Huntingtin-interacting protein 1 is overexpressed in prostate and colon cancer and is critical for cellular survival

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Huntingtin-interacting protein 1 (HIP1) is a cofactor in clathrin-mediated vesicle trafficking. It was first implicated in cancer biology as part of a chromosomal translocation in leukemia. Here we report that HIP1 is expressed in prostate and colon tumor cells, but not in corresponding benign epithelia. The relationship between HIP1 expression in primary prostate cancer and clinical outcomes was evaluated with tissue microarrays. HIP1 expression was significantly associated with prostate cancer progression and metastasis. Conversely, primary prostate cancers lacking HIP1 expression consistently showed no progression after radical prostatectomy. In addition, the expression of HIP1 was elevated in prostate tumors from the transgenic mouse model of prostate cancer (TRAMP). At the molecular level, expression of a dominant negative mutant of HIP1 led to caspase-9–dependent apoptosis, suggesting that HIP1 is a cellular survival factor. Thus, HIP1 may play a role in tumorigenesis by allowing the survival of precancerous or cancerous cells. HIP1 might accomplish this via regulation of clathrin-mediated trafficking, a fundamental cellular pathway that has not previously been associated with tumorigenesis. HIP1 represents a putative prognostic factor for prostate cancer and a potential therapy target in prostate as well as colon cancers.

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Introduction

Originally identified as a protein that interacts with huntingtin, the product of the gene mutated in Huntington disease (1, 2), huntingtin-interacting protein 1 (HIP1) is a 116-kDa protein that belongs to an evolutionarily conserved family of proteins that includes *Sla2p*, HIP1-related (HIP1r), and HIP1. Sla2p, also known as End4p, is a yeast protein involved in clathrin-mediated endocytosis, cytoskeletal organization, and cellular survival (3, 4). HIP1r was the first human homologue of *Sla2p* to be identified as a cofactor in clathrin-mediated endocytosis (5, 6). More recently, HIP1 itself has been shown to be involved in clathrin-mediated trafficking (7–10). The domain structure of all three proteins contains an epsin N-terminal homology (ENTH) domain, a leucine zipper motif, and a carboxyl terminus homologous to TALIN. ENTH domains bind to polyphosphoinositide signaling lipids and have so far only been found in cofactors of clathrin-mediated trafficking (11, 12). This lipid binding is thought to localize the cofactors, including HIP1, to areas of receptor-mediated endocytosis to assist in clathrin lattice formation.

HIP1 and its interaction with huntingtin (htt) may be important in clathrin trafficking and cell survival. The function of htt itself is unknown, but it has been shown to colocalize with and copurify with clathrin-coated vesicles (7, 13, 14). Expression of htt is necessary for cellular survival, as demonstrated by the murine knockout of Hdh, which results in increased apoptosis in multiple tissues (15). In addition to its role in clathrin trafficking, a role for HIP1 in cell survival or growth is suggested by the fact that the mRNA of HIP1 is elevated in various tumor cell lines compared with that in their normal tissue derivatives (16), as well as by genetic evidence from *HIP1* knockout mice. These mice exhibit increased apoptosis in postmeiotic spermatids of the testis. This is due to an intrinsic loss of HIP1 expression as the latter cells are the only cells of the testis that express HIP1 (9). If the HIP1/htt interaction is physiologic, it is possible that HIP1 recruits htt to clathrin-coated vesicles and that this subcellular localization of htt is necessary for cell survival.

In light of the proposed involvement of the htt/HIP1 pathway in cell survival and apoptosis, it is interesting to note that individuals with Huntington disease have a lower incidence of cancer compared with their nonaffected relatives (17). This inverse correlation suggests

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Nonstandard abbreviations used: huntingtin-interacting protein 1 (HIP1); HIP1-related (HIP1r); epsin N-terminal homology (ENTH); huntingtin (htt); prostatic intraepithelial neoplasia (PIN); transgenic mouse model of prostate cancer (TRAMP); National Cancer Institute (NCI); prostrate specific antigen (PSA); tissue microarray (TMA).

that disruption of the same fundamental pathway may result in both neuronal apoptosis (Huntington disease) and cancer cell apoptosis (diminished incidence of cancer). If such is the case, and HIP1 is necessary for some cell types to survive, the expression of HIP1 would be expected to be altered in tumor tissues, as compared with non-tumor tissue.

Here, using 60 cancer cell lines, immunohistochemistry, and primary tissue microarrays (TMAs) with clinical follow-up of 114 prostate cancer patients, we report the surprising result that the HIP1 protein was specifically overexpressed in prostate and colon cancer, which represent tissues of origin where HIP1 is not normally expressed. The frequency of expression of HIP1 was highest in metastatic prostate cancer, and was higher in clinically localized cancer than in prostatic intraepethelial neoplasia, thus correlating with the severity of the neoplastic lesion. In clinically localized prostate cancer, expression of HIP1 was correlated with aggressive pathology. There were no events of prostate specific antigen (PSA) recurrence in patients with HIP1-negative tumors, and HIP1 staining was a significant predictor of PSA recurrence in both univariate and multivariable models. In addition, HIP1 overexpression in human prostate cancer was recapitulated in tumors of the transgenic mouse model of prostate cancer (TRAMP) mouse (18). Finally, expression of a dominant negative mutant of HIP1 led to apoptosis in a caspase-9–dependent and caspase-8–independent fashion. These data suggest that HIP1 expression contributes to tumorigenesis by maintaining survival of cells resulting in a selective growth advantage, and they indicate a possible role for HIP1 as both a prognostic marker and a therapy target in human prostate cancer.

Methods

Western blot analysis of NCI 60 cell lines. Cell pellets kindly provided by Richard Camalier of the National Cancer Institute (NCI) were dissolved in lysis buffer (20 mM Tris [pH 7.4], 1% Triton, 150 mM NaCl, and protease inhibitors). Extracts (50 μg) were then subjected to SDS-PAGE and transferred to nitrocellulose for Western blot analysis.

Immunohistochemistry and tissue microarrays. The high-density TMA of multiple primary tumors and the TARP1 were obtained from the NCI. High-density TMAs for prostate tumors were assembled as previously described (19). Tumor samples from patients with clinically localized prostate cancer were obtained in the Institutional Review Board–approved University of Michigan Prostate Specialized Program of Research Excellence (SPORE) tumor bank. Cancer survival among these subjects was monitored by annual serum PSA testing, and PSA recurrence was defined as a postoperative rise in PSA to levels greater than 0.2 ng/ml. Metastatic prostate tumors were collected from a series of rapid autopsies performed at the University of Michigan on men who died of metastatic prostate cancer. Autopsies were performed 4–6 hours after death (20). Standard biotin-avidin–complex immunohistochemistry was performed. Immunostaining intensity was scored as absent, weak, moderate, or high. In order to qualify as high, staining intensity had to be equivalent to that observed in endothelia of small blood vessels of the tissue. There was no staining when secondary antibody was used alone as a negative control. These TMAs had been previously validated for control proteins.

