The tumor suppressor function of the von Hippel-Lindau protein (pVHL) has previously been linked to its role in regulating hypoxia-inducible factor levels. However, VHL gene mutations suggest a hypoxia-inducible factor-independent function for the N-terminal acidic domain in tumor suppression. Here, we report that phosphorylation of the N-terminal acidic domain of pVHL by casein kinase-2 is essential for its tumor suppressor function. This post-translational modification did not affect the levels of hypoxia-inducible factor; however, it did change the binding of pVHL to another known binding partner, fibronectin. Cells expressing phospho-defective mutants caused improper fibronectin matrix deposition and demonstrated retarded tumor formation in mice. We propose that phosphorylation of the acidic domain plays a role in the regulation of proper fibronectin matrix deposition and that this may be relevant for the development of VHL-associated malignancies.

In some anatomical venues, neoplastic transformation occurs upon biallelic inactivation of the von Hippel-Lindau (VHL) gene (1). In their lifetime, patients carrying a mutated VHL gene are predisposed to multiple tumors in a number of organs, including the retina, cerebellum, spinal cord, adrenal gland, and kidney. Despite this phenotypic heterogeneity, VHL patients can be segregated based on their likelihood of developing pheochromocytoma. Type 1 VHL patients are at low risk for developing pheochromocytoma, but do develop clear-cell renal cell carcinoma (RCC). Type 2 patients develop pheochromocytoma and are further subdivided. Type 2A patients have low risk for clear-cell RCC, and type 2B patients have high risk for clear-cell RCC. Type 1, 2A, and 2B patients develop the two cardinal features of VHL disease: retinal and central nervous system hemangioblastoma. However, type 2C patients exclusively develop pheochromocytoma (2, 3).

pVHL forms a multiprotein complex (VEC) with elongin C, elongin B, cul2, and Rbx-1 (reviewed in Ref. 4). VEC functions as a ubiquitin-activating enzyme (E3) to target the \( \alpha \) subunit of a heterodimeric transcription factor, hypoxia-inducible factor (HIF), for polyubiquitination (5–7). Polyubiquitin-tagged HIF-\( \alpha \) is subsequently degraded by the 26 S proteasome. The VHL protein (pVHL) functions as the target recognition moiety of the VEC complex and specifically recognizes prolyl-hydroxylated HIF-\( \alpha \) subunits (8, 9). This post-translational modification of HIF-\( \alpha \) requires oxoglutarate, iron, and oxygen (8–10). Thus, ubiquitin-mediated destruction of HIF-\( \alpha \) occurs selectively under normoxic conditions. Accordingly, biallelic inactivation of the VHL gene through mutation, deletion, or promoter silencing generally leads to the stabilization of HIF-\( \alpha \) and thereby promotes the up-regulation of numerous HIF target genes, resulting in an inappropriate triggering of the hypoxic response under normal oxygen tension (4). However, type 2C patient mutations do not affect the E3 function of pVHL, and hence, HIF regulation is normal; yet these patients nevertheless develop pheochromocytoma (3). Therefore, we hypothesized that another function of pVHL is essential for its tumor suppressor activity.

The VHL gene produces two wild-type isoforms: a full-length 30-kDa form (pVHL30) and a shorter 19-kDa form (pVHL19) that is generated by alternative translational initiation at Met\(^{54} \) (11–13). Both pVHL30 and pVHL19 form an active VEC complex, and both proteins have been shown to suppress tumor formation independently in nude mouse xenograft assays (12–14). However, analyzing known mutations in VHL patients revealed a number of disease-associated mutations in the acidic domain that would not affect the transcription and function of the pVHL19 isoform (VHL Mutations Database, available at www.umd.be:2020/). Hence, although the functional significance of the extra N-terminal acidic domain of pVHL30 remains unclear, this region in pVHL clearly contributes to its tumor suppressor function.

Here, we show that pVHL30 is phosphorylated within the N-terminal acidic domain by casein kinase-2 (CK2). The phos-
Phosphorylation status of pVHL30 did not affect E3 function. Ablation of CK2-specific phosphorylation sites or inhibition of CK2 activity increased affinity for fibronectin, yet resulted in decreased deposition of extracellular fibronectin. Interestingly, cells expressing phospho-defective pVHL delayed the onset of tumor formation in a severe combined immunodeficiency (SCID) mouse xenograft assay. Hence, CK2-mediated phosphorylation represents an HIF-independent tumor suppressor function of the acidic domain of pVHL.

