kb) was used as a control for equal loading of RNA samples.

Luciferase assay

Dual-luciferase assay was performed with extracts of transiently transfected HeLa cells as described elsewhere [21, 22]. Both firefly and sea pansy luciferase reporter enzymes were simultaneously expressed in the transfected cells. The activity of sea pansy enzyme was used to normalize the readouts of firefly luciferase activity.

Confocal microscopy

HeLa cells were cultured and transfected as previously described [18, 23]. Laser-scanning confocal immunofluorescence microscopy was carried out as detailed elsewhere [23, 24]. Images were captured at 63 \times magnification on MRC1024 system (BioRad) with the help of the LaserSharp software. For leptomycin B treatment, HeLa cells were incubated with leptomycin B at a final concentration of 15 nM for 5 hours.

Mouse monoclonal anti-V5 antibody was purchased from Invitrogen. Mouse monoclonal anti-BD antibody (clone RK5C1) was from Santa-Cruz.

Co-immunoprecipitation

MCF-7 cells grown to about 70% confluence in 10-cm dishes were transfected with KLHDC1- or KLHDC2-expressing plasmid. After 24 hours cells were lysed in HEPES lysis buffer [50 mM HEPES (pH 7.5), 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.01% Triton X-100 plus protease inhibitor cocktails]. For each transfection the cell lysate was immunoprecipitated with 1 μ l anti-V5 antiserum as previously described [18].

Rabbit polyclonal anti-V5 antiserum and mouse monoclonal anti-actin antibody (clone AC-40) were purchased from Sigma.

Results

KLHDC1 and KLHDC2 represent two subfamilies of kelch repeat proteins in vertebrates

The characterization of human *KLHDC2* [15] facilitates the cloning of its orthologs and paralogs. On one hand, a comparison with the amino acid sequence of *KLHDC2* led to the identification of mouse and rat orthologs. More than 95% of the 406 amino acid residues in murine *Klhdc2* are identical to those in the human counterpart. In addition, chicken *Klhdc2* and pufferfish *klhdc2*, which share 84% and 58% identity with human *KLHDC2*, respectively, were identified from the working draft of their genome sequences. On the other hand, we also found a novel human kelch repeat protein closely related to *KLHDC2*. This protein was subsequently named as *KLHDC1* by the HUGO Gene Nomenclature Committee.

We assembled full-length *KLHDC1* cDNA from human EST clones homologous to *KLHDC2*. The human *KLHDC1* sequence was determined and a complete coding region was identified. *KLHDC1* and *KLHDC2* share 47% identity and 64% similarity in an overlap of 352 amino acid residues (Fig. 1). Both proteins consist entirely of six kelch repeats that form a β -propeller. Notably, orthologs of *KLHDC1* were also found in mouse, rat and other vertebrates. For example, the mouse *Klhdc1* and pufferfish *klhdc1* share 85% and 51% identity with the human protein. Because a homology search in the current databases yielded additional *KLHDC1/2*-related sequences in vertebrates, we generated a consensus parsimony tree of representative *KLHDC1/2* homologs from various vertebrate species (Fig. 2) in order to understand the genetic relationship.

In this phylogenetic tree, human *KLHDC1* clusters with murine and other vertebrate

Klhdc1 proteins, while KLHDC2 groups with its vertebrate orthologs (Fig. 2). The bootstrap supports for these clusters are statistically significant (97-99%). While each vertebrate species had two KLHDC1/2 homologs, KLHDC1/2 orthologs were not found in invertebrates and other lower organisms. Thus, KLHDC1 and KLHDC2 likely represent two closely-related vertebrate subfamilies of kelch repeat proteins. It will be of interest to compare and contrast the biological activities of KLHDC1 and KLHDC2.

Chromosomal location and genomic organization of KLHDC1/2 loci

Chromosomal map locations of *KLHDC1* and *KLHDC2* loci in human genome Build 35.1 were determined by sequence alignment. Using cDNA information we identified genomic clones that contain the *KLHDC1* and *KLHDC2* loci. Interestingly, both loci map to chromosome 14q21.3, forming a small gene cluster, in which *KLHDC1* and *KLHDC2* are arranged in a head-to-tail orientation (Fig. 3A). The human *KLHDC1/2* genes were noted to be closely linked to the loci of DNA polymerase $\epsilon 2$ (*POLE2*) and serologically defined colon cancer antigen 1 (*SDCCAG1*). The distance between *KLHDC1* and *KLHDC2* is within 15 kb. Notably, the map locations of *Klhdc1* and *Klhdc2* genes in mouse genome Build 34.1 are highly conserved (Fig. 3B). In the genome of pufferfish *Tetraodon nigroviridis*, while *klhdc1* and *klhdc2* loci are also adjacent to each other and closely linked to *pole2* and *sdccag1* loci, the order and orientation of genes are different (Fig. 3C). In particular, pufferfish *pole2* locus lies between *klhdc1* and *klhdc2*, which transcribe towards opposite directions. The high degree of conservation at the levels of amino acid sequence and of genomic organization suggests that the separation of *klhdc1* and *klhdc2* genes was an early event during the evolution of vertebrates.

Human *KLHDC1* locus spans 60 kb, whereas mouse *Klhdc1* and pufferfish *klhdc1* are 42 kb and 3 kb in length, respectively (Fig. 3). However, the sizes of exons in *KLHDC1* and *KLHDC2* genes are relatively conserved in human, mouse and pufferfish genomes (Fig. 3D and Fig. 3E). The genes contain 12-13 exons varying in size from 53 bp to 1.6 kb. Many exons are of the same size in all three species.

Expression of KLHDC1 and KLHDC2 transcripts in human tissues

To assess the expression pattern of *KLHDC1* and *KLHDC2* mRNA in human tissues, we performed Northern blotting (Fig. 4). Both *KLHDC1* and *KLHDC2* were abundantly expressed in skeletal muscle (Fig. 4A and Fig. 4B, lane 3). A relatively high level of *KLHDC2* mRNA was also found in heart (Fig. 4B, lane 2). Meanwhile, weak expression of *KLHDC1* and *KLHDC2* transcripts was detected in most other tissues. However, they were apparently absent in peripheral blood leukocytes (PBL; Fig. 4A and Fig. 4B, lane 12). In consistent with our unpublished data, previous reports [15, 25] and microarray analysis (<http://www.genecards.org>) indicated that *KLHDC1* and *KLHDC2* are also expressed at moderate-to-high levels in pancreas and liver. Taken together, both *KLHDC1* and *KLHDC2* are ubiquitously expressed in human tissues albeit in low abundance. The similar expression patterns of *KLHDC1* and *KLHDC2* suggest that they might serve related function *in vivo*.

