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pVHL Negatively Regulates Antiviral Signaling by Targeting MAVS for Proteasomal Degradation

Juan Du,*1 Dawei Zhang,*1 Wei Zhang,*1 Gang Ouyang,* Jing Wang,* Xing Liu,* Shun Li,* Wei Ji,* Wei Liu,† and Wuhan Xiao*‡

The von Hippel–Lindau (VHL) gene is a well-defined tumor suppressor linked to human heredity cancer syndromes. As a component of the VHL-elongin B/C E3 ligase complex, pVHL performs its tumor function by targeting proteins for proteasomal degradation. It is largely unknown whether pVHL functions in antiviral immunity. In this article, we identify that pVHL negatively regulates innate antiviral immunity, which acts mainly by inducing degradation of mitochondrial antiviral-signaling protein (MAVS, also known as Cardif, IPS-1, or VISA). Overexpression of pVHL abrogated the cellular response to viral infection, whereas knockdown of pVHL exerted the opposite effect. pVHL targeted the K420 residue of MAVS to catalyze the formation of K48-linked polyubiquitin chains, leading to proteasomal degradation of MAVS. After viral infection, Mavs levels remained low in wild type zebrafish embryos but became much higher in vhl-deficient (vhl−/−) zebrafish embryos. Higher MAVS levels correlated with a greatly exaggerated antiviral response. In this work, we demonstrate that pVHL exhibits a previously unknown role in innate antiviral immunity. The Journal of Immunology, 2015, 195: 1782–1790.

The von Hippel–Lindau (VHL) gene is a classic tumor suppressor. Inactivation of VHL is linked to human hereditary VHL diseases that are autosomal-dominant, neoplastic diseases (1). VHL diseases are associated with various tumor types, including clear cell renal cell carcinomas, CNS and retinal hemangioblastomas, pheochromocytomas, and pancreatic neuroendocrine tumors, in addition to pancreatic and renal cysts (2, 3). The VHL tumor suppressor protein (pVHL) forms a ternary complex with elongin C and elongin B termed VHL-elongin B/C (VCB) complex, which resembles the yeast Skp1-Cullen-1/Cdc53-F-box protein complex (4). The VCB complex and the similar Skp1-Cullen-1/Cdc53-F-box protein complex both possess ubiquitin ligase activities and are capable of targeting proteins for proteasomal degradation.

The best-characterized function of pVHL is acting as a substrate recognition subunit of the VCB E3 ligase complex that targets the proline hydroxylated hypoxia-induced factor (HIF)-1α for proteasomal degradation under hypoxia conditions, which is thought to be the major pVHL mechanism for tumor suppression (5).

other than HIF-α have been tentatively identified, including estrogen receptor α (6), Kruppel-like factor receptor 4 (7), ERK5 (8), and androgen receptor (9), to rationalize the multiple symptoms exhibited in VHL disease. However, apart from HIF-α, the mechanisms and downstream effects of pVHL-induced ubiquitination of non–HIF-α targets are still obscure. Identification of additional targets of pVHL action and elucidation of their biological effects will shed new light on the physiological functions of pVHL.

Mitochondrial antiviral-signaling protein (MAVS, also known as Cardif, IPS-1, or VISA) was the first identified mitochondrial protein linked to innate antiviral immunity (10–13). As an adaptor protein, MAVS anchors itself to the mitochondrial outer membrane, where it interacts with Mda5 or RIG-1 sensor proteins that had been triggered by viral infection (14). Mda5 and RIG-1 are two cytoplasmic RNA sensors that recognize viral RNA released during viral replication (15). The interaction of the N-terminal CARD domain of MAVS with RIG-1 CARDs or Mda5 CARDs activates MAVS. Activated MAVS then signals downstream to the kinases IKK and TBK1, which induce the activities of transcription factors, including NF-κB and IFN regulatory factor 3 (IRF3), eventually leading to the production of type I IFNs and proinflammatory cytokines (14, 16).

