A Novel Cas Family Member, HEPL, Regulates FAK and Cell Spreading

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For over a decade, p130Cas/BCAR1, HEF1/NEDD9/Cas-L, and Efs/Sin have defined the Cas (Crk-associated substrate) scaffolding protein family. Cas proteins mediate integrin-dependent signals at focal adhesions, regulating cell invasion and survival; at least one family member, HEF1, regulates mitosis. We here report a previously undescribed novel branch of the Cas protein family, designated HEPL (for HEF1-Efs-p130Cas-like). The HEPL branch is evolutionarily conserved through jawed vertebrates, and HEPL is found in some species lacking other members of the Cas family. The human HEPL mRNA and protein are selectively expressed in specific primary tissues and cancer cell lines, and HEPL maintains Cas family function in localization to focal adhesions, as well as regulation of FAK activity, focal adhesion integrity, and cell spreading. It has recently been demonstrated that upregulation of HEF1 expression marks and induces metastasis, whereas high endogenous levels of p130Cas are associated with poor prognosis in breast cancer, emphasizing the clinical relevance of Cas proteins. Better understanding of the complete protein family should help inform prediction of cancer incidence and prognosis.

INTRODUCTION

The Cas (Crk-associated substrate) scaffolding protein family contains three defined members: p130Cas/BCAR1 (Sakai *et al.,* 1994; Brinkman *et al.,* 2000); HEF1/Cas-L/NEDD9 (Kumar *et al.,* 1992; Law *et al.,* 1996; Minegishi *et al.,* 1996), and Efs/Sin (Ishino *et al.,* 1995; Alexandropoulos and Baltimore, 1996). Elevated expression of p130Cas/BCAR1 has been linked to poor prognosis in breast cancer (van der Flier *et al.,* 2000), whereas overexpression of HEF1/NEDD9/Cas-L recently been found to potently induce metastatic melanoma (Kim *et al.,* 2006). Mechanistically, the best-studied functions of the Cas family proteins include regulation of attachment-dependent survival signaling or anoikis and regulation of cell motility and invasion, although there is evidence for additional roles for some of these proteins in control of cell cycle, growth factor signaling, cell differentiation, and bacterial and viral infection (reviewed in Defilippi *et al.,* 2006; Singh *et al.,* 2007). These many functions reflect the ability of the Cas proteins to interact with multiple partner proteins, as the predominant structural feature of Cas proteins is their possession of numerous protein interaction domains (discussed in O'Neill *et al.,* 2000), allowing them to act as scaffolding proteins for different functional complexes.

An important current issue in understanding cancer pathogenesis is that of why different oncogenes and tumor suppressors are selectively targeted in tumors arising from different tissue sources. For example, although elevation of HEF1/Cas-L/NEDD9 induces metastasis in melanomas, reduced levels of the same gene have been reported in breast

cancers that metastasize aggressively to the lung (Minn *et al.,* 2005; see also O'Neill *et al.,* 2007 for discussion). Undoubtedly, the differing physiology and complement of expressed genes in differing precursor cell types imposes distinct requirements for the type of genetic or epigenetic change required to make a cell cancerous. For protein families, another relevant issue is likely to be that for a given cell type, the expression of one family member may condition the impact of modulating the expression of a paralogous family member with overlapping biological activities. The complexity of cellular signaling networks currently emerging through systems-level analysis (Mak *et al.,* 2007) emphasizes the importance of exactly defining the composition, expression, and functional properties of protein family groups.

In this study, we have identified a previously unreported but evolutionarily conserved member of the Cas group, which we have termed HEPL (HEF1-Efs-p130Cas-like). We show that the HEPL mRNA and protein are expressed in cultured cell lines and tumors, and that HEPL has biological activities similar to those of other family members in influencing cell attachment and movement. The identification of HEPL provides an important context for further studies of this increasingly important protein group.

MATERIALS AND METHODS

Genomic and Structural Analysis of the Cas Protein Family

Details of sequence collection and processing are provided in the legend to Supplementary Figure 1. Dendrograms showing family relationships were displayed using the Treeview program. The HEPL SH3 domain was modeled using as template the high-resolution crystal structure (1.1Å) of the SH3 domain of p130Cas (PDB code 1WYX; Wisniewska *et al.,* 2005). The rat p130Cas structure (PDB code 1Z23; Briknarova *et al.,* 2005) was used to model the four-helix bundle region. Homology modeling was initiated using a multiple-round PSI-BLAST (Altschul *et al.,* 1997) sequence search using HEPL and p130Cas sequences to build profiles. The profiles were used to identify

suitable templates in the Protein Data Bank (PDB; Berman *et al.,* 2000). The profile/template match was refined using secondary structure predictions from PSIPRED (Jones, 1999). Conserved backbone and side chain residues were copied from the template structure, whereas divergent residues were rebuilt using SCWRL3 (Canutescu *et al.,* 2003). LOOPY (Xiang *et al.,* 2002) was used to build loops at points of insertion and deletion. Molecular graphics and 3D structural manipulation was performed using Chimera (Pettersen *et al.,* 2004). The Cas multiple sequence alignment was overlaid with secondary structure prediction rendered by MolIDE (Canutescu and Dunbrack, 2005).

Quantitative Real-Time PCR Assays

Total RNA was isolated using an RNeasy kit (Qiagen, Chatsworth, CA). Contaminating DNA was removed using TURBO DNA-*free* (Ambion, Austin, TX). RNA was quantified using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) in combination with a RNA 6000 Nano LabChip (Agilent Technologies). See Supplementary Tables 1 and 2 for technical details of PCR assays. Ambion's First Choice human total RNA survey panel was used as a source of RNA from 20 different normal tissues.

HEPL Plasmids and Small Interfering RNA and Cell Culture

HEPL was cloned using conventional molecular biology techniques by combining sequences from Human MGC verified full-length cDNA (Clone 5205865, Open Biosystems, Huntsville, AL) and human genomic DNA. Hemagglutinin (HA)-epitope tagged HEF1, HEPL, FAK, and negative controls (empty vector or Δ BioB, an extensively truncated *Escherichia coli* BioB) were expressed from pcDNA3.1-6HA for transfections. Cell lines were cultured under standard conditions, in DMEM or in RPMI-1640 plus 10% fetal bovine serum (FBS) supplemented with antibiotics, as specified by the ATCC (Manassas, VA). Scrambled (control) small interfering (siRNA) and siRNA duplexes against HEPL (NM_020356) and HEF1 were made by Dharmacon Research (Lafayette, CO). HEPL-directed siRNAs were used both as a Smartpool and four individual deconvoluted sequences, as described in Results. Plasmid transfections were done using LipofectAMINE-Plus reagent (Invitrogen, Carlsbad, CA) and siRNA transfections using the Cell Line Nucleofector Kit V from Amaxa Biosystems (Gaithersburg, MD).

