von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF

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von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome caused by germ line mutation of the von Hippel-Lindau tumor suppressor gene (VHL). Tumors observed in this disorder include retinal and central nervous system hemangioblastomas, clear cell renal carcinomas and pheochromocytomas. The VHL gene product, pVHL, is a component of a ubiquitin ligase which targets the transcription factor known as hypoxia-inducible factor (HIF) for degradation in the presence of oxygen. pVHL also plays roles in the control of extracellular matrix formation and cell-cycle exit. Different VHL mutations confer different site-specific risks of cancer. Type 2C VHL mutations confer an increased risk of pheochromocytoma without the other stigmata of VHL disease. Here we report that the products of such type 2C VHL alleles retain the ability to down regulate HIF but are defective for promotion of fibronectin matrix assembly. Furthermore, pVHL L188V, a well studied type 2C mutant, retained the ability to suppress renal carcinoma growth in vivo. These studies strengthen the notion that HIF deregulation plays a causal role in hemangioblastoma and renal carcinoma, and raises the possibility that abnormal fibronectin matrix assembly contributes to pheochromocytoma pathogenesis in the setting of VHL disease.

INTRODUCTION

von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome in which blood vessel tumors of the retina and central nervous system, called hemangioblastomas, are typical features (1,2). It appears that all patients with a clinical diagnosis of VHL disease harbor a germ line mutation of the VHL gene located on chromosome 3p25 (3). Tumor development in this disorder is linked to loss or inactivation of the remaining wild-type VHL allele. Thus, the VHL gene behaves as a tumor suppressor gene.

The product of the VHL gene, pVHL, forms a multimeric complex that contains, at a minimum, elongin B, elongin C, Cul2 and Rbx1 (also called ROC1 or Hrt1) (2,4). This complex regulates the abundance of a sequence-specific, DNA-binding, transcriptional activator called hypoxia-inducible factor (HIF) (5,6). HIF binds to DNA as a heterodimer containing an α subunit (such as HIF1α) and a β subunit (such as HIF1β, also called aryl hydrocarbon receptor nuclear transporter). pVHL polyubiquitinates HIFα subunits when oxygen is available, which targets them for degradation by the 26S proteasome (6–9). Cells lacking pVHL are unable to degrade HIFα subunits and consequently overproduce HIF target genes. Among these are genes implicated in angiogenesis, such as VEGF (vascular endothelial growth factor) and PDGF B (platelet-derived growth factor B chain) (6,10–12). Thus, in the simplest model, the vascular nature of VHL-associated neoplasms is due to the overproduction of angiogenic peptides encoded by HIF-responsive genes. In keeping with this idea, forced overproduction of VEGF in the mouse brain causes the development of hemangioblastoma-like blood vessels (13).

Genotype–phenotype correlations are emerging in VHL disease. Some families with VHL disease display an increased risk of clear cell carcinomas of the kidney and pheochromocytomas in addition to hemangioblastoma (14–18). VHL families have been described which manifest a low (type 1) or high risk (type 2) of pheochromocytoma. Type 2 VHL disease can be associated with a low (type 2A) or high risk (type 2B) of renal carcinoma. Finally, some VHL families (type 2C) present as familial pheochromocytoma without hemangioblastoma or renal cell carcinoma (19–23). In general, type 2 disease is associated with subtle VHL mutations, such as missense mutations, that would be predicted to give rise to conformationally intact pVHL mutants (15–18,24,25). In contrast, type 1 disease is often associated with mutations that should grossly alter the structure of pVHL or lead to complete absence of pVHL. This latter set of observations has led to speculation that type 2 mutants acquire a ‘gain of function’ that is linked to the induction of pheochromocytoma (14,26). Alternatively, it is possible that complete loss of pVHL function is incompatible with pheochromocytoma development.

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In addition to regulation of HIF, pVHL has been implicated in the control of extracellular matrix formation and the cell-cycle (27–31). Whether these functions directly or indirectly relate to its ability to regulate HIF is still not clear. It is conceivable that pVHL performs multiple functions and that some of these functions are tissue-specific. If true, it might be possible to understand the clinical phenotype associated with a particular pVHL mutant through an analysis of its various biochemical functions. In this regard, we previously noted that a pVHL mutant associated with type 2C disease, pVHL L188V, preserved the ability to bind to and ubiquitinate HIF (8) and could downregulate HIF target genes (32). In this report we show that a panel of type 2C pVHL mutants retain the ability to interact with HIF and to downregulate HIF target genes when reintroduced into pVHL-defective tumor cells. This provides a biologically plausible mechanism to account for the low risk of hemangioblastoma associated with these mutants. Furthermore, we show that pVHL L188V can suppress renal carcinoma growth in vivo, suggesting that deregulation of HIF contributes to pVHL-defective renal tumorigenesis. In contrast, we show that pVHL L188V, like classical null pVHL mutants, is defective at promoting extracellular fibronectin matrix assembly. This might suggest that abnormal extracellular matrix formation following pVHL inactivation contributes to the development of pheochromocytoma in the setting of VHL disease.