Studies have shown that ubiquitination-mediated protein degradation plays a crucial role in the modulation of MAVS-mediated signaling (17). This regulation is initiated after three enzymes catalyze the covalent attachment of ubiquitin moieties to MAVS: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) that recognizes MAVS specifically (17). E3 ligase RNF125 can ubiquitinate and trigger degradation of Mda5, RIG-1, and MAVS to suppress the RIG-1–like-helicase-mediated response (18). The E3 ligase TRIM25 induces ubiquitination and proteasomal degradation of MAVS to suppress type I IFN production (19). The E3 ligase Smurf2 negatively modulates the antiviral response by targeting MAVS for proteasomal degradation (20). In addition, some factors have been shown to mediate degradation of MAVS through their effects on other E3 ligases. PCBP2 recruits the E3 ligase AIP4 to polyubiquitinate and degrade MAVS (21). Ndfip1 negatively modulates the antiviral response by enhancing Smurf1 E3 ligase–mediated MAVS degradation (22).
As a classic tumor suppressor with E3 ligase activity, pVHL has not previously been linked to the antiviral response. In this study, we identify pVHL as a negative regulator in MAVS-mediated antiviral signaling. Overexpression of pVHL abrogated the cellular response to viral infection, whereas knockdown of pVHL exerted the opposite effect. pVHL targeted the K420 residue of MAVS to catalyze the formation of K48-linked polyubiquitin chains, leading to proteasomal degradation of MAVS. MAVS levels remained low in wild type (WT) zebrafish embryos after viral infection but became much higher in vhl-deficient (vhl−/−) zebrafish embryos; the latter result was accompanied by a greatly exaggerated antiviral response. Collectively, the data presented in this work point to a previously unknown function of pVHL in innate antiviral immunity.

Materials and Methods

Cell line and culture conditions

HEK293T, Cos7, and HCT116 cells were originally obtained from American Type Culture Collection and were cultured in DMEM (HyClone) with 10% FBS and grown at 37°C in a humidified incubator containing 5% CO2.

Plasmid constructions

RIG-1, Mda5, MAVS, and TBK1 expression vectors were purchased from Addgene and subcloned into different expression vectors by PCR amplification. IRF3 and mouse MAVS were amplified by PCR from human or mouse cDNA pool. IRF-E reporter was kindly provided by Hong-Bing Shu (23). VHL expression vectors, pSUPER-VHL–short hairpin RNA (shRNA)-1 and pSUPER-VHL-shRNA2, have been described previously (9). Zebrafish nuvoa and vhl were amplified by PCR from zebrafish cDNA pool.

Abs and reagents

The Abs used are as follows: Anti-Myc (9E10; Santa Cruz), anti-GAPDH (0411; Santa Cruz), Anti-HA (Covance), Anti-Flag (F1804; Sigma), Anti-$\alpha$-tubulin (EPR1333; Epitomics), anti-MAVS (166583; Santa Cruz), VHL (AbClonal). Zebrafish Mavs Ab (anti-DrMavs) was developed by injecting rabbits with GST-tagged partial zebrafish Mavs.

Mito-Tracker Green kit was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu). MG132 was purchased from Calbiochem. Ni-NTA beads were purchased from Novagen.

Virus production, titer measurement, and infection

Sendai virus (SeV), vesicular stomatitis virus (VSV)-GFP, and HSV-1 viruses were propagated in Vero cells. Postinfection for 24 h, the supernatant was collected and was clarified by centrifugation. Viral titers were determined by plaque assay on Vero cells. SeV was provided by Xinwen Chen, VSV-GFP was provided by Minzhou Chen, and HSV-1 was provided by Chunfu Zheng.

For HEK293T cell infection, the cells were incubated with DMEM without FBS containing SeV, HSV-1, or VSV-GFP at multiplicity of infection (MOI) from 1 to 100. Postinfection for 1 h, the cells were washed twice and then were cultured cells in DMEM containing 1–10% FBS.

Luciferase reporter assays

Cells were grown in 24-well plates and transfected with various amounts of plasmids by VigoFect (Vigorous Biotech, Beijing, China), as well as with pTR-Renilla used as an internal control. After the cells were transfected for 18–24 h, the luciferase activity was determined by dual-luciferase reporter assay system (Promega). Data were normalized to Renilla luciferase. Data are reported as mean ± SEM from three independent experiments performed in triplicate. The statistical analysis was performed using GraphPad Prism 5 (unpaired t test; GraphPad Software, San Diego, CA).

