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FOR THE RECORD

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The long form of pVHL is artifactually modified by serine protease inhibitor AEBSF

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Abstract

von Hippel–Lindau protein (pVHL) is the tumor suppressor responsible for ubiquitylating the hypoxia-inducible factor (HIF) family of transcription factors for degradation under normoxic conditions. There are two major pVHL isoforms with the shorter isoform ($pVHL_{19}$) lacking the acidic domain present in the N-terminus of the longer isoform ($pVHL_{30}$). Although both isoforms can degrade HIF and suppress tumor formation in experimental systems, previous research suggests that $pVHL_{30}$ can undergo posttranslational modifications (PTM) and interact with unique proteins. Indeed, $pVHL_{30}$ has long been observed to migrate as two species on a reducing polyacrylamide gel, indicating the presence of an uncharacterized PTM on the slower-migrating $pVHL_{30}$ without an identifiable biological consequence. Thus, there has been considerable effort to elucidate the exclusive biological activity of $pVHL_{30}$, if any, by first defining the unique features of the slower-migrating species. We show here that the migration of $pVHL_{30}$, but not $pVHL_{19}$, is retarded by 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), an irreversible serine protease inhibitor commonly found in protease inhibitor cocktails.

KEYWORDS

AEBSF, gel electrophoresis, hypoxia-inducible factor (HIF), isoform-specific, serine protease inhibitor, von Hippel–Lindau (VHL)

1 | INTRODUCTION

von Hippel–Lindau protein (pVHL) is the substrate-conferring F-box component of an E3 ubiquitin ligase complex. The best characterized target of this complex is the alpha subunit of the hypoxia-inducible factor (HIF) transcription factor. Under normoxic conditions, HIFα is rapidly hydroxylated by prolyl hydroxylase (PHD) enzymes, which use molecular oxygen as a co-substrate.[1] Hydroxylated HIFα is then bound by pVHL and targeted for degradation via the ubiquitin-proteasome pathway.[2,3] Under hypoxic conditions, HIFα escapes recognition via pVHL and is consequently stabilized to dimerize with HIFβ to form a functional transcription factor that positively regulates factors governing cellular response and adaptation to hypoxia such as glycolysis, erythropoiesis and vasculogenesis. Mutations in the components of the HIF pathway, including pVHL, PHD2, and HIF2α, underlie numerous cancerous and non-cancerous pathologies.[4–7]

There are two isoforms of pVHL. $pVHL_{30}$ represents the full-length amino acid chain (1–213) encoded by the *VHL* gene. $pVHL_{19}$ is a shorter isoform that is translated from an internal methionine (54–213).[8,9] It is still unclear whether there is a functional significance to having two isoforms. Both $pVHL_{30}$ and $pVHL_{19}$ possess the alpha

domain necessary for forming an E3 complex and the beta domain that mediates HIFα binding.[10,11] Further, it has been shown that both isoforms can regulate HIFα levels and suppress tumor growth.[8,9] The first 53 amino acids of $pVHL_{30}$, absent in $pVHL_{19}$, encode a repetitive stretch of acidic residues. On the basis of multiple sequence alignments, it appears that the long form of pVHL lacks the acidic domain in non-mammalian species.[12] Within kingdom mammalia, different orders possess a different number of acidic repeats; primates (7–8 units), cingulata (5–6 units), artiodactyla (4–5 units), proboscidea (2–3 units), and lagomorpha (1 unit).[12]

Several unique properties have been attributed to $pVHL_{30}$ and, by extension, to this acidic domain. First, several studies have suggested that $pVHL_{30}$ is localized primarily to the cytoplasm and $pVHL_{19}$ to the nucleus.[8,13] Second, three serine residues (33, 38, 43) present in the acidic domain have been shown to be phosphorylated by casein kinase II (CK2).[14] Phosphorylation by CK2 is suggested to be important for $pVHL_{30}$ tumor suppressor function. Third, $pVHL_{30}$ but not $pVHL_{19}$ has been shown to interact with p14/ARF, a tumor suppressor that positively regulated p53 by inhibiting E3 ubiquitin ligase MDM2.[12,15] These bodies of work suggest that the acidic domain of $pVHL_{30}$ may be biologically active.

In previous publications, it is apparent that $pVHL_{30}$ can migrate as a doublet on an acrylamide gel.[2,16–26] The nature of the slower-migrating band is unknown. We hypothesized that the elucidation of the posttranslational modification (PTM) of $pVHL_{30}$ may help shed light on a potentially unique function of $pVHL_{30}$.