Differential activity of KLHDC1 and KLHDC2 in the inhibition of LZIP

We have previously shown that *KLHDC2* inhibits LZIP-mediated transcription through direct interaction with LZIP [15]. To compare the influence of *KLHDC1* and *KLHDC2*

on LZIP-dependent transcription, we performed luciferase reporter assays. We noted that the expression of either KLHDC2 or KLHDC1 did not affect the basal transcriptional activity in BD-expressing HeLa cells (Fig. 5, columns 1-3). Consistent with our previous finding [15], the co-expression of KLHDC2 with BD-LZIP led to substantial inhibition of LZIP-dependent expression of the luciferase reporter (Fig. 5, compare column 5 to column 4). In contrast, the expression of KLHDC1 had no influence on LZIP-mediated activation of the luciferase gene (Fig. 5, compare column 6 to column 4). Hence, human KLHDC1 and KLHDC2 have differential LZIP-inhibiting activity in cultured cells. KLHDC1 is incapable of suppressing LZIP-dependent transcription.

Differential subcellular localization of KLHDC1 and KLHDC2

Above we have demonstrated the differential activity of KLHDC1 and KLHDC2 in the inhibition of LZIP (Fig. 5). To further investigate the underlying mechanism, we next queried for the subcellular localization of KLHDC1. We have previously shown that KLHDC2 localizes predominantly to the nucleus, where it interacts with and inhibits the active form of LZIP [15]. In this study, we first verified the nuclear localization of V5-tagged KLHDC2 using an anti-V5 antibody (Fig. 6, panels A-C). Significant overlapping of KLHDC2-specific fluorescent signal with propidium iodide staining (Fig. 6, panel C) indicates that KLHDC2 is indeed a nuclear protein. In contrast, KLHDC1 was apparently found in the nucleus-excluded region (Fig. 6, panels D-F). The cytoplasmic localization of KLHDC1 was further corroborated by the staining patterns of two artificially constructed proteins fused to Gal4 DNA binding domain (BD): BD-VP16-KLHDC1 and BD-cJun-KLHDC1 (Fig. 6, panels G-L). BD contains a nuclear localization signal and is

ambiently found in the nucleus [26]. VP16 and c-Jun are potent transcription factors and constitutively localize to the nucleus [27, 28]. In fact they are usually capable of driving their fusion or dimerization partners efficiently into the nucleus [26, 28]. However, both BD-VP16-KLHDC1 and BD-cJun-KLHDC1 fusions localized predominantly to the cytoplasm (Fig. 6, panels G-L), suggesting that the KLHDC1 moiety in these proteins can sufficiently prevent BD-VP16 and BD-cJun from entering the nucleus. In further support of this, the addition of KLHDC1 to either N or C terminus of VP16 or c-Jun resulted in cytoplasmic retention of the fusion protein (data not shown). Plausibly, KLHDC1 is sequestered in the cytoplasm by as-yet unidentified partner protein(s).

We were unable to identify nuclear import and export signals in KLHDC1 or KLHDC2, but we could not rule out the possibility that more efficient or rapid export of KLHDC1 might account for its cytoplasmic localization. To compare the nuclear retention properties of KLHDC1 and KLHDC2, we treated KLHDC2- and KLHDC1-expressing cells with leptomycin B, an inhibitor of CRM1-mediated nuclear export. If KLHDC1 is exported more rapidly from the nucleus in a CRM1-dependent manner, treatment with leptomycin B would block this export and lead to nuclear retention of the protein. On the other hand, if the distribution of KLHDC1 in the nucleus and the cytoplasm is not influenced by leptomycin B, CRM1-dependent nuclear export is unlikely a critical factor in determining the cytoplasmic localization of KLHDC1. We observed that the treatment of KLHDC2-expressing cells with leptomycin B led to the disappearance of residual KLHDC2-specific fluorescent signal in the cytoplasm (Fig. 7, compare panel D to panel A). In sharp contrast, the cytoplasmic staining pattern of KLHDC1 did not alter after leptomycin B treatment (Fig. 7, panel J compared to panel G).

These results suggest that KLHDC1 is not substantially imported into the nucleus. This is consistent with our model that KLHDC1 is retained in the cytoplasm by its partner protein(s).

Neither KLHDC1 nor KLHDC2 associates with actin in vivo

The high abundance of KLHDC1 and KLHDC2 in skeletal muscles (Fig. 4) raises an interesting question as to whether these two kelch repeat proteins may have a specific function in muscle cells. Because many kelch repeat proteins associate with actin and modulate actin cytoskeleton [2, 5], it will be of interest to see whether KLHDC1 and KLHDC2 might interact with actin. In this regard, differential affinity to actin might also cause cytoplasmic retention of KLHDC1 as seen in Fig. 6 and Fig. 7. Thus, we compared the actin-binding activities of KLHDC1 and KLHDC2 by co-immunoprecipitation (Fig. 8). While both β -actin and KLHDC1/2 are abundant in the cell lysates, β -actin was not found in the anti-V5 immunoprecipitates containing KLHDC1 or KLHDC2 (Fig. 8A, lanes 6 and 8 compared to lanes 5 and 7, respectively). Hence, neither KLHDC1 nor KLHDC2 associates with actin in cultured MCF7 cells.

Discussion

In this study we identified a novel kelch repeat protein called KLHDC1 and we compared the sequence, expression, subcellular localization and activity of KLHDC1 and KLHDC2. KLHDC1 is strikingly homologous to KLHDC2, a nuclear inhibitor of LZIP transcription factor (Fig. 1). Both *KLHDC1* and *KLHDC2* loci mapped to the same gene cluster on chromosome 14q21.3 and they situated next to each other in a head-to-tail orientation (Fig. 3). Orthologs of KLHDC1 and KLHDC2 were commonly found in other vertebrates. Phylogenetic analysis suggests that they represent two closely-related subfamilies of proteins composed entirely of kelch repeats (Fig. 2). *KLHDC1* and *KLHDC2* transcripts were abundantly expressed in skeletal muscles, and they were also found in low abundance in many other human tissues, except that high level of *KLHDC2* mRNA was expressed in heart (Fig. 4). While the sequence and genomic structure of *KLHDC1* and *KLHDC2* genes are highly conserved, KLHDC1 and KLHDC2 showed differential activity in the inhibition of LZIP transcription factor (Fig. 5) and localized to different subcellular compartments (Fig. 6 and Fig. 7). Neither KLHDC1 nor KLHDC2 associates with actin in cultured cells (Fig. 8), but KLHDC1 is likely sequestered in the cytoplasm by unidentified partner protein(s). In contrast to KLHDC2 which is predominantly in the nucleus and potently inhibits LZIP-dependent transcriptional activity [15], KLHDC1 is primarily a cytoplasmic protein (Fig. 6 and Fig. 7) that has no influence on LZIP-mediated transcriptional activation (Fig. 5).

The kelch repeats represent one of commonest modules that mediate protein-protein interactions in eukaryotes [2]. Although kelch domains adopt similar tertiary structures known as β -propeller, they are remarkably versatile in partner selection and binding [2].