Data analysis. To avoid bias, readers of the microarrays were blinded to the clinical data. Heterogeneity was reflected in the prostate cancer microarrays by the fact that each patient with prostate cancer had multiple microarray samples (average of four per patient) that did not display the same levels of HIP1 staining (supplementary Table 1). The highest level of HIP1 expression from multiple tumor specimens was therefore used to represent the overall patient score. Next, the expression of HIP1 was characterized as a dichotomous variable. Specifically, each patient received a score of either 0, for absent expression, or a score of 1, if expression was weak, moderate, or high. Any cases where there was only a single specimen available were excluded from analysis. SPSS software (SPSS Science, Chicago, Illinois, USA) was used to compare HIP1 staining with known clinical and histopathologic prognosticators as well as outcome data. The significance of correlations was examined using Pearson's χ-squared, likelihood ratios, and Spearman's correlation. We assumed statistical significance for findings where P was less than 0.05. The dichotomous categories of HIP1 staining were compared with measures in the clinically linked database, which included Gleason score, pathologic stage (pT3 = extra prostatic extension or seminal vesicle invasion, two measures of local spread), preoperative PSA, PSA recurrence, and clinical stage. For multivariate modeling, Cox regression analyses were performed using HIP1 expression, Gleason score (using 7 or greater as predictive of a bad outcome; see supplementary Figure 3a), preoperative PSA (using 4 as a cutoff; see supplementary Figure 3b), and tumor stage (using pT3 as predictive of a bad outcome; see supplementary Figure 3c) as covariates. Because of the complete separation in HIP1 expression, a likelihood ratio test was used to examine its role in predicting recurrence in the context of Gleason scores, preoperative PSA values, and tumor stage at diagnosis. Subsequently, a second run was performed, with only the three traditional pathologic indicators as covariates. A comparison of the –2 log likelihood values obtained was carried out to determine whether HIP1 expression was a significant independent predictor.

Prostate cancer in TRAMP mice. TRAMP mice were genotyped by PCR (18) and observed for the development of tumors by palpation. Normal and tumor tissue was obtained from TRAMP mice prostates, placed in OCT, and snap-frozen in liquid nitrogen. Tissue was subsequently recovered from the blocks, homogenized, and placed in lysis buffer A (9). These protein extracts

were run on standard denaturing SDS-PAGE, and Western blotting was performed using the anti-HIP1 mAb's 4B10 and 1B11.

Plasmids and HIP1 constructs. Upon identification of a previously unknown 5′ exon (9), the construct pcDNA3/HIP1 was modified in order to include this exon. Using primers HIP1.ex1.5′ (5′-GGCCGTGGTACCCCCGGGGCAGCCGAGGGC-3′) and HIP1.ex2.3′ (5′-TTCCTGCGTATTAATGGCCTTATTGATGCTGACAGTCTGAGTTCCGCTCGAAGCTCTCGCGCTCCGCC-3′), and human genomic DNA (Promega Corp., Madison, Wisconsin, USA) as template, these exons were amplified by polymerase chain reaction. Subsequently, the PCR product was digested with KpnI and cloned between the KpnI site of the vector and the AsnI site in HIP1 exon 2. This plasmid, designated pcDNA3/FLHIP1, was sequenced and confirmed to correspond to known HIP1 sequences 3′ of the newly identified exon. Furthermore, the sequence of exon 1 was identical with the human genomic sequence of the human HIP1 gene in the National Center for Biotechnology Information database (accession no. AC004491). In order to generate pcDNA3/HIP1/ΔE, the full-length HIP1 construct was digested with KpnI and HpaI (positions 1 and 596, respectively) to excise the ENTH domain, blunted with Klenow polymerase (New England Biolabs, Beverly, Massachusetts, USA) and religated. pcDNA3/HIP1/ΔLD was generated by ligating a PCR fragment containing the first 766 bp of the HIP1 gene (generated with primers HIP1.ATG.5′ [5′-TCGGCTCCTCGCGAATTCATGGATCGGATGGCCAGC-3′] and HIP1.784-823.H3R1.3′ [5′-GTGGAGTTTGAAGAGAAGCTTGACAGTGTAGTCATAAA-3′]) to the EcoRI site at position 1336 in HIP1. pcDNA3/HIP1/ΔC was generated by digesting pcDNA3/FLHIP1 with EcoRI and BamHI (sites at positions 1336 and 2510, respectively), blunting the ends with Klenow, and subsequently religating. The resulting construct was sequenced and shown to be in-frame. HIP1/ΔT was generated by deletion of the sequences of HIP1 C-terminal to the BamHI site (position 2510). FLHIP1-myc-his was generated by subcloning FLHIP1 into the pcDNA3.1/myc-his vector. pcDNA3/DN Casp8, pcDNA3/DN Casp9-myc, and pEF-Bos/β-gal were the kind gift of Gabriel Nunez (University of Michigan).

Antibodies. The polyclonal HIP1 antibody pcHIP1, was generated using the antigen 3′-HIP1 (containing the coiled coil, leucine zipper, and TALIN homology domains starting at amino acid 416) and has been previously described (21). The HIP1 mAb's HIP1/4B10 and HIP1/1B11 were generated using the same antigen and standard methods. HIP1/1B11 was found to recognize human HIP1 and mouse HIP1, whereas HIP1/4B10 recognized only human HIP1. In addition, it recognized an epitope between amino acids 750 and 780 of HIP1 (I.F. Mizukami and T.S. Ross, unpublished observations). Commercial antibodies were obtained from the following sources: monoclonal myc, Invitrogen Corp. (Carlsbad, California, USA); polyclonal caspase-9, Cayman Chemical Co. (Ann Arbor, Michigan, USA); monoclonal caspase-8, Cell Signaling Technologies (Beverly, Massachusetts, USA); and monoclonal adaptin-γ (AP1), Transduction Laboratories (San Diego, California, USA).