RESULTS

We previously showed that a type 2C pVHL mutant, pVHL L188V, retains the ability to bind to HIF (8). Furthermore, pVHL L188V, like wild-type pVHL, can direct the poly-ubiquitination of HIF in vitro and in vivo leading to decreased activation of HIF target genes (8,32). To ask whether this is a general property of type 2C mutants, we made plasmids encoding pVHL R64P, pVHL V84L and pVHL F119S (21–23). These pVHL mutants are the predicted products of VHL alleles that have been identified in cases of apparent type 2C VHL disease (Table 1).

Structurally, pVHL protein contains two functional subdomains called α and β (26). The α domain binds to elongin C which, in turn, binds to elongin B and Cul2. The β domain binds to HIFα subunits. The residues affected by the four type 2C mutants affect different portions of pVHL (Fig. 1). R64 is located on the surface adjacent to a cluster of frequently mutated β domain residues which includes Y98. V84 and F119 contact one another and are located in the hydrophobic core of the β domain. Finally, L188V is buried in the α domain core.

Table 1. Type 2C pVHL mutants used in this study

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<th>Mutation</th>
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<tr>
<td>R64P</td>
<td>2</td>
<td>21</td>
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<tr>
<td>V84L</td>
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<td>1</td>
<td>23</td>
</tr>
<tr>
<td>L188V</td>
<td>2 families</td>
<td>19</td>
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Figure 1. Localization of pVHL residues affected by type 2C VHL mutations. (A) Localization of R64. Surface view of pVHL with R64 shown in blue and frequently mutated β domain surface residues shown in red. (B) Localization of V84 and F119. V84 and F119 are shown in red, core residues in gray, and frequently mutated β domain surface residues in orange. (C) Localization of L188. Shown is region of pVHL α domain that participates in elongin C binding. pVHL and elongin C main chains are shown in red and blue, respectively. pVHL side chains are shown in yellow except for L188, which is in red. Figures were generated with Swiss PDB Viewer (62).
tated with anti-Gal4 antibody and bound pVHL was detected by fluorography (Fig. 2A). As expected, both wild-type pVHL and pVHL L188V bound to the HIF1α ODD, whereas pVHL Y98H and pVHL C162F did not. These latter two mutants, bearing missense mutations of the β and α domains, respectively, are associated with a high risk of retinal and central nervous system hemangioblastoma. The failure of C162F to bind to HIF might be due to the importance of elongin binding with respect to proper pVHL folding as well as to conformational effects related to the bulky phenylalanine substitution (34,35). The type 2C mutant pVHL V84L, like pVHL L188V, bound to the HIF1α ODD with seemingly wild-type efficiency. The other two type 2C mutants, R64P and F119S, also retained the ability to bind to the ODD although at reduced levels compared to wild-type pVHL.

Next, these same pVHL proteins were tested for their ability to polyubiquitinate HIF in vitro (Fig. 2B). 35S-labeled Gal4-HIF ODD was incubated with a cytosolic extract prepared from a pVHL-defective renal carcinoma line (786-O) in the presence of an energy regenerating system, ubiquitin, and the indicated pVHL proteins produced by in vitro translation. All of the type 2C pVHL mutants were able to measurably polyubiquitinate HIF1α ODD in contrast to pVHL Y98H and pVHL C162F. Notably, HIF polyubiquitination activity by the type 2C mutants mirrored their ability to bind to HIF. Qualitatively similar results were observed in assays performed with full-length HIF1α (data not shown).