Kelch repeat proteins are numerous and ancient [2, 5]. While other kelch repeat proteins are found in organisms ranging from yeast to human, KLHDC1 and KLHDC2 exist only in vertebrates and they belong to a subfamily of kelch repeat proteins consisting entirely of β -propeller structure. This subfamily is only seen in animals. The highly conserved organization of *KLHDC1* and *KLHDC2* loci in vertebrate genomes implicates that they might probably be generated by duplication of a primordial gene in the ancestor of vertebrate animals. The expansion of the family of kelch repeat proteins in multicellular animals has been documented previously [2, 29]. This expansion is accompanied with the emergence of kelch repeat proteins that are critically involved in cell signaling and transcriptional regulation [5]. In light of this, it will be of particularly great interest to elucidate the physiological roles of KLHDC1 and KLHDC2.

It is noteworthy that the β -propeller domain of KLHDC1 and KLHDC2 is particularly similar to that of HCF1 and HCF2 [15]. Our previous work demonstrates that KLHDC2 interacts with transcription factor LZIP [15], in a manner similar to the proteolytic fragments of HCF1 that contain the kelch domain only [30, 31]. Currently we do not understand the molecular basis of the differential activity of KLHDC1 and KLHDC2. However, these two proteins resemble another pair of human kelch repeat proteins HCF1 and HCF2, which have shown different subcellular localization and activity in cultured cells [5]. While HCF1 is an exclusively nuclear protein, HCF2 can be found predominantly in the cytoplasm [32]. In addition, whereas HCF1 efficiently binds with and coactivates VP16 and Oct1, HCF2 binds to these transcription factors inefficiently and it does not support their transcriptional activity [32, 33].

LZIP and closely related transcription factors normally reside in the endoplasmic reticulum and undergo nuclear translocation upon proteolytic activation [18, 34, 35]. KLHDC2 interacts with the activated form of LZIP in the nucleus and inhibits its transcriptional activity [15]. As a predominantly cytoplasmic protein, KLHDC1 has no chance to interact with and inhibit active LZIP in the nucleus. This might explain the inability of KLHDC1 to down-regulate LZIP-dependent transcription (Fig. 5). While we did not detect a direct interaction between KLHDC1 and LZIP (data not shown), we still could not rule out the possibility that KLHDC1 could bind to other transcription factors of the same subfamily [18] in the cytoplasm.

The kelch domains are thought to provide various contact sites for protein-protein interaction. One important route towards characterizing the physiological roles of KLHDC1 and KLHDC2 is to identify their protein partners. In particular, it will be of importance to compare the spectra of KLHDC1 and KLHDC2 partners obtained through yeast two-hybrid screening or co-immunoprecipitation. The characterization of these protein partners will likely derive additional mechanistic insight into the differential subcellular localization and activity of KLHDC1 and KLHDC2.

Acknowledgments

We thank G.S. Hayward for gift of plasmid and A.C.S. Chun for critical reading of the manuscript. D.-Y. Jin is a Leukemia and Lymphoma Society Scholar. This work was supported by a grant to D.-Y. Jin from the Hong Kong Research Grants Council (Project HKU 7294/02M).

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Figure legends

Fig. 1. Sequence alignment of representative vertebrate KLHDC1 and KLHDC2 proteins. Perfect matches and conservative substitutions were boxed in black and gray, respectively. Human HCF-1 sequence (HCFC1) was included for comparison and the start positions of the six kelch repeats were also highlighted. GenBank identification numbers (gi) are as follows: human KLHDC1, 28380069; mouse Klhdc1, 30142701; pufferfish klhdc1, 47229997; human KLHDC2, 7657301; human HCFC1, 4885403.

Fig. 2. Phylogenetic tree of vertebrate KLHDC1-/KLHDC2-related proteins. Phylogeny was inferred from protein sequences aligned with the PILEUP program in the Wisconsin package using parsimony computed with the PROTPARS program in the PHYLIP package. SEQBOOT and CONSENSUS programs in the same package were used to perform bootstrap replication and to generate the majority rule consensus tree from 100 replicates. Numbers on the nodes are bootstrap confidence probabilities (%). Human kelch repeat protein p40 was used as an outgroup in this tree. GenBank identification numbers (gi) are as follows: chicken Klhdc1, 50748920; mouse Klhdc1, 30142701; rat Klhdc1, 62650770; human KLHDC1, 28380069; pufferfish klhdc1, 47229997; chicken Klhdc2, 60302820; mouse Klhdc2, 13542753; rat Klhdc2, 34865313; human KLHDC2, 7657301; pufferfish klhdc2, 47229999; human p40, 33695109.

Fig. 3. Genomic organization of vertebrate *KLHDC1* and *KLHDC2* loci. A. Schematic delineation of human *KLHDC1* and *KLHDC2* at chromosome 14q21.3. Arrows indicate orientation of transcription. Map locations were based on human genome sequence Build

35.1. *POLE2*, DNA polymerase $\epsilon 2$ (p59 subunit); *SDCCAG1*, serologically defined colon cancer antigen 1; Tel, telomeric; Cen, centromeric. B. Schematic diagram of mouse *Klhdc1* and *Klhdc2* at chromosome 12. Map locations were based on mouse genome sequence Build 34.1. C. Schematic delineation of pufferfish *klhdc1* and *klhdc2* at chromosome 10. Map locations were based on pufferfish genome sequence provided by Genoscope, the French National Sequencing Center (<http://www.genoscope.cns.fr>). D. Comparison of the genomic structure of human (H), mouse (M) and pufferfish (P) *KLHDC1* locus. The sizes of 13 exons are shown in nucleotides. E. Comparison of the genomic structure of human (H), mouse (M) and pufferfish (P) *KLHDC2* locus. The sizes of 12 (pufferfish) or 13 (human and mouse) exons are shown in nucleotides.

Fig. 4. Northern blot analysis of *KLHDC1* (A) and *KLHDC2* (B) mRNA in human tissues. *KLHDC1*- and *KLHDC2*-specific probes hybridized to mRNA species migrating at 2.7 kb and 2 kb, respectively. The same blot was first probed for *KLHDC1*, stripped, and then re-probed for *KLHDC2*. β -actin-specific hybridization was performed on this blot after stripping the *KLHDC2* signal. Blots were from CLONTECH and all lanes contain $\sim 2 \mu\text{g}$ of polyadenylated RNA. *KLHDC1* and *KLHDC2* transcripts are indicated. PBL: peripheral blood leukocytes.

Fig. 5. Differential activity of *KLHDC2* and *KLHDC1* in the inhibition of LZIP-mediated transcription. HeLa cells were transfected with pGal-Luc reporter plasmid and expression vectors for the indicated proteins. Expression of the V5-tagged *KLHDC1* and *KLHDC2* proteins in transfected cells was verified by Western blotting. Results are

representative of three independent experiments and the error bars indicate *S.E.* Luc activity: relative firefly luciferase activity in arbitrary units normalized to sea pansy luciferase activity.