Immunoprecipitation and Western blot analysis. For co-immunoprecipitation assays, 60–80% confluent 293T cells in 100-mm plates were transfected with 5 μg of DNA, respectively, using the LipofectAMINE system (Invitrogen). Cells were harvested at 18–22 hours after transfection, and lysis buffer A was used in co-immunoprecipitation analyses. One milligram of protein, as specified in the figure legends, was immunoprecipitated with 10 μl of the anti-myc mAb overnight at 4°C, incubated on a rotator for 30 minutes with protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA), and run on 7% polyacrylamide gels. These gels were transferred to nitrocellulose, blotted with the manufacturer's recommended dilutions of the antibodies (1:5,000 for myc, 1:1,000 for HIP1/4B10, 1:2,000 for pcHIP1), and visualized by enhanced chemiluminescence. For analysis of cellular extracts overexpressing HIP1 and mutant constructs, 10 μg of protein was loaded per lane when examining the expression of HIP1 proteins; 100 μg per lane was used for the Western blot analysis of DN caspase-8 and DN caspase-9. Both mutants have the active cysteine in each caspase mutated to a serine (22).

Apoptosis assays. Apoptosis was determined by co-transfecting cells with HIP1 mutant plasmids and other constructs, as well as pEF-Bos/β-gal, which contains the β-galactosidase gene under the control of the EF1 promoter (23). Two micrograms of experimental plasmid DNA was used (1 μg of each construct, with pcDNA3 added to transfections that involved single experimental plasmid) in each well of a six-well plate. Transfections were done with LipofectAMINE PLUS (Invitrogen) in triplicate in 293T cells that were 60–80% confluent at the time of transfection. At 10–15 hours after transfection, cells were fixed in 1× PBS, 2% paraformaldehyde, and 0.8% glutaraldehyde. After washing with PBS, cells were incubated with a stain solution containing X-gal (Roche Molecular Biochemicals, Indianapolis, Indiana, USA) for 30 minutes to 1 hour. Apoptotic profiles were defined as cells with extensive blebbing, shrinkage, and rounding. The number of apoptotic profiles among cells that were stained blue by X-gal was counted and expressed as a percentage of total blue cells that were apoptotic. By this technique, only cells that are adherent to the plate are counted (22).

TUNEL assays. 293T cells were transfected as above with full-length HIP1 and HIP1/ΔE. After allowing for expression of the protein (20 hours), cells were fixed in PBS with 4% paraformaldehyde for 1 hour and permeabilized in 0.1% Triton X-100. Cells were then labeled with fluorescein-tagged dUTP using the InSitu Cell Death Kit (Roche Molecular Biochemicals), following manufacturer's instructions. Following TUNEL label-

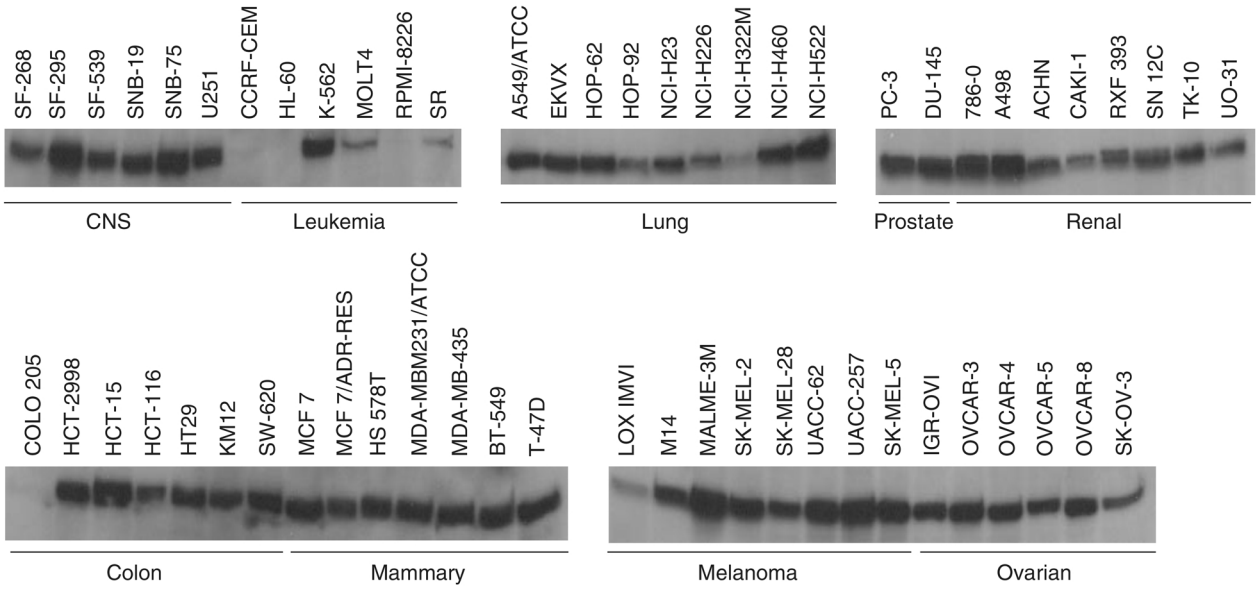

Figure 1
Western blot analysis of NCI 60–cancer cell line screen. Protein extracts (50 μg) derived from the 60 cell lines were separated on a 6% polyacrylamide gel, transferred to nitrocellulose, and blotted with a mix of the anti-HIP1 mAb's 4B10 and 1B11.

ing, cells were labeled with HIP1/4B10, followed Texas Red–tagged secondary antibody. Labeling was visualized by confocal microscopy (Carl Zeiss MicroImaging Inc., Thornwood, New York, USA) as previously described (9). The proportion of transfected cells (labeled red) that were also TUNEL-positive (green) was counted and reported.

Prostate sample acquisition. Clinical samples came from a series of patients treated at the University of Michigan. Tumor samples for outcome analyses were derived from a series of consecutive patients who underwent prostatectomy for prostate-confined (stage I and II) cancer in 1995 and 1996. Prostatectomy was the primary therapy for these patients. They received no hormonal or radiation therapy prior to relapse. To obtain metastatic cases of prostate cancer (see Figure 5a), a rapid autopsy protocol was used. Informed consent was obtained from patients with advanced hormone-refractory prostate cancer for autopsy and tissue procurement prior to death. The median time from death to autopsy was 3 hours. Metastatic prostate cancer samples were then collected, fixed in formalin, embedded in paraffin, and used to generate TMAs. Clinical and pathologic data for all patients were collected with Institutional Review Board approval.

Results

HIP1 is overexpressed in a majority of epithelial-derived cancer cell lines. To complement the analysis of HIP1 mRNA expression in tumor cell lines (16), we decided to investigate protein levels by creating mAb's specific to HIP1. The specificity of the antibodies to HIP1 was confirmed by comparing Western blot analyses of murine embryonic fibroblast extracts from embryos with HIP1 deleted with those from their wild-type littermates (data not shown). Two mAb's were found to be useful for Western blot analysis of HIP1, as well as for immunohistochemistry of human tissue. These antibodies were designated HIP1/4B10 and HIP1/1B11.