Antibodies and Immunoprecipitation

Rabbit polyclonal antibody to HEPL was generated using a peptide corresponding to HEPL amino acids 773-786 (by Zymed Laboratories, San Francisco, CA). Antibody was purified from sera using the NAb Protein A Spin Purification Kit (Pierce Biotechnology, Rockford, IL). Other antibodies included anti-HA mAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-paxillin and anti-p130Cas (BD Transduction Laboratories, Carlsbad, CA), anti-HEF1 (2G9; Pugacheva and Golemis, 2005), anti-FAK[pY^{397}] (Biosource, Nevelle, Belgium), anti-gelsolin (BD Biosciences, San Jose, CA), Alexa Fluor 488– and 568–conjugated anti-mouse (Molecular Probes, Eugene, OR), and anti-mouse and anti-rabbit IgG antibodies conjugated to HRP (Amersham Biotech, Buckinghamshire, England). For immunoprecipitations, transfected cells were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology) and immunoprecipitated with either anti-HA or anti-HEPL Abs, using Immobilized Protein A/G Agarose (Pierce Biotechnology). To establish HEPL and FAK interaction, HA-epitope tagged HEF1, HEPL, FAK, and negative control (Δ BioB, an extensively truncated *E. coli* BioB) were expressed from pcDNA3.1-6HA for transfections in 293T cells and immunoprecipitated with anti-FAK mAb, clone 4.47 (Millipore, Bedford, MA).

To study cell adhesion–dependent tyrosine phosphorylation, trypsinized HOP-62 cells were either maintained in suspension in serum-free medium for 45 min at 37°C or subsequently were replated on fibronectin (4 μg/cm²; Chemicon International, Temecula, CA)-coated dishes for 30 min. Experiments were performed in parallel in the presence or absence of 10 μM PP2 (Calbiochem, San Diego, CA). Cell lysates were prepared using M-PER Mammalian Protein Extraction Reagent supplemented with protease inhibitors and Halt Phosphatase Inhibitor Cocktail (Pierce Biotechnology), immunoprecipitated with antibodies to HEPL or HEF1, and immunoblotted with anti-phosphotyrosine mAb (BD Transduction Laboratories).

Yeast Two-Hybrid Analysis

The modified Interaction Trap form of two-hybrid system was used to study HEPL protein interactions, using reagents and approaches as described in (Serebriiskii *et al.,* 2002). The LexA-fused HEPL SH3 domain (aa 1-148) was used to assess interactions with B42 activation domain-fused FAK C-terminus (aa 688-997). LexA fused to the SHC PTB domain and to a B42-ΔBioB, and B42-fused Raf and B42-ΔBioB were used as nonspecific negative controls. Expression of all protein fusions was analyzed by Western blot.

Immunofluorescence, Cell Spreading, Cell Migration, Cell Size, Cell Cycle, and Apoptosis Assays

Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.2% Triton X-100 for 5 min and blocked with 3% BSA in PBS. After incubation with primary antibodies, cells were stained with either Alexa Fluor 488– or 568–conjugated secondary antibodies. Epifluorescence microscopy was performed using an inverted Nikon TE300 microscope (Melville, NY). Confocal microscopy was performed using a Radiance 2000 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). All images were acquired as 12-bit images with a Spot RT monochrome camera (Diagnostic Instruments, Sterling Heights, MI). For cell spreading analysis, cells were transfected with indicated plasmids or siRNAs for 18–48 h before fixation, as indicated. Anti-paxillin mAb was used to mark focal adhesions and outline cells. Cell area measurements were made using MetaMorph or MetaVue software (Molecular Devices, Universal Imaging, Downingtown, PA) software to score pixels within cell perimeters.

To measure motility, movement of siRNA-treated HOP62 cells plated in six-well tissue culture dishes was monitored with a Nikon TE300 microscope using 10× NA 0.25 PlanA objective, and images were collected with CCD video camera (Roper Scientific, Trenton, NJ) at 20-min intervals over a 12-h period and then digitized and stored as image stacks using MetaMorph software. Velocity and persistence of migratory directionality (D/T) were determined by tracking the positions of cell nuclei using the Track Point function of MetaMorph.

Apoptosis was measured using an APOPercentage apoptosis assay kit (Biocolor, Belfast, Northern Ireland, United Kingdom) and Western blot to measure appearance of cleaved gelsolin. Cell cycle compartmentalization was measured using a Guava Personal Cell Analysis (PCA) System (Guava Technologies, Hayward, CA). Treatment for 48 h with 200 μM etoposide (Sigma-Aldrich, St. Louis, MO) or 10 nM dasatinib (a gift of Dr. Andrew Godwin) was used as positive control for apoptosis assays. All calculations of statistical significance were made using the GraphPad InStat software package (San Diego, CA) and STATA software (StataCorp, College Station, TX). Approaches included unpaired *t* tests, ANOVA analysis, and generalized linear models estimated using generalized estimating equations (GEE).

RESULTS

Prediction of a New Cas Family Member, HEPL

Using the p130Cas, HEF1, and Efs protein and mRNA sequences in reiterative BLAST analysis against genomic sequences and EST resources, we searched for Cas-related sequences in an evolutionarily diverse group of organisms (Figures 1, A and B), and Supplementary Figure 1). No protein strongly related to the Cas family was identified in *Saccharomyces cerevisiae* or *Caenorhabditis elegans*, whereas a single ancestral family member was detected in arthropods, echinoderms, and primitive chordates. The family branches in gnathostomes to produce the three previously characterized mammalian family members. Unexpectedly, we detected a completely novel member of the Cas family that was conserved as an ortholog group from gnathostomes through mammals. We have designated this ortholog group as HEPL. Dendrogram analysis indicates that the HEPL does not diverge at a significantly more rapid rate than other Cas branches, suggesting that it maintains a biological function, rather than representing a pseudogene (Figure 1B).