2 | RESULTS

2.1 | $pVHL_{30}$, but not $pVHL_{19}$, migrates as a doublet

In previous publications,[17,18] we employed an in vitro transcription and translation (IVTT) system where rabbit reticulocyte lysate is used to produce HA-$pVHL_{30}$. The programmed rabbit reticulocyte is then incubated with biotinylated HIF1αOH peptides immobilized on streptavidin agarose beads. By pulling down on the beads, we can visualize levels of associated pVHL via western blotting. Curiously, we observed that a 10% input of HA-$pVHL_{30}$ migrated as a single species on 10% acrylamide gel while HA-$pVHL_{30}$ pulled down by HIF1αOH peptide migrated as a doublet (Figure 1a). In other experiments, we noted that HA-$pVHL_{19}$ does not migrate as a doublet when pulled down via HIF1αOH (Figure 1b). By conducting immunoprecipitation experiments with 3xFLAG-$pVHL_{30}$, we realized that this construct migrated as a singlet (Figure 1c). We considered multiple possibilities. One was that the HA tag promoted PTM of $pVHL_{30}$. The second possibility was that the N-terminal 3xFLAG tag, which is larger than an HA tag, prevented the PTM of $pVHL_{30}$. To test the latter hypothesis directly, we cloned a C-terminal 3xFLAG $pVHL_{30}$. Indeed, this construct migrated as a doublet (Figure 1c). Further, when we added hydroxylated and unhydroxylated peptide to the pulldown buffer, we found no modulation in the proportion of the slower- and faster-migrating species. These results suggested that the pVHL modification was independent of an interaction with HIFα (Figure 1c). To further interrogate the role of N-terminal tags on the modification of the $pVHL_{30}$ acidic domain, we cloned an untagged $pVHL_{30}$ construct. Much like the HA-tagged construct, untagged $pVHL_{30}$ migrated as a doublet (Figure 1d).

We observed a similar phenomenon when HEK293A cells were transiently transfected with either $pVHL_{30}$ or $pVHL_{19}$ (Figure 1e). Our first hypothesis was that the shift in apparent molecular weight may represent phosphorylation of serine residues 33, 38, and 43.[14] However, site-directed mutagenesis of these three serine residues to alanine did not abolish the slower-migrating band (Figure 1e). At this point in our investigation, we hypothesized that a novel PTM localized to the acidic domain of $pVHL_{30}$ was being catalyzed by an active enzyme present in both rabbit reticulocyte lysate and HEK293A cells.

2.2 | AEBSF retards $pVHL_{30}$ migration

We further completed a series of biochemical experiments by omitting and altering certain conditions of the pVHL pulldown experiment. We found that the slower-migrating band does not appear when the pulldown is conducted in a buffer with neutral pH (Figure S1). The slower-migrating band becomes incrementally more prominent with increasing alkalinity of the pulldown buffer (Figure S1). Notably, $pVHL_{30}$ migrated as a singlet when protease inhibitor cocktail was omitted from the pulldown buffer (Figure 2a). The addition of a protease inhibitor cocktail to the rabbit reticulocyte lysate yielded the slower-migrating $pVHL_{30}$ species when directly resolved on an acrylamide gel (Figure 2b). We hypothesized that the protease inhibitor cocktail may be inhibiting an enzyme that removes a PTM from $pVHL_{30}$ or that the modified $pVHL_{30}$ species is proteolytically degraded. However, further attempts to probe the conditions necessary for the modification of $pVHL_{30}$ were hampered by the inability to modify the rabbit reticulocyte lysate without also directly affecting $pVHL_{30}$ translation/stability. To circumvent this issue, we purified