Fig. 6. Subcellular localizations of KLHDC2 and KLHDC1 in HeLa cells. pcDNA-KLHDC2 (panels A-C), pcDNA-KLHDC1 (panels D-F), pBD-VP16-KLHDC1 (panels G-I) and pBD-cJun-KLHDC1 plasmids were individually transfected into HeLa cells. Cells were fixed and stained for KLHDC1/2 with mouse monoclonal anti-V5 (α -V5; panels A and D) or anti-BD (α -BD; panels G and J) antibody. Nuclear morphology was visualized by counterstaining with propidium iodide (PI; panels B, E, H and K). Green (KLHDC1/2) and red (PI) fluorescent signals were overlaid by computer assistance (panels C, F, I and L). Co-localizations are in yellow. The same fields of cells are shown in panels A-C, D-F, G-I and J-L. Results represent three independent experiments and 100 transfected cells from each experiment were quantitated. While $88 \pm 5\%$ of KLHDC1-expressing cells showed predominantly cytoplasmic staining of KLHDC1, $86 \pm 7\%$ of KLHDC2-positive cells showed predominantly nuclear staining of KLHDC2. The nuclear localization of BD-VP16 and BD-c-Jun has been shown elsewhere [36, 37] and verified in our experiment (data not shown).

Fig. 7. Subcellular localization of KLHDC2 and KLHDC1 in HeLa cells treated with leptomycin B (LMB). Cells were transfected with either pcDNA-KLHDC2 (panels A-F) or pcDNA-KLHDC1 (panels G-L). LMB was added to a final concentration of 15 nM

and cells were incubated for additional 5 hours (panels D-F and J-L). Immunostaining was then performed as in Fig. 5.

Fig. 8. Neither KLHDC2 nor KLHDC1 associates with actin. MCF-7 cells grown to about 70% confluence in 10-cm dishes were either mock transfected (lanes 1 and 2) or transfected with expression plasmid for V5-tagged β -galactosidase (lanes 3 and 4), KLHDC2 (lanes 5 and 6) or KLHDC2 (lanes 7 and 8). Twenty-four hours after transfection, cells were lysed in HEPES lysis buffer and immunoprecipitated with rabbit polyclonal anti-V5. The protein complexes were subsequently resolved in 10% SDS-PAGE followed by Western blotting using mouse anti-actin (A). Western blot analysis with mouse anti-V5 was also performed to detect V5-tagged proteins in the immunoprecipitates (B). Lanes 1, 3, 5 and 7 contain 1/10 input materials, while the immunoprecipitates (IP) are in lanes 2, 4, 6 and 8. Asterisk (*) indicates the heavy chain of IgG.

Repeat 1 →

1 -----MADSO~~L~~FCVAE--ERSGHCAVVDGNFLYVWGGYASIEDN---EVYLPNDEIWF
1 -----MADPO~~A~~FCVAE--ERSGHCAVVDGHFLYVWGGYVSIEDN---EVYLPNDEMWF
1 -----MOYEPVMD~~P~~GFQGHFSELVAR--ERSGHTAVV~~E~~GHRLYVWGGYMSVADD---EVFLPNDEIWF
1 -MADGNEDLRADDL~~P~~GFAPAFESYE~~S~~MELACPAERSGHVAVSDGRHMFVWGGYKSNQVRGLYDFYLPREELWF
1 MASAVSPANLPAVLLQ~~P~~RRWKR~~V~~VGWSGPVPRPRRHGHRAVAIKELIVVFGGGNE-----GIVDELHF

Repeat 2 →

48 **TYDIDSGLWRMHLMEGE**LPASMSGSCGACINGKLYIFGGYDDKG--YSNRLYFVNLRTRDETYIWEKITDF
48 **TYDIDSGLWKMHLMEGE**LPPSMSGSCGACIHGRLYVFGGYDDKG--YSNRLYFVNLRTRDTYIWEKITDF
58 **AYDLNRGVWEVFMHMGD**TTPSMSGTCGCSVNGHMYIFGGCDDNG--QTNMLYCVNLT--DGKYTWRRIVPG
70 **IYNMETGRWKKINTEGD**VPPSMSGSCAVCVDRVLYLFGGHHSRG--NTNKFYMLDSRSTDRVLOWERID-C
61 **VYNTATNQWFI**PAVRGDIPPGCAAYGFVCDGTRL~~L~~VFGGMVEY~~G~~KYSNDLYELQASRWEWKRLKAKTP-K

Repeat 3 →

117 **EGQPP**TPRDKLSCWVYKDRLIYFGGYCRRHSELQD---CFDVHDAS--WEEQIFWGW~~H~~NDVHIFD~~T~~KTQT
117 **DGQPP**TPRDKLSCWVYKDRLIYFGGYGYRRHSELQE---CFDVHDAS--WEEQIFWGW~~H~~NDVHVF~~T~~KTTRT
125 **YGSPPS**PRDKLSCWVYETRLVYFGGYGHKLLTDVDSRNRSEITVDEAS--WVEDVFWGW~~N~~NEVHVF~~D~~PMNSS
138 **QGITPPSSKDKL**GVVYK~~N~~KLIEFGGYGYLPEDKVLG---TEFDETSFWNSSSHPRG~~W~~NDHVHIL~~D~~TEFT
130 **NGPPP**CPRLGHSFSLVGNKCYLFGGLANDSEDPKNN---IPRYLNDLYI~~L~~LELRPS

Repeat 4 →

183 ----WFOPEIKGGVPPOPRAAHTCAVLGNKG-----YIFGGRVLQTRMNDLHYLNLDTW~~T~~WSGRITINGE
183 ----WSOPEIKGGVPPOPRAAHS~~C~~AVLGNKG-----YVFGGRVLQTRMNDLHYLNLDTW~~V~~WSGRISVNGE
194 ----WSEPKTING-RAPAPRAA~~H~~SATLGH~~R~~G-----YICGGRVME~~T~~RTSDVHCLDFE~~T~~WTWT-EITPASP
205 ----WSQPIITG-KAPSPRAA~~H~~ACATVGNRG-----FVFGGRYRDARMNDLHYLNLDTW~~E~~WN-ELIPQGI
183 **GVVAWDIPITYG-VLPP**RESH~~T~~AVVYTEKDNKSKLVYIGGMSGCRLGLDLWTLDID~~T~~L~~T~~WN-KPSLSGV