Human *HEPL* localizes to chromosome 20q13.31 and is annotated in Unigene as C20orf32. Comparison of the human HEPL protein sequence with the other three human Cas family members (Supplementary Figure 1) shows overall identity with other family members up to 26% and similarity up to 42%. Human HEPL is 786 amino acids (aa), versus 870 aa for p130Cas, 834 aa for HEF1, and 561 aa for Efs. For the three well-studied Cas proteins, a highly conserved amino-terminal SH3 domain is followed by a moderately conserved region encompassing multiple SH2-binding sites, a serine-rich region shown to encompass a four-helix bundle in p130Cas (Briknarova *et al.,* 2005), and a well-conserved carboxy-terminal domain that contributes to focal adhesion targeting (Nakamoto *et al.,* 1997; Harte *et al.,* 2000; O'Neill and Golemis, 2001; Figure 1C). Although the HEPL proteins maintain a recognizable SH3 domain, this domain has the lowest overall similarity to other SH3 domains in the Cas group. Human HEPL has a limited number of candidate SH2-binding sites, in contrast to p130Cas and HEF1. The HEPL carboxy-terminus has a short region of detectable Cas
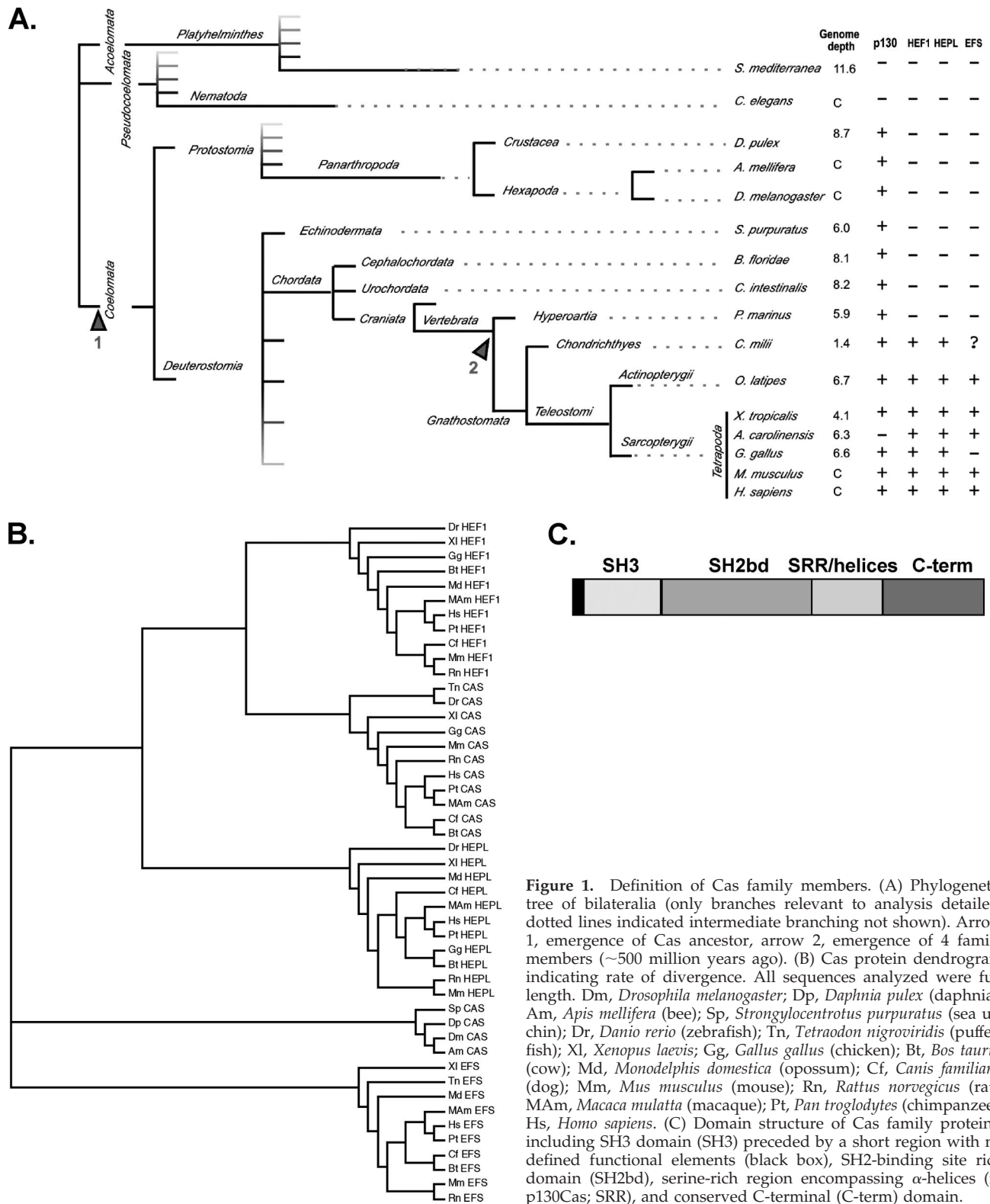
	Genome depth	p130	HEF1	HEPL	EFS
S. mediterranea	11.6	−	−	−	−
C. elegans	C	−	−	−	−
D. pulex	8.7	+	−	−	−
A. mellifera	C	+	−	−	−
D. melanogaster	C	+	−	−	−
S. purpuratus	6.0	+	−	−	−
B. floridae	8.1	+	−	−	−
C. intestinalis	8.2	+	−	−	−
P. marinus	5.9	+	−	−	−
C. milii	1.4	+	+	+	?
O. latipes	6.7	+	+	+	+
X. tropicalis	4.1	+	+	+	+
A. carolinensis	6.3	−	+	+	+
G. gallus	6.6	+	+	+	−
M. musculus	C	+	+	+	+
H. sapiens	C	+	+	+	+

Figure 1. Definition of Cas family members. (A) Phylogenetic tree of bilateralia (only branches relevant to analysis detailed; dotted lines indicated intermediate branching not shown). Arrow 1, emergence of Cas ancestor, arrow 2, emergence of 4 family members (~500 million years ago). (B) Cas protein dendrogram indicating rate of divergence. All sequences analyzed were full length. Dm, *Drosophila melanogaster*; Dp, *Daphnia pulex* (daphnia); Am, *Apis mellifera* (bee); Sp, *Strongylocentrotus purpuratus* (sea urchin); Dr, *Danio rerio* (zebrafish); Tn, *Tetraodon nigroviridis* (pufferfish); Xl, *Xenopus laevis*; Gg, *Gallus gallus* (chicken); Bt, *Bos taurus* (cow); Md, *Monodelphis domestica* (opossum); Cf, *Canis familiaris* (dog); Mm, *Mus musculus* (mouse); Rn, *Rattus norvegicus* (rat); MAm, *Macaca mulatta* (macaque); Pt, *Pan troglodytes* (chimpanzee); Hs, *Homo sapiens*. (C) Domain structure of Cas family proteins, including SH3 domain (SH3) preceded by a short region with no defined functional elements (black box), SH2-binding site rich domain (SH2bd), serine-rich region encompassing α-helices (in p130Cas; SRR), and conserved C-terminal (C-term) domain.

family homology (res ~670 to the carboxy-terminus), but otherwise lacks obvious similarity at the level of primary sequence. Flanking the carboxy-terminal domain, HEPL also lacks a YDYVHL sequence conserved among the other three Cas family proteins (bold, Supplementary Figure 1) that is an important binding site for the Src SH2 domain (Tachibana *et al.*, 1997), possibly suggesting reduced functionality.