**MATERIALS AND METHODS**

**Cell Culture**—Human kidney 293T cells and 786-O clear-cell RCC cells were cultured in RPMI 1640 medium or Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (Sigma) supplemented with penicillin, streptomycin, and glutamine and maintained at 37 °C in a humidified 5% CO2 atmosphere. 786-O subclones stably expressing hemagglutinin (HA)-tagged wild-type pVHL30 (pVHL30(WT); 786-WT cells), HA-pVHL30(AAA) (pVHL30 triple mutant with Ser33, Ser38, and Ser43 mutated to alanines; 786-AAA cells), or empty plasmid (786-MOCK cells) were generated as described previously (15).

**Antibodies**—Anti-Gal4 DNA-binding domain (RK5C1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-HA (hybridoma supernatant, 3D7) (34), an-ti-Gal4 DNA-binding domain (RK5C1; Santa Cruz Biotechnology, Inc.), and anti-fibronectin (TaKaRa Biomedicals, Shiga, Japan) polyclonal antibodies were obtained from the indicated companies.

**Plasmids**—pVHL30 and pVHL19 were cloned into a pcDNA3 vector (Stratagene, La Jolla, CA) containing an N-terminal VSV or HA tag. The mammalian expression plasmid pRC-CMV-HA-pVHL(WT) was described previously (15), and pRC-CMV-HA-pVHL(AAA) was generated using the QuikChange site-directed mutagenesis kit (Stratagene). The mammalian expression plasmid pRc-CMV-HA-pVHL(WT) was described previously (15), and pRc-CMV-HA-pVHL(AAA) was generated (Stratagene, La Jolla, CA) containing an N-terminal VSV or HA tag.

**Immunoprecipitation and Immunoblotting**—For immunoprecipitation, protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.), and anti-fibronectin (TaKaRa Biomedicals, Shiga, Japan) polyclonal antibodies were obtained from the indicated companies.

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**RESULTS**

The Acidic Domain of pVHL30 Is Phosphorylated by CK2—To determine whether the two naturally occurring pVHL isoforms are phosphorylated, 293T cells were transfected with VSV-tagged pVHL30 and pVHL19 and metabolically labeled with [32P]orthophosphate. Cells were then lysed, immunoprecipitated with anti-VSV antibody, resolved by SDSPAGE, and immunoblotted with anti-VSV antibody (Fig. 1A). Autoradiography of the immunoblot demonstrated that, although VSV-pVHL30 incorporated [32P]orthophosphate, VSV-pVHL19 did not (Fig. 1A). Computer-based scanning of full-length pVHL30 indicated multiple potential phosphorylation sites over the entire open reading frame and three predicted CK2 phosphorylation sites in the acidic domain at Ser33, Ser38, and Ser43 (data not shown). Thus, we generated the triple mutant pVHL30(AAA) with Ser-to-Ala substitutions at each of these positions and found that pVHL30(AAA) was no longer capable of incorporating [32P]orthophosphate (Fig. 1B). These results suggest that, in an overexpression system, pVHL30, but not pVHL19, is phosphorylated at one or more of the serine residues within the acidic domain. To determine the physiologic relevance of this finding, we metabolically labeled 293T cells with [32P]orthophosphate. Cells were then lysed, immunoprecipitated with anti-pVHL antibody, resolved by SDS-PAGE, and blotted (Fig. 1C). Autoradiograph—