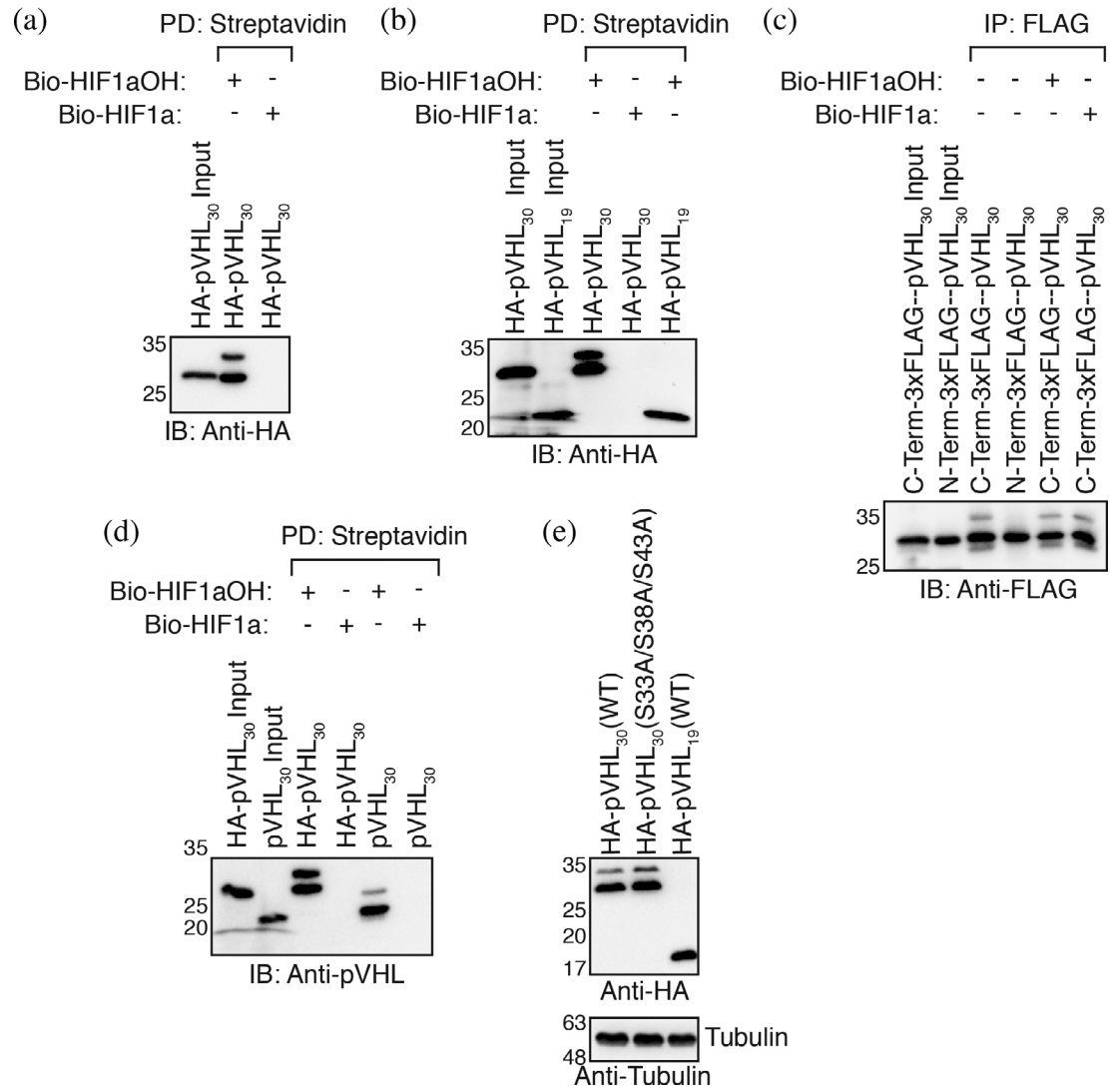

FIGURE 1 $pVHL_{30}$ migrates as a doublet. (a, b, and d) Biotinylated HIF1α peptides were immobilized on streptavidin-agarose beads and incubated with in vitro transcribed and translated (IVTT) pVHL in EBC buffer supplemented with protease inhibitors. Streptavidin beads were pulled down (PD), and levels of pVHL were visualized via immmunoblotting (IB). (c) IVTT pVHL was incubated in EBC buffer supplemented with protease inhibitors with or without HIF1α peptides. 3xFLAG-pVHL was immunoprecipitated (IP) with an anti-FLAG antibody, and levels of pVHL were visualized via IB. (e) HEK293a cells were transiently transfected HA-pVHL constructs for 48 hr before being lysed in EBC buffer supplemented with protease inhibitors. Hundred micrograms of total protein was resolved via SDS-PAGE, and levels of pVHL were visualized via immunoblotting

recombinant $pVHL_{30}$ and $pVHL_{19}$ in complex with elongin B and elongin C from BL21 *Escherichia coli*. $pVHL_{19}$ migrated as a singlet even when incubated with protease inhibitor and rabbit reticulocyte lysate (Figure 2c). Conversely, $pVHL_{30}$ was purified as a singlet but migrated as a doublet when incubated with protease inhibitor and rabbit reticulocyte (Figure 2c). This process was temperature dependent as the proportion of modified $pVHL_{30}$ was increased when the incubation temperature was increased from 4°C to 30°C (Figure 2c). Unexpectedly, the presence of a protease inhibitor cocktail was both necessary and sufficient for the modification of $pVHL_{30}$ (Figure 2c).

We next screened individual protease inhibitors and observed that incubation with the serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) alone is sufficient to obtain two migratory species of $pVHL_{30}$ (Figure 2d). Incubation of $pVHL_{30}$ with other protease inhibitors, including E64, leupeptin, bestatin, and pepstatin, did not induce a slower-migratory shift in $pVHL_{30}$ (Figure 2d). Under increasing concentrations of AEBSF, $pVHL_{30}$ began to migrate in a laddering pattern (Figure 2e). $pVHL_{19}$ also began to migrate as multiple species but only under higher concentrations of AEBSF. These results demonstrate that AEBSF covalently modifies $pVHL_{30}$ at concentrations