Repeat 5 →

Repeat 6 →

244 **SPKHRSWHTLTP**IADDKLEL~~C~~GGLSADNIPLSDGWIHNVT~~T~~NCWKQLTHLPKT--RPRLWHTACLGKENE
244 **SPKHRSWHTLTAI**TDDKLELFGGLNADNIPLSDGWIHNIT~~T~~NCWKQLRHLPYT--RPRLWHTACLGKENE
253 **VPVGRSWHTLTA**VSDHSLLELFGGLSVD~~C~~NPMSDGLWLLDVEKKTWREVDHPPFN--KPRLWHTACQGRDAD
264 **CPVGRSWHSL**TPVSSDHLLELFGGF~~T~~TDKQPLSDAWTYCISKNEWIOFNHPYTE--KPRLWHTACASDEGE
251 **APLPRSLHSATTIG-NKMY**VEGGWVP--LVMD~~D~~VKVATHEKEWKCTNTLACLNLDTMA~~W~~E~~T~~ILMDTLED

312 **IMVFGGSKDDLLALD**-TGH~~C~~NDLLIFQTOPYSL~~L~~RSCLDCIGK---NSIMLESQISL~~L~~PPKLLQOVLK~~K~~I
312 **IMVFGGSKDNLFLD**-TGH~~C~~NDLLIFQTOPYSL~~L~~RSCLDCIGK---NAIILK~~S~~QISL~~L~~PPKLLQOVLK~~K~~I
321 **VIVFGGSCDYILLVD**-TGH~~C~~NDALV~~F~~QTOPYPLFRICEDYI~~A~~KNVKNYEALRN~~S~~QLPRLPSKLLTAVQARM
332 **VIVFGGCANNLLVH**HRAAHSNEILIFSVQPKSLVRLSLEAVIC--FKEMLANSWNCL~~E~~PHLLHSV~~N~~QRF
317 **NI**PRARAGHCAVAIN-----TRIVYI~~W~~SGRD-GYRKAWNNQVCC~~K~~DLWYLETEKPPPPARVQLV~~R~~RANTNSL

378 **TFWAAANHREEQ**RVQKEETENKYQWISSN KLHDC1-human
378 **TFWTAANRKEQ**RI~~R~~KEETENQPRVSSC Klhdc1-mouse
390 **CEY**----- KLhdc1-pufferfish
399 **GSNNTSGS**----- KLHDC2-human
381 **EVS**WGA~~V~~ATA----- HCF1-human

Figure 1

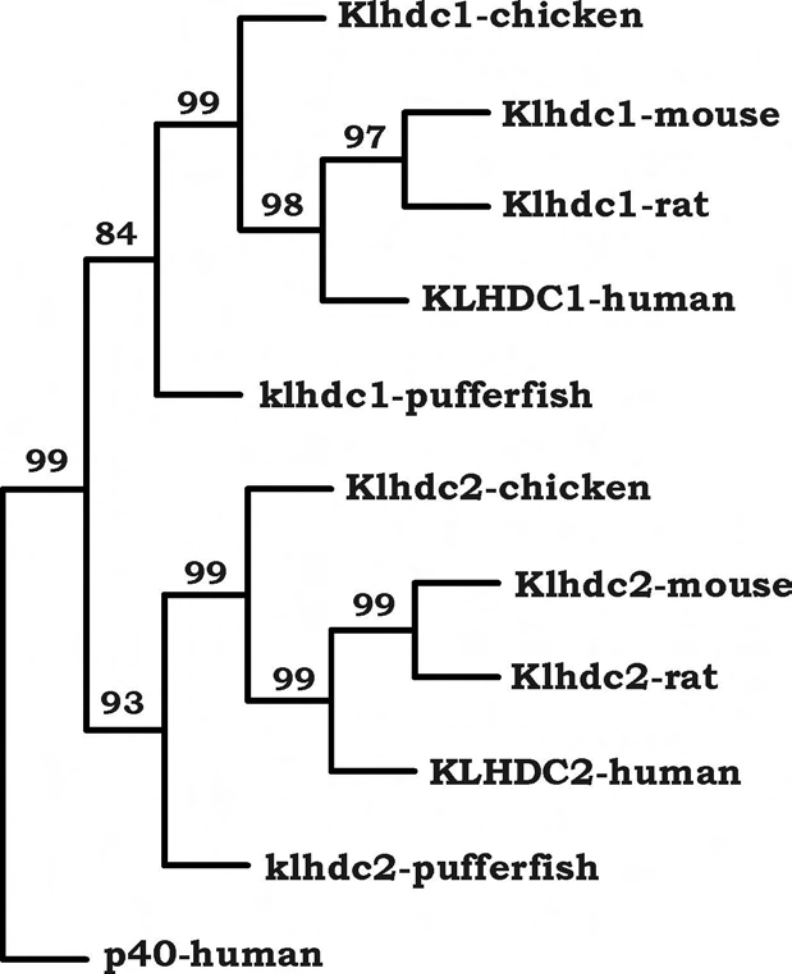
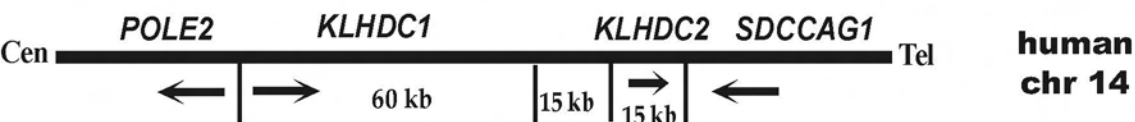
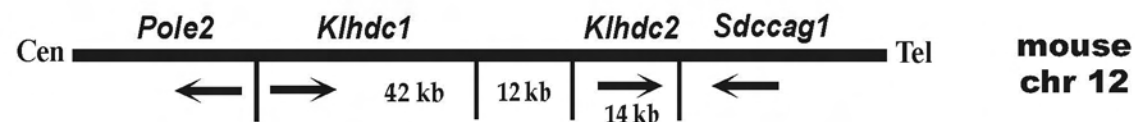
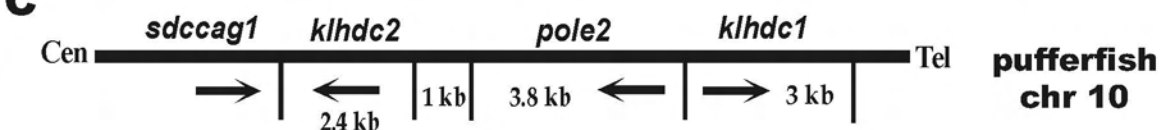


Figure 2

A**B****C****D**

	1	2	3	4	5	6	7	8	9	10	11	12	13
H	124	71	118	119	79	84	84	59	112	73	85	53	1564
M	182	71	118	119	79	84	84	59	112	73	85	53	829
P	117	71	118	113	88	84	81	59	110	73	85	53	373

E

	1	2	3	4	5	6	7	8	9	10	11	12	13
H	469	80	118	116	82	84	81	59	110	73	85	53	308
M	380	80	118	116	82	84	81	59	112	73	85	53	312
P	141	80	118	113	256	81	59	110	76	88	53	124	