To better assess whether the predicted HEPL proteins maintain important features of the Cas family, we used molecular modeling to compare the Cas proteins based on
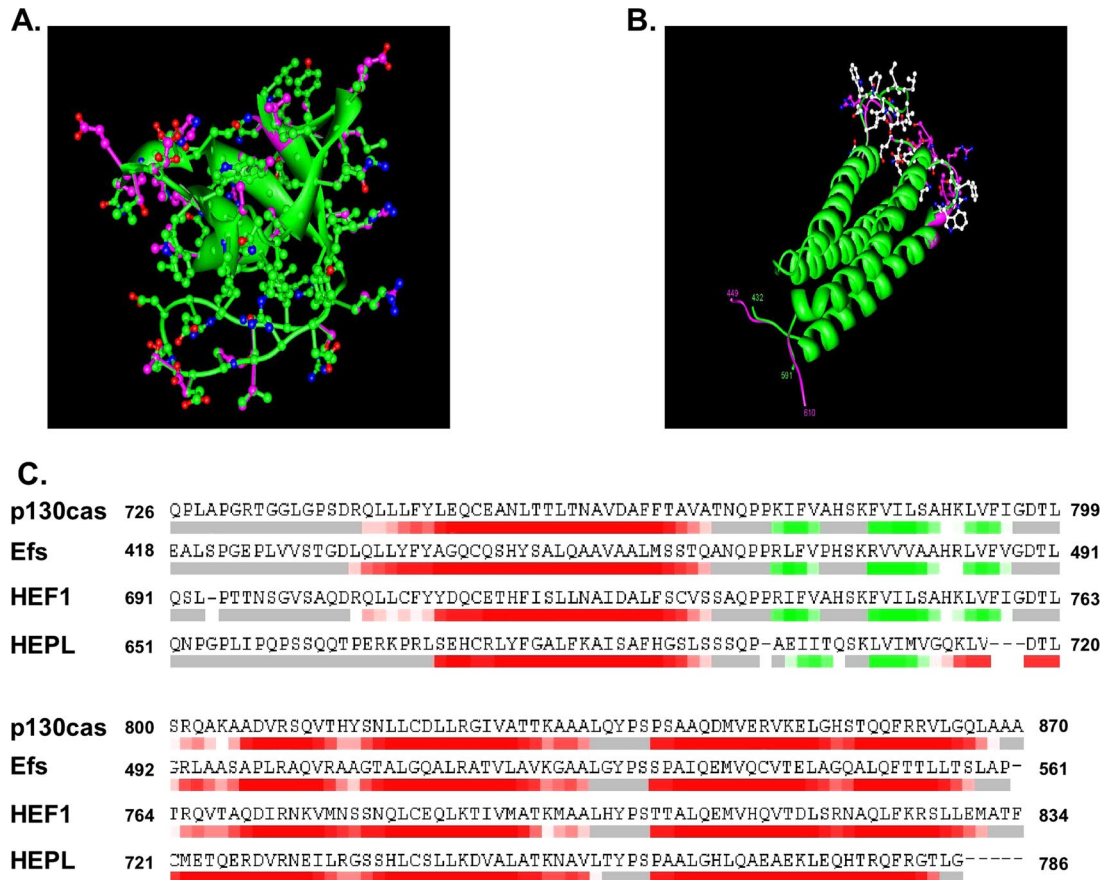
Figure 2. Modeled secondary and tertiary structure of Cas domains. (A) Superposition of a model of the SH3 region of human HEPL with the crystal structure of the same domain from p130Cas. The HEPL sequence alignment with the template had an e value of $4.00e^{-32}$, 52% sequence identity, 70% positives, and no gaps, comprising residues 13-79 (3-69 in the template sequence). The backbone is shown in green for both proteins. For the side chains, oxygen atoms are red and nitrogen atoms are blue. Carbons in p130Cas are purple and carbons in HEPL are green. (B) Superposition of models of the serine-rich region of human p130Cas (purple ribbon) and human HEPL (green ribbon). HEPL alignment with the template showed an e value of $7.00e^{-53}$, 28% sequence identity, 48% positives, 8% gaps and contained the 432-591 residue range (4-161 in the template). Because the p130Cas and HEPL helix regions are highly similar, only the HEPL ribbons are shown. Two divergent loop regions are shown with side chains. Oxygen atoms are red and nitrogen atoms are blue. Carbons in p130Cas are purple and carbons in HEPL are white. (C) Predicted Cas family carboxy-terminal secondary structure. Red bar, predicted helical regions (with lighter shades corresponding to lower certainty of prediction); green bar, β -sheets; gray bar, ambiguous.

predicted secondary and tertiary structure, using structures of p130Cas as templates (Briknarova *et al.*, 2005; Wisniewska *et al.*, 2005). Figure 2A demonstrates that HEPL and p130Cas are predicted to fold almost identically within the SH3 domain. Further, despite only 28% primary sequence identity, the predicted secondary structure for residues 432-591 of HEPL is extremely similar to that for residues 449-610 for p130Cas, implying a well-conserved fold (Figure 2B). At present, no adequate template exists in PDB to create a tertiary model for the Cas carboxy-terminus. However, comparison of the predicted secondary structure for the four Cas proteins reveals a strikingly similar periodicity of α -helices and β-sheets (Figure 2C) that is again compatible with the idea of a conserved tertiary structure.

Expression of Endogenous HEPL

The evolutionary conservation of HEPL suggested that it encoded a functional protein product rather than an unexpressed pseudogene. To test this directly, we first used quantitative RT-PCR to analyze HEPL expression in mRNAs prepared from 20 human tissues (Figure 3A). HEPL was most abundant in lung and spleen and was detected at lower

levels in additional tissues. These results were in accord with online resources in NCBI/Unigene (Wheeler *et al.*, 2004) that estimate the relative abundance of mRNAs based on their frequency of isolation in sequencing of tissue-specific libraries (results not shown). We next used quantitative RT-PCR to analyze mRNAs prepared from a panel of commonly used laboratory cell lines derived from diverse cell lineages (but enriched in carcinomas and leukemias, in accord with the expression prediction), to establish the general abundance of HEPL mRNA in cultured cells.

