

# Ubiquitin/SUMO Modification Regulates VHL Protein Stability and Nucleocytoplasmic Localization

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## Abstract

Functional inactivation of the von Hippel-Lindau (VHL) tumor suppressor protein is linked to the development of several forms of cancer as well as oncogenic progression like sporadic renal clear-cell carcinomas (RCC). Despite the critical role played by VHL in destruction of hypoxia-inducible factor  $\alpha$  (HIF $\alpha$ ) via ubiquitin-mediated proteolysis, very little is known about the post-translational modification which regulates VHL activity. Our previous study showed that the SUMO E3 ligase PIASy interacts with VHL and induces VHL SUMOylation on lysine residue 171 (Cai et al, *PLoS ONE*, 2010, 5(3):e9720). Here we further report that VHL also undergoes ubiquitylation on both lysine residues 171 and 196, which is blocked by PIASy. Moreover, using a VHL-SUMO1 or ubiquitin fusion protein, we found that ubiquitylated VHL is localized predominantly in the cytoplasm, while SUMOylated VHL results in increased VHL protein stability and nuclear redistribution. Interestingly, substitution of lysine 171 and 196 to arginine of VHL abrogates its inhibitory function on the transcriptional activity of HIF $\alpha$ , and tube formation *in vitro*. This demonstrates that post-translational modifications like ubiquitylation and SUMOylation contributes to VHL protein stability and nucleocytoplasmic shuttling, and that the overall function of VHL in tumor suppression may require a precise and dynamically regulated process which involves protein modification.

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## Introduction

The von Hippel-Lindau (VHL) gene was identified as a tumor suppressor in 1993 [1]. Germ-line mutations in this gene causes *VHL* hereditary cancer syndrome, which is characterized by the development of tumors of the central nervous system (CNS), kidney, eye, and pancreas [2,3]. Somatic mutations of the VHL gene also has been widely demonstrated in the majority of sporadic renal clear-cell carcinomas (RCCs, the most common form of adult kidney cancer) [4–6], and cerebellar hemangioblastomas [7]. Reintroduction of wild type VHL into *VHL*<sup>-/-</sup> renal carcinoma cells (RCC) has been shown to sufficiently suppresses tumor formation *in vivo* [8]. In the 3D protein structure, the VHL protein contains two functional domains,  $\alpha$  and  $\beta$ . The  $\alpha$  domain binds to elongin C and the  $\beta$  domain acts as the substrate-recognition site for targeting proteins [9]. Many studies have demonstrated that VHL is a multi-purpose adaptor protein that engages in regulation of the extracellular matrix [10,11], cellular differentiation [12], cell cycle [13], cell survival, apoptosis [14–16], and senescence [17]. However, the main function of VHL is viewed as an adapter for targeting hypoxia-inducible factor (HIF)  $\alpha$  subunit for proteolytic degradation [18,19].

In the presence of oxygen, HIF $\alpha$  is hydroxylated by prolyl hydroxylase (PHD) and then binds to VHL for proteasome-mediated degradation through the formation of EC<sub>2</sub>V (Elongin BC-Cul2-VHL) E3 ubiquitin ligase complex [18–20]. Under hypoxic environment, this hydroxylation-mediated degradation pathway is

blocked, and results in HIF $\alpha$  translocation and accumulation in the nucleus, where it binds with the constitutively expressed HIF $\beta$  to form a heterodimer and transactivates hypoxia-responsive genes (including Glut-1 and VEGF) that are implicated in cellular metabolism, angiogenesis, invasion, and metastasis [21,22]. Thus, loss function of VHL or hypoxic conditions will lead to HIF $\alpha$  accumulation and will also impair several other VHL-modulated biological pathways associated with tumor suppression.

Degradation of nuclear substrates by the ubiquitylation dependent system often requires nuclear-cytoplasmic trafficking of both the E3 ubiquitin ligase and the substrate proteins [23]. As a ubiquitin E3 ligase, it has been viewed that VHL is dynamic in controlling the degradation of HIF $\alpha$ , and several physiological cues can modulate the function of VHL within this setting [24]. For instance, VHL is predominantly nuclear at low cell density and cytoplasmic at high cell density [25]. Additionally, upon transcriptional arrest or low pH, VHL will accumulate in the nucleus [26,27]. Although the biological significance of this is unclear, the evidence supports the notion that nucleocytoplasmic shuttling of VHL may be important for its antitumor effects. Notably, by using various nuclear import or export sequences fused with VHL, a previous study has indirectly showed that specific subcellular localization affects the antitumor properties of VHL [28].

To date protein posttranslational modification by ubiquitin or other ubiquitin-like molecules (i.e. SUMO, NEDD) has emerged as an important strategy for dynamically regulating target proteins involved in regulation of diverse cellular processes, including protein

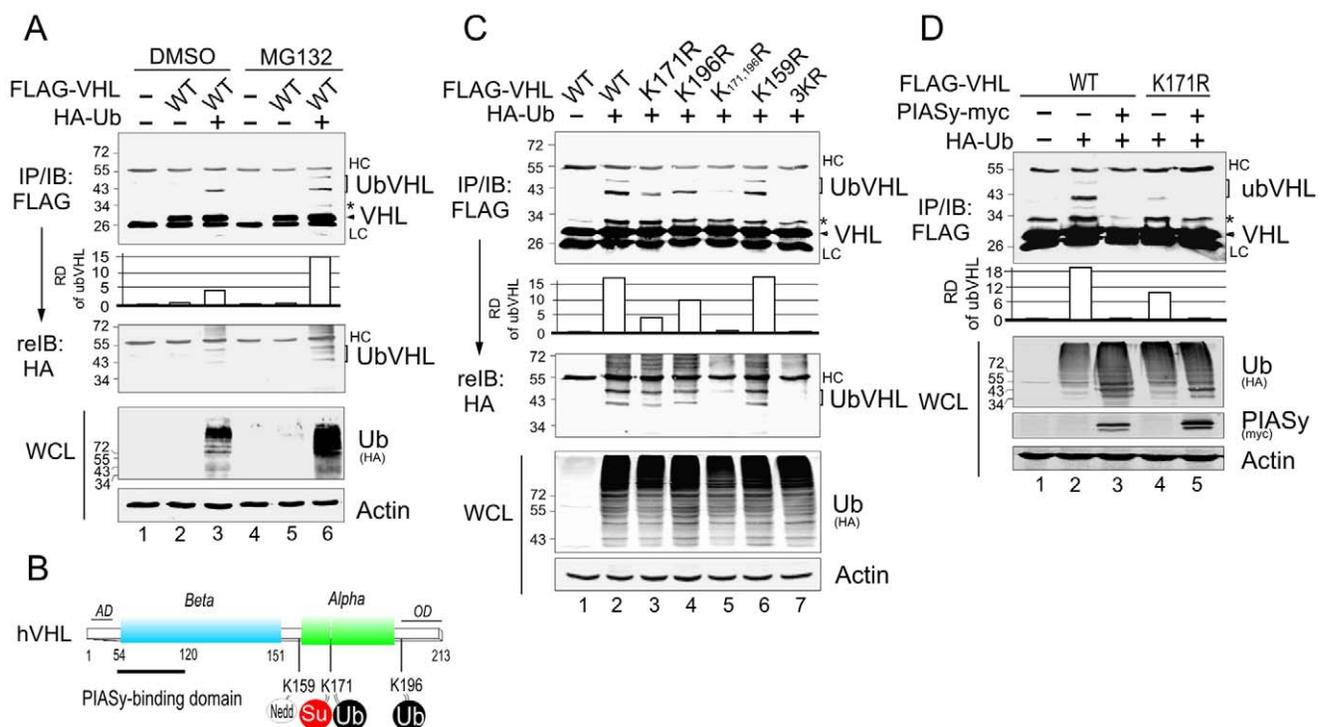
relocalization, stability and stress response [29–31]. We and other groups have previously found that SUMOylation or Neddylolation of VHL is able to affect its function in tumor suppression [32–34]. To determine whether the posttranslational modification of VHL affects its protein stability and nucleocytoplasmic redistribution, we further investigated the modification of VHL by mutation of specific lysine residues. Our previous finding showed that PIASy (a SUMO E3 ligase) induces VHL SUMOylation [32]. In the present study, we further showed that VHL is also ubiquitylated on both lysines 171 and 196, and that coexpression of PIASy prevents VHL ubiquitylation. Furthermore, we also demonstrated that VHL with ubiquitin or SUMO modification at high cell density exhibited distinct subcellular distribution and protein stability. The mutation of VHL lysine 171/196 which abrogates its ubiquitin and SUMO modification disabled its function related to inhibition of HIF $\alpha$  transcriptional activity and tube formation. Therefore, the ubiquitin/SUMO modification of VHL allows for precise regulation of VHL nucleocytoplasmic trafficking, and disruption of this process can impair its antitumor effects.