Western blot analysis of extracts from the 60 cancer cell lines of the NCI's in vitro anticancer drug screen (24) demonstrated that HIP1 was highly expressed in most of the cancer cell lines (50 of 53) derived from solid tumors (Figure 1). The cancers represented included colon, breast, melanoma, ovarian, prostate, kidney, and lung. Analysis of clathrin, AP1, and actin served as loading controls and did not correlate with HIP1 expression (supplementary Figure 1).

HIP1 expression in primary human tissues and tumors. To examine whether the expression level of HIP1 in cell lines corresponded with the behavior of tumors in vivo, we used standard immunohistochemistry to examine the expression of HIP1 in primary human normal and cancerous tissue. First, we examined expression in archived normal human tissues. HIP1 was expressed most highly in the endothelium of small blood vessels (Figure 2a). HIP1 was also expressed in several secretory epithelia, including breast ductal epithelium and gastric epithelium, and highly expressed in kidney distal tubular epithelium (Figures 2, b, c, and d, respectively). Other tissues that expressed HIP1 at high levels included the lung alveolar epithelia, cardiac muscle, choroid plexus of the brain, various peripheral nervous system ganglia, and liver (data not shown). We have also shown previously that HIP1 is expressed specifically in the postmeiotic spermatids of the seminiferous tubules of the testis (9). The normal epithelium of the colon and prostate did not express HIP1 (Figure 2, e and f, respectively).

Next, we used TMAs (25) to study the expression of HIP1 in primary tumor samples. Using the NCI's TMA

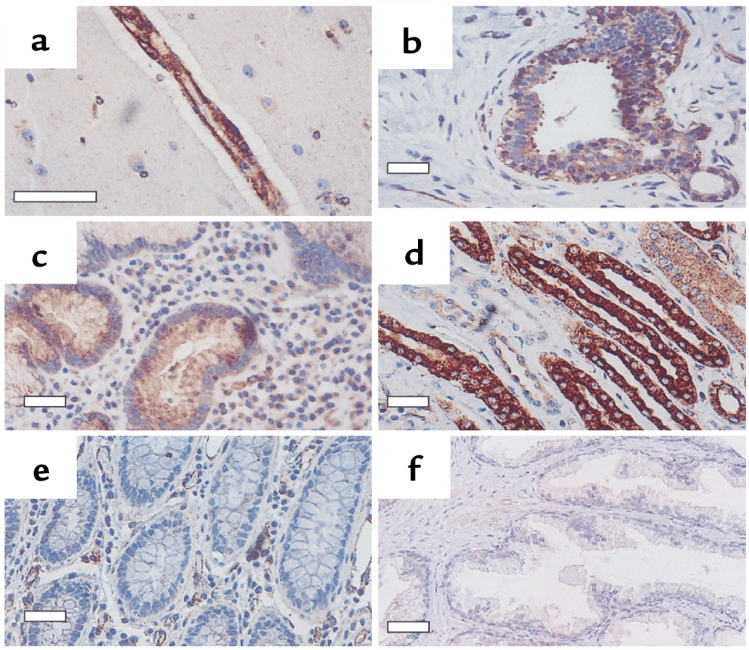

Figure 2
Expression of HIP1 in normal human tissues. (**a**) Endothelium of a cortical blood vessel shown in transverse section. (**b**) Breast ductal epithelia displayed moderate HIP1 staining. (**c**) Stomach epithelial cells displayed moderate HIP1 staining. (**d**) HIP1 was highly expressed in the distal renal tubules, seen here interspersed with weakly stained proximal renal tubules. (**e** and **f**) Colonic columnar epithelium lacked HIP1 staining (**e**), as did normal prostatic epithelium (**f**). The high staining of the stroma of the colon (**e**) is mostly endothelia of the blood vessels. Magnification, ×400. Scale bar, 50 μm. There was no staining when secondary antibody was used alone as a negative control.

that includes multiple tumor types, designated TARP1, we found HIP1 was moderately to highly expressed in a great percentage of CNS, breast, colon, lung, melanoma, ovarian, and prostate cancers (Figure 3). The highest frequencies of expression were found in ovarian cancer and melanoma. In contrast, there was no detectable HIP1 expression in over 80% of primary lymphoma samples, with the remaining 20% having low levels of expression. Thus, we find concordance between HIP1 expression in cell lines and that in primary tumor tissue, suggesting that HIP1 expression is an important contributory event in oncogenesis. We have used these arrays for expression analysis of multiple other proteins, and did not find any that were expressed in tumor tissue in the pattern observed for HIP1 (data not shown).

HIP1 is a specific marker for colon and prostate cancer. Given that benign colonic and prostatic epithelia did not express HIP1 (Figure 2, e and f), while their corresponding neoplastic derivatives did, we undertook a more careful study of these tissues. First, anti-HIP1 mAb's were used to stain colon cancers. Standard slides from archival tissue samples were obtained from 25 patients with well-differentiated colon cancers. On staining with either of the anti-HIP1 antibodies (4B10 or 1B11), benign tissue was found to not express HIP1, while adjacent colon tumors had high levels of HIP1 expression (Figure 4a). Quantitatively, benign epithelium of the colon never had moderate or high expression, whereas 48% of the well-differentiated colon tumors had moderate or high HIP1 expression (Figure 4b). Eighty-four percent of benign colon