HEPL mRNA was detected in the majority of the cell lines (15 of 26), with highest levels of HEPL in the leukemia and ovarian cell lines (Figure 3B). As reference, HEF1 and p130Cas were readily detected in most of the cell lines examined, although p130Cas was not detected in most of the lymphoma/leukemia cell lines analyzed. In contrast, although very abundant in one breast carcinoma cell line (T47D), the Efs mRNA was only detected in 6 of the 26 cell lines assessed. Because increased expression of some Cas proteins has been linked to cancer progression, we investigated the relative expression of HEPL mRNA in a series of normal primary human ovarian surface epithelial (HOSE)
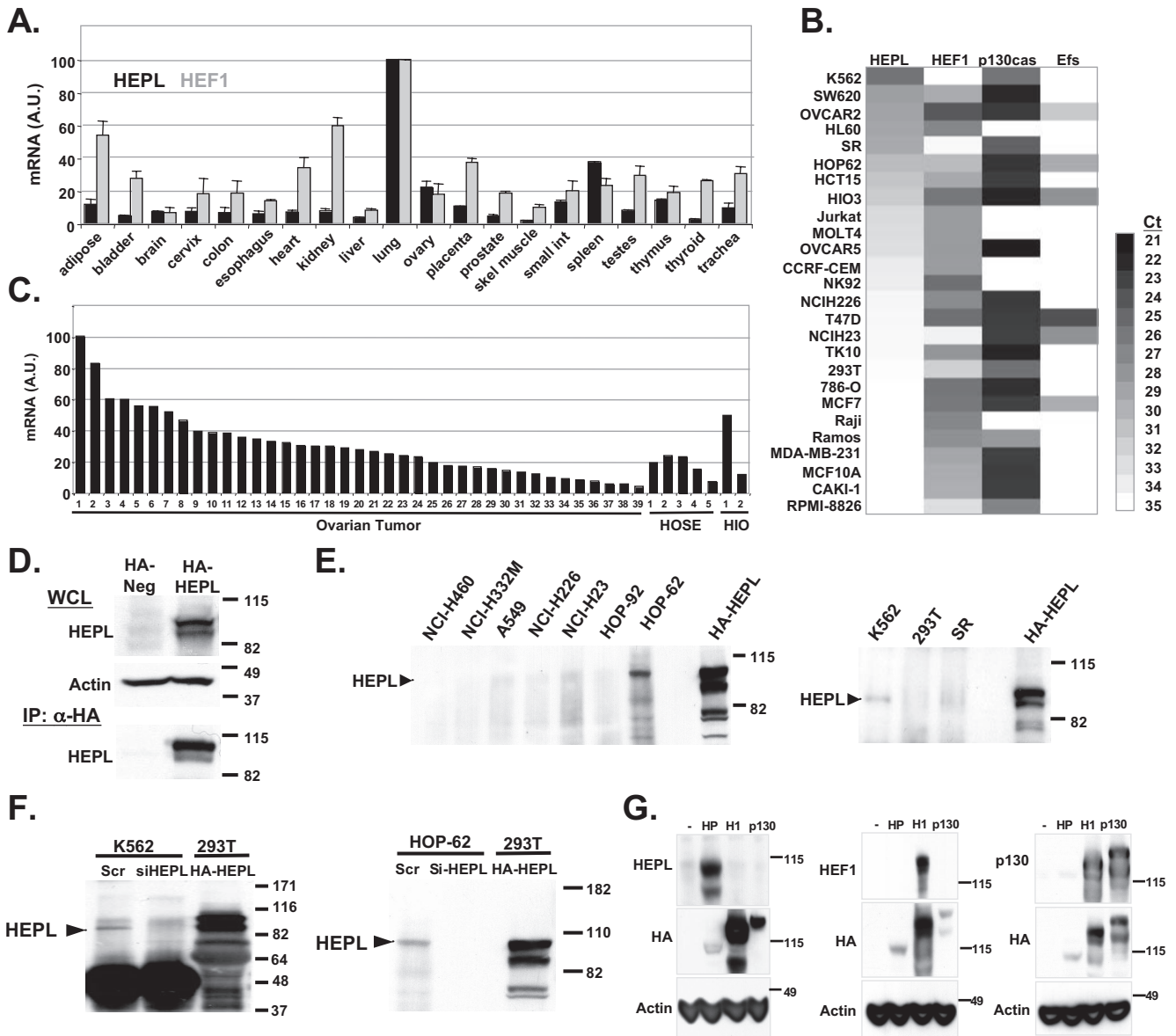
Figure 3. HEPL is most abundant in blood cells and lung tissue. (A) Relative abundance of HEPL transcript assessed by RT-PCR in cDNA prepared from primary human tissues, expressed in arbitrary units (AU). Four human cell lines are included for reference of expression levels. (B) Expression of HEPL and other Cas mRNAs in human cell lines. cDNA made with 100 ng (shown) or 20 ng (similar results; not shown) of total RNA of each sample was used for relative quantification of the transcripts using Taqman assays. The figure displays intensity-coded cycle threshold (Ct) values, reflecting the number of PCR cycles required for a significant fluorescence level above the baseline: Ct values of ≥35 indicate little or no gene expression. (C) Detection of HEPL and other Cas transcripts in nontransformed, immortalized, and transformed ovarian cell lines and tumors; data prepared as in B. (D) HA-HEPL or HA-fused to a negative control protein (*Materials and Methods*) were transfected into 293T cells, and whole cell lysates (WCL) were used directly for Western analysis with antibodies to HEPL or actin (as loading control) or for immunoprecipitation (IP) with antibody to HA before Western analysis with anti-HEPL. HEPL doublet seen preferentially in WCL over IP likely reflects a population of mixed HA-HEPL and HEPL with HA cleaved because of proteolysis. (E) Antibody to HEPL was used to probe immunoprecipitates from cell lysates as indicated; WCL of 293T cells transfected with HA-HEPL is a sizing control. (F) IPs of K562 or HOP-62 cells transfected with siRNA to HEPL (siHEPL) or control scrambled (Scr) siRNA were probed with antibody to HEPL; WCL from 293T expressing HA-HEPL provides a sizing control in each case. (G) Western blots of 293 cells expressing HA-HEPL (HP), -HEF1 (H1), -p130Cas (p130), or -negative control (−), sequentially probed with antibodies indicated.

cells, human SV40-immortalized ovarian (HIO) cell lines, and primary ovarian tumor tissue (Figure 3C). From this preliminary analysis, HEPL mRNA expression levels did not correlate significantly with ovarian transformation status.