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blot analysis were performed as described previously (24). Anti-Flag and anti-HA Ab–conjugated agarose beads were purchased from Sigma. The Fiji Film LAS4000 mini-luminescence image analyzer was used to photograph the blots. Multi Gauge V3.0 was used for quantifying the protein levels based on the band density obtained in Western blot analysis.

Semi-quantitative real-time PCR

The total RNA was extracted from HEK293T cells by TRizol reagent (Invitrogen) and the total RNA extracted from zebrafish embryos by SV Total RNA Isolation System (Promega). The cDNA synthesis was carried out using a first-strand cDNA synthesis kit (Fermentas).

The primers used for RT-PCR are as follows: human IFN-β: 5′-CAT-TACCCTAGGCAAGGA-3′ and 5′-CAATGTGCACTGCCAGG-3′; human IFN-stimulated gene 15 (ISG15): 5′-ATGGCTCGTGACGCGG-3′ and 5′-TTACCTCCGGCCAGCA-3′; human GAPDH (internal control): 5′-AGGCACATGCTGACAGAC-3′ and 5′-GCCCAAACTGACAACATC-3′; zebrafish (ifn-1): 5′-AGGTTTCAGCTCAGCATTG-3′ and 5′-TCCGATGTTACCGTATCAG-3′; zebrafish (ifn-3): 5′-TTACTCTTCTGGTGAGATGAT-3′ and 5′-GGATGAAAGCCGCTGCTG-3′; zebrafish ef1α (internal control): 5′-CTTCCACGGTCAGCTG-3′ and 5′-CCGGCT-AGCATACCCCTCC-3′ (25).

Confocal microscopy

The Cos-7 cells were transfected with GFP-tagged VHL expression vector for 24 h, then were infected with SeV for 8 h. Subsequently, the cells were incubated with the MitoTracker Green (Beyotime Institute of Biotechnology) for 45 min and were photographed under a Zeiss confocal microscope (LSM-710).

Generation of zebrafish vhl mutant and spring viremia of carp virus infection assays

One pair of zebrafish vhl knockout TALEN was designed based on the exon 1 sequence of zebrafish vhl gene. The left target sequence is 5′-CAACGTCTGTGCTTGTTGT-3′; the right target sequence is 5′-CCA-GACGGGCTTGTGAC-3′. Vhl-TALENs were constructed by FastTAL EN Assembly kit (Sidansai Biotechnology, Shanghai, China) and the mRNAs of vhl-TALENs were synthesized by Amplicap Sp6 High Yield Message Maker kit (Cell Script).

The synthesized mRNA was injected into zebrafish embryos at one-cell stage. For determining the efficiency of TALEN-mediated vhl knockout, the total DNA was extracted from injected embryos at 24-h postfertilization stage; PCR amplification was conducted and the products were checked by non-denaturation PAGE gel. If the positive results were observed, this batch of embryos was matured until they matured (F0). F0 fishs were cross-bred with WT zebrafish to obtain F1. If F1 zebrafish were confirmed that one copy of vhl was knocked out, then the corresponding F2 DNA was extracted from the F2 tails, and PCR products were examined by non-denaturation PAGE gel assay at first; then the positive PCR products were further confirmed by sequence analysis. The vhl heterozygous zebrafish (vhl+/−) were bred for generating vhl homozygous null embryos (vhl−/−). The vhl homozygous null embryos could be selected by their morphology after 5 d postfertilization (dpf), which were also further genotyped by sequence analysis.

Spring viremia of carp virus (SVCV) were propagated in carp epithelium papulosum cyprinid cells (originally obtained from American Type Culture Collection) at 28°C. After one round of frozen (−70°C) and thawed, the supernatant was collected and was clarified by centrifugation. Viral titers were determined by 50% tissue culture infection dose assay (TCID50) assay on epithelium papulosum cyprinid cells. Vhl heterozygous zebrafish (vhl+/−) were mated and vhl homozygous null embryos (vhl−/−) were selected based on morphology at 5-dpf stage. Two mutant lines were used for viral infection assays, and their corresponding sibling WT embryos (vhl+/+) were used for the control. For viral infection of zebrafish embryos, ∼2 × 107 TCID50/ml SVCV were added to fish water in six-well plates at triplicate.