Figure 3

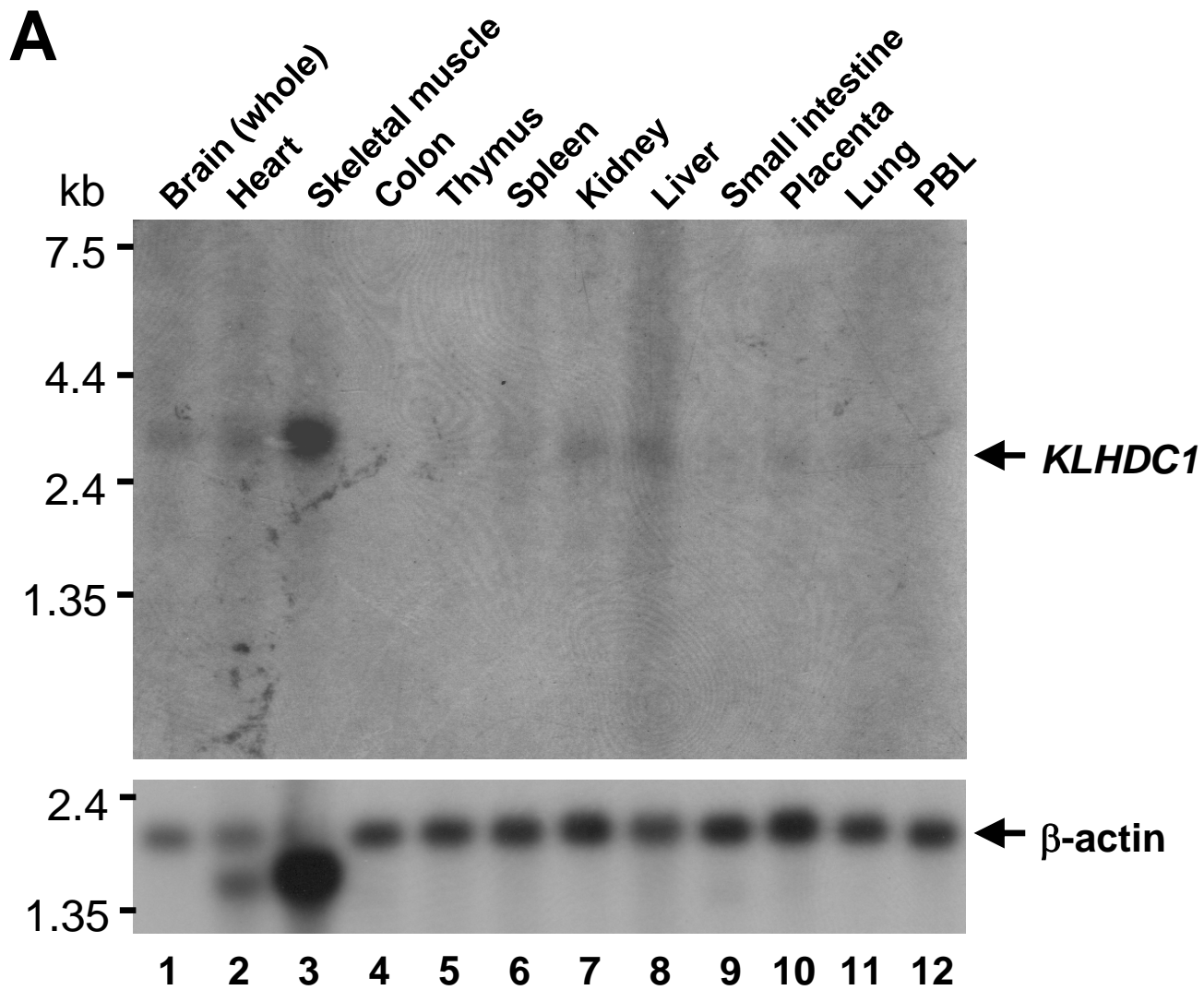


Figure 4A

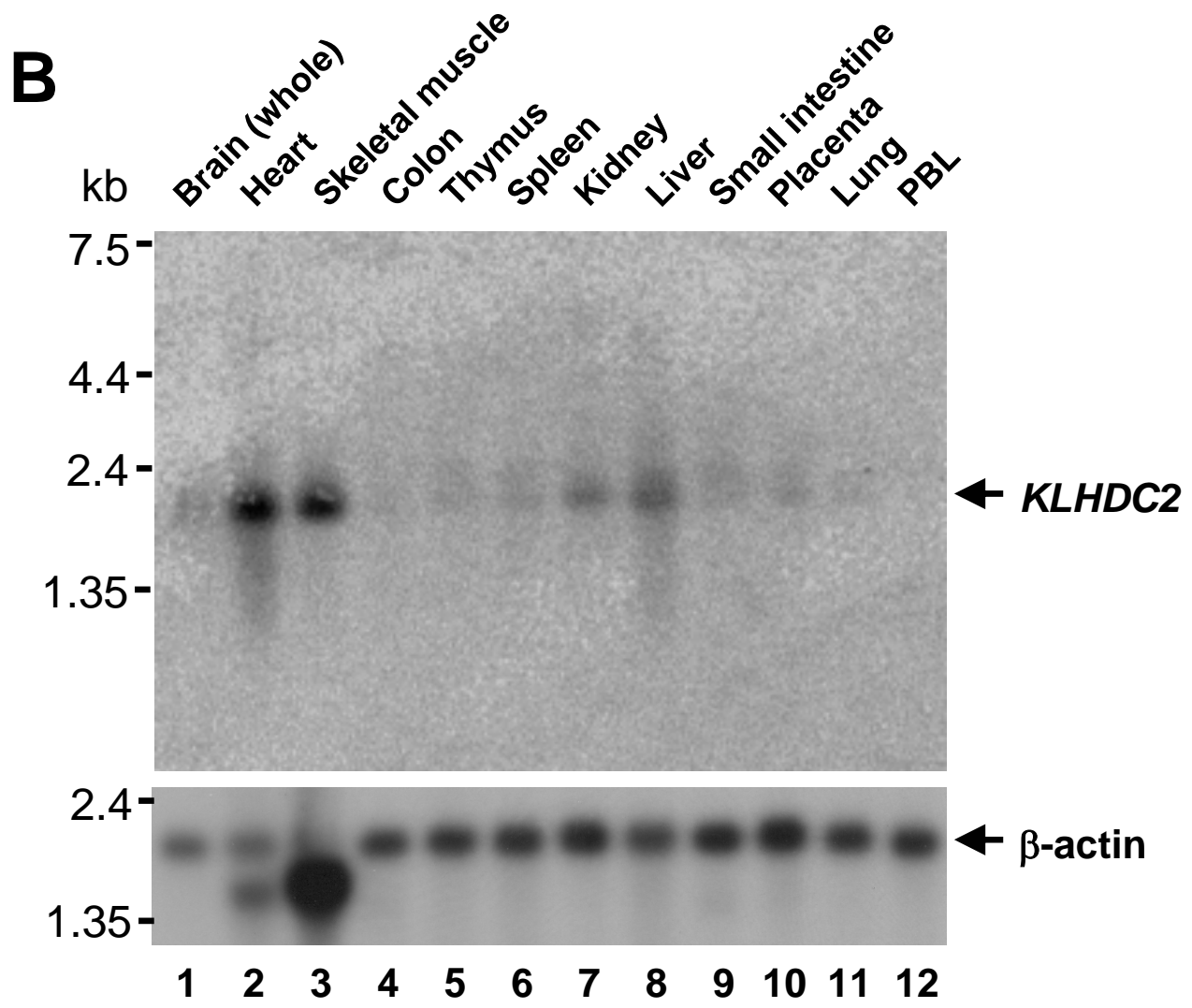