## Results

### Lysine residues 171 and 196 of VHL are targeted for ubiquitylation

Previous studies have demonstrated that VHL is a major player in the ubiquitylation system by acting as an ubiquitin E3 ligase.

However, recent studies has shown that VHL is targeted for proteasomal degradation by cellular or viral proteins (like E2-EFP UCP and KSHV LANA) [35,36]. To investigate whether VHL itself can be ubiquitylated, we overexpressed FLAG-tagged VHL in the presence or absence of HA-tagged ubiquitin in HEK293 cells followed by treatment with or without proteasome inhibitor MG132, and then performed immunoprecipitation and immunoblotting with FLAG antibody. The results showed that VHL had two slower-migrating bands (ubVHL) which appeared when coexpressed with ubiquitin (Figure 1A, compare lane 3 with 2), and the increase intensity of the modified ubVHL bands consistent with higher levels of ubiquitin after proteasomal inhibitor MG132 treatment, further proving that ubVHL is the isoform of VHL with ubiquitin modification (Figure 1A, compare lane 6 with 3). To determine which lysine residues are required for the ubiquitylation of VHL, we generated a series of VHL mutants (K171R, K196R, K<sub>171,196</sub>R, K159R, and 3KR) based on all the lysine residues with potential modification (like SUMOylation and Neddylolation indicated in Figure 1B), and then coexpressed with exogenous HA-tagged ubiquitin in HEK293 cells. Figure 1C showed that among three mutants (K159R, K171R, and K196R) with the single residue mutation, only K171R and K196R showed a significant reduction in the intensity of the modified isoform ubVHL when compared to wild type VHL. Furthermore, the double mutant K<sub>171,196</sub>R, similar to the triple mutant 3KR, almost completely lost the modified bands (Figure 1C, compare lane 5



**Figure 1. VHL can be ubiquitylated on lysines 171 and 196.** (A) The level of ubiquitylated VHL is increased by proteasomal inhibitor treatment. HEK293 cells were individually cotransfected with expression vector encoding the indicated proteins in the top panel. Forty-eight hour posttransfection, the cells were treated with or without 20  $\mu$ M MG132 for 2 hrs before harvest. Cell extracts were subjected to immunoprecipitated (IP) and immunoblotting (IB) as indicated in the figure. The relative quantitation of VHL ubiquitylation (ubVHL) was presented at the bottom. (B) Schematic representation of VHL  $\alpha$  and  $\beta$  domains with three potentially modification lysines K159, K171 and K196. Su, SUMOylation; Ub, ubiquitylation; Nedd, Neddylolation; AD, acidic domain; OD, oligomerization domain. (C) Lysine 171 and 196 but not 159 of VHL occurs ubiquitylation in vivo. HEK293 cells cotransfected with expression vector encoding the indicated proteins in the top panel, were treated with 20  $\mu$ M MG132 for 2 hrs before harvest and subjected to immunoprecipitated (IP) and immunoblotting (IB) as indicated in the figure. (D) PIASy suppresses VHL ubiquitylation. As performed in panel C, HEK293 cells were transfected with indicated plasmids. WCL, whole cell lysate; HC, heavy chain; LC, light chain. The asterisk denotes uncharacterized protein band.  
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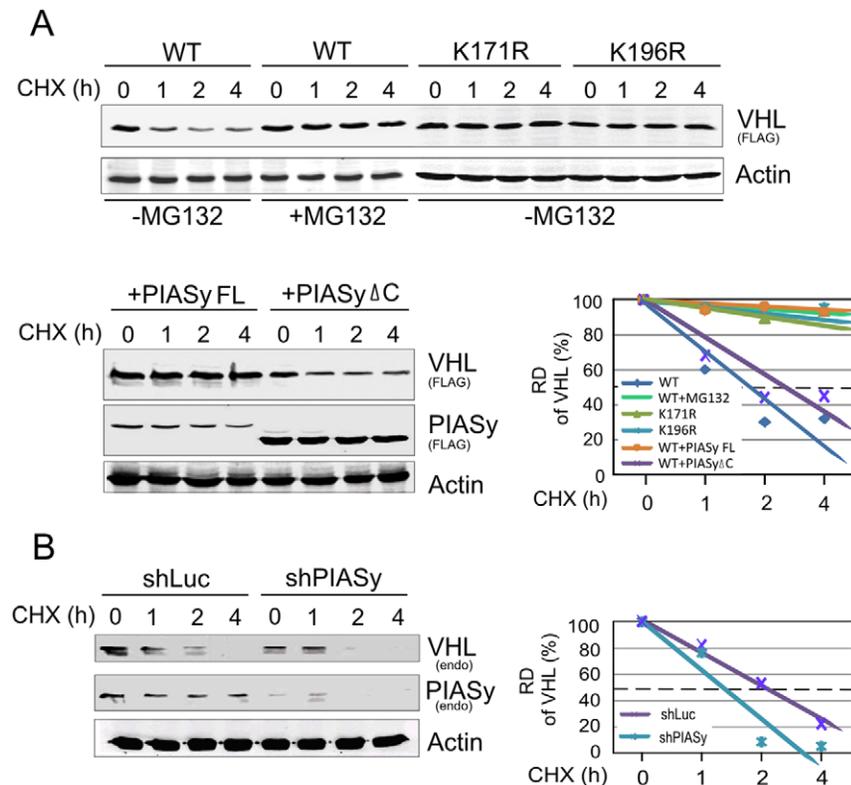
with 7). This further confirms that both the lysine 171 and 196 residues are the major residues for ubiquitin modification but not lysine 159. The re-immunoblotting results showing that the double (K<sub>171,196</sub>R) and triple (3KR) mutants present less association with ubiquitin than wild type of VHL (Figure 1C, middle panel), suggest that lysines 171 and 196 modification contribute to the ubiquitylation function of VHL.