Results

pVHL negatively regulates MAVS-mediated signaling

In an attempt to define the role of pVHL in viral infection, we initially performed luciferase reporter assays using IRF-E (23), a reporter driven by five copies of IRF-E of the ISG54 gene promoter, which is activated by IRF3 (26, 27). As expected, SeV infection (MOI = 1) enhanced IRF-E reporter activity dramatically in HEK293T cells (Fig. 1A). However, ectopic expression of pVHL inhibited the enhancement of activity of the IRF-E reporter by SeV infection (Fig. 1A). This phenomenon suggests that pVHL might negatively regulate cellular innate antiviral immune response.

Cells use pattern-recognition receptors to sense viral nucleic acids. RIG-1–like helicases, including RIG-1 and Mda5, function as cytoplasmic RNA sensors and share a common adaptor protein MAVS. Upon viral infection, RIG-1–like helicases bind to viral RNA and activate MAVS, which, in turn, activates the IRF3 and
NF-κB to induce type I IFN expression. TBK1 acts as an upstream factor of IRF3 and is required for induction of IFN-α triggered by overexpression of MAVS (12). To define the specific step whereby pVHL affects the cellular antiviral innate immune signaling, we performed subsequent coexpression experiments. When RIG-1, Mda5, MAVS, TBK1, or IRF3 were overexpressed in HEK293T cells without coexpression of pVHL, the activity of an IRF-E reporter was upregulated significantly (Fig. 1B–F). However, coexpression of pVHL together with RIG-1, MDA5, or MAVS appreciably suppressed the enhancement of IRF-E activity observed when no pVHL was present (Fig. 1B–D). In contrast, coexpression of pVHL together with TBK1 or IRF3 had no obvious inhibitory effect on the activity of IRF-E reporter (Fig. 1B, 1F). These results imply that pVHL might negatively regulate cellular antiviral innate immune response through its effect on MAVS, RIG-1, or MDA5, but pVHL absolutely does not suppress this response through any effect on TBK1 and IRF3.

**pVHL induces the degradation of MAVS**

Given that pVHL has been well defined as a component of the VHL-elongin B/C E3 ligase complex, we examined whether pVHL could affect the protein stabilities of RIG-1, MDA5, and MAVS. Coexpression of pVHL together with RIG-1 or MDA5 resulted in a mild reduction of RIG-1 or MDA5 protein levels, but coexpression of pVHL together with MAVS caused a dramatic reduction in MAVS protein levels, consistent with pVHL’s suppressive effect on the cellular antiviral innate immune response (Fig. 2A). Because MAVS acts as the common adaptor used by RIG-1 and MDA5 to mediate the cellular antiviral innate immune response, we focused our subsequent assays on the effect of pVHL on MAVS. As shown in Fig. 2B, coexpression of either human MAVS or mouse MAVS together with an increasing amount of human pVHL in HEK293T cells resulted in a dramatic reduction of MAVS protein levels. Furthermore, overexpression of pVHL in HEK293T cells also caused reduction of endogenous MAVS induced by SeV infection (Fig. 2C). Notably, knockdown of endogenous pVHL by VHL–shRNA-1 and VHL–shRNA-2 in HEK293T cells caused endogenous MAVS to increase, as compared with the control (GFP-shRNA; Fig. 2D). Taken together, these results suggest that pVHL promotes MAVS degradation.