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**Fig. 2.** CK2 phosphorylates pVHL30. A, in vitro translated VSV-pVHL30(WT) and HA-pVHL30(AAA) were analyzed in an in vitro kinase assay (IVK). After translation, proteins were immunoprecipitated with anti-VSV or anti-HA antibody, and CK2α was added in the presence of [γ-32P]ATP. The proteins were immunoblotted (IB) with anti-VSV or anti-HA antibody (lower panel) and visualized by autoradiography (upper panel). Casein was used as a positive control. B, in vitro translated HA-pVHL30(WT) and the indicated mutations were immunoprecipitated with anti-HA antibody and treated as described for A. Upper panel, CK2-mediated phosphorylation; lower panel, anti-HA immunoblot of the in vitro translated pVHL used in the in vitro kinase assay. C, 786-WT cell lysates were immunoprecipitated (IP) with anti-receptor protein-tyrosine phosphatase-μ mAb (α-RPTP-μ) as a negative control or anti-pVHL antibody, and bound proteins were separated by SDS-PAGE and immunoblotted with anti-CK2α antibody (upper panel) or anti-pVHL antibody (lower panel). The asterisk indicates the antibody light chain, wcl, whole cell lysate. D, 293T cells were lysed and used for endogenous immunoprecipitation of CK2α, pVHL, or receptor protein-tyrosine phosphatase-μ (used as an aspecific control). The samples were incubated with [γ-32P]ATP, separated by SDS-PAGE, and blotted. Bands were visualized by autoradiography. E, 293T cells were lysed, and the lysate was submitted to gel filtration. Fractions of different sizes were collected, and after trichloroacetic acid precipitation, the proteins were submitted to Western blot analysis using anti-CK2α or anti-pVHL antibody. F, 293T cells were transfected with HA-pVHL30(WT). In vitro kinase assays of anti-HA immunoprecipitations were performed without or with increasing concentrations of the indicated CK2-specific inhibitors (upper panels). The anti-HA immunoblot indicates equal loading (lower panels). TBB, 4,5,6,7-tetra-bromobenzotriazolol.

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SCID Mouse Xenograft Assay—Multiple 786-O subclones expressing pVHL(WT), pVHL(AAA), or empty plasmid were grown to ~90% confluence in a humidified 5% CO2 atmosphere at 37 °C. Cells were harvested with a solution of 0.25% trypsin and 1 mM EDTA. Cells (2 × 10⁶) in 50 μl of 1× PBS were injected intramuscularly into the left hind leg of male SCID mice (Charles River Laboratories, Inc.). Tumor growth was assessed and measured weekly by carefully passing the tumor-bearing leg through a series of holes of decreasing diameter (0.5-mm decrements) in a plastic rod.

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To address directly whether CK2 is responsible for phosphorylation of pVHL30 at the predicted serine residues, we performed an in vitro kinase assay using in vitro translated VSV-pVHL30(WT) and HA-pVHL30(AAA) with or without recombinant CK2α, the catalytic subunit of CK2 (Fig. 2A). Autoradiography of the in vitro kinase assay clearly demonstrated that pVHL30(WT), but not pVHL30(AAA), was phosphorylated by CK2α (Fig. 2A). Casein was used as a positive control for the CK2-mediated in vitro kinase assays (Fig. 2A). To determine the exact site(s) of pVHL30 phosphorylation, single and double Ser-to-Ala mutants were generated and analyzed using the in vitro kinase assay (Fig. 2B). All in vitro translated single Ser-to-Ala mutants were phosphorylated by recombinant CK2α. It should be noted, however, that pVHL30(S38A) had a noticeably weaker phosphorylation signal. All double Ser-to-Ala mutants showed significantly weaker phosphorylation profiles compared with their wild-type counterpart (Fig. 2B). These data suggest that all three serine residues can serve as CK2-mediated phosphorylation sites and that phosphorylation of any one site appears to be temporally and spatially independent of the phosphorylation status of the other two sites.