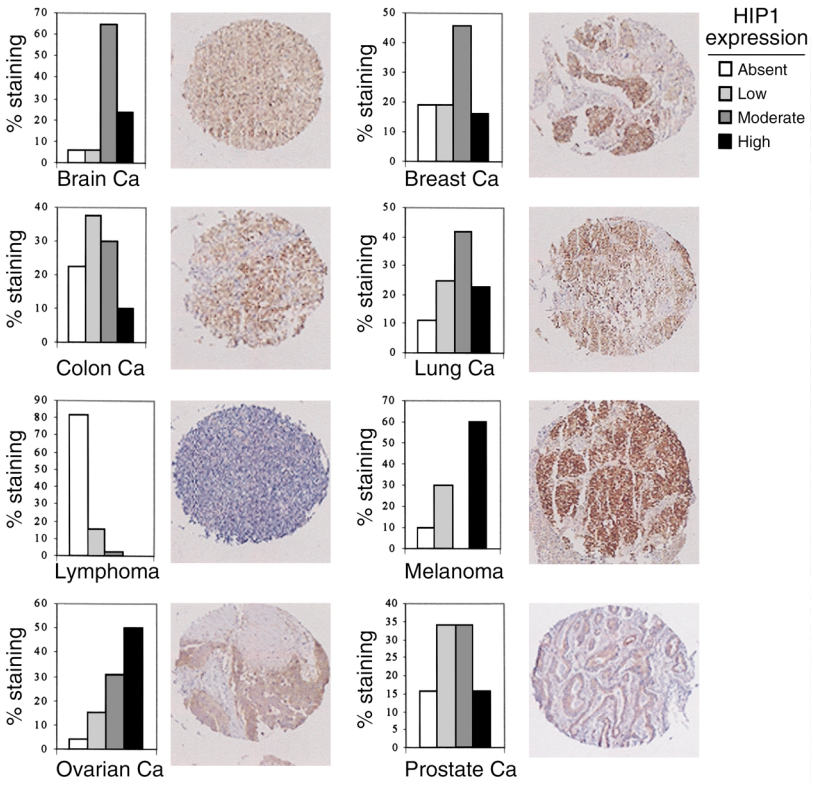

Figure 3
Immunohistochemical analysis of the multitumor tissue microarray TARP1. Microarray slides were subjected to immunohistochemical analysis with the anti-HIP1 mAb 4B10. Ca, cancer.

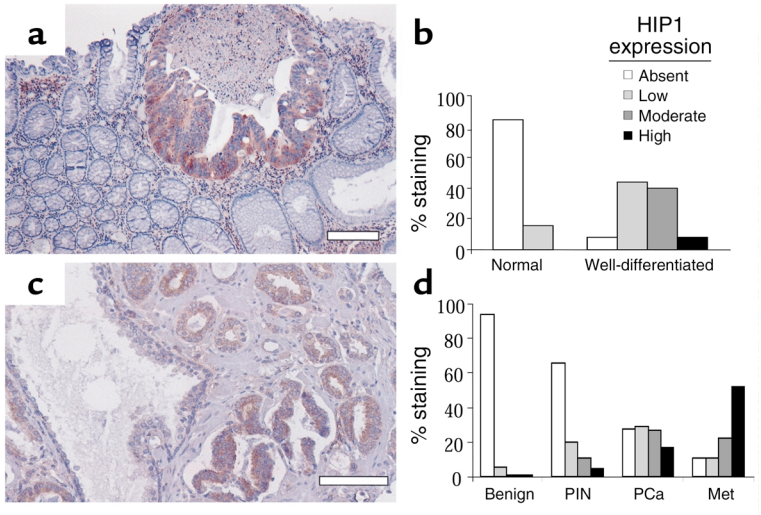

Figure 4
Immunohistochemical analysis of colon and prostate tumors. In separate experiments, slides were stained with either of the monoclonal anti-HIP1 antibodies 4B10 and 1B11, with similar results. (**a**) Low-power photomicrograph of a representative specimen from the analysis of colon cancer. There were many benign glands (lower left), which did not stain for HIP1, and there was a large well-differentiated tumor (upper right), which expressed HIP1 at high levels. (**b**) Histogram of immunohistochemical score distribution obtained from analysis of colon cancer slides (n = 25 patients). (**c**) Low-power photomicrograph of a representative sample from the prostate tumor microarray, demonstrating both the benign, large gland–forming prostatic epithelium (left) and several smaller, neoplastic glands (right). The benign epithelium did not stain for HIP1, while the neoplastic glands demonstrated moderate to high HIP1 expression. (**d**) Histogram of immunohistochemical score distribution (HIP1 expression) obtained for benign prostate, PIN, prostate cancer (PCa), and metastatic prostate cancer (Met) specimens. Two hundred sixteen benign, 174 PIN, 463 prostate cancer, and 136 metastatic prostate cancer specimens were analyzed. Magnification, ×100. Scale bar, 150 μm. Fifty percent of the spots were independently reviewed by a clinical pathologist (M.A. Rubin) and an oncologist (T.S. Ross), with 90% concordance of scores. In addition, the slides were read in their entirety during two independent reading sessions by D.S. Rao, T.S. Hyun, and T.S. Ross. There was 95% concordance of score assignments from these two reading sessions.

epithelium samples did not express HIP1, compared with 8% of the colon tumor samples.

In addition to colon cancer, prostate cancer demonstrated moderate to high levels of HIP1 expression, while benign epithelia did not express HIP1 (Figures 3 and 2f, respectively). To study aberrant HIP1 expression in more detail, three prostate TMAs (19) containing a total of 853 tissue samples from 114 patients with localized prostate cancer were stained with HIP1 mAb's. These samples included benign prostate tissue, PIN, and clinically localized prostate cancer. In addition, a separate tissue microarray that contained 135 tumor samples from 14 patients with hormone-refractory metastatic prostate cancer was examined for HIP1 expression. Striking differences were noted in staining between neoplastic and benign epithelia. Figure 4c shows a sample containing prostate cancer from the prostate tissue microarray that demonstrated high HIP1 expression, juxtaposed to non–HIP1-expressing benign prostatic epithelium (left side). Ninety-five percent of normal epithelium samples did not express HIP1. The remaining 5% had weak levels of expression, and there was no moderate or high expression in any of the normal epithelia examined. Among neoplastic lesions, the precursor of prostate cancer, PIN, had the lowest proportion of samples with moderate or strong levels of HIP1 expression (25% of the 230 samples). Clinically localized prostate cancer had an intermediate proportion (51% of the 463 samples), and hormone-refractory metastatic prostate cancer had the highest proportion of samples expressing moderate to strong levels of HIP1 (70% of the 135 samples). The difference in levels of HIP1 in progressively more advanced prostate cancer was statistically significant (Figure 4d; Pearson's χ-squared, $P < 0.0001$).

Clinical outcome is predicted by HIP1 expression in prostate cancer. It was noteworthy that there were significant numbers of patients with colon cancer, PIN, and clinically localized prostate cancer whose tumors did not express HIP1, similar to normal epithelia, which lacks HIP1 expression. We hypothesized that levels of HIP1 expression relate to clinical progression or metastasis as a clinical manifestation of the biologic effects of HIP1. To test this hypothesis, we first compared HIP1 expression in prostate cancer samples from patients with metastatic disease with HIP1 expression in samples from early-stage prostate cancer patients and patients with precancerous prostatic pathology (high-grade PIN). We found that prostate cancer metastases expressed HIP1 in all cases evaluated (100%), whereas 88% of tumors from patients with localized prostate cancer expressed HIP1 and 50% of tumors from patients with PIN expressed HIP1 (Figure 5a). These frequencies were significantly different among, and correlated with, the different tissue types (Pearson's χ-squared, $P < 0.001$; and Spearman's correlation = 0.664, $P < 0.001$).