To gain a more complete picture of pVHL in cellular antiviral innate immunity, we further examined the effect of pVHL on RIG-1 and MDA5. Coexpression of RIG-1 together with an increasing amount of pVHL in HEK293T cells resulted in a reduction of RIG-1 protein levels (Supplemental Fig. 1A). Notably, the reduction of RIG-1

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**FIGURE 1.** pVHL negatively regulates MAVS-mediated signaling. (A) pVHL suppresses IRF-E reporter activity activated by SeV infection in a dose-dependent manner in HEK293T cells. HEK293T cells were transfected with the IRF-E luciferase reporter (0.1 μg/well) together with an increasing amount of Myc-VHL vector (0.1, 0.2, and 0.4 μg/well). After 24 h, the cells were infected by SeV (MOI = 1) for 8 h and then luciferase assays were performed. (B) pVHL suppresses IRF-E reporter activity activated by overexpression of RIG-1 in a dose-dependent manner in HEK293T cells. HEK293T cells were transfected with IRF-E luciferase reporter (0.1 μg/well) and Flag-RIG-1 vector (0.2 μg/well) together with an increasing amount of Myc-VHL vector (0.1, 0.2, and 0.4 μg/well). After 18–24 h, luciferase assays were performed. (C) pVHL suppresses IRF-E reporter activity resulting from overexpression of Mda5 in a dose-dependent manner in HEK293T cells. HEK293T cells were transfected with IRF-E luciferase reporter (0.1 μg/well) and Flag-MDA5 vector (0.2 μg/well) together with an increasing amount of Myc-VHL vector (0.1, 0.2, and 0.4 μg/well). After 18–24 h, luciferase assays were performed. (D) pVHL suppresses IRF-E reporter activity resulting from overexpression of MAVS in a dose-dependent manner in HEK293T cells. HEK293T cells were transfected with IRF-E luciferase reporter (0.1 μg/well) and Flag-MAVS vector (0.2 μg/well) together with an increasing amount of Myc-VHL vector (0.1, 0.2, and 0.4 μg/well). After 18–24 h, luciferase assays were performed. (E) pVHL has no obvious effect on IRF-E reporter activity resulting from overexpression of TBK1 in HEK293T cells. HEK293T cells were transfected with IRF-E luciferase reporter (0.1 μg/well) and HA-TBK1 vector (0.2 μg/well) together with an increasing amount of Myc-VHL vector (0.1, 0.2, and 0.4 μg/well). After 18–24 h, luciferase assays were performed. (F) pVHL has no obvious effect on IRF-E reporter activity activated by overexpression of IRF3 in HEK293T cells. HEK293T cells were transfected with IRF-E luciferase reporter (0.1 μg/well) and Myc-IRF3 vector (0.2 μg/well) together with an increasing amount of Myc-VHL vector (0.1, 0.2, and 0.4 μg/well). After 18–24 h, luciferase assays were performed. Data are presented as mean ± SEM of three independent experiments performed in triplicate.
by pVHL was not as dramatic as that of MAVS by pVHL (Fig. 2B versus Supplemental Fig. 1A). However, coexpression of MDA5 together with an increasing amount of pVHL in HEK293T cells did not cause an obvious reduction of MDA5 protein levels (Supplemental Fig. 1D). These results suggest that pVHL might also promote RIG-1 degradation, but not of MDA5.

pVHL interacts with MAVS
To determine whether pVHL acts as an E3 ligase for mediating MAVS degradation, we subsequently examined the interaction between pVHL and MAVS. We transfected Myc-tagged VHL (Myc-VHL) with Flag-tagged MAVS (Flag-MAVS) or Flag empty vector into HEK293T cells and then performed coimmunoprecipitation assays. Flag-MAVS could efficiently pull down Myc-VHL (Fig. 3A), indicating that pVHL interacts with MAVS. To determine whether these proteins interact endogenously, we performed coimmunoprecipitation assays from HEK293T cell extracts in the absence or presence of SeV infection using a polyclonal Ab against pVHL. The results show that endogenous MAVS could interact with pVHL when the cells were infected by SeV (MOI = 1). For unknown reasons, we could not express MAVS in bacteria in our laboratory (data not shown); therefore, we could not determine whether pVHL interacts with MAVS directly.

To determine which domains of MAVS bind to pVHL, we performed domain mapping using coimmunoprecipitation assays. The C terminus of MAVS (aa 361–540) was observed to interact with pVHL and required the transmembrane domain of MAVS for the interaction (Fig. 3A–E). Moreover, we mapped the domains of pVHL that bind to MAVS. The full-length pVHL could interact with MAVS, but the C terminus appeared to be necessary for strong binding to MAVS (Fig. 3F, 3G). Together, these results suggest that pVHL interacts with MAVS.