During the course of these in vitro kinase assays, we observed that, even without adding exogenous CK2α, anti-pVHL30(WT) immunoprecipitates derived from 293T cells exhibited endogenous kinase activity capable of phosphorylating casein (data not shown). Likewise, immunoprecipitates of pVHL30(AAA), which themselves could not be phosphorylated (Figs. 1A and 2, A and B), were also capable of phosphorylating purified casein (data not shown). These data suggest that CK2 exists in a complex with pVHL30 irrespective of its phosphorylation status. To further verify that the pVHL30-associated kinase is CK2α, 786-O RCC subclones stably expressing HA-pVHL30(WT) (786-WT cells) were lysed and immunoprecipitated with anti-pVHL, anti-CK2α, or anti-receptor protein-tyrosine phosphatase-μ (used as a negative control) antibody (Fig. 2C). Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-CK2α or anti-pVHL antibody, which showed that CK2α associated with pVHL30 (Fig. 2C). To determine whether this interaction occurs in the absence of overexpression, we performed immunoprecipitation using anti-CK2α, anti-pVHL, or anti-receptor protein-tyrosine phosphatase-μ (used as a negative control) antibody; an in vitro kinase assay; SDS-PAGE; and visualization by autoradiography. Kinase activity was detected in immunoprecipitates of pVHL and CK2α, which showed phosphorylation of endogenous pVHL30 (Fig. 2D, first and second lanes). We also observed phosphorylated proteins comigrating with CK2α (Fig. 2D), suggesting autophosphorylation of CK2α. These results suggest that pVHL30 and CK2α interact under a physiological condition. Furthermore, we performed a size gel filtration assay to separate endogenous multiprotein complexes from 293T lysate according to their size. After performing the gel filtration according to size, proteins were concentrated by trichloroacetic acid precipitation and immunoblotted for pVHL and CK2α. In the fraction containing pVHL30, we did find CK2α (Fig. 2E), again supporting the notion that pVHL and CK2α are present in the same protein complex.

We next examined the effects of the CK2-specific inhibitors daidzein and 4,5,6,7-tetramethoxyflavone on phosphorylation of pVHL30. To this end, anti-PA immunoprecipitates from 293T cells transfected with HA-pVHL30 were prepared and used in an in vitro kinase assay in the presence of 0, 10, 30, and 100 μM daidzein or 4,5,6,7-tetramethoxyflavone (Fig. 2F). We observed dose-dependent inhibition of pVHL30 phosphorylation (Fig. 2F). Collectively, these results show that CK2α binds and phosphorylates pVHL30.

**VEC Complex Formation and Function Are Independent of pVHL30 Phosphorylation**—To address whether the pVHL30-(AAA) mutant is capable of forming an E3 complex, we tested the ability of pVHL30(AAA) to bind to Cul2, a component of the VEC complex that acts as a scaffold. 786-WT, 786-AAA, or 786-MOCK cells were lysed, immunoprecipitated with anti-HA antibody (upper panel), anti-Cul2 antibody as a loading control (middle panel), or anti-HA antibody (lower panel). ERS/ub-Ald-ub, energy regeneration system/ubiquitin-aldehyde-ubiquitin; HIF1α(ODD)Ub(ub), polyubiquitinated HIF(ODD).

We next asked whether pVHL30(AAA) can ubiquitinate HIF-
1α(ODD) by performing an in vitro ubiquitination assay. S100 extracts prepared from 786-MOCK cells lacking pVHL and supplemented with in vitro translated empty plasmid failed to support the ubiquitination of 35S-labeled HA-HIF-1α(ODD) (Fig. 3B, first lane). However, S100 extracts supplemented with increasing amounts of either in vitro translated HA-pVHL30(WT) (Fig. 3B, second and third lanes) or in vitro translated HA-pVHL30(ΔAA) (fourth fifth and lanes) ubiquitinated 35S-labeled HA-HIF-1α(ODD). As expected, S100 extracts made from 786-WT cells supported the ubiquitination of 35S-labeled HA-HIF-1α(ODD) (Fig. 3B, sixth lane). Furthermore, whole cell extract made from 786-AAA cells down-regulated the expression of the HIF target gene GLUT1 under normal oxygen tension similarly to 786-WT cells (Fig. 3C). Whole cell extract made from 786-MOCK cells showed overexpression of GLUT1 under normal oxygen tension. These results indicate that phosphorylation of pVHL30 by CK2 is not required for participation in the VEC complex to ubiquitinate HIF-α.