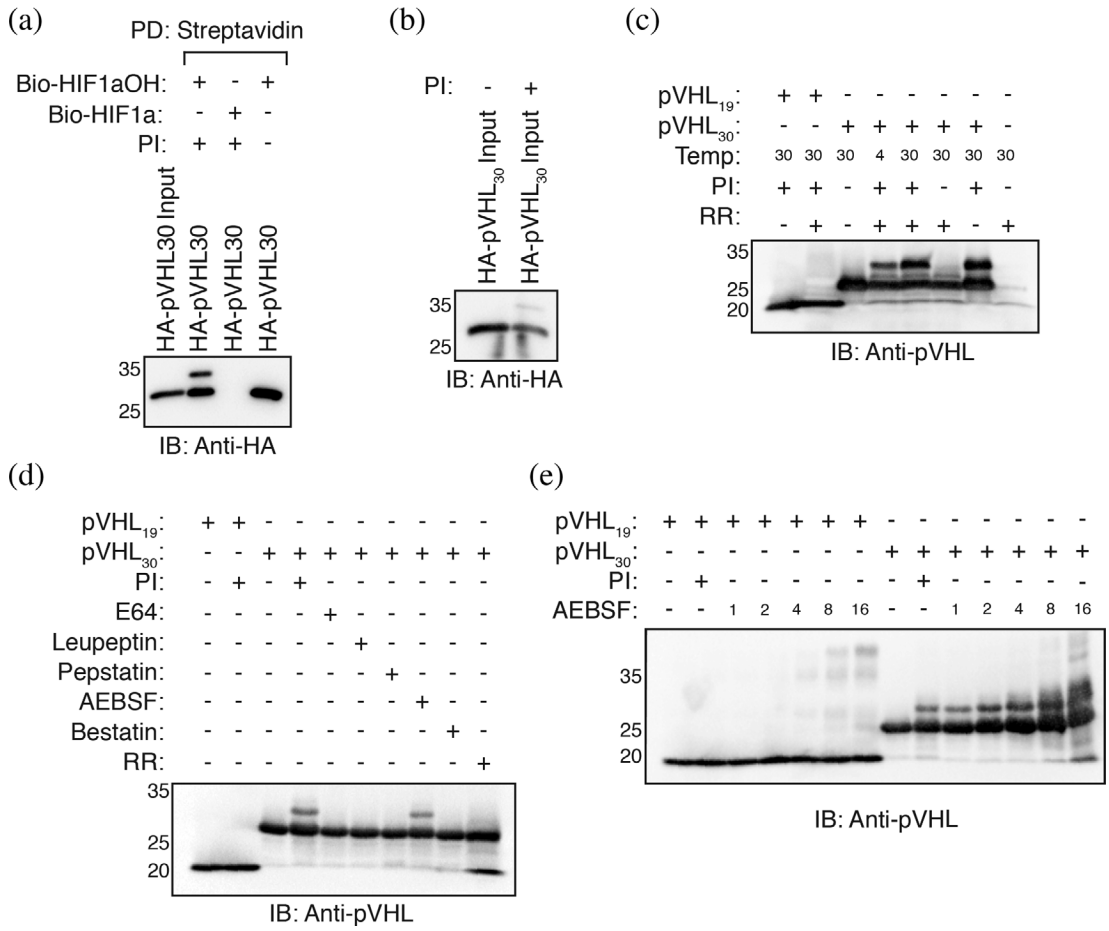

FIGURE 2 AEBSF covalently modifies $pVHL_{30}$. (a) Biotinylated HIF1α peptides were immobilized on streptavidin- agarose beads and incubated with in vitro transcribed and translated (IVTT) pVHL in EBC buffer supplemented with or without protease inhibitors. Streptavidin beads were pulled down (PD), and levels of pVHL were visualized via immmunoblotting (IB). (b) Rabbit reticulocyte lystate was programmed with a HA-$pVHL_{30}$ construct in the presence or absence of protease inhibitors. Two microliter of rabbit reticulocyte lysate was resolved via SDS-PAGE. (c–e) 2 μg of purified pVHL complex ($pVHL_{19}$/pVHL30, elongin B, and elongin C) was incubated at 30°C for 2 hr with or without rabbit reticulocyte lysate and with or without protease inhibitors. One microgram of protein was resolved via SDS-PAGE, and levels of pVHL were visualized via immunoblotting

normally used in experiments. As AEBSF predominantly modifies serine and tyrosine residues,[27] and mutation of the three serine residues found in the acidic domain failed to prevent $pVHL_{30}$ modification (Figure 1e), we hypothesized that AEBSF may be modifying the lone tyrosine residue found in the acidic domain (Tyr23). However, Y23F mutation did not prevent $pVHL_{30}$ modification (Figure S2). The first 53 amino acids of $pVHL_{30}$ lack lysine and histidine residues that are other possible sites of AEBSF modification.[27]

We next aimed to identify the modified site by mass spectrometry. AEBSF-modified $pVHL_{30}$ was enzymatically digested using trypsin and samples were analyzed by LC–MS. As AEBSF treatment results in a modification with a calculated mass shift of 183.035, we used an open search with this mass shift as a potential modification on any amino acid, in addition to other typical post-translational modifications (see Section 4). This resulted in the identification of 11 unique peptides, representing sequence coverage of approximately 50%. Based on the in silico tryptic digestion of $pVHL_{30}$, the identified peptides correspond to the majority of predicted MS-amenable peptides in the protein sequence. None of the identified peptides, however, mapped to the N-terminal region of the protein. This was not surprising, considering that no tryptic cleavage site is present within the first 63 amino acid residues. Moreover, digestion with a different protease such as Glu-C would be of little use considering that over one-third (21/60) of the first 60 amino acids of the protein are glutamic acid residues. However, we did identify a peptide (IAHQ[+183]R) with a putative AEBSF-modification near the C-terminus of the protein (Figure S3). Interestingly, AEBSF appears to modify a glutamine residue at position 209 in this case. To the best

of our knowledge, glutamine residues have not been reported to be covalently modified by AEBSF. As the C-terminus region is common to both $pVHL_{19}$ and $pVHL_{30}$, this modification is unlikely to explain the slower migration seen specifically with $pVHL_{30}$.