In addition, we further confirmed that pVHL could also interact with RIG-1 and MDA5 (Supplemental Fig. 1B, 1E).

pVHL promotes MAVS proteasomal degradation by targeting lysine 420 residue of MAVS
To determine whether pVHL mediates proteasomal degradation of MAVS, we initially used the proteasome inhibitor, MG132, to examine whether it could block pVHL-mediated MAVS degradation. As shown in Fig. 4A, addition of MG132 could effectively block degradation of MAVS induced by pVHL. Subsequently, we performed ubiquitination assays to determine whether pVHL could catalyze polyubiquitin chain formation on MAVS. As shown in Fig. 4B, only pVHL could indeed induce MAVS polyubiquitination, but Smurf1 and Nedd4 did not do so, suggesting that pVHL serves as a specific E3 ligase of MAVS.

To determine which lysine sites are catalyzed by pVHL to form polyubiquitin chains, we first did a sequence alignment for mouse, rat, human, and zebrafish MAVS proteins (Supplemental Fig. 2). Among mouse, rat, and human MAVS proteins, lysine sites including K7, K10, K325, K331, K348, K371, and K420 are evolutionarily conserved (Supplemental Fig. 2). We made three mutants (K325/331R, K371R, and K420R) with lysine mutated to arginine and examined the effect of pVHL on the function of these mutants using IRF-E luciferase reporter assays. As shown in Fig. 4C, overexpression of pVHL still suppressed the induction role of the K325/331R and K371R mutants on IRF-E luciferase reporter activity. However, overexpression of pVHL had no obvious effect on the induction role of the mutant K420R on IRF-E luciferase reporter activity (Fig. 4C), suggesting that the K420 residue of MAVS might be the target site of pVHL. To confirm that K420 residue of MAVS is the polyubiquitination target site catalyzed by pVHL, we performed ubiquitination assays. Overexpression of pVHL could promote polyubiquitination of the MAVS mutant K371R, as well as the WT MAVS, but not of the K420R mutant (Fig. 4D). Notably, overexpression of pVHL did not induce degradation of the K420R mutant (Fig. 4E). Taken together, these data suggest that pVHL promotes MAVS proteasomal degradation by targeting lysine 420 residue of MAVS.

FIGURE 2. pVHL induces the degradation of MAVS. (A) Overexpression of HA-VHL (2 μg/well) caused ectopically expressed MAVS (2 μg/well) to be dramatically reduced in HEK293T cells. (B) Overexpression of pVHL (2 μg/well) caused ectopically expressed human MAVS (2 and 3 μg/well), as well as mouse MAVS (2 and 3 μg/well), to be reduced in a dose-dependent manner in HEK293T cells. (C) The endogenous MAVS induced by SeV infection (MOI = 1) in HEK293T cells was reduced by ectopic expression of VHL in a dose-dependent manner (0.5, 1, 2, and 4 μg/well). Lane 1, without SeV addition and pVHL overexpression; lanes 2–6, with SeV addition and an increasing amount of pVHL overexpression. (D) Knockdown of endogenous VHL in HCT116 cells by VHL–shRNA-1 and VHL–shRNA-2 (4 μg/well) caused an increase in endogenous MAVS. Data are presented based on three independent experiments. IB, immunoblotting.
targeting lysine 420 residue of MAVS for addition of a polyubiquitin chain. Interestingly, consistent with the notion that pVHL induces RIG-1 degradation, pVHL could also catalyze RIG-1 to form polyubiquitin chains (Supplemental Fig. 1C).

pVHL negatively regulates cellular antiviral response

To assess the functional importance of pVHL-mediated MAVS degradation, we examined the effect of pVHL on the cellular antiviral response. When HEK293T cells were infected with fluorescently labeled VSV (VSV-eGFP) at MOI = 10, no obvious difference was observed between the cells transfected with Myc empty vector (control) and the cells transfected with Myc-VHL expression vector (MOI = 10; Fig. 5A, left two panels). However, when HEK293T cells were infected with VSV-eGFP at MOI = 1, overexpression of pVHL caused more cells to be infected by VSV-eGFP as assessed by number of GFP+ cells (MOI = 1; Fig. 5A, right two panels). In contrast, when pVHL was knocked down in HEK293T cells by transfection with either pSUPER-VHL1 or pSUPER-VHL2, the number of cells infected by VSV-eGFP at an MOI = 10 was greatly reduced (MOI = 10; Fig. 5B).