Figure 4B

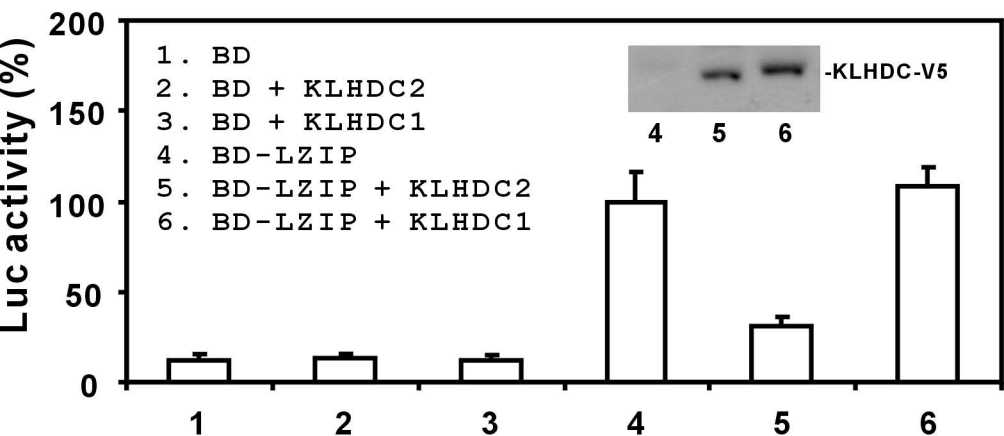


Figure 5

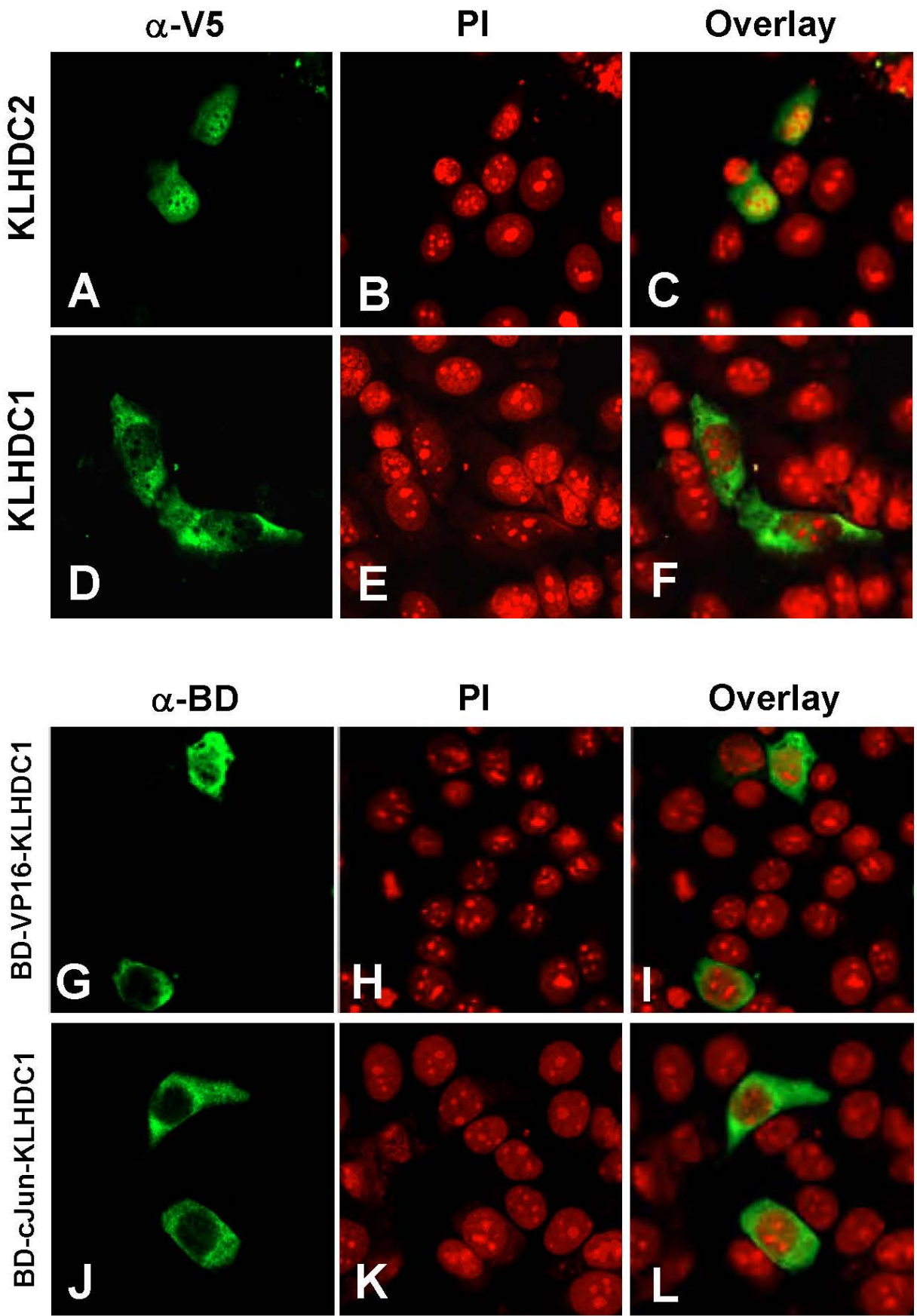