Together, these results suggested that type 2C pVHL mutants might downregulate HIF. To test this, 786-O cells were stably transduced to produce hemagglutinin (HA)-tagged versions of the indicated pVHL species. Due to pVHL loss, 786-O overproduce HIF2α under normoxic conditions (6). Clonal lines were metabolically labeled with [35S]methionine and immunoprecipitated with an anti-HA antibody under stringent wash conditions (Fig. 3). pVHL-associated proteins were resolved by SDS–PAGE and detected by fluorography. All of the type 2C pVHL mutants, in contrast to pVHL C162F, retained the ability to bind to elongin B, elongin C and Cul2. Note that HIF 2α was not detected bound to wild-type pVHL, presumably because it is rapidly ubiquitinated and degraded. In keeping with this idea, HIF2α can be detected in assays such as these by using cells in which either the proteasome (6) or E1 ubiquitin-activating enzyme are inactivated (H. Yang and W.G. Kaelin Jr, unpublished data). In contrast, variable amounts of HIF2α were detected in association with the pVHL 2C mutants. Among several possibilities, this could be due to altered ubiquitination activity leading to a slower off-rate for binding.

In keeping with our earlier results with pVHL L188V, all of the type 2C pVHL mutants suppressed steady-state HIF2α levels in these cells under normoxic conditions as determined by immunoblot analysis (Fig. 4). To ask whether this translated into suppression of HIF target gene expression, we measured the levels of the glucose transporter GLUT1, which is the product of a HIF-responsive gene, by immunoblot analysis (Fig. 4). As expected, wild-type pVHL suppressed the accumulation of GLUT1, whereas pVHL C162F did not. It should be noted that the amount of pVHL in the cells producing wild-type pVHL exceeds by ~10-fold the amount of pVHL required for regulation of HIF and HIF-target gene expression (36). Thus, the inability of C162F to regulate HIF and GLUT1 is not due to inadequate protein levels. All of the type 2C pVHL mutants reproducibly suppressed GLUT1 levels below those observed in the parental pVHL-defective cells or in subclones transfected to produce pVHL mutants associated with classic VHL disease such as C162F, L158P or Y98H (Fig. 4 and data not shown).

pVHL binds to fibronectin and cells lacking pVHL are unable to properly assemble an extracellular fibronectin matrix (27). All of the pVHL 2C mutants displayed diminished binding to fibronectin compared with wild-type pVHL when assayed under stringent wash conditions (Fig. 3), suggesting that this biochemical abnormality might be causally linked to pheochromocytoma development in VHL disease. It is not known whether pVHL binds directly or indirectly to...
fibronectin, nor is it known how, mechanistically, this interaction affects the fate of secreted fibronectin. To address the first question, whole-cell extracts were resolved by SDS–PAGE and transferred to nitrocellulose. The filters were then probed with anti-fibronectin antibody or with recombinant pVHL/elongin C/elongin B complexes purified to crystallographic homogeneity. In these assays, VBC bound directly to fibronectin (Fig. 5). Similarly, VBC bound directly to fibronectin present in anti-fibronectin or anti-pVHL immunoprecipitates (data not shown).

These data suggested that pVHL binds directly to fibronectin and that type 2C mutants are at least partially defective for fibronectin binding. To examine the potential functional significance of these findings further, we elected to focus our attention on the pVHL L188V mutant for two reasons. First, among the type 2C mutants, pVHL L188V most closely resembled wild-type pVHL in biochemical and functional assays related to elongin/Cul2 binding and HIF ubiquitination. Second, more type 2C patients with the L188V VHL mutation have been identified than with any of other type 2C mutation (Table 1). It is possible that the phenotypes assigned to these other mutations will change with longer clinical followup or with the identification of additional patients at risk.

Fibronectin matrix deposition can be scored by an ELISA or by immunofluorescence (27). The former requires the deposition of insoluble, immunoreactive, fibronectin to a plastic surface, whereas the latter also requires that the fibronectin form visualizable macroscopic arrays. As expected, wild-type pVHL restored fibronectin matrix deposition in both assays, whereas pVHL C162F did not (Fig. 6). In contrast, pVHL L188V scored positively in the ELISA but was defective when measured in the immunofluorescence assay. Based on these
studies, we conclude that pVHL L188V retains the ability to regulate hypoxia-inducible genes but is partially defective with respect to promoting extracellular fibronectin matrix formation. Restoration of wild-type pVHL function in pVHL-defective renal carcinoma cells suppresses their ability to form tumors in nude mice (11,37). Since renal cancer is not a component of type 2C VHL disease, we asked whether pVHL L188V retained this activity. To this end, 786-O cells stably transduced to produce pVHL L188V (right flank) or transfected with the empty expression plasmid (clone 3; squares). The y-axis shows the product of the two longest orthogonal tumor measurements, and the x-axis shows weeks following subcutaneous injection.