These results suggested that HIP1 expression is a late event in the progression of prostate cancer, as expression was seen to increase going from PIN to advanced hormone-refractory prostate cancer. This led us to question whether HIP1 expression could distinguish prostate cancers lacking the biological capacity for progression or systemic metastasis from more aggressive cancers. To explore this, we examined associations between expression of HIP1 in primary tumors and progression after treatment limited to the primary tumor (radical prostatectomy); progression was designated by "PSA failure" (defined as a PSA elevated to more than 0.2 ng/ml following radical prostatectomy). A PSA failure predicts recurrence of cancer, and is therefore commonly used as a measure of progression-free survival. Strikingly, none of the men with HIP1-negative prostate cancer developed a PSA recurrence (Figure 5b). In contrast, 28% of the patients whose tumors expressed HIP1 had PSA recurrences. By univariate analysis, HIP1 was a significant predictor of recurrence-free survival (log rank test, $P = 0.05$; univariate Cox regression analysis, $P = 0.01$).

The contribution of HIP1 expression to PSA recurrence (in the context of Gleason scores, preoperative PSA values, and clinical stage) was next explored by multivariable modeling using Cox regression analysis. HIP1 expression predicted PSA recurrence independent of PSA, Gleason score, and stage of disease (multivariable Cox regression analysis; likelihood ratio test, $P = 0.04$). Since PSA recurrences are thought to be caused by clinically undetectable or occult metastases, these data suggest that HIP1 expression can distinguish aggressive cancers that have the propensity for metastasis and progression from prostatic cancers that lack the biological requirements for tumor growth beyond the primary tumor.

Preoperative PSA level is a well-known predictor of clinical outcome, and in our patient sample, PSA less than 4.0 ng/ml did predict PSA-free survival (supplementary Figure 3b) but was not significantly associated with HIP1 protein expression (Pearson correlation, $P = 0.408$). This is interesting in light of the fact that HIP1 may be more representative of the underlying biology of the tumor, as evidenced by the lack of its expression in benign tissue. PSA, on the other hand, is elevated in a number of conditions that enlarge the prostate, including both cancer and benign prostatic hypertrophy. It is therefore consistent that both are predictive of recurrence but do not correlate with each other. Thus HIP1, in addition to being an independent predictor of clinical outcome, is unique in that it may represent a convergence of cancer biology and the ability to predict clinical outcome.

HIP1 is overexpressed in TRAMP, and a mutant of HIP1 promotes apoptosis. In an effort to determine whether HIP1 expression was an active component of tumorigenesis, we looked at its expression in the well-established transgenic mouse model of prostate cancer (TRAMP) (18), and at the effect of expression of HIP1 and HIP1 mutants on cellular survival. TRAMP mice were created by use of a transgene in which the rat probasin promoter drives expression of SV40 early genes (T and t; Tag) (18). SV40 Tag interacts with p53 and the retinoblastoma gene product Rb, abrogating their tumor suppressor gene functions. HIP1 expression was elevated in 50% of prostate tumors, compared to benign tissue, in TRAMP mice ($n = 10$). (Figure 6a), suggesting that its expression is a prevalent "second hit" in these mice. The heterogeneity of HIP1 expression is reminiscent of that seen in human prostate cancers. Analysis of AP1 and clathrin expression demonstrated basal levels of expression in normal prostates, which were unchanged in tumor specimens. This demonstrated that not all proteins involved in clathrin-mediated trafficking were upregulated at the protein level during prostate tumorigenesis, while HIP1 specifically was (Figure 6a; lower two panels). We have also not found altered expression of HIP1r in the tumors of the TRAMP mice (data not shown).

Because of prior reports of HIP1 as a proapoptotic molecule (26, 27), we originally created a number of mutants to determine which domains were important in promoting apoptosis (Figure 7a). Briefly, stepwise deletions were made to delete individual domains of the HIP1 protein, namely, the ENTH domain, the clathrin- and AP2-binding domain, the central coiled coil domain, and the TALIN homology domain. Contrary to the previous reports, we found that the full-length HIP1 and most mutants were not proapoptotic but that an N-terminally truncated mutant

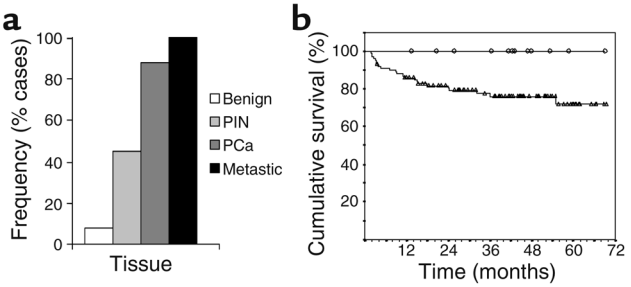

Figure 5
Relationship of HIP1 expression with clinical outcome. A tissue microarray derived from prostatic tumors surgically resected from 121 patients and a tissue microarray derived from hormone-refractory metastatic prostate cancer from 15 patients were used. (**a**) Analysis of HIP1 expression in individual patients showed that there were progressively higher frequencies of HIP1 expression in benign ($n = 89$), PIN ($n = 89$), prostate-confined prostate cancer ($n = 114$), and metastatic cases ($n = 14$). These differences are statistically significant (Pearson's χ-squared, $P < 0.001$), and the presence of HIP1 expression correlated significantly with the ordinal categories of benign, PIN, prostate cancer, and metastatic (Spearman's correlation coefficient, 0.664, $P < 0.001$). (**b**) Kaplan-Meier survival analysis illustrates progression-free survival after radical prostatectomy stratified by prostate-confined tumors that did not express HIP1 (circles) with tumors that did express HIP1 (triangles). Tumors that had zero HIP1 staining ($n = 14$, circles) did not have a prostate cancer recurrence. On the other hand, 28% of patients with HIP1 staining ($n = 100$) went on to relapse.