### VHL ubiquitylation is blocked by PIASy and its protein stability and nuclear localization is increased

Previously we reported that SUMOylation occurs on lysine 171 of VHL [32]. To determine whether PIASy could prevent VHL ubiquitylation in addition to stimulating SUMOylation, we studied the effects of PIASy on both wild type VHL and the SUMOylation-deficient mutant K171R (that could still be ubiquitylated to some extent on lysine 196). Figure 1D showed that coexpression of PIASy completely suppressed the ubiquitylation of VHL on either lysine 171 or 196. This suggests that PIASy plays a role in stabilization of VHL by blocking ubiquitylation. To further assess the role of PIASy on VHL protein stability, we used cycloheximide to block protein synthesis and examined the stability of wild type VHL when cotransfected with either the plasmid expressing full-length (FL) PIASy or a PIASy mutant which lacks the ability to bind VHL ( $\Delta$ C) in HEK293 cells. As shown in Figure 2A, VHL stability was significantly increased in the presence of wild type

PIASy but not its mutant which lacks VHL-binding ability. The overall role of PIASy on VHL stability was further confirmed by the observation that the half life of endogenous VHL is decreased when endogenous PIASy expression is knocked-down (Figure 2B). Similarly, to determine the effect of ubiquitylation on VHL protein stability, we utilized the same strategy along with or without treatment with the proteasomal inhibitor MG132 to test the stability of wild type VHL and its ubiquitylated-site specific mutants (K171R or K196R). The results showed that MG132 treatment significantly increases the half life of wild type VHL when compared with no MG132 treatment. Consistently, the mutation of ubiquitylated lysine residue 171 or 196 leads to increased stability of wild type VHL (Figure 2A, lower panels). Therefore, these results indicate that in addition to SUMOylation, the lysine 171 of VHL is also targeted for ubiquitylation and degradation, and further supports a role for lysine 196 as an alternative target for VHL ubiquitylation, and that PIASy can function to increase VHL stability by blocking its ubiquitylation.

A number of studies have shown that the process of ubiquitin and ubiquitin-like modification not only increases target protein stability but also contributes to nuclear localization of target proteins [29–31]. To explore whether the subcellular localization of VHL is influenced by interaction with PIASy and its deficiency of ubiquitin/SUMO modification, we investigated the subcellular localization of wild type VHL and its K171R mutant in the

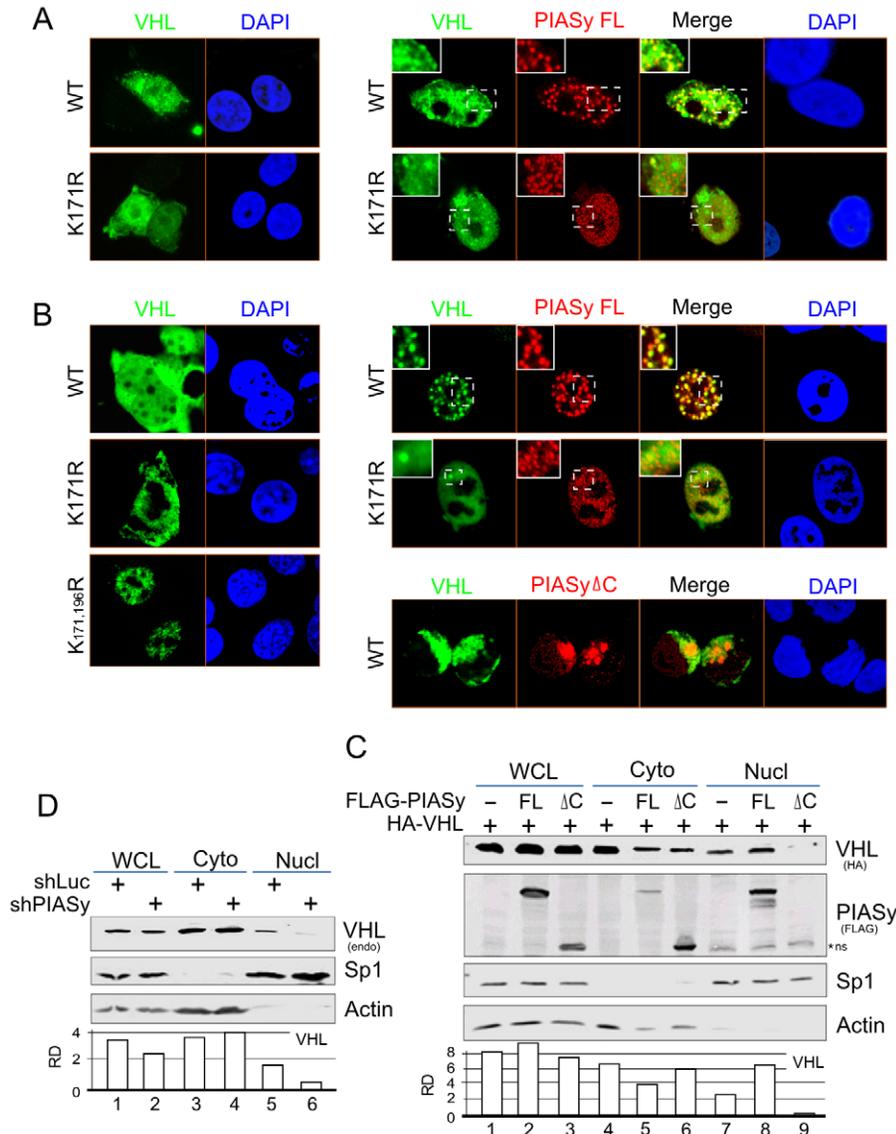


**Figure 2. The VHL protein stability is enhanced by lysine 196 mutation and PIASy coexpression.** (A) Proteasomal inhibitor MG132, Lys196 mutation or PIASy coexpression increases VHL protein stability. HEK293 cells were cotransfected with plasmids expressing wild type (WT) VHL, VHL plus full length PIASy or its deletion mutant ( $\Delta$ C) of VHL-binding domain, or VHL K196R alone. 48 h post-transfection, cell lysates treated with cycloheximide (CHX, 100  $\mu$ g/ml) for 0, 1, 2 and 4 hours in the presence or absence of MG132 was subjected to immunoblot as indicated.  $\beta$ -Actin immunoblotting was used as the loading control. The relative quantitation of VHL is shown in bottom panel. (B) PIASy knockdown reduces VHL protein stability. HEK293 cells were individually infected by lentivirus expressing small hair RNA against PIASy (shPIASy) or luciferase (shLuc). The infected cells after puromycin (1  $\mu$ g/ml) selection were treated with cycloheximide, lysated and then subjected to immunoblot as described in panel A.