To analyze HEPL at the protein level, we cloned the gene and prepared antibody against HEPL-derived peptide sequences that specifically recognized overexpressed epitope-tagged HEPL (Figure 3D). Using this antibody, we have found that HOP-62, K562, and SR, cell lines predicted by mRNA analysis to contain relatively abundant levels of HEPL, contained a protein species of ~105 kDa, whereas lower levels of a similarly migrating species are detected in a number of other cell lines (Figure 3E). This species was removed by treatment of cells with an siRNA targeted to
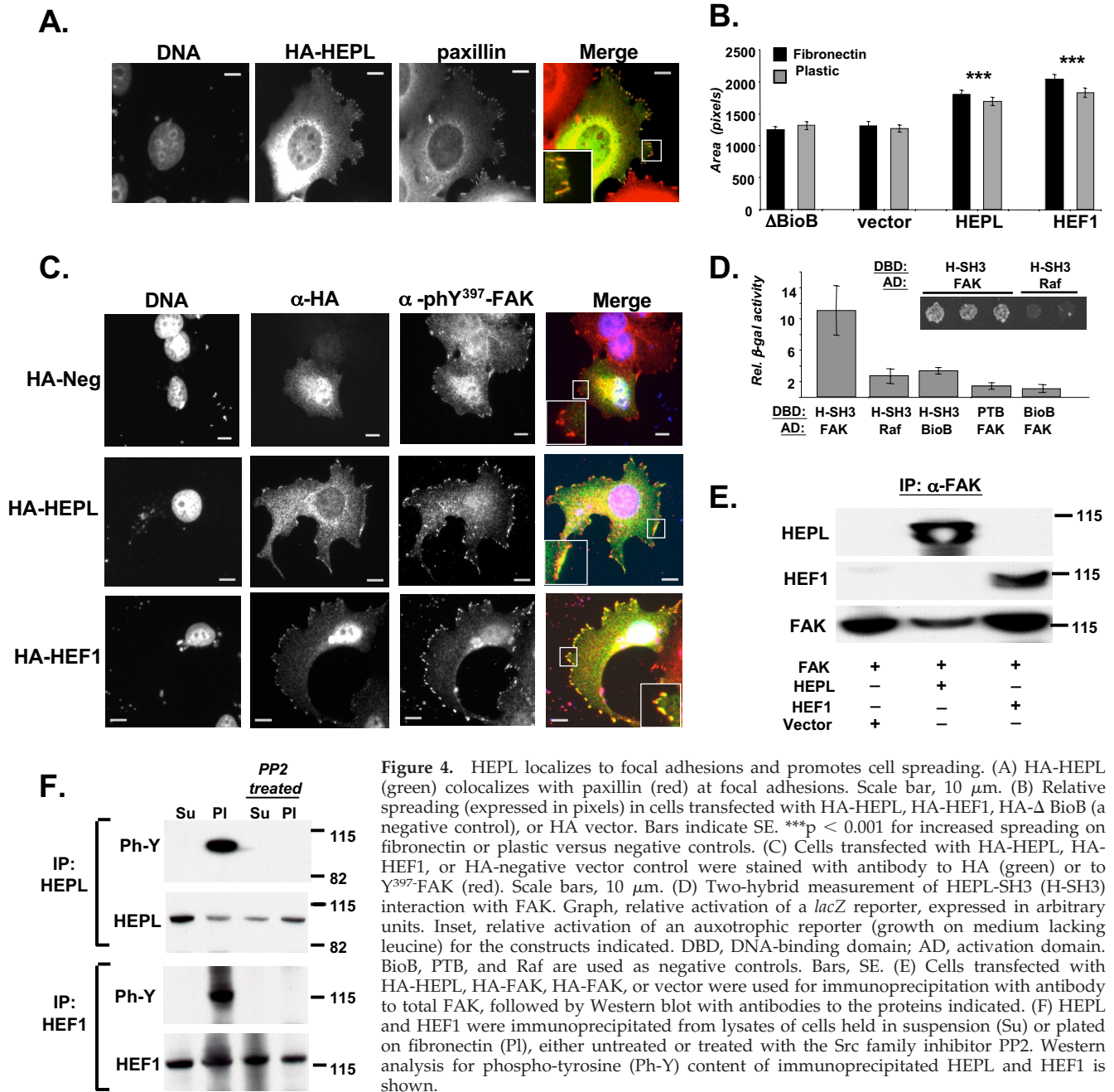
A.

B.

C.

D.

E.

F.

Figure 4. HEPL localizes to focal adhesions and promotes cell spreading. (A) HA-HEPL (green) colocalizes with paxillin (red) at focal adhesions. Scale bar, 10 μm. (B) Relative spreading (expressed in pixels) in cells transfected with HA-HEPL, HA-HEF1, HA-Δ BioB (a negative control), or HA vector. Bars indicate SE. ***p $<$ 0.001 for increased spreading on fibronectin or plastic versus negative controls. (C) Cells transfected with HA-HEPL, HA-HEF1, or HA-negative vector control were stained with antibody to HA (green) or to Y^{397}-FAK (red). Scale bars, 10 μm. (D) Two-hybrid measurement of HEPL-SH3 (H-SH3) interaction with FAK. Graph, relative activation of a *lacZ* reporter, expressed in arbitrary units. Inset, relative activation of an auxotrophic reporter (growth on medium lacking leucine) for the constructs indicated. DBD, DNA-binding domain; AD, activation domain. BioB, PTB, and Raf are used as negative controls. Bars, SE. (E) Cells transfected with HA-HEPL, HA-FAK, HA-FAK, or vector were used for immunoprecipitation with antibody to total FAK, followed by Western blot with antibodies to the proteins indicated. (F) HEPL and HEF1 were immunoprecipitated from lysates of cells held in suspension (Su) or plated on fibronectin (Pl), either untreated or treated with the Src family inhibitor PP2. Western analysis for phospho-tyrosine (Ph-Y) content of immunoprecipitated HEPL and HEF1 is shown.

HEPL (Figure 3F). Together, these data indicate that HEPL is a bona fide new member of the Cas family. Based on our analysis to date, antibodies to the more widely studied Cas family members p130Cas and HEF1 do not cross-react with HEPL (Figure 3G), suggesting the presence of HEPL may mask phenotypes associated with depletion of other family members.