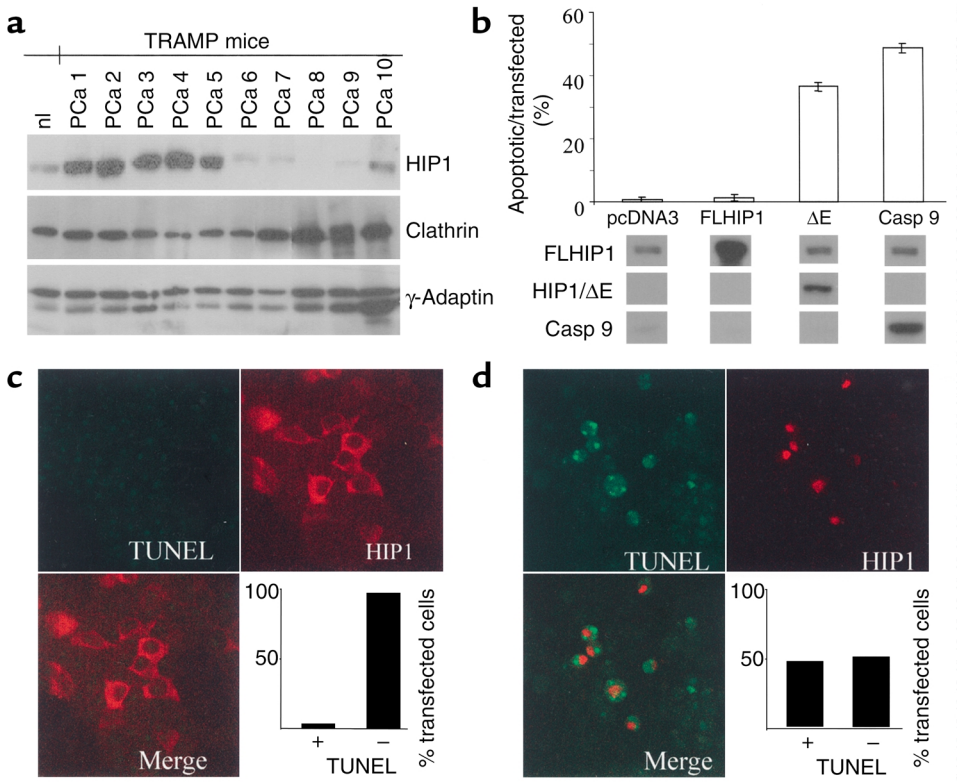

Figure 6
Full-length HIP1 is overexpressed specifically in tumor tissue from TRAMP mice and is not proapoptotic. (**a**) Western blot analysis of normal and prostate cancer tissue from a mouse model of prostate cancer, TRAMP. Normal and prostate cancer tissue was immunoblotted using mAb's to HIP1, clathrin heavy chain (clathrin), and adaptin-γ, a subunit of the clathrin-associated adaptor protein AP1. nl, normal prostate. (**b**) A mutant of HIP1 lacking a critical N-terminal ENTH domain, designated HIP1/ΔE, causes cell death. Vector (pcDNA3), full-length HIP1 (FLHIP1), HIP/ΔE (ΔE), and caspase-9 (casp-9) were transfected into 293T cells. Cell death was assayed by cellular morphology following transfection of the plasmid of interest. Western blot analysis was used to confirm expression of the various proteins. (**c** and **d**) TUNEL assays on transfected cells confirm that HIP1/ΔE–mediated cell death is apoptotic. Following transfection, cells were subjected to TUNEL assays with fluorescein-tagged dUTP and immunofluorescent labeling with HIP1 mAb. Confocal image analysis was carried out, and results for TUNEL labeling of full-length HIP1 and HIP1/ΔE –transfected cells are shown in **c** and **d** respectively, as TUNEL-positive cells (green), HIP1-immunofluorescent cells (red), and merged images. Bar graphs in **c** and **d** show the percentage of transfected cells (i.e., those staining red) that are also TUNEL-positive (green).

designated HIP1/ΔE did induce cell death (Figure 6b and data not shown). Thirty-nine percent of HIP1/ΔE–expressing cells and, as expected, 50% of caspase-9–expressing cells were apoptotic by morphologic criteria as described previously (22). This finding was confirmed by testing HIP1- and HIP1/ΔE–transfected cells by TUNEL assay (Figure 6, c and d). Expression of full-length HIP1 did not induce apoptosis, as less than 2% of HIP1-transfected cells were labeled by TUNEL (Figure 6c). On the other hand, HIP1/ΔE caused massive apoptosis, with 50% of HIP1/ΔE–transfected cells labeled by TUNEL (Figure 6d).

HIP1/ΔE functions as a dominant negative mutant and causes apoptosis via caspase-9. There are two major pathways in a cell that lead to apoptosis: the extrinsic, or death receptor–mediated, pathway, and the intrinsic, mitochondrial pathway (28). The former pathway is dependent on caspase-8, while the latter pathway is dependent on caspase-9. Having confirmed that the N-terminally truncated mutant HIP1/ΔE caused apoptosis while full-length HIP1 did not, we wanted to understand which apoptotic pathway was activated. To test this, we used dominant negative caspase-9 and dominant negative caspase-8 constructs and found that inhibition of the caspase-9 pathway rescued HIP1/ΔE–mediated apoptosis, whereas inhibition of the caspase-8 pathway did not (Figure 7b). The expression of HIP1/ΔE and the dominant negative caspase mutants in these experiments was confirmed by Western blot analysis (supplementary Figure 2a).

Since 293T cells (like the cancer cell lines) express large amounts of endogenous HIP1, we suspected that HIP1/ΔE may be functioning as a dominant negative mutant. To determine whether HIP1/ΔE–induced apoptosis resulted from the disruption of a HIP1-mediated pathway, we tested the ability of full-length HIP1 and various mutants to rescue HIP1/ΔE–mediated apoptosis. The wild-type HIP1 protein and all mutant forms of HIP1 except HIP1/ΔC corrected apoptosis (Figure 7c). Expression of endogenous HIP1, HIP1/ΔE,

and the other HIP1 mutants was confirmed by Western blot (supplementary Figure 2b). The HIP1/ΔC mutant lacks the central coiled coil domain, which is thought to mediate protein-protein interactions. Deletion of the coiled coil domain in the HIP1/ΔC mutant abrogated the ability to associate with FLHIP1. This was demonstrated by the lack of the ability of the HIP1/ΔC protein to interact with a myc-tagged full-length HIP1 protein in co-immunoprecipitation assays (Figure 7d, lane 5 vs. lanes 2, 3, 4, and 6). Therefore, the correction of HIP1/ΔE–mediated apoptosis was dependent on the ability of the HIP1 full-length and mutant proteins to interact with wild-type HIP1. This suggests that HIP1/ΔE binds to endogenous HIP1, sequestering it from performing its normal role in clathrin trafficking and resulting in cell death. The role of HIP1 in this fundamental pathway is not known but likely involves a nonredundant function in some cells that is necessary for their survival.