Phosphorylation of pVHL30 Regulates Binding of Fibronectin and Secretion into Extracellular Space—Interestingly, HA-pVHL30(ΔAA) bound strikingly more fibronectin (~3-fold, as measured by densitometry) compared with HA-pVHL30(WT) (Fig. 4A). Furthermore, the addition of increasing amounts of purified recombinant fibronectin during lysis of radiolabeled cells resulted in greater reduction of fibronectin bound to HA-pVHL30(WT) compared with HA-pVHL30(ΔAA) (Fig. 4, B and C). For example, whereas we observed no appreciable effect on HA-pVHL30(ΔAA) binding to fibronectin, HA-pVHL30(WT) binding to fibronectin was reduced by 40% upon the addition of 0.1 μg of purified fibronectin, as measured by densitometry (Fig. 4C). The addition of 1 μg of purified fibronectin almost completely competed against the binding of endogenous fibronectin to HA-pVHL30(ΔAA), but negligibly affected the binding of endogenous fibronectin to non-phosphorylatable HA-pVHL30(ΔAA). Collectively, these data imply that dephosphorylation of pVHL30 enhances its ability to bind fibronectin or reduces its ability to release fibronectin.

We next asked whether inhibition of CK2 activity would increase the binding of pVHL30(WT) to fibronectin. During metabolic labeling, 786-WT cells were treated with increasing concentrations of the CK2-specific inhibitor daidzein. Cells were then lysed, immunoprecipitated with anti-HA mAb, resolved by SDS-PAGE, and visualized by autoradiography. Upper panel, bands at the molecular mass of fibronectin; lower panel, bands at the molecular mass of pVHL indicating equal loading. IB, immunoblot.

![Image](http://www.jbc.org/)

**Fig. 4. Inhibition of CK2-mediated phosphorylation of pVHL30 increases binding to fibronectin.** A, the indicated 786-O subclones were radiolabeled with [35S]methionine. Cells were lysed and immunoprecipitated (IP) with anti-HA antibody. The bound proteins were resolved by SDS-PAGE and visualized by autoradiography. B, the indicated radiolabeled cells were lysed in the presence of increasing amounts of purified recombinant fibronectin (rFibronectin) and immunoprecipitated with anti-HA antibody. Bound proteins were resolved by SDS-PAGE and visualized by autoradiography. C, are shown the results from densitometry of B. 100% binding represents pVHL30-bound fibronectin in the absence of competing exogenous purified fibronectin. D, 786-O subclones ectopically expressing HA-pVHL30(WT) were radiolabeled with [35S]methionine in the presence of increasing concentrations of the CK2-specific inhibitor daidzein. Cells were lysed and immunoprecipitated with anti-HA antibody, resolved by SDS-PAGE, and visualized by autoradiography. Upper panel, bands at the molecular mass of fibronectin; lower panel, bands at the molecular mass of pVHL indicating equal loading. IB, immunoblot.
resolved by SDS-PAGE, and immunoblotted (IB) with anti-fibronectin lysates were prepared after 30 min of trypsin/EDTA treatment at 37 °C.

786-WT, and 786-AAA cells were grown for 4 days, and whole cell of the matrix were taken using a confocal microscope.

The relative amounts of fibronectin deposited by cells were normalized against the mean of readings obtained from wells containing cell culture medium alone for the indicated period. B, 786-WT and 786-AAA cells were grown on coverslips for 4 days, fixed using ice-cold methanol, and stained for fibronectin. Thin slices (0.6 μm) of the cells above the level of the matrix were taken using a confocal microscope. C, 786-MOCK, 786-WT, and 786-AAA cells were grown for 4 days, and whole cell lysates were prepared after 30 min of trypsin/EDTA treatment at 37 °C, resolved by SDS-PAGE, and immunoblotted (IB) with anti-fibronectin antibody (α-FN; upper panel) and anti-mitogen-activated protein kinase antibody (α-MAPK) as a loading control (lower panel).

by pVHL(AAA) resulted in less fibronectin deposition compared with 786-WT cells (Fig. 5A). Anti-fibronectin immunofluorescence by confocal microscopy demonstrated the expected fibronectin staining in the cytoplasm and along the cell periphery in 786-WT cells (Fig. 5B). However, 786-AAA cells showed excessive perinuclear fibronectin staining as well as punctate staining along the cell cortex (Fig. 5B). Concordantly, 786-AAA cells showed more intracellular fibronectin expression compared with 786-WT cells (Fig. 5C). 786-WT and 786-AAA cells showed similar de novo translation of fibronectin as measured by 35S metabolic labeling (data not shown). These data suggest that, although 786-AAA cells are capable of producing fibronectin, they are less capable of releasing fibronectin into the extracellular space, which would account for the increased intracellular accumulation of fibronectin.