Table 2. pVHL L188V suppresses renal carcinoma growth in vivo

<table>
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</tr>
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<td>Clone 3</td>
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<td>8</td>
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Data represent a summary of nude mouse xenograft assays. Measurements were taken at 13 weeks or at the time of death for tumors measuring >2 cm in any dimension. All animals survived for >10 weeks.

Figure 6. pVHL L188V exhibits a qualitative defect in fibronectin matrix assembly. (A) Fibronectin ELISA data for 786-O cells stably transfected to produce the indicated pVHL proteins. Signal strength reflects the amount of insoluble fibronectin deposited by the cells. CAKI-1 (VHL+/+) renal carcinoma cells were included in these experiments. (B) Anti-fibronectin immunofluorescence staining of 786-O subclones producing the indicated pVHL proteins. Note the characteristic fibrillar fibronectin material associated with clones producing wild-type pVHL.
The VHL gene is ubiquitously expressed, and yet VHL inactivation has only been implicated in the development of a narrow subset of human tumors such as hemangioblastoma, renal cell carcinoma and pheochromocytoma (38,39). Furthermore, different germ line VHL mutations are associated with different site-specific cancer risks. The goal of this study was to begin to understand the biochemical basis for these genotype–phenotype correlations. Our assumption is that the differences in site-specific cancer risk associated with various mutant VHL alleles reflect differences in the biochemical activities of their protein products. Specifically, we hypothesize that pVHL performs multiple functions, some of which may be tissue-specific, and that disease phenotype is determined by the degree to which these various functions are quantitatively or qualitatively altered. In this regard, we found that the products of type 2C VHL alleles, which are associated with a ‘pheochromocytoma only’ variant of VHL disease, retain the ability to bind to and ubiquitinate the HIF1α oxygen-dependent degradation domain. These two activities mirrored each other in vitro, suggesting that HIF binding, but not recruitment of the elongins and Cul2, was limited for ubiquitination by these mutants. In keeping with this idea, all of the type 2C pVHL mutants bound to the elongins and Cul2 with seemingly wild-type efficiency. Furthermore, all of the type 2C mutants down-regulated HIF when reintroduced into pVHL-defective tumor cells. In contrast, all four type 2C pVHL mutants exhibited decreased fibronectin binding. For the best documented and studied of these mutants, pVHL L188V, this was associated with an impaired ability to restore extracellular fibronectin matrix assembly. Finally, pVHL L188V retained the ability to suppress renal carcinoma growth in vivo.

Based on these findings, we suggest that deregulation of HIF following VHL inactivation plays a causal role in the development of VHL-associated hemangioblastoma and renal carcinoma, but is not strictly required for VHL-associated pheochromocytoma. In short, we propose that the low risk of hemangioblastoma and renal cell carcinoma associated with type 2C VHL alleles reflects that fact that their protein products retain the ability to suppress HIF.

A growing body of evidence also supports a critical role for HIF in the pathogenesis of hemangioblastoma and renal cell carcinoma. In hemangioblastoma, it is the stromal cell component that has sustained biallelic VHL inactivation (40,41). These cells overproduce growth factors such as TGFβ, VEGF and PDGF B, which are the products of HIF target genes (41–47) (S.Lee, personal communication). There is evidence that TGFβ and its receptor, EGFR, may participate in an autocrine loop in hemangioblastoma (42,43). Overproduction of VEGF and PDGF B almost certainly contributes to the proliferation of endothelial cells and pericytes, respectively, which give rise to the blood vessels which are characteristic of these tumors (48). In support of this idea, localized overproduction of VEGF in the brain causes blood vessel proliferations reminiscent of hemangioblastomas (13).

In the kidney, biallelic VHL inactivation causes renal cysts but is not sufficient for tumor development (49). It is presumed that additional mutations at non-VHL loci are responsible for the conversion of cysts to frank carcinomas. In general, renal cysts are characterized by abnormal epithelial proliferation and abnormal cell-matrix interactions. With respect to the former, TGFβ is a renal epithelial mitogen that is suspected of playing a role in renal carcinogenesis (50–55). With respect to the latter, pVHL plays a role in matrix control, both through its effects on fibronectin and through its effects on the secretion of enzymes which regulate matrix turnover (27–29). Among these are matrix metalloproteinases and tissue inhibitor of metalloproteinases. Whether any of these genes are also under the control of HIF is not known. Finally, renal carcinomas are notoriously vascular and overproduce angiogenic peptides including VEGF (56–58). Thus, VHL inactivation, and deregulation of HIF, may be responsible for the angiogenic phenotype of renal cell carcinoma and facilitate tumor growth even if VHL inactivation is not sufficient for renal carcinogenesis. The ability of pVHL L188V to suppress the growth of pVHL-defective renal carcinoma cells in nude mouse assays is consistent with this idea.