3 | DISCUSSION

PTMs have long been recognized as an important means of regulating protein activity, localization, stability, and function. Numerous pVHL PTMs have been reported, including phosphorylation,[14,28,29] SUMOylation,[19] ubiquitylation,[30] neddylation,[16,31] and N-terminal cleavage.[32] Of these potential PTMs, phosphorylation of serine residues 33, 38, and 43 and cleavage C-terminal to Tyr23 are localized to the N-terminal acidic domain unique to $pVHL_{30}$. Thus, we were intrigued to study a slower migrating $pVHL_{30}$ species (Figure 1a), especially once the potential modification was found to be localized to the acidic domain, and the possibility of serine phosphorylation was ruled out (Figure 1b,e). Experiments with both IVTT $pVHL_{30}$ and purified $pVHL_{30}$–elongin B–elongin C complex show that the slower migrating $pVHL_{30}$ species is an AEBSF-related artifact.

Other proteins, including amyloid beta,[33] transthyretin,[34] and the ζ of the T cell receptor,[33–35] have been found to be covalently modified by AEBSF. In the aforementioned cases, the modification by AEBSF was detected by mass spectrometry. In the case of the T cell receptor, the protein modification was not observable by SDS-PAGE. Two-dimensional electrophoresis studies of human plasma revealed that AEBSF alters the migration profile of several proteins.[27] AEBSF inhibits serine proteases through covalent modification of serine residues in the active site, thus forming a sulfonate ester. AEBSF has also been reported to modify serine residues in other chemical contexts in addition to tyrosine, histidine, lysine, and N-terminal amine nucleophiles.[27] Interestingly, we identified a Gln209 as being covalently modified by AEBSF. This modification, however, is unlikely to cause the slower migration associated with $pVHL_{30}$ as it also found in $pVHL_{19}$. It is likely that modification of a residue unique to $pVHL_{30}$ retards protein migration, but no such site was identified in our MS/MS experiment. It is also possible that the acidic domain of $pVHL_{30}$ interacts with the core of the pVHL protein and activates a nucleophile that otherwise would not react with AEBSF.

AEBSF preferentially modifies active, nucleophilic serine residues, which has led to the development of AEBSF as an activity-based probe (ABP) for serine proteases.[36] Interestingly, further use of AEBSF as an ABP uncovered the specific labeling of functional tyrosine residues in the active site of glutathione-s-transferase enzymes.[37] This work has been extended to designing ABPs that specifically react with specific tyrosine residues in the active site of the mRNA-decapping scavenger enzyme DcpS.[38] Thus, AEBSF as ABP could be useful for specifically labeling $pVHL_{30}$ and not $pVHL_{19}$. However, this might be a challenge given that we found a residue common to both $pVHL_{19}$ and $pVHL_{30}$ that can react with AEBSF.

In addition to identifying a putitive and artifactual PTM of $pVHL_{30}$, we uncovered several conditions that prevent this modification. The presence of modified $pVHL_{30}$ is more pronounced under alkaline condition. This might explain why the $pVHL_{30}$ doublet is not observable in experiments where buffer conditions are more neutral.[39] Further, the use of a N-terminal 3xFLAG tag prevents modification of $pVHL_{30}$ by AEBSF (Figure 1d). This observation suggests that the lengthy 3xFLAG tag interacts with the acidic domain. This notion is supported by the observation that intracellular truncation of the $pVHL_{30}$ acidic domain, hypothesized to be catalyzed by chymotrypsin, is inhibited by N-terminal HA and 3xFLAG tags.[32]

Here, we show that the migration of $pVHL_{30}$ is artifactually modified by the protease inhibitor AEBSF. This phenomenon is dependent on the first 53 amino acids unique to the longer isoform of pVHL. Since it is unknown whether the artifactual, slower-migrating species of $pVHL_{30}$ has or influences any biological activity, any investigation into the potential function of the acidic domain, which is predicted to be disordered,[12] should avoid the use of any N-terminal tags.

4 | MATERIALS AND METHODS

4.1 | Plasmids

Construction of the following plasmids has been described previously; pcDNA3-HA-VHL_{30}(WT),[40] pcDNA3-3xFLAG-VHL_{30}(WT), pACYCDuet-1 plasmid encoding untagged elongin B and elongin C(17–112), pGEX-4T-1-GST-VHL_{19}.[41] pcDNA3-HA-VHL_{19}(WT), pcDNA3-VHL_{30}(WT), and pGEX-4T-1-GST-VHL_{30} were subcloned from pcDNA3-HA-VHL_{30}(WT). pcDNA3-HA-VHL_{30}(S33A/S38A/S43A) was generated by site-directed mutagenesis of pcDNA-HA-VHL_{30}(WT). C-terminal tagged pcDNA3-3xFLAG-VHL_{30}(WT) was generated by amplifying VHL_{30} from pcDNA3-HA-VHL_{30}(WT) with a reverse primer containing 3xFLAG and subcloning into an empty pcDNA3 vector.

4.2 | Antibodies

Anti-HA (C29F4; 1:2,000 dilution) was obtained from Cell Signaling Technology. Anti-FLAG (F1804; 1:5,000)

and Anti-tubulin (T5168; 1:5,000) were obtained from Sigma-Aldrich. Anti-VHL (sc-135657, 1:1,500) was obtained from Santa Cruz.

4.3 | Peptides

HIF-1α (556–564; DLDLEMLAPYIPMDDDFQL) peptides with N-terminal biotinylation and C-terminal amidation modifications were custom synthesized by Genscript. All peptides were reconstituted to 2 mg/mL, as measured by A280, using sterile DMSO, aliquoted, and stored at −80°C.