Figure 6

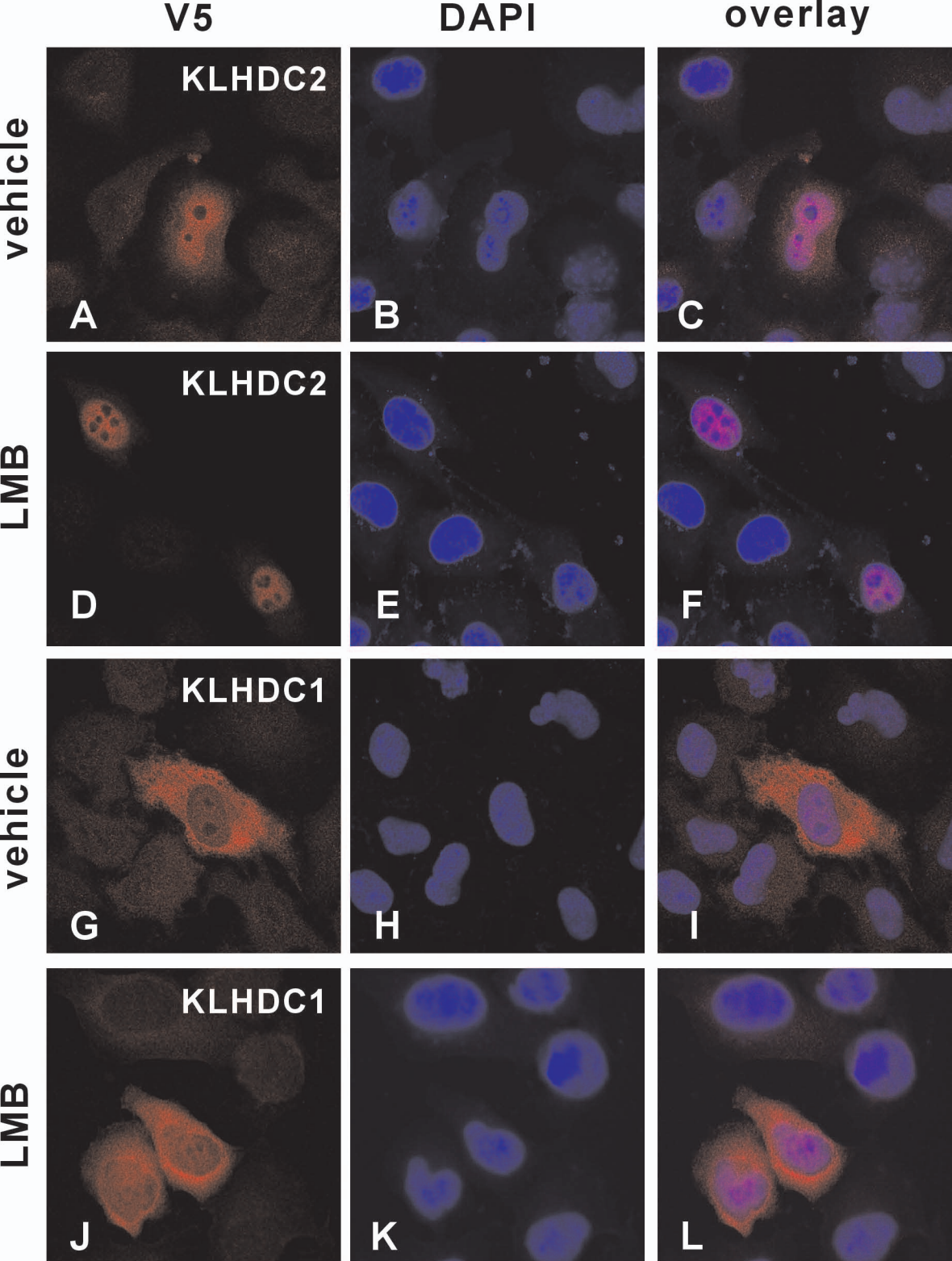


Figure 7

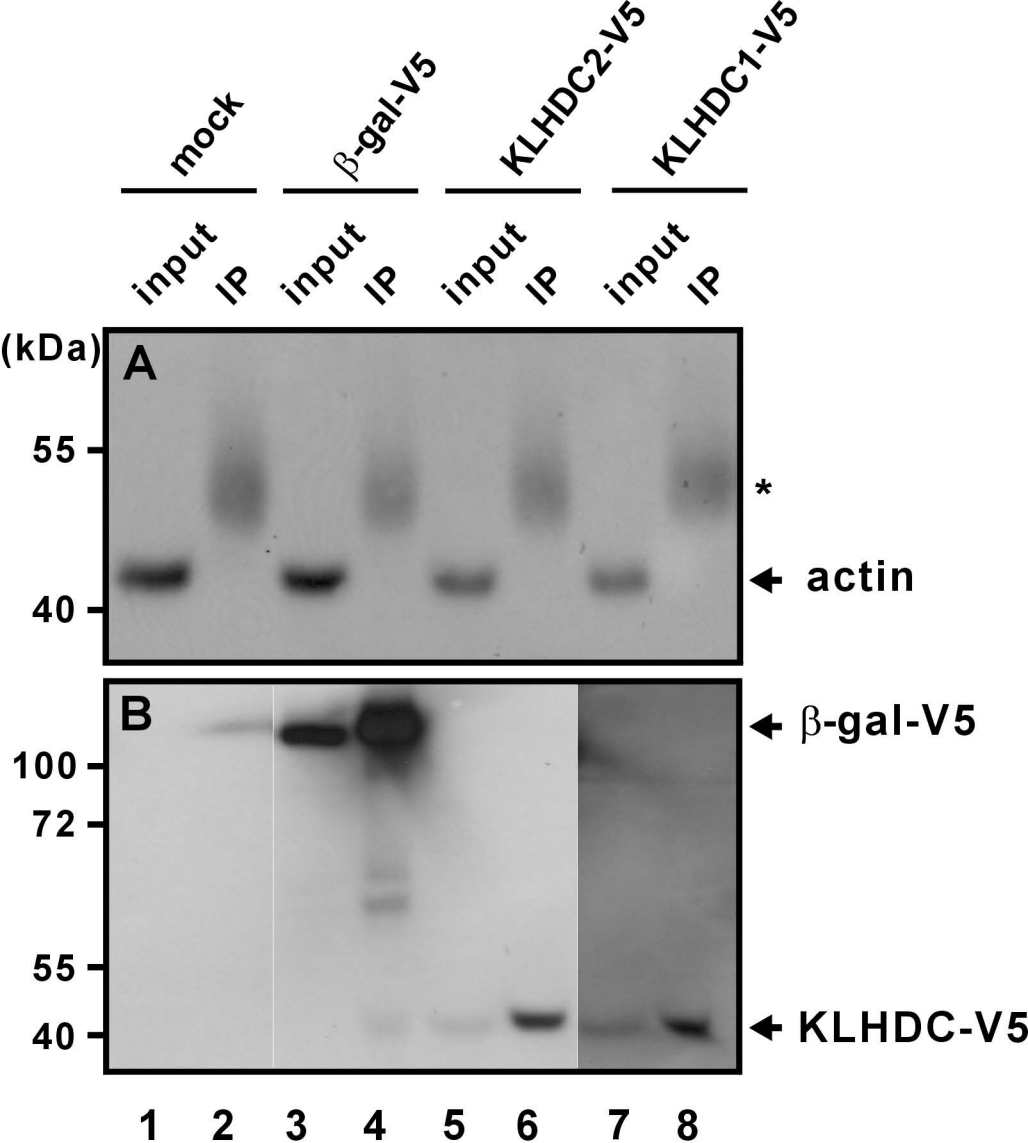


Figure 8