According to the Knudson 2-hit model, one would anticipate that somatic mutations leading to biallelic VHL inactivation would be a common feature of the sporadic counterparts of the tumors seen in VHL disease. This appears to be true for sporadic hemangioblastoma and renal carcinoma, but not sporadic pheochromocytoma (23,38,39,59), suggesting that the pathogenic role of VHL inactivation in pheochromocytoma might differ from its role in hemangioblastoma and renal cell carcinoma. In this context, it is intriguing that type 2C pVHL mutants retain the ability to downregulate HIF but are defective with respect to promoting fibronectin matrix assembly. This suggests that fibronectin matrix assembly might play an especially important role in pheochromocytoma development, although clearly more work needs to be done in this area. For example, it is still possible that some as yet unappreciated pVHL function is similarly affected by the limited number of type 2C mutations available for study.

Clearly, a link between loss of fibronectin matrix assembly and pheochromocytoma would not explain why type 1 VHL mutations confer a low risk of pheochromocytoma, since some of these mutations are associated with complete loss of pVHL function. We speculate that either the cell which gives rise to pheochromocytoma cannot tolerate complete loss of pVHL function, or that pVHL mutants linked to pheochromocytoma acquire an as yet unknown gain of function. To date, however, we have no biochemical or cellular evidence for such a gain of function following overproduction of type 2C pVHL mutants in rat pheochromocytoma cells (data not shown).

Likewise, it is not clear why germline inactivation of VHL can cause pheochromocytoma, whereas VHL inactivation is rare in sporadic pheochromocytoma. It is possible that VHL inactivation must occur during a critical developmental window or must be associated with a ‘field-defect’ in order to give rise to pheochromocytoma. This latter idea is intriguing given a potential role for pVHL in extracellular matrix control. Clearly, it will be important to determine how, mechanistically, pVHL promotes fibronectin matrix assembly. We have found that pVHL can bind directly to fibronectin. One model, which remains to be proven, is that malfolded or malprocessed fibronectin can undergo retrograde transport to the cytosolic surface of the ER membrane and interact with pVHL. The cytosolic surface of the ER membrane is a known platform for ubiquitination (60), although we have no direct evidence that pVHL can polyubiquitinate fibronectin.
It is clear that additional patients, and longer follow-up, will be necessary to firmly establish that all of the alleles studied here cause phaeochromocytoma without the other stigmata of VHL disease. In this regard, we note that two of the four pVHL mutants we tested are partially defective for HIF ubiquitination in vitro. One wonders whether the corresponding VHL alleles might be partially penetrant with respect to the risk of vascular tumors. In short, while the current classification scheme might be partially penetrant with respect to the risk of vascular tumors, in VHL disease will hopefully shed new light on the functions of pVHL as well as provide patients with prognostically useful information.

MATERIALS AND METHODS

Plasmids

pRc-CMV-HA-VHL, pRc-CMV-HA-VHL Y98H, pRc-CMV-VA-HHL C162F, pRc-CMV-HA-VHL L188V and GAL4-HA-HIF2 ODD were described previously (8,32,37,61). Plasmids were verified by direct DNA sequence analysis.

Cell culture, metabolic labeling and transfection

Renal carcinoma cells lines were grown in DMEM supplemented with 10% Fetalclone (Hyclone) in a humidified atmosphere at 37°C in the presence of 10% CO₂, Caki-1 (VHL +/+), renal carcinoma cells were obtained from the American Type Culture Collection. 786-O cells and subclones stably producing wild-type pVHL, pVHL Y98H, pVHL C162F or pVHL L188V were described previously (32,37,61). Additional 786-O subclones producing either these pVHL proteins, or pVHL R64P, pVHL V84L or pVHL F119S were made in the same way using a Clontech Site-Directed Mutagenesis Kit, according to the manufacturer’s instructions. Plasmids were verified by direct DNA sequence analysis.

For metabolic labeling, subconfluent cells were transfected with plasmids encoding the indicated pVHL protein using Fugene (Roche Diagnostics). Forty-eight hours later, G418 (Life Technologies) was added to a final concentration of 1 mg/ml. Immunoprecipitation and immunoblot analysis were performed basically as described previously, with minor modifications (27).

Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot analysis were performed as described previously, with minor modifications (27). For immunoprecipitation of metabolically labeled cell extracts, cells grown in 100 mm plastic dishes were washed twice with PBS and then lysed by incubation in 1 ml of EBC buffer (50 mM Tris pH 8, 120 mM NaCl, 0.5% Nonidet P-40 buffer) supplemented with aprotinin, PMSF, leupeptin, sodium orthovandate and sodium fluoride for 30 min. Following clarification by centrifugation at 14,000 g for 15 min at 4°C, 500 µl of extract was incubated with 2 µg of anti-HA antibody with rocking for 1 h at 4°C. Immunoprecipitates were recovered on Protein A-Sepharose and washed five times with NETN 900 (20 mM Tris pH 8, 900 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and once with NETN 125. Bound proteins were eluted by boiling in SDS-containing sample buffer and resolved by SDS-PAGE.

For GLUT1 immunoblot analysis, 50 µg of total cell extract, as determined by the Bradford method, was resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked for 1 h in the presence of 5% bovine serum albumin (BSA) and then incubated with anti-GLUT1 (1:1000 in TBS (10 mM Tris pH 8, 150 mM NaCl) plus 4% BSA) for 1 h. Following three washes with TBS, the bound antibody was detected using an alkaline phosphatase-conjugated goat anti-rabbit.

Far western analysis

Far western analysis using purified pVHL/elongin B/elongin C complexes was performed exactly as described previously (8).

Immunofluorescence

Cell were grown in six-well plates on coverslips (5 x 10⁵ cells/well). Five days later, the cells were rinsed twice with PBS and fixed in chilled 95% ethanol for 1 h at −80°C. Following ethanol aspiration the coverslips were air dried at 4°C and then blocked in PBS plus 1% BSA for 1 h at 37°C followed by overnight incubation with 200 µl of mouse monoclonal anti-fibronectin antibody (1:5000 v/v PBS + 1% BSA) at 4°C. Following three washes with PBS, 500 µl of rhodamine-conjugated goat anti-mouse (1:500 v/v + 1% BSA) was added to the coverslips for 1 h at 37°C. After three additional washes with PBS, the coverslips were stained with DAPI, mounted, and photographed using a Nikon Eclipse E680 fluorescence microscope.

Fibronectin ELISA

Fibronectin ELISA was performed as described previously (27). Briefly, cells were grown in 96-well plates (2 x 10⁴ cells/well). Mock wells contained the same media without cells. Five days later, the culture media was aspirated and replaced with PBS with 2 mM EDTA. Following release of the cells from plastic, as determined by direct microscopic inspection, the cells were aspirated and the wells were blocked by incubation in PBS plus 1% BSA for 1 h at 37°C and then incubated with rabbit anti-fibronectin antibody (1:500 v/v in PBS + 1% BSA) for 1 h at 37°C. Following three washes with PBS, alkaline-phosphatase
goat anti-rabbit (1:10 000 in PBS + 1% BSA) was added for 1 h at 37°C. The wells were washed three times with PBS, p-nitrophenylphosphate (Sigma) [5 mg/ml in DEA buffer (Kiregaard and Perry Laboratories)] was added, and absorbance at 405 nM was measured 30 min later. Values were corrected for the background signal obtained for mock wells.

**In vitro ubiquitination assay**

Ubiquitylation reactions were performed as described previously (8). Briefly, 35S-labeled in vitro Gal4-HIF ODD translate (4 µl) was made using the TNT-coupled transcription/translation system (Promega) and incubated in the presence of a 786-O cell S100 extract (100–200 µg) supplemented with 8 µg/µl ubiquitin (Sigma), 100 ng/µl ubiquitin aldehyde (BostonBiochem), energy-regenerating system (20 mM Tris pH 7.4, 2 mM ATP, 5 mM MgCl2, 40 mM creatine phosphate, 0.5 µg/µl creatine kinase) and 2.5 µM MG262 (BostonBiochem) in a reaction volume of 20–30 µl for 1.5–2 h at 30°C. Where indicated, 2 µl of the indicated in vitro translated pVHL proteins were added. Products were immunoprecipitated with anti-Gal4 antibody and resolved by SDS–PAGE.

**Nude mouse xenograft assay**

Nude mouse xenograft assays were performed as described previously (37). Approximately 10^7 viable tumor cells, as determined by trypan blue exclusion, were injected subcutaneously per flank of nu/nu mice. Tumors were measured using calipers weekly by an animal technician who was unaware of the genotypes of the cells.

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