4.4 | Protein expression and purification

BL21(DE3) *E. coli* cells were co-transformed with a dual expression construct encoding untagged elongin B (full-length, 1–118) and elongin C (17–112) and a construct encoding either N-terminal GST-tagged $pVHL_{19}$ or N-terminal GST-tagged $pVHL_{30}$. Bacterial cultures (1 L) were grown to an OD_{600} of approximately 0.6. Expression of the pVHL–elongin B–elongin C complex was induced via addition of IPTG (final concentration of 1 mM). Following induction, cells were grown for an additional 3.5 hr at a temperature of 37°C. Bacterial pellets were resuspended in 20 mM HEPES pH 7.4, 200 mM NaCl freshly supplemented with 10 mM DTT and lysed using a cell disruption unit at a pressure of 30 kPSI. Cell lysate was cleared via centrifugation (34,000*g* for 40 min). Cleared lysate was applied to a column of glutathione sepharose resin (GE Life Sciences). The column was washed with 20 mM HEPES pH 7.4, 200 mM NaCl and protein was eluted with 20 mM HEPES pH 7.4, 200 mM NaCl supplemented with 10 mM reduced glutathione. Protein solution was concentrated using a 4-mL centrifugal concentrator with a molecular weight cut-off of 3,500 Da (Pall Corporation). The concentrated protein solution was purified on a Superdex 200 10/300 size exclusion chromatography column equilibrated with 20 mM HEPES pH 7.4, 200 mM NaCl, and 1 mM DTT. The monomeric VBC complex was diluted to a concentration of 0.7 mg/mL. The GST-tag was cleaved via incubation (60 hr) with 1 U thrombin per mg of VBC protein at 4°C. Free GST was removed by applying the protein solution to regenerated glutathione sepharose resin. The protein solution was concentrated as discussed above and size exclusion chromatography was employed to purify soluble, monomeric VBC complex. Purity was confirmed via SDS-PAGE analysis. Aliquots of protein were frozen at −80°C at a concentration between 1 and 2 mg/mL.

4.5 | In vitro binding assay

The in vitro pVHL-HIF-1α binding assay was performed according to a previously published protocol.[42] VHL constructs was expressed in a transcription and translation (TNT) rabbit reticulocyte lysate system (Promega, Cat. No. L1170) and incubated with 2 μg of biotinylated HIF-1α peptide (hydroxylated or unhydroxylated), immobilized on streptavidin agarose beads, in either 500 μL of EBC buffer (50 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.5% [v/v] NP-40) supplemented with protease inhibitors for 2 hr at 4°C. Following incubation, beads were washed ×5 with NETN buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% [v/v] NP-40). Biotinylated peptide was pulled down via streptavidin agarose beads and protein was eluted by boiling beads in sample buffer. Samples were resolved on a 10% acrylamide gel via SDS-PAGE.

4.6 | In vitro VHL modification assay

Two μg of purified pVHL–elongin B–elongin C was incubated in 20 μL of 50 mM Tris–HCl pH 8.0, 120 mM NaCl for 2 hr at 30°C. Two microliter of rabbit reticulocyte was added to indicated reaction conditions. For individual protease inhibitors, the following working concentrations were utilized; leupeptin (100 μM), pepstatin A (10 μM), E-64 (10 μM), AEBSF (1 mM), bestatin hydrochloride (10 μM). After incubation, the reaction was stopped with sample buffer. Samples were resolved on a 10% acrylamide gel via SDS-PAGE.

4.7 | Cell culture and transfection

HEK293a cells were obtained from American Type Culture Collection (ATCC). Cells were maintained in DMEM (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (Wisent) and were grown at 37°C in a humidified, 5% CO_2 atmosphere. The indicated plasmids were transiently transfected into HEK293a cells using polyethylenimine.

4.8 | Liquid chromatography–mass spectrometry

Purified pVHL was incubated with AEBSF as indicated above. To avoid inhibition of trypsin activity by excess AEBSF, samples were subjected to buffer exchange in Amicon Ultra 3 kDa MWCO columns (Millipore) using 50 mM NH_4HCO_3. Samples were next reduced with DTT

(5 mM, 20 min, 60°C) and alkylated with iodoacetamide (10 mM, 30 min, room temperature, light protected). Protein digestion was performed with sequencing-grade, TPCK-treated, modified trypsin (Promega) for 16 hr at 37°C. The resulting peptide samples were desalted using C18 chromatography columns and lyophilized. Peptides were re-suspended in 0.1% formic acid analyzed by LC–MS.