Discussion

In this report we describe the unexpected discovery of aberrant HIP1 expression in human prostate and colon cancer. We also provide data showing that HIP1 is necessary for cellular survival. This is consistent with HIP1 overexpression in human cancer cell lines and tissues, as its maintenance of survival could provide a selective growth advantage to cancer cells. However, both results described here contrast with a report that shows that HIP1 promotes apoptosis in a caspase-8–independent fashion (26) and another study that reports that HIP1 causes apoptosis in a caspase-8–dependent fashion (27). The primary reason for the discrepancy may be that these two previous reports use a different HIP1 cDNA. This cDNA lacks an N-terminal sequence that we have recently identified (9). It is not clear whether this difference is due to the existence of alternative splice forms of HIP1 mRNA. We speculate that the cDNA used in the previous reports is functionally similar to the dominant negative mutant HIP1/ΔE described herein. Similar to our results with HIP1, the N-terminus of the yeast ortholog of HIP1, *Sla2p*, has been shown to be necessary for survival (4). Thus, the N-terminus of these proteins, containing the ENTH domain, may be critical in mediating their role(s) in cellular survival or death pathways.

We found that expression of HIP1 in human prostate tumors had a significant association with prostate cancer progression. This was evidenced by a lack of expression in the benign epithelium, intermediate expression levels in precancerous prostate lesions (high-grade PIN), and high frequency of expression in metastatic prostate cancer. In addition, progression after prostatectomy did not occur in any of the 14 prostate cancer cases that lacked HIP1 expression, suggesting that HIP1 may distinguish aggressive prostate cancers from those with a more indolent clinical course.

It is intriguing to speculate that, similar to HIP1 expression in prostate cancer, the stratification of colonic adenocarcinomas based on HIP1 expression may have independent predictive value with regard to both tumor aggressiveness and patient survival. Because the vast majority of colonic tumors produce a similar histologic picture, it is difficult to obtain prognostic information from routine histologic analysis.

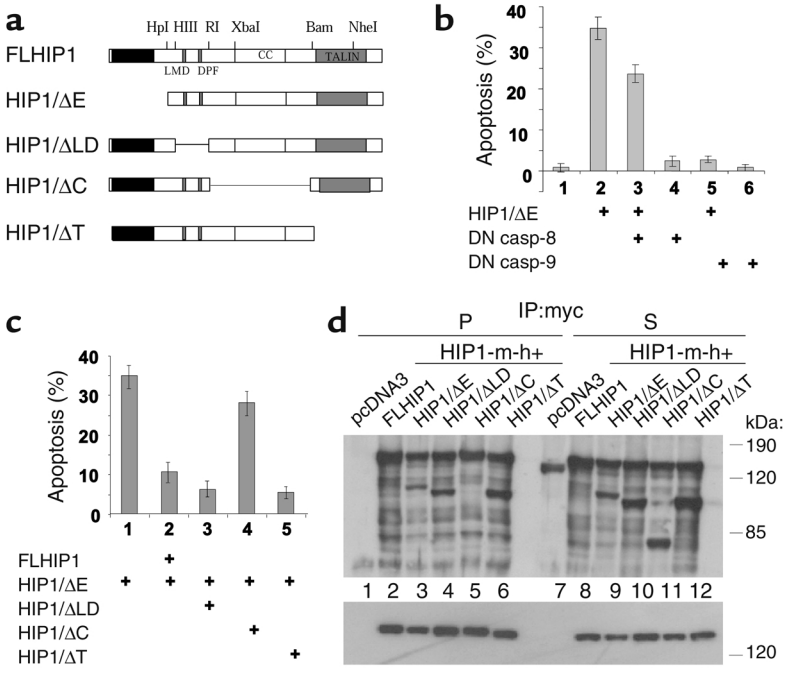

Figure 7

HIP1/ΔE–mediated apoptosis is dependent on caspase-9 and functions as a dominant negative mutant. (**a**) Schematic of HIP1 mutants. HIP1 is a multidomain protein with an N-terminal ENTH domain; LMDMD (LMD) and DPF motifs that bind to clathrin and AP2, respectively; a central coiled coil (CC) domain with multiple interaction motifs; and a C-terminal TALIN homology domain. Each of these domains was deleted to yield the respective deletion mutants, HIP1/ΔE, HIP1/ΔLD, HIP1/ΔC, and HIP1/ΔT. (**b**) Correction of HIP1/ΔE–mediated apoptosis by dominant negative caspase-9 (DN casp-9) but not dominant negative caspase-8 (DN casp-8). Cells were cotransfected with HIP1/ΔE and either of the dominant negative caspases as indicated under the graph. Controls included vector-transfected cells (bar 1) and dominant negative caspases alone (bars 4 and 6). Apoptosis was assayed as in Figure 6b. (**c**) HIP1/ΔE–mediated apoptosis was rescued by full-length HIP1 and all HIP1 mutants except HIP1/ΔC. As in **b**, cells were cotransfected with various combinations of plasmids as indicated under the graph. (**d**) Interaction of all HIP1 mutants except HIP1/ΔC with full-length HIP1. Myc-tagged full-length HIP1 was cotransfected with various mutants as indicated. Cell extracts were immunoprecipitated with myc antibody, and pellets (P) and supernatants (S) were collected. These were then immunoblotted with HIP1 polyclonal antibody (top panel) and myc antibody (bottom panel). Note that endogenous HIP1 is co-immunoprecipitated with myc-tagged HIP1 in all lanes.

Therefore, it will be of considerable interest to correlate clinical outcomes from a large cohort of patients with colon cancer with tumor HIP1 expression. If expression does correlate with reduced survival, as is true for prostate cancer, this single molecular marker may be one of the most useful clinical tools for prognostication in the care of patients with two of the most common forms of cancer.

The likelihood of a contributory role for HIP1 in the development of cancer is strengthened by its overexpression in prostate cancers of the TRAMP mouse and data suggesting that wild-type HIP1 expression maintains cell survival. However, it remains possible that HIP1 overexpression, although specific to prostate and colon cancer, is a consequence of tumor formation. We are working to determine whether HIP1 expression is causal by using *HIP1* transgenic and knockout mice to discover whether expression of HIP1 is sufficient for formation of tumors in vivo.

Finally, the implications of HIP1 expression in cancer progression are exciting. As a factor necessary for cellular survival, HIP1 overexpression in cancer may reduce the growth factor dependence of cancer cells by dysregulating cell surface growth factor receptor density or growth factor secretion, as a consequence of its role in clathrin-mediated trafficking. By reducing dependence on growth factors, cancer cells can gain a proliferative advantage, which could allow for additional mutations to accumulate in surviving cells. If HIP1 proves to be necessary for cellular transformation, HIP1 function in clathrin trafficking may provide a novel pathway for design of anticancer drugs.

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