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presence or absence of PIASy coexpression by immunofluorescence assays. In contrast to wild type VHL, the localization pattern of K171R mutant showed a slight increase in nuclear localization (Figure 3A and B, left panels). Moreover, when PIASy was coexpressed, the K171R mutant dramatically localized to the nucleus but lost the punctate foci which overlapped with PIASy in 786-O cells (Figure 3A and B, left panels). Unexpectedly, we also observed that the foci staining pattern of PIASy in the coexpression of wild type VHL is larger and less than in the K171R mutant (Figure 3B, right panels, the enlarged region). Similar results were also seen in the HEK293 cells with less translocation efficiency (Figure 3A). In addition, the evidence of VHL-interacting domain deletion of PIASy lost its effect on the

induction of VHL nuclear localization (Figure 3B, right lower panel), further indicating that the punctate foci which represent VHL staining are the dominant modified isoform of VHL on lysine 171 due to PIASy induction. However, SUMO modification of VHL is not a critical prerequisite for PIASy-mediated VHL nuclear localization but may be important for localization in specific subnuclear compartments. To further confirm that PIASy does induce VHL nuclear localization, we individually extracted nucleus and cytoplasm fractions from 786-O cells cotransfected with full length PIASy, its mutant ( $\Delta C$ ) of the VHL-interacting domain deletion or the empty vector alone in the presence of wild type VHL. Consistent with the results from the immunofluorescence assays, the nuclear distribution of wild type VHL was greatly



**Figure 3. PIASy alters VHL subcellular localization.** Immunofluorescence assay of HEK293 (A) or 786-O (B) cells transfected with wild type (WT) HA-VHL, its mutant K171R in the presence and absence of PIASy-RFP (red) coexpression were cultured on coverslips, fixed with 3% paraformaldehyde, and then subjected to immunofluorescence assay followed by mouse anti-HA antibody (green) and nuclear staining (blue) with DAPI as indicated. (C) Immunoblotting analysis. 786-O cells expressing HA-VHL in the presence or absence of PIASy-FLAG coexpression were subjected to nucleus (Nucl) and cytoplasm (Cyto) proteins extract followed by immunoblotting against HA and FLAG antibodies. Nuclear protein Sp1 and cytoplasm protein  $\beta$ -actin were blotted as fraction positive control. FL, full length;  $\Delta C$ , the carboxyl domain deletion; ns, non-specific band. (D) PIASy knockdown reduces endogenous VHL nuclear distribution. HEK293 cells with constitutively knockdown against PIASy (shPIASy) or luciferase (shLuc) were performed nucleus (Nucl) and cytoplasm (Cyto) fraction assays as described in panel C. doi:10.1371/journal.pone.0012636.g003

enhanced by full length PIASy coexpression, and disrupted by the deletion of VHL-interacting domain of PIASy (Figure 3C). Moreover, the lentivirus-mediated knockdown inhibition of endogenous PIASy expression in 293 cells dramatically reduced nuclear localization of endogenous VHL (Figure 3D). Taken together, these results further strengthen our hypothesis that PIASy facilitates VHL nuclear localization and that SUMO modification of VHL contributes to its colocalization with PIASy in the subcellular nuclear compartment.

### Ubiquitin/SUMO modification distinctly impairs VHL protein stability and subcellular distribution

Despite the fact that some physiological conditions are able to induce the constitutive shuttling of VHL between the nucleus and cytoplasm compartment [26,27], the underlying molecular mechanism still remains unclear. To explore whether ubiquitin modification of VHL leads to nuclear export and SUMO modification results in nuclear import, as well as whether ubiquitylation and SUMOylation of VHL exhibits distinct protein stability, we engineered the ubiquitin and SUMO modified VHL (VHL-Ub<sup>AGG</sup> and VHL-SUMO1<sup>ΔC4</sup>) with a mutation in lysine 171 by utilizing an artificially fused protein strategy [32]. The glycines which are critical for generating the covalent link were removed in both fused ubiquitin and SUMO proteins to exclude the possibility of the fusion protein acting as a ubiquitin or SUMO like protein. The results showed that ubiquitylated VHL (VHL-Ub<sup>AGG</sup>) was exclusively located in the cytoplasm, while SUMOylated VHL (VHL-SUMO1<sup>ΔC4</sup>) predominantly existed in the nucleus (Figure 4A). This provides evidence that ubiquitin and SUMO modification of VHL can regulate VHL specific subcellular localization. Consistently, the protein stability of VHL where monitored by ubiquitin or SUMO modification and showed reduced stability of VHL-Ub<sup>AGG</sup> compared to VHL-SUMO1<sup>ΔC4</sup> and may explain why VHL was more stable once lysine 171 was mutated (Figure 4B).

Moreover, to determine whether the SUMO/Ubiquitin modification on lysine 171 of VHL influences the further ubiquitylation on lysine 196 and alters VHL subcellular distribution, we performed the ubiquitylation assays by individually coexpressing ubiquitylated (VHL-Ub<sup>AGG</sup>) and SUMOylated (VHL-SUMO1<sup>ΔC4</sup>) form of VHL with or without lysine 196 mutation in the presence of HA-Ub. The results showed that ubiquitin but not SUMO1 modification increases the lysine 196 ubiquitylation which is significantly abolished by lysine 196 mutation (Figure 4C). Coordinately, in the nuclear and cytoplasm fractions, the result showed that mutation of lysine 196 not only increases the nuclear localization of SUMO1-modified VHL (VHL-SUMO1<sup>ΔC4</sup>) but also ubiquitin-modified VHL (VHL-Ub<sup>AGG</sup>) (Figure 4D). Interestingly, we also observed that the enhancement of nuclear localization of VHL with either SUMO1 or ubiquitin modification caused by the mutation at lysine 196 occurs with increase oligomerization and are mostly distributed in the cytoplasm compartment (Figure 4D, and the oligomerization definition referred to [32]). This further corroborates the hypothesis that localization in the nuclear compartment is a prerequisite for inducing oligomerization despite the fact that oligomerized VHL eventually goes to cytoplasm.

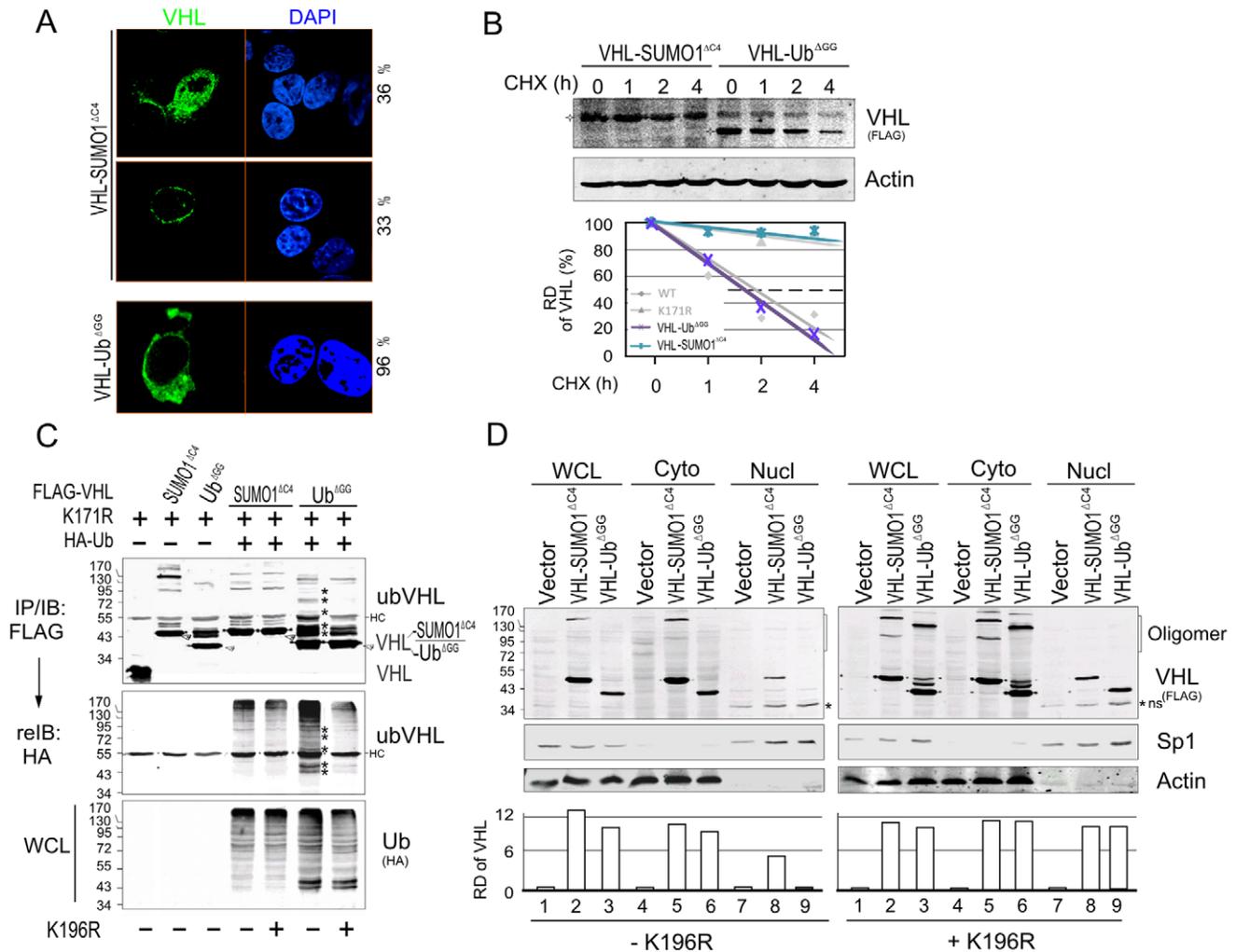
### Mutation of lysine residues 171/196 abolishes the inhibitory function of VHL on the transcriptional activity of HIF $\alpha$ as well as tube formation