Liquid chromatography was conducted using a C18 pre-column (Acclaim PepMap 100, 2 cm × 75 μm ID, Thermo Scientific) and a C18 analytical column (Acclaim PepMap RSLC, 50 cm × 75 μm ID, Thermo Scientific), running a 120 min reversed-phase gradient (0–40% ACN in 0.1% formic acid) at 225 nL/min on an EASY-nLC1200 pump (Thermo Scientific). Mass spectrometry was performed on a Q-Exactive HF instrument (Thermo Scientific). An MS scan was performed with a resolution of 60,000 followed by up to 20 MS/MS scans (minimum ion count of 1,000 for activation) using higher energy collision induced dissociation (HCD) fragmentation. Dynamic exclusion was set for 5 s (10 ppm; exclusion list size = 500). Raw data was analyzed for identification of peptides and post-translational modifications using the FragPipe platform.[43] Spectra were searched against the $pVHL_{30}$ protein sequence. Search parameters specified a parent ion mass tolerance of 10 ppm, and an MS/MS fragment ion tolerance of 0.4 Da, with up to two missed cleavages allowed for trypsin (excluding K/RP). Variable modifications of 0.98 on N or Q, 15.99 on M, 57.02 on C and 183.04 (corresponding to the mass difference resulting from the AEBSF-induced modification) on any amino acid were set in the search parameters. Spectra were manually inspected for quality assurance and confirmation of proper assignment of modifications.

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AUTHOR CONTRIBUTIONS

Daniel Tarade: Conceptualization; data curation; formal analysis; investigation; methodology; supervision; validation; visualization; writing-original draft; writing-review and editing. **Shelley He:** Formal analysis; investigation; methodology; validation; visualization; writing-review and editing. **Jonathan St-Germain:** Data curation; formal analysis; investigation; methodology; validation; visualization; writing-review and editing. **Avi Petroff:** Investigation; validation. **Anya Murphy:** Investigation. **Brian Raught:** Resources; supervision; writing-review and editing. **Michael Ohh:** Conceptualization; formal analysis; funding acquisition; methodology; resources; supervision; writing-original draft; writing-review and editing.

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REFERENCES

1. Epstein AC, Gleadle JM, McNeill LA, et al. *C. elegans* egl-9 and mammalian homologs define a family of dioxygenases that regulate hif by prolyl hydroxylation. Cell. 2001;107:43–54.
2. Ivan M, Kondo K, Yang H, et al. Hifalpha targeted for VHL-mediated destruction by proline hydroxylation: Implications for o2 sensing. Science. 2001;292:464–468.
3. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science. 2001;292:468–472.
4. Percy MJ, Furlow PW, Lucas GS, et al. A gain-of-function mutation in the hif2a gene in familial erythrocytosis. N Engl J Med. 2008;358:162–168.
5. Zhuang Z, Yang C, Lorenzo F, et al. Somatic hif2a gain-of-function mutations in paraganglioma with polycythemia. N Engl J Med. 2012;367:922–930.
6. Ladroue C, Carcenac R, Leporrier M, et al. Phd2 mutation and congenital erythrocytosis with paraganglioma. N Engl J Med. 2008;359:2685–2692.
7. Latif F, Tory K, Gnarra J, et al. Identification of the von Hippel-Lindau disease tumor suppressor gene. Science. 1993;260:1317–1320.
8. Iliopoulos O, Ohh M, Kaelin WG Jr. Pvhl19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. Proc Natl Acad Sci U S A. 1998;95:11661–11666.
9. Schoenfeld A, Davidowitz EJ, Burk RD. A second major native von Hippel-Lindau gene product, initiated from an internal translation start site, functions as a tumor suppressor. Proc Natl Acad Sci U S A. 1998;95:8817–8822.
10. Stebbins CE, Kaelin WG Jr, Pavletich NP. Structure of the VHL-ElonginC-ElonginB complex: Implications for VHL tumor suppressor function. Science. 1999;284:455–461.
11. Min JH, Yang H, Ivan M, Gertler F, Kaelin WG Jr, Pavletich NP. Structure of an hif-1alpha-pvhl complex: Hydroxyproline recognition in signaling. Science. 2002;296:1886–1889.
12. Minervini G, Mazzotta GM, Masiero A, et al. Isoform-specific interactions of the von Hippel-Lindau tumor suppressor protein. Sci Rep. 2015;5:12605.
13. Hergovich A, Lisztwan J, Barry R, Ballschmieter P, Krek W. Regulation of microtubule stability by the von Hippel-Lindau tumour suppressor protein pvhl. Nat Cell Biol. 2003;5:64–70.
14. Lolkema MP, Gervais ML, Snijckers CM, et al. Tumor suppression by the von Hippel-Lindau protein requires phosphorylation of the acidic domain. J Biol Chem. 2005;280:22205–22211.
15. Lai Y, Song M, Hakala K, Weintraub ST, Shiio Y. Proteomic dissection of the von Hippel–Lindau (VHL) interactome. J Proteome Res. 2011;10:5175–5182.