HEPL Conserves Cas Family Functions in FAK Regulation and Cell Spreading

The best-defined action of Cas family proteins is as intermediates in integrin-dependent attachment signaling, regulating cell attachment, spreading, and migration. Although antibody to endogenous HEPL worked poorly in immunofluorescence analysis, HA-HEPL transfected into MCF7 cells (which express a low level of HEPL mRNA) colocalized with paxillin at focal adhesions (Figure 4A), comparable to other Cas proteins. HA-HEPL–transfected cells spread to a greater degree than control or vector-transfected cells, but to a lesser degree than cells transfected with HA-HEF1 (Figure 4B). Also suggesting a less potent action for HA-HEPL than HA-HEF1, levels of Y^{397}-phosphorylated (activated) FAK were strongly increased at focal adhesions in HA-HEF1 cells. Interestingly, in HA-HEPL transfected cells, only a subpopulation of the cells ($\sim$15–20%) showed increased levels of Y^{397}-phosphorylated FAK, whereas the remainder of the population remained at the levels of the negative control cells (Figure 4C).
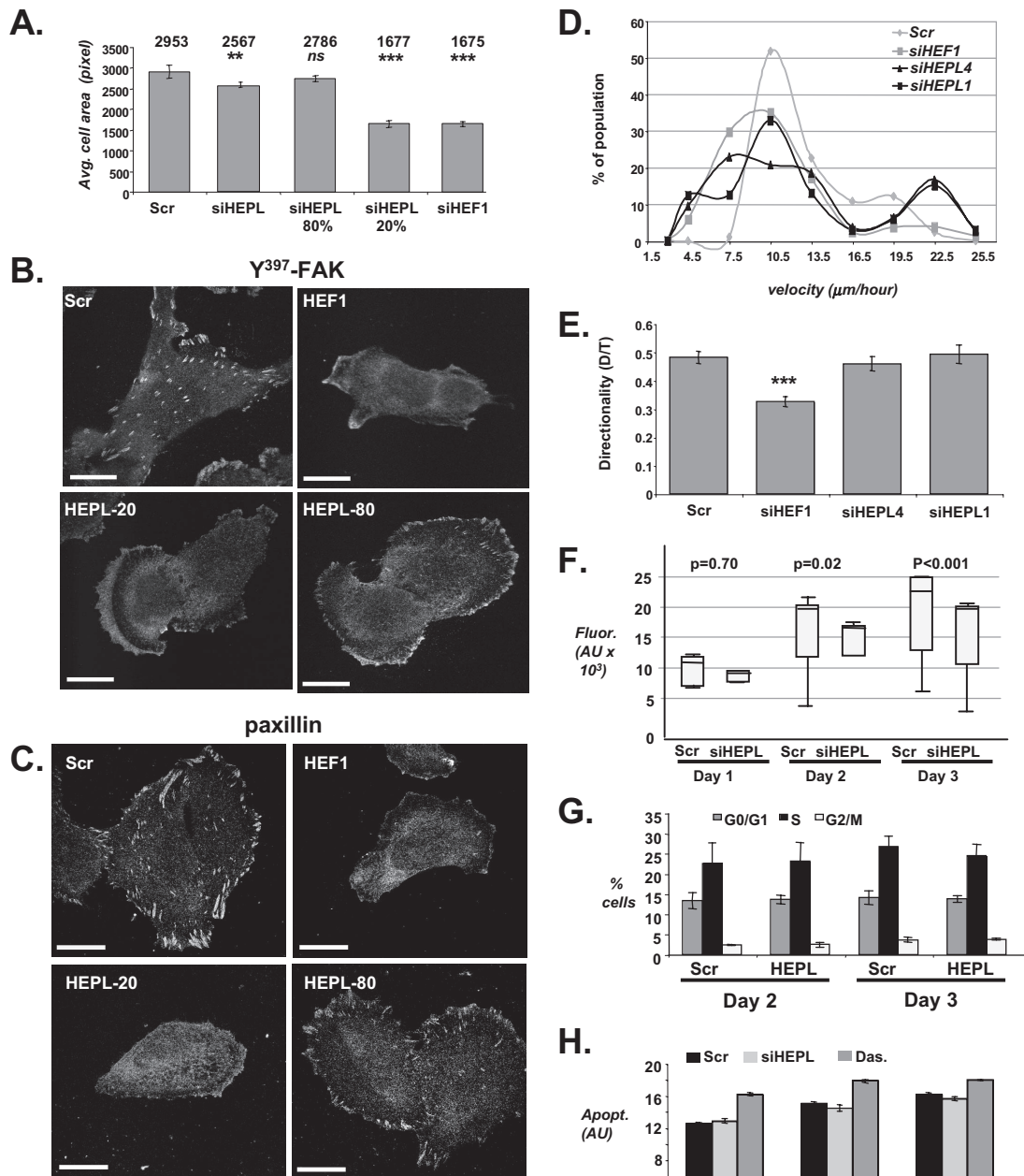
Figure 5. Depletion of HEPL influences FAK activation, cell spreading, and motility. (A) Relative spreading (expressed in pixels) in cells transfected with siRNA to HEPL, HEF1, or scrambled control (Scr). Bars, SE. **p < 0.01; ***p < 0.001; ns, nonsignificant (p > 0.05). HEPL-depleted cells staining weakly (20%) or more intensely (80%) for Y^{397}-FAK are presented grouped, and separately. For this and subsequent panels in this figure, a Dharmacon Smartpool containing four HEPL targeting siRNAs was used. We have separately confirmed results using individual deconvoluted siRNAs to HEPL (Supplementary Figure 2) and shown that siHEPL1 and siHEPL4, but not siHEPL2 and siHEPL3, deplete HEPL and generate equivalent results. (B and C) Confocal images of HOP62 cells transfected with siRNA to HEPL, HEF1, or scrambled control (Scr) were stained with antibody to Y^{397}-FAK (B) or paxillin (C). Two different representative HEPL-depleted cells, corresponding to the 20 and 80% phenotypes, are shown. All scale bars, 20 μm. (D) The migration of individual cells was tracked in movies taken from 36 to 48 h after treatment with siRNAs as indicated. Cells were binned into different groups based on average velocity over 12 h. For each group ~80 cells were analyzed in two independent experiments. (E) The ability of siRNA-treated cells to maintain directional movement was calculated from the movies analyzed in D. D/T indicates the average radial distance (D) individual siRNA-treated cells move from position at the beginning of the assay in comparison to the total (T) distance the cells move during the period of observation. ***p < 0.001; other values not significant versus Scr. (F) Proliferation of K562 cells treated with siRNA to HEPL (siHEPL) or control scrambled (Scr) siRNA, as assessed by Alamar blue staining 1, 2, or 3 d after depletion, with

Our modeling experiments had suggested that the HEPL SH3 domain would bind FAK (Figure 2A). Using a two-hybrid approach, we confirmed a direct interaction between the HEPL SH3 domain and the FAK C-terminus (Figure 4D). This result paralleled previous demonstrations of interactions between the HEF1 and other Cas family SH3 domains with proline-rich SH3 domain–binding motifs in this region (Polte and Hanks, 1995; Law *et al.,* 1996) and suggested that overexpressed HEPL activates FAK based on direct interaction. Finally, like HEF1, full-length HEPL was immunoprecipitated with antibody to FAK from cells transfected with plasmids expressing each protein (Figure 4E). A common characteristic of Cas family proteins is their phosphorylation by Src family kinases during the cell attachment process, which provides a docking site for FAK and contributes to assembly of signaling complexes at focal adhesions (O'Neill *et al.,* 2000). HEPL lacks the YDYVHL site that coordinates docking with the Src SH2 domain for other family members (Tachibana *et al.,* 1997). Nevertheless, attachment-induced Src phosphorylation of HEPL was observed (Figure 4F), suggesting that additional interactions between HEPL, FAK, and Src are sufficient to drive this modification.

We next analyzed focal adhesions and cell spreading in HOP-62 cells from which HEPL had been depleted by siRNA (Figure 5A). In the total population of siRNA transfected cells, HEPL depletion reduced cell spreading ($p < 0.01$), although to a significantly smaller degree than HEF1 depletion ($p < 0.001$). On closer inspection, the HEPL-depleted population differed from both scrambled siRNA control- and HEF1-depleted cells in its heterogeneous nature. A subpopulation of ~20% of HEPL-depleted cells showed reduced and/or differentially localized staining for Y^{397}-phosphorylated FAK (Figure 5B), with residual staining diffusely distributed at the cell periphery rather than in discrete focal adhesions. Subsequent staining with antibody to paxillin (Figure 5C) revealed a similar population of ~20% of cells that had greatly reduced paxillin staining. Reanalyzing spreading data for strongly versus weakly Y^{397}-FAK staining HEPL-depleted cells (Figure 5A) revealed a clear segregation of weak staining with reduced spreading. By contrast, almost all cells with depleted HEF1 were less spread than Scr control cells and had reduced Y^{397}-phosphorylated FAK, to the same degree as the 20% of responsive HEPL-depleted cells.