VHL functions as a tumor suppressor by targeting specific proteins, including HIF $\alpha$  for degradation [24]. To ascertain if

mutation of ubiquitylated (K171 and 196) or SUMOylated (K171) site is sufficient to inactivate the antitumor properties of VHL in cancer cells, we tested whether mutation of lysine 171/196 resulted in disruption of VHL on inhibition of the transcriptional activity of HIF $\alpha$ . The VHL-deficient 786-O cells were transiently transfected with vector, vector expressing wild-type VHL or its mutants along with HIF $\alpha$  responsive element driven luciferase reporter. The results of the reporter assay showed that except for the K159R mutant, both the K171R and K196R mutants disrupted the inhibition of the transcriptional activity of HIF $\alpha$  when compared to WT (Figure 5A). The double mutant of lysine 171/196 (K<sub>171,196</sub>R) and the triple mutant 159/171/196 (3KR) showed the greatest efficiency of disruption of VHL inhibitory activities (Figure 5A). Concordantly, *in vitro* endothelial tube formation assay using conditioned medium from the supernatants of the vector, VHL or K<sub>171,196</sub>R -expressing 786-O cells, showed that the K<sub>171,196</sub>R mutation markedly reduced the inhibition of tube formation compared to wild type VHL (Figure 5B). Thus, these results demonstrate that modification on lysines 171/196 is critical for VHL to function as a tumor suppressor.

## Discussion

Functional inactivation of VHL is not only able to induce the von-Hippel Lindau familial cancer syndrome but can also result in sporadic renal carcinomas, and sporadic hemangioblastomas of CNS [37]. VHL has been shown to associate with the inhibition of several cellular processes including angiogenesis, cell cycle exit [38,39] and fibronectin matrix assembly [10,11]. Importantly, the ability of VHL to induce substrate proteolysis as a ubiquitin E3 ligase is critical to its function [18]. Previous studies have shown that the function of a ubiquitin ligase can be regulated by controlling the ligase or its substrate using a number of strategies including posttranslational modifications, interactions with regulatory factors, or subcellular localization [24]. Recently, VHL was shown to be Neddylated on lysine 159 and associated with the fibronectin matrix assembly and suppression of tumor development [33,34], however, these activities are not related to its E3 ligase activity. Therefore, the posttranslational modifications status in regulation of VHL E3 ligase activity remains understudied. Our previous studies have demonstrated that PIASy interacts with VHL and induces VHL SUMOylation [32], and that the viral protein LANA is able to promote VHL ubiquitylation and degradation [36]. In this work, we further demonstrated that VHL is ubiquitylated on lysine 171 and 196, and that enhanced expression of the SUMO E3 ligase PIASy not only blocks VHL ubiquitylation, but also increases VHL nuclear localization which leads to protein stabilization. By using SUMO1 or ubiquitin fusion VHL proteins, we have further showed that SUMO1 and ubiquitin modified isoform of VHL exhibits reverse nucleocytoplasmic distribution. This suggests that reversible modification of SUMO1 and ubiquitin is a strategy for dynamic regulation of VHL nucleocytoplasmic shuttling. Interestingly, a single or double mutation of lysine 171 and 196 or fusion with ubiquitin or SUMO1 in VHL resulted in a decrease in HIF $\alpha$  inhibition when compared to wild type VHL. The most significant of inactivation was the SUMO1 modification followed by the double lysine 171/196 mutation and the single mutation (Figure 5C). In addition, the evidence of the increased nuclear localization of VHL-Ub<sup>AGG</sup> with lysine 196 mutation suggested that the regulation of lysine 196 is involved in controlling the process of VHL nucleus – cytoplasm shuttling, and that this regulation may be associated with PIASy interaction since we observed that VHL-Ub<sup>AGG</sup> interacts with PIASy in our previous studies [32]. These findings