16. Russell RC, Ohh M. Nedd8 acts as a 'molecular switch' defining the functional selectivity of VHL. EMBO Rep. 2008;9:486–491.
17. Tarade D, Robinson CM, Lee JE, Ohh M. Hif-2α-pvhl complex reveals broad genotype-phenotype correlations in hif-2α-driven disease. Nat Commun. 2018;9:3359.
18. Tarade D, Ohh M. The hif and other quandaries in VHL disease. Oncogene. 2018;37:139–147.
19. Cai Q, Verma SC, Kumar P, Ma M, Robertson ES. Hypoxia inactivates the VHL tumor suppressor through PIASy-mediated sumo modification. PLoS One. 2010;5:e9720.
20. Cai Q, Robertson ES. Ubiquitin/sumo modification regulates VHL protein stability and nucleocytoplasmic localization. PLoS One. 2010;5:e12636.
21. Yang H, Minamishima YA, Yan Q, et al. Pvhl acts as an adaptor to promote the inhibitory phosphorylation of the nf-κb agonist card9 by ck2. Mol Cell. 2007;28:15–27.
22. Lee SB, Frattini V, Bansal M, et al. An id2-dependent mechanism for VHL inactivation in cancer. Nature. 2016;529:172–177.
23. Yu X, Chen S, Hou P, Wang M, Chen Y, Guo D. Vhl negatively regulates sars coronavirus replication by modulating nsp16 ubiquitination and stability. Biochem Biophys Res Commun. 2015;459:270–276.
24. Cho HK, Kim SY, Kim KH, Kim HH, Cheong J. Tumor suppressor protein VHL inhibits hedgehog–gli activation through suppression of gli1 nuclear localization. FEBS Lett. 2013;587:826–832.
25. Yuen J, Cockman M, Sullivan M, et al. The VHL tumor suppressor inhibits expression of the igf1r and its loss induces igf1r upregulation in human clear cell renal carcinoma. Oncogene. 2007;26:6499–6508.
26. Kim D, Choi Y, Han B, et al. Cancer cells promote survival through depletion of the von Hippel–Lindau tumor suppressor by protein crosslinking. Oncogene. 2011;30:4780–4790.
27. Schuchard MD, Mehigh RJ, Cockrill SL, et al. Artifactual isoform profile modification following treatment of human plasma or serum with protease inhibitor, monitored by 2-dimensional electrophoresis and mass spectrometry. Biotechniques. 2005;39:239–247.
28. Ampofo E, Kietzmann T, Zimmer A, Jakupovic M, Montenarh M, Götz C. Phosphorylation of the von Hippel–Lindau protein (VHL) by protein kinase ck2 reduces its protein stability and affects p53 and hif-1α mediated transcription. Intl J Biochem Cell Biol. 2010;42:1729–1735.
29. Hergovich A, Lisztwan J, Thoma CR, Wirbelauer C, Barry RE, Krek W. Priming-dependent phosphorylation and regulation of the tumor suppressor pvhl by glycogen synthase kinase 3. Mol Cell Biol. 2006;26:5784–5796.
30. Metcalf J, Bradshaw P, Komosa M, Greer S, Meyn MS, Ohh M. K63-ubiquitylation of VHL by socs1 mediates DNA double-strand break repair. Oncogene. 2014;33:1055–1065.
31. Stickle NH, Chung J, Klco JM, Hill RP, Kaelin WG Jr, Ohh M. Pvhl modification by nedd8 is required for fibronectin matrix assembly and suppression of tumor development. Mol Cell Biol. 2004;24:3251–3261.
32. German P, Bai S, Liu X-D, et al. Phosphorylation-dependent cleavage regulates von Hippel Lindau proteostasis and function. Oncogene. 2016;35:4973–4980.
33. Conboy JJ, Wood KG, Lame ME, Durham RA, Geoghegan KF. Modification of amyloid-β (1–40) by a protease inhibitor creates risk of error in mass spectrometric quantitation of amyloid-β (1–42). Analyt Biochem. 2008;382:147–149.
34. Bergen HR III, Klug MG, Bolander ME, Muddiman DC. Informed use of proteolytic inhibitors in biomarker discovery. Rapid Commun Mass Spectrom. 2004;18:1001–1002.
35. Sweeney B, Proudfoot K, Parton A, King L, Slocombe P, Perry M. Purification of the t-cell receptor ζ-chain: Covalent modification by 4-(2-aminoethyl)-benzenesulfonyl fluoride. Anal Biochem. 1997;245:107–109.
36. Shannon DA, Gu C, McLaughlin CJ, Kaiser M, van der Hoorn RA, Weerapana E. Sulfonyl fluoride analogues as activity-based probes for serine proteases. Chembiochem. 2012;13:2327–2330.
37. Gu C, Shannon DA, Colby T, et al. Chemical proteomics with sulfonyl fluoride probes reveals selective labeling of functional tyrosines in glutathione transferases. Chem Biol. 2013;20:541–548.
38. Hett EC, Xu H, Geoghegan KF, et al. Rational targeting of active-site tyrosine residues using sulfonyl fluoride probes. ACS Chem Biol. 2015;10:1094–1098.
39. Guo J, Chakraborty AA, Liu P, et al. Pvhl suppresses kinase activity of akt in a proline-hydroxylation–dependent manner. Science. 2016;353:929–932.
40. Ohh M, Park CW, Ivan M, et al. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol. 2000;2:423–427.
41. Heir P, Srikumar T, Bikopoulos G, et al. Oxygen-dependent regulation of erythropoietin receptor turnover and signaling. J Biol Chem. 2016;291:7357–7372.
42. Heir P, Ohh M. Hydroxylation-dependent interaction of substrates to the von hippel-Lindau tumor suppressor protein (VHL). Tumor Microenviron Methods Protoc. 2016;1458:87–94.
43. Kong AT, Leprevost FV, Avtonomov DM, Mellacheruvu D, Nesvizhskii AI. Msfragger: Ultrafast and comprehensive peptide identification in mass spectrometry–based proteomics. Nat Methods. 2017;14:513–520.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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