To further confirm the role of pVHL in the cellular antiviral response, we examined the expression of IFN-β, a marker gene induced by viral infection (28). As shown in Fig. 5C, overexpression of pVHL in HEK293T cells suppressed IFN-β induction by SeV. In contrast, overexpression of pVHL in HEK293T cells had no obvious effect on IFN-β induction by HSV-1, a DNA virus. As reported, MAVS acts as adaptor protein to transmit signals from both RIG-1 and MDA5, which function as cytoplasmic RNA sensors that recognize viral RNA released during virus replication (29). Thus, the observation that overexpression of pVHL only suppressed IFN-β induction by RNA virus but not by a DNA virus are consistent with the role of pVHL in promoting MAVS degradation. Conversely, knockdown of pVHL in HEK293T cells by either pSUPER-VHL1 or pSUPER-VHL2 enhanced IFN-β induction by VSV infection (Fig. 5D).

Furthermore, we examined the expression of ISG15, a ubiquitin-like modifier gene induced by type I IFN that has been shown to mediate protection in a number of different viral infection models (30). As shown in Fig. 5E, overexpression of pVHL in HEK293T cells suppressed ISG15 expression induced by SeV.
FIGURE 4. pVHL promotes MAVS proteasomal degradation by targeting lysine 420 of MAVS. (A) The proteasome inhibitor, MG132, blocks VHL-mediated MAVS degradation. HEK293T cells were transfected with the indicated expression vectors (2 μg/well); after 18–24 h, the cells were harvested for Western blot analysis; 6 h before cell harvest, MG132 (20 μM) was added to the culture medium; DMSO was used as a carrier control. (B) pVHL catalyzes MAVS to form polyubiquitin chains. HEK293T cells were transfected with the indicated expression vectors (His-ubiquitin, 3 μg/well; Flag-MAVS, 6 μg/well; Myc-VHL/Myc-Smurfl/Myc-Nedd4, 3 μg/well); the cell lysates underwent affinity purification using Ni²⁺-NTA resin with anti-Flag Ab used for detection. (C) pVHL (0.2 μg/well) suppresses IRF-E reporter (0.1 μg/well) activity activated by WT MAVS (0.2 μg/well), K325/K331R mutant (0.2 μg/well), and K371R mutant (0.2 μg/well), but not by the K420R mutant (0.2 μg/well) in HEK293T cells. Data are presented as mean ± SEM of three independent experiments performed in triplicate. (D) pVHL (3 μg/well) catalyzes formation of polyubiquitin chains on WT MAVS (6 μg/well) and the K371R mutant (6 μg/well), but not on the K420R mutant (6 μg/well). (E) pVHL (2 μg/well) induces degradation of WT MAVS (2 μg/well), but not of K420R mutant (2 μg/well). Data are presented based on three independent experiments. WCL, whole cell lysates.

Interestingly, without virus infection, RFP-tagged pVHL mainly distributed in cytoplasm but was outside of mitochondria (Fig. 5F, white arrows). However, SeV infection caused translocation of pVHL to mitochondria completely (Fig. 5G, white arrows), further highlighting the role of pVHL in inducing MAVS degradation and negatively regulating antiviral immunity (31). Taken together, these data suggest that pVHL negatively regulates cellular antiviral response.

Knockout of vhl in zebrafish enhances the antiviral response

To determine the physiological role of pVHL in vivo in response to viral infection, we took advantage of the zebrafish model by using infection with the SVCV (32, 33). Using the TALEN technique, we generated two zebrafish lines with mutated vhl (mutant 1 and mutant 2; Supplemental Fig. 3) (34). In the mutant 1, two nucleotides in exon 1 of vhl were deleted (Supplemental Fig. 3C). In the mutant 2, two nucleotides were inserted in exon 1 (Supplemental Fig. 3C).