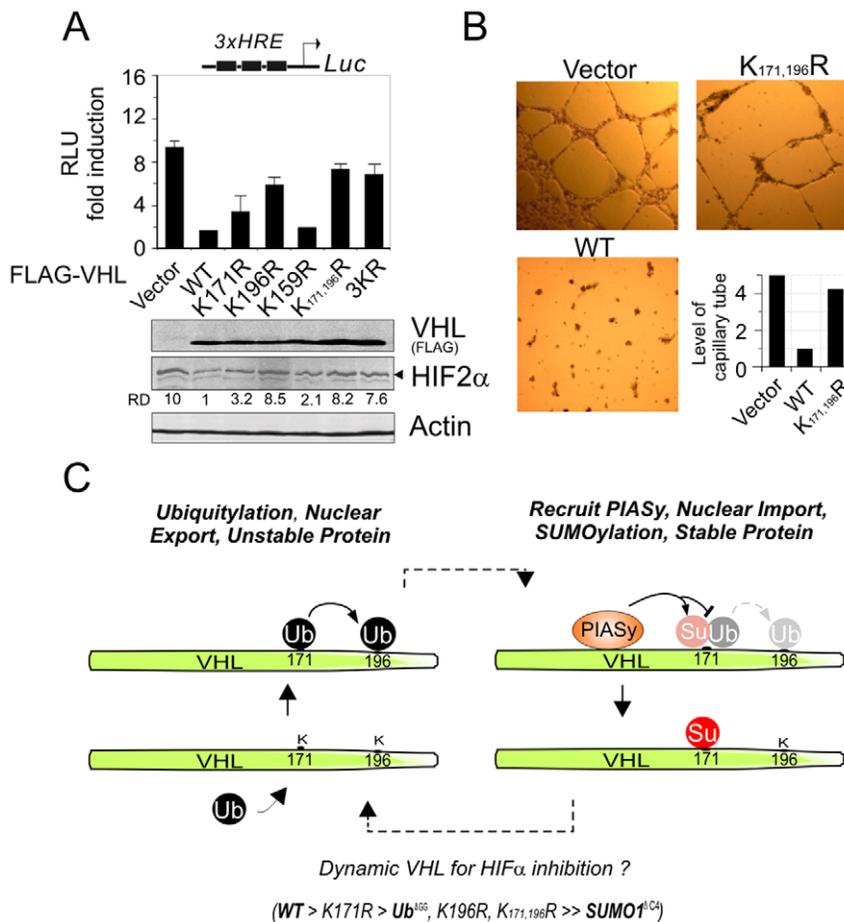
**Figure 4. Ubiquitin/SUMO1 modification of VHL presents opposite effect on the VHL protein stability and subcellular localization.** (A) The effect of SUMO1 and ubiquitin modification on the VHL subcellular localization. 786-O cells transfected with VHL-SUMO1<sup>ΔC4</sup> or VHL-Ub<sup>ΔGG</sup> were cultured on coverslips, fixed with 3% paraformaldehyde, and then subjected to immunofluorescent assay followed by mouse anti-FLAG antibody (green) and nuclear staining (blue) with DAPI as indicated. The percentage of cell staining pattern is calculated by total twenty positive staining cells of each sample. (B) The effect of SUMO1 and ubiquitin modification on VHL protein stability. 786-O cells were cotransfected with plasmids expressing VHL-SUMO1<sup>ΔC4</sup> or VHL-Ub<sup>ΔGG</sup> alone. 48 h post-transfection, cell lysates treated with cycloheximide (CHX, 100 μg/ml) for 0, 1, 2 and 4 hours was subjected to immunoblot as indicated. β-Actin immunoblotting was used as the loading control. The relative quantitation of VHL is shown in bottom panel. (C) Ubiquitin but not SUMO1 modification of VHL induces the ubiquitylation on lysine 196. 786-O cells were individually cotransfected with expression vector encoding the indicated protein in the top panel. Forty-eight hour posttransfection, the cells were treated with 20 μM MG132 for 2 hrs before harvest. Cell extracts were subjected to immunoprecipitated (IP) and immunoblotting (IB) as indicated in the figure. Blank arrows indicate the native VHL-SUMO1<sup>ΔC4</sup> or VHL-Ub<sup>ΔGG</sup>; ubVHL, ubiquitylated VHL. The position of ubiquitylated VHL is highlighted by asterisks. WCL, whole cell lysate; HC, heavy chain. (D) Immunoblotting analysis of nuclear and cytoplasm fraction of VHL with SUMO1/ubiquitin modification. 786-O cells transfected with VHL-SUMO1<sup>ΔC4</sup> or VHL-Ub<sup>ΔGG</sup> were subjected to nuclear (Nucl) and cytoplasm (Cyto) proteins extract followed by immunoblotting against FLAG antibody. Nuclear protein Sp1 and cytoplasm protein β-actin were blotted as fraction positive control. ns, non-specific band.

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are consistent with a prior study [26], and strongly suggest that any steps in this dynamic regulatory process which involves posttranslational modification will contribute to VHL nucleocytoplasmic shuttling (Figure 5C).

To date, one of the difficulties in separating many of the discrete cellular roles of VHL is that individual mutations in the gene result in widespread changes to the protein's function [4–6]. Consistent with the previous reports [40], we found that although mutation of ubiquitylated-sites increase VHL stability, the mutants also significantly influenced the ubiquitin ligase activity of VHL toward HIF1α. The explanation of this

phenomenon could be attributed to the ubiquitylation of VHL related with proteasomal degradation. The ability of VHL to traffic between nucleus and cytoplasm is decreased, even as the mutation of the ubiquitylated site stabilizes VHL. Recently, Jung et al reported that the cellular protein E2-EPF UCP degrades VHL and stabilizes HIF [35]. Our present study further provides evidence that deficiency of posttranslational modifications at these lysines can disrupt VHL ability to target HIF1α. Meanwhile, we also observed that PIASy expression and SUMO modification can block VHL ubiquitylation, increase its protein stability and nuclear localization. However, it remains to be



**Figure 5. The lysine 171/196 mutation abolishes the inhibitory function of VHL as a tumor suppressor.** (A) The lysine 171/196 mutation of VHL attenuates its inhibition on transcriptional activity of HIF-responsive reporter. 786-O cells were transiently transfected with wild type VHL or its mutants in the presence of HRE-luciferase reporter. Empty vector was used as control. Data are presented as means ± SD of three independent experiments. The levels of exogenous VHL and endogenous HIF2α were individually detected by immunoblotting against FLAG and HIF2α. RD, relative density. (B) The lysine 171/196 mutation of VHL loss its inhibitory effect on endothelial tube formation *in vitro*. The conditioned medium from 786-O cells expressing vector, wt VHL, or K<sub>171,196</sub>R, was tested for tube formation assay *in vitro*. Photographs were taken at 24 hr post-incubation. The quantitation presents the average of the pattern/value association criterion from 5 random view fields per well. (C) A proposed model for the dynamic regulation of VHL protein stability and nucleocytoplasmic distribution in tumor suppression. In some circumstance, interaction of VHL with PIASy results in VHL nuclear localization, SUMOylation (Su) and stability for blocking ubiquitylation (Ub) of VHL. Meanwhile, PIASy dissociation with VHL or attenuation of VHL SUMOylation facilitates VHL nuclear export, ubiquitylation and instability. This dynamic process of VHL with reversible modification acts in a concert to inhibit HIFα. The ability of VHL with different isoforms on inhibition of HIFα transcriptional activity is indicated at the bottom.

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determined whether the deubiquitylation process is involved in competition between these two modification states. Further experiments will be required to explore these questions in more detail.

Protein ubiquitin and ubiquitin-like posttranslational modification has emerged as an important strategy for reversible modification of many proteins that play regulatory roles in diverse cellular processes, including protein relocalization, stability, transcriptional control and stress response [29–31]. Many reports have suggested that posttranslational modifications are an important cellular strategy that enables the cell to react to intracellular or environmental changes caused by exposure to stress factors such as hypoxia [37,41]. Our previous studies have shown that PIASy expression in response to hypoxic stress negatively regulates VHL function as a tumor suppressor [32]. Here we further show that a single lysine mutation also impairs the ability of VHL in terms of its inhibition of HIFα activity although

the level is reduced when compared with the SUMOylated isoform. Therefore, there are many critical steps required for VHL to fully function as a tumor suppressor protein, and the trafficking between the nucleus and cytoplasm could be one of these critical steps. Any block to this step will affect its antitumor activity. This notion has been supported by three points of evidence: 1) Failure of VHL to continuously shuttle between the nuclear and cytoplasmic compartments leads to the stabilization of HIF1α [27]; 2) Oxygen-dependent degradation of nuclear HIF1α is dependent upon the dynamic nuclear-cytoplasmic trafficking of VHL [26]; and 3) Acidosis blocks VHL nuclear-cytoplasmic shuttling [42]. However, the impact of posttranslational modification on VHL nuclear and cytoplasmic localization still remains unclear. Our observation that SUMOylated VHL localizes to the nucleus, while ubiquitylated VHL is cytoplasmic, suggests that posttranslational modification may affect VHL subcellular localization and its inhibitory function on HIFα.

Our data now demonstrate that SUMOylation and not ubiquitylation at the C terminus of VHL contributes to the nuclear import of VHL, and supports a model in which SUMOylation of the C terminus of VHL, in a region close to both the nuclear export and oligomerization sequences, blocks the nuclear export signal and allows interaction of VHL with the nuclear import machinery. Thus, the ability of VHL to shuttle between the nucleus and the cytoplasm may contribute to its antitumor property under specific physiologic conditions.