Phosphorylation of pVHL30 Is Required for Tumor Suppression in SCID Mice—To determine whether phosphorylation plays a role in tumor suppression, multiple 786-O subclones expressing pVHL(WT), pVHL(AAA), or empty plasmid were injected intramuscularly into the left hind legs of SCID mice. Tumor take and size were monitored and measured weekly (Fig. 6). After ~3–4 weeks, the mice injected with 786-MOCK cells began to form tumors (15/15), as expected. The mice injected with 786-AAA cells began to develop tumors after 7–8 weeks (15/15), whereas the mice injected with multiple 786-WT subclones were tumor-free (0/14). These results demonstrate that phosphorylation of pVHL30 within the first 53 amino acids have a direct role in tumor suppression.

DISCUSSION

Not all VHL disease-causing mutations result in the dysregulation of HIF activity (2, 3, 17). For example, type 2C-associated mutations such as L188V and K159E have been shown to have wild-type or “normal” E3 activity to target HIF-α subunits for oxygen-dependent polyubiquitination. However, every VHL disease-causing mutant tested to date has shown a failure to bind fibronectin, resulting in reduction of extracellular fibronectin matrix assembly (2, 3, 17). Here, we have shown that inhibition of pVHL phosphorylation, either by Ser-to-Ala substitutions or by treatment with CK2-specific inhibitors, resulted in markedly increased binding of pVHL30 to fibronectin while maintaining wild-type E3 function. However, this increased binding led to reduced fibronectin deposition into the extracellular space. The improper deposition of fibronectin into the extracellular space or interference with its proper function has been correlated with the development of dysplasia in Xenopus, and fibronectin has been shown to influence the malignant behavior of tumor cells in experimental mouse models (18, 19). Thus, we postulate that the tumor suppressor function of pVHL30 phosphorylation is related to the regulation of proper fibronectin deposition.

The tumor suppressor function of pVHL that we have described here requires the N-terminal acidic domain and is independent of the regulation of HIF-α. Multiple studies on the role of stabilized HIF in tumorigenesis using the identical cell system that we have described here have been recently published. Collectively, these previous studies suggest that HIF-2α is the effector of tumorigenesis upon loss of functional pVHL. Kondo et al. (20) and Maranchie et al. (21) addressed whether overexpressed stabilized HIF can counteract tumor suppression by pVHL. Kondo et al. (22) have shown that 786-O cells expressing stable HIF-2α are able to override pVHL-mediated tumor suppression. However, these previous studies did not address the more physiologically relevant question of whether the suppression of endogenous HIF-2α would be sufficient to...
Phosphorylation of von Hippel-Lindau Protein

abrogate tumor development in these patient-derived RCC cells (786-O RCC). Kondo et al. (22) and Zimmer et al. (23) did address this question using HIF-2α RNA interference; their data indeed support an oncogenic role for HIF-2α. However, these experimental data do not exclude further tumor suppressor functions for pVHL. The in vivo experiments described in these previous studies were terminated after 8–10 weeks, whereas our experiments demonstrated that this time point is only when 786-O cells deficient in CK2-mediated phosphorylation begin to form detectable tumors (Fig. 6). Moreover, tumor growth in the absence of HIF-2α is retarded, but not completely inhibited (22). On the basis of these findings and our data, we propose that, although HIF-2α is an important regulator of tumor growth, it is not the only effecter of tumorigenesis in pVHL-deficient cancers. These data led us to hypothesize that an additional tumor suppressor function is linked to phosphorylation of the acidic domain of pVHL.

The regulation of fibronectin by pVHL is tightly linked to the development of VHL disease. It follows then that there are multiple levels of fibronectin regulation by pVHL30. First, pVHL30 positively regulates transcription of the gene encoding fibronectin (FN1) in an HIF-independent manner (24). Second, as shown here, phosphorylation of the N-terminal acidic domain of pVHL30 mediates the engagement and/or disengagement of fibronectin. Third, modification of pVHL30 by the ubiquitin-like molecule NEDD8 is linked to fibronectin binding, and inhibition of pVHL30 neddylation prohibits fibronectin binding (18). Thus, fibronectin deposition by pVHL seems to be highly regulated and process is unique to the pVHL30

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