An important function of Cas proteins is regulation of cell migration (Klemke *et al.,* 1998; van Seventer *et al.,* 2001; Fashena *et al.,* 2002). Analysis of live cell images of HEPL- or HEF1-depleted cells indicated that depletion distorted migration profiles relative to Scr-depleted controls. HEF1 depletion uniformly reduced cellular velocity ($p < 0.001$, Figure 5D). Interestingly, the phenotype observed with HEPL

depletion was more complex. Two distinct HEPL-depleting siRNAs caused appearance of a population of slow-moving cells, although for one of the siRNAs the effect was not statistically significant (velocity $<9 \mu m/h$, $p < 0.05$ and $p = 0.15$). However, the HEPL-depleting siRNAs each also unexpectedly caused appearance of a faster-moving (velocity $<18 \mu m/h$) group of cells, corresponding to ~15% of the population ($p < 0.05$ and $p < 0.02$). Greater velocity has been reported to be associated with greater cell spreading in some cell types with manipulated Cas proteins (e.g., Fashena *et al.,* 2002) for HEF1 in MCF7 cells), although there are examples of cell movement where a connection between spreading and velocity is not observed (Friedl and Wolf, 2003). No "highly spread" population was detected with HEPL-depleted cells (results not shown). As further difference from the Cas group, whereas depletion of HEF1 reduced directionality of movement of cells, no such effect was seen with HEPL depletion (Figure 5E).

Some members of the Cas family, such as HEF1, also play important roles in regulation of apoptosis and proliferation (e.g., Law *et al.,* 2000; O'Neill and Golemis, 2001; Pugacheva and Golemis, 2005; Dadke *et al.,* 2006). siRNA depletion of HEPL from K562 cells led to a slightly slower accumulation of cells over 3 days (Figure 5F), although the general compartmentalization of cells in the G1, S, and G2/M phases of cell cycle was not significantly affected (Figure 5G). HEPL depletion did not influence the level of apoptotic cells in the population (Figure 5. H and I). As siRNA depletion rarely exceeds 90–95%, a definitive determination that HEPL does not affect cell cycle or apoptosis requires a gene knockout; however, at present, the most demonstrable activity of HEPL is at focal adhesions, as with p130Cas.

DISCUSSION

Although the Cas protein family has been studied for over a decade, this study represents the first extensive examination of the Cas proteins utilizing genomic resources. The newly identified HEPL proteins define a legitimate novel branch of the Cas family. HEPL proteins conserve important functional domains required for interaction with FAK, for targeting to focal adhesions, and for regulating cell spreading and FAK activation. Although human HEPL is expressed in a relatively limited subset of cell types relative to p130Cas and HEF1, based on mRNA analysis it appears to be as prevalent as Efs/Sin in cultured cell lines.

HEPL does not appear to be as biologically active as HEF1, based on a number of criteria presented above. Particularly in control of FAK activation, only a minority of cells respond either to overexpressed HEPL or to depleted HEPL, under conditions where almost all cells respond to similarly manipulated HEF1. siRNA depletion typically introduces siRNA into >90% of cells, and our analysis of HEPL siRNA-transfected cells confirmed >75% depletion in practice, excluding the trivial explanation of incomplete depletion. Rather, we expect the difference may relate to cell-specific variability in the intrinsic expression level of the additional Cas family members within HOP-62 cells: single cell analyses are beginning to demonstrate that this is an important property governing average gene expression in cell populations (e.g., Levsky and Singer, 2003; Mar *et al.,* 2006). We propose that typically within cells expressing multiple Cas family members, HEPL may make a minor contribution to regulation of cell growth properties. Part of the reduced biological activity of HEPL may arise from lack of a key motif for Src recognition (YDYVHL; Tachibana *et al.,* 1997). We have shown that HEPL is still phosphorylated by Src

Figure 5 (cont). data represented using Tukey box-plots. Statistical significance was assessed by use of a GEE-estimated model assuming a Gamma distribution with log link and an exchangeable correlation over time. (G) Guava analysis of cell cycle compartmentalization of Scr or siHEPL-transfected cells analyzed in F. Note that the high percentage of cells in S is characteristic of K562 cells. (H) Analysis of apoptosis in K562 cells treated with siRNA to HEPL or control scrambled (Scr) siRNA, or with dasatinib as a positive control. Apoptosis detected by Apoptag is reported in arbitrary units (AU); difference between dasatinib and other samples is statistically significant ($p < 0.001$). (I) Western blot of full-length and cleaved (**) gelsolin in cells transfected 3 d previously with Scr or HEPL siRNA, or treated with dasatinib or etoposide as positive controls to induce apoptosis. β-actin provides a loading control. Similar results were obtained for PARP cleavage (not shown).

family kinases during cell attachment, suggesting this motif is not essential for an interaction with Src, presumably because of the presence of multiple interaction interfaces joining HEPL, FAK, and Src; however, Cas proteins also reciprocally contribute to Src activation in the attachment process (e.g., Alexandropoulos and Baltimore, 1996), and this function may be limited.

Intriguingly, HEPL activity qualitatively differs from other Cas proteins in at least one important way, in the regulation of migration. Although loss or depletion of p130Cas and HEF1 reduces cell migration (e.g., Natarajan *et al.,* 2006), HEPL depletion induced faster migration in at least a subset of cells. The reason for this is so far unknown; however, an intriguing possibility is that through possession of some but not all Cas family functions, HEPL may weakly oppose the action of other Cas family proteins via action as a "dominant negative." Particularly in a cell background low in other Cas proteins, HEPL may be important. Hence, it is important for experiments involving knockdown or knockout of Cas family proteins to subsequently consider HEPL status in interpreting phenotypes. Separately, for each of the Cas proteins, some interacting partners have been described unique to that family member; the interaction profile of HEPL has not yet been explored, but may include novel interactors and intracellular roles.

Intriguingly, the region of chromosome 20 encompassing HEPL is included as an amplicon in many solid tumors (Dessen *et al.,* 2002). It is hence possible that as with p130Cas and HEF1 (Singh *et al.,* 2007), altered expression of HEPL contributes to the pathogenesis of cancers or other diseases. Our data demonstrate that HEPL overexpression is sufficient to increase cell spreading and FAK activation, phenotypes associated with increased tumor invasiveness. Based on its expression profile, in nontransformed cells HEPL may be most relevant to the normal function of the hematopoietic system and the lung. As all the Cas proteins have the potential to interact with multiple partner proteins, sometimes in large complexes, the presence of additional family members might also be expected to induce cell- and tissue–type differences in complex assembly and stoichiometry. Clearly, future studies of the Cas group should consider the possible role of redundant HEPL function in evaluating knockdown, knockout, or overexpression phenotypes. In sum, this study suggests ample new ground for further investigation.

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