## Materials and Methods

### Cell culture and transfection

Human renal carcinoma *VHL*-null cell lines 786-O and embryonic kidney (HEK) 293 cells were described as previously [36], and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS, Hyclone), 4  $\mu$ M L-glutamine, penicillin, and streptomycin [36]. Cells were incubated at 37°C in a humidified environmental incubator supplemented with 5% CO<sub>2</sub>. Ten million cells with 400  $\mu$ l medium were transfected by electroporation with a Bio-Rad Gene Pulser in 0.4 cm-gap cuvettes at 210 Volts and 975 microfarads.

### Plasmids, antibodies and reagents

Plasmid FLAG-VHL was provided individually by Joan W Conaway (Stowers Institute for Medical Research, USA). Plasmids encoding human PIASy with FLAG tag were gifts from Stefan Müller (Max Planck Institute of Biochemistry, Germany). PIASy $\Delta$ C-FLAG (1-233) and PIASy-myc (1-492) were generated by ligating *Bam*HI/*Eco*RV PCR fragments into pcDNA-FLAG and pA3M vector, respectively. Full length PIASy-RFP was prepared by ligating *Kpn*I/*Bam*HI PCR fragments into pDs-Red-N1 vector. VHL-Ub<sup>AGG</sup> and VHL-SUMO1<sup>AC4</sup> were generated by in-frame ligation of *Bam*HI/*Xba*I PCR fragments to the downstream of FLAG-VHL(K171R). FLAG-VHL (K171R), (K196R), (K<sub>171,196</sub>R), (K159R), and (3KR), as well as VHL-Ub<sup>AGG</sup> (K196R) and VHL-SUMO1<sup>AC4</sup> (K196R) were individually generated by PCR site-directed mutagenesis. All constructs were confirmed by direct DNA sequencing. Plasmids of pGL2-HRE (HIF $\alpha$ -target reporter), and HA-Ub were previously described [36,43]. The VHL antibodies were from Cell signaling technology Inc. HIF2 $\alpha$  antibodies were from Novus Biologicals Inc. PIASy (I-19) and Sp1 (1C6) was purchased from Santa Cruz Biotech. Inc. Other antibodies used were anti-myc (9E10), anti-FLAG (M2), anti-HA (12CA5), and  $\beta$ -actin (Cell signaling technology). Proteasome inhibitor MG132 was purchased from Biomol International, and Cyclohexamide (C4859, Sigma Inc., St. Louis, MO).

### Immunofluorescence assay

Cells on coverslips were washed three times with PBS and then fixed in 3% paraformaldehyde for 20 min at room temperature. After fixation, cells were washed three times in PBS and permeabilized in PBS containing 0.2% fish skin gelatin (G-7765, Sigma), 0.2% Triton X-100 for 5 min, and then incubated for 1 h at room temperature with primary antibodies in blocking solution. Cells were washed three times with PBS and incubated for 30 min at room temperature with fluorescently labeled Alexa fluors-488 or 594 against mouse or rabbit (Molecular Probes Inc., Eugene, OR) in blocking solution. Coverslips were washed three times with PBS and slides were mounted with Prolong antifade mounting medium with 0.5  $\mu$ M DAPI (4', 6'-diamidino-2-phenylindole). Fluorescence confocal microscopy was performed with an Olympus

microscope using FluoView™ FV300 software (Olympus, Melville, NY).

### Immunoblotting

Cells were harvested and washed once with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin A) on ice and homogenized. The cell lysates were resuspended in 50  $\mu$ l of 1 $\times$  SDS Laemmli buffer and heated 95°C for 5 minutes. The sample was subjected to SDS-PAGE and transferred to a membrane that was probed with specific antibody.

### Determination of VHL stability

786-O cells were cotransfected FLAG-VHL with or without FLAG-PIASy as described above. After 24 h transfection, cells were incubated with 100  $\mu$ M cyclohexamide for 0 to 4 hour and then harvested. Equal amounts of total proteins from each treatment were taken to perform western blot analysis.

### Fractionation of nuclear or cytoplasm proteins

Twenty million transfected cells were harvested and washed twice with ice-cold PBS followed by resuspending the cell pellet in a hypotonic buffer A (10 mM HEPES-K<sup>+</sup> pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 DTT) in the presence of protease inhibitor cocktail (PIC: 1 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin A). Cells were pelleted by spinning at 1000 rpm  $\times$ 5 min. The cells were lysated in ice-cold 0.5% NP-40 containing buffer A with PIC on ice for 10 min. The nuclei were pelleted by 3,000 rpm  $\times$ 2 min at 4°C. The supernatant (cytoplasm protein) harvested and frozen at -80°C for use. The nuclear pellets were washed twice with buffer A (without NP-40), followed by resuspending in buffer C (20 mM HEPES-K<sup>+</sup> pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 0.5 DTT, 25% Glycerol) with PIC. Nuclei were incubated on ice for 30 min, and vortex periodically. Supernatant containing nuclear protein were collected by spinning at 14,500 rpm for 5 min at 4°C and then snap frozen for further use. Antibodies against nuclear protein Sp1 and cytoplasm protein  $\beta$ -Actin were used as markers.

### RNA interference

The PIASy shRNA (5'-GTACTTAAACGGACTGGGA-3') sequence was inserted into lentivirus pGIPz vector according to the manufacturer's instructions (Clontech). HEK293 cells were individually transduction by lentivirus packaged from Core T which cotransfected with Rev, VSVG and gp expressing plasmids, and selection by 1  $\mu$ g/ml Puromycin. pGIPz vector with luciferase (shLuc) target (5'-TGC GTT GCT AGT ACC AAC-3') sequence was used as control, and the RNA interfering efficiency was assessed by western blot analysis.

### Luciferase reporter assay

The luciferase reporter assays were performed as described previously [36]. After transfection for 48 h, cells were lysed in 200  $\mu$ l of reporter lysis buffer (Promega, Inc., Madison, WI). Luciferase activities and  $\beta$ -galactosidase were individually measured using luciferase assay reagent (Promega, Inc., Madison, WI) and the Opticomp Luminometer (MGM Instruments, Inc. Hamden, CT) according to the suppliers' instructions. Luciferase activities were normalized with  $\beta$ -galactosidase activities. Relative luciferase activity (RLU) was expressed as fold activation relative to the reporter construct alone. Assays were performed in triplicate.

## Endothelial tube formation assay

Human umbilical vascular endothelial cells (HUVEC) were purchased (Cambrex) and maintained in EBM-2 medium supplemented with EGM-2. Tube formation assay on extracellular BD matrigel was performed according to the manufacturer's protocol with minor modifications. Briefly,  $2 \times 10^4$  HUVEC resuspended with 500  $\mu$ l of EBM-2 medium were seeded on Matrixgel solidified in 48-well tissue culture plate. Conditioned medium (1 ml) from the supernatant of each 786-O stable cells was added, respectively. Cells were incubated in a CO<sub>2</sub> incubator for 24 hrs at 37°C and then examined for tube formation with a light microscope.

## References

- Latif F, Tory K, Gnarr J, Yao M, Duh FM, et al. (1993) Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 260: 1317–1320.
- van der Harst E, de Krijger RR, Dinjens WN, Weeks LE, Bonjer HJ, et al. (1998) Germline mutations in the vhl gene in patients presenting with pheochromocytomas. *Int J Cancer* 77: 337–340.
- Zbar B, Kishida T, Chen F, Schmidt L, Maher ER, et al. (1996) Germline mutations in the Von Hippel-Lindau disease (VHL) gene in families from North America, Europe, and Japan. *Hum Mutat* 8: 348–357.
- Kaelin WG, Jr., Maher ER (1998) The VHL tumour-suppressor gene paradigm. *Trends Genet* 14: 423–426.
- Linchan WM, Lerman MI, Zbar B (1995) Identification of the von Hippel-Lindau (VHL) gene. Its role in renal cancer. *Jama* 273: 564–570.
- Kim WY, Kaelin WG (2004) Role of VHL gene mutation in human cancer. *J Clin Oncol* 22: 4991–5004.
- Gnarr JR, Tory K, Weng Y, Schmidt L, Wei MH, et al. (1994) Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet* 7: 85–90.
- Iliopoulos O, Kibel A, Gray S, Kaelin WG, Jr. (1995) Tumour suppression by the human von Hippel-Lindau gene product. *Nat Med* 1: 822–826.
- Stebbins CE, Kaelin WG, Jr., Pavletich NP (1999) Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. *Science* 284: 455–461.
- Ohh M, Yauch RL, Lonergan KM, Whaley JM, Stemmer-Rachamimov AO, et al. (1998) The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol Cell* 1: 959–968.
- Hergovich A, Lisztwan J, Barry R, Ballschmieter P, Krek W (2003) Regulation of microtubule stability by the von Hippel-Lindau tumour suppressor protein pVHL. *Nat Cell Biol* 5: 64–70.
- Davidowitz EJ, Schoenfeld AR, Burk RD (2001) VHL induces renal cell differentiation and growth arrest through integration of cell-cell and cell-extracellular matrix signaling. *Mol Cell Biol* 21: 865–874.
- Pause A, Lee S, Lonergan KM, Klausner RD (1998) The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum withdrawal. *Proc Natl Acad Sci U S A* 95: 993–998.
- Mikhaylova O, Ignacak ML, Barankiewicz TJ, Harbaugh SV, Yi Y, et al. (2008) The von Hippel-Lindau tumor suppressor protein and Egl-9-Type proline hydroxylases regulate the large subunit of RNA polymerase II in response to oxidative stress. *Mol Cell Biol* 28: 2701–2717.
- Yang H, Minamishima YA, Yan Q, Schlisio S, Ebert BL, et al. (2007) pVHL acts as an adaptor to promote the inhibitory phosphorylation of the NF-kappaB agonist Card9 by CK2. *Mol Cell* 28: 15–27.
- Chitalia VC, Foy RL, Bachschmid MM, Zeng L, Panchenko MV, et al. (2008) Jade-1 inhibits Wnt signalling by ubiquitylating beta-catenin and mediates Wnt pathway inhibition by pVHL. *Nat Cell Biol* 10: 1208–1216.
- Young AP, Schlisio S, Minamishima YA, Zhang Q, Li L, et al. (2008) VHL loss activates a HIF-independent senescence programme mediated by Rb and p400. *Nat Cell Biol* 10: 361–369.
- Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, et al. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399: 271–275.
- Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, et al. (2000) Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol* 2: 423–427.
- Bruick RK, McKnight SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294: 1337–1340.
- Semenza GL (2001) HIF-1 and mechanisms of hypoxia sensing. *Curr Opin Cell Biol* 13: 167–171.
- Pouyssegur J, Dayan F, Mazure NM (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 441: 437–443.
- Scheffner M (1999) Moving protein heads for breakdown. *Nature* 398: 103–104.
- Petroski MD, Deshaies RJ (2005) Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* 6: 9–20.
- Lee S, Chen DY, Humphrey JS, Gnarr JR, Linchan WM, et al. (1996) Nuclear/cytoplasmic localization of the von Hippel-Lindau tumor suppressor gene product is determined by cell density. *Proc Natl Acad Sci U S A* 93: 1770–1775.
- Lee S, Neumann M, Stearman R, Stauber R, Pause A, et al. (1999) Transcription-dependent nuclear-cytoplasmic trafficking is required for the function of the von Hippel-Lindau tumor suppressor protein. *Mol Cell Biol* 19: 1486–1497.
- Mekhail K, Khacho M, Carrigan A, Hache RR, Gunaratnam L, et al. (2005) Regulation of ubiquitin ligase dynamics by the nucleolus. *J Cell Biol* 170: 733–744.
- Lewis MD, Roberts BJ (2003) Role of nuclear and cytoplasmic localization in the tumour-suppressor activity of the von Hippel-Lindau protein. *Oncogene* 22: 3992–3997.
- Hay RT (2005) SUMO: a history of modification. *Mol Cell* 18: 1–12.
- Schmidt D, Muller S (2002) Members of the PIAS family act as SUMO ligases for c-Jun and p53 and repress p53 activity. *Proc Natl Acad Sci U S A* 99: 2872–2877.
- Wei F, Scholer HR, Atchison ML (2007) Sumoylation of Oct4 enhances its stability, DNA binding, and transactivation. *J Biol Chem* 282: 21551–21560.
- Cai Q, Verma SC, Kumar P, Ma M, Robertson ES (2010) Hypoxia inactivates the VHL tumor suppressor through PIASy-mediated SUMO modification. *PLoS One* 5: e9720.
- Stickle NH, Chung J, Klco JM, Hill RP, Kaelin WG, Jr., et al. (2004) pVHL modification by NEDD8 is required for fibronectin matrix assembly and suppression of tumor development. *Mol Cell Biol* 24: 3251–3261.
- Russell RC, Ohh M (2008) NEDD8 acts as a 'molecular switch' defining the functional selectivity of VHL. *EMBO Rep* 9: 486–491.
- Jung CR, Hwang KS, Yoo J, Cho WK, Kim JM, et al. (2006) E2-EPF UCP targets pVHL for degradation and associates with tumor growth and metastasis. *Nat Med* 12: 809–816.
- Cai QL, Knight JS, Verma SC, Zald P, Robertson ES (2006) E2-EPF ubiquitin complex is recruited by KSHV latent antigen LANA for degradation of the VHL and p53 tumor suppressors. *PLoS Pathog* 2: e116.
- Haglund K, Dikic I (2005) Ubiquitylation and cell signaling. *Embo J* 24: 3353–3359.
- Chen F, Kishida T, Duh FM, Renbaum P, Orcutt ML, et al. (1995) Suppression of growth of renal carcinoma cells by the von Hippel-Lindau tumor suppressor gene. *Cancer Res* 55: 4804–4807.
- Kaelin WG (2007) Von hippel-lindau disease. *Annu Rev Pathol* 2: 145–173.
- Knauth K, Bex C, Jemth P, Buchberger A (2006) Renal cell carcinoma risk in type 2 von Hippel-Lindau disease correlates with defects in pVHL stability and HIF-1alpha interactions. *Oncogene* 25: 370–377.
- Hershko A, Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* 67: 425–479.
- Mekhail K, Gunaratnam L, Bonicalzi ME, Lee S (2004) HIF activation by pH-dependent nucleolar sequestration of VHL. *Nat Cell Biol* 6: 642–647.
- Cai Q, Murakami M, Si H, Robertson ES (2007) A potential alpha-helix motif in the amino terminus of LANA encoded by Kaposi's sarcoma-associated herpesvirus is critical for nuclear accumulation of HIF-1alpha in normoxia. *J Virol* 81: 10413–10423.

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## Author Contributions

Conceived and designed the experiments: QC ESR. Performed the experiments: QC. Analyzed the data: QC ESR. Wrote the paper: QC ESR.