Similar to reported zebrafish vhl mutants generated by N-ethyl-N-nitrosourea treatment (35), these two mutants displayed a severe hyperventilation and cardiophysiological response and developed polycythemia (35) (Supplemental Fig. 3D). The homozygous null embryos (vhl−/−) survived up to 8–11 d and ultimately developed severe edema and died (35) (Supplemental Fig. 3D).

At 5 dpf, based on the general morphology (hyperventilation, polycythemia, etc.), it was easy to separate homozygous null embryos (vhl−/−) from vhl+/− and vhl+/+ offspring produced by mating adult heterozygous zebrafish (vhl+/−; Supplemental Fig. 2D).

Therefore, we used 5 dpf embryos for viral infection assays. SVCV infection could cause obvious pathological effects in both vhl-deficient zebrafish embryos (vhl−/−; mutant 1 and mutant 2) and WT embryos (vhl+/+; Fig. 6A, left two lanes). Interestingly, SVCV infection enhanced the endogenous Mavs protein level, resulting in much higher levels in the homozygous embryos (vhl−/−) compared with that observed in WT embryos (vhl+/+; Fig. 6B, left two lanes). Importantly, SVCV infection enhanced the endogenous Mavs protein level, resulting in much higher levels in the homozygous embryos (vhl−/−) compared with that observed in WT embryos (vhl+/+; Fig. 6B, right two lanes) (32, 33). Of note, the homozygous embryos (vhl−/−; mutant 1) had the highest level of ifn1, a well-defined gene for zebrafish response to viral infection (33, 36), but the heterozygous embryos (vhl+/−) had a middle level of ifn1, and WT embryos (vhl+/+) had a lowest level of ifn1 (Fig. 6C). This expression pattern is correlated with the level of vhl in zebrafish embryos, implying the physiological role of vhl in antiviral immune response.

To further determine the role of vhl in response to viral infection, we examined expression of ifn1 and ifn3, two well-defined genes for zebrafish in response to viral infection (33, 36). Moreover, SVCV infection caused ifn1 and ifn3 to increase much more dramatically in vhl-deficient zebrafish embryos (vhl−/−; mutant 1) than in WT.
embryos (vhl++; Fig. 6D, 6E). Taken together, these data suggest that zebrafish vhl negatively regulates the antiviral response probably through mediation of Mavs degradation in vivo.

**Discussion**

Since VHL was identified in 1993 as the genetic basis for VHL disease, it has proved to be of widespread interest (1, 37). pVHL has been well characterized and performs its tumor suppressive function by targeting hypoxia-inducible factors for ubiquitination and proteasomal degradation, which accounts for the cellular oxygen sensing (10, 38). Large phenotypic variations exist in VHL patients. Coupled with the fact that some VHL mutants can still cause VHL diseases while retaining the ability to induce degradation of hypoxia-inducible factors, mechanisms other than those mediating HIF-degradation have been proposed to explain pVHL function (1). However, these mechanisms still need to be further defined (10).

Although the impact of HIFs on immune responses has been observed (39), the mechanisms by which VHL affects the cellular immunity response, either directly or through HIFs, is still largely unknown. Interestingly, it was reported that pVHL could inhibit NF-κB phosphorylation through its effect on the NF-κB agonist...

**FIGURE 5.** pVHL negatively regulates the cellular antiviral response. (A) Overexpression of pVHL in HEK293T cells enhances VSV infection. HEK293T cells were transfected with a Myc-VHL expression vector (0.5 μg/well) or Myc empty vector control (0.5 μg/well); after 24 h, VSV-eGFP viruses were added to culture medium (MOI = 10 or MOI = 1); 8 h later, the cells were checked and photographed under a Nikon-TE2000U inverted fluorescent microscope. (B) Knockdown of pVHL by pSUPER-VHL1 and pSUPER-VHL2 in HEK293T cells diminishes VSV viral infection. HEK293T cells were transfected with pSUPER-VHL1 (1 μg/well), pSUPER-VHL2 (1 μg/well), or pSUPER empty vector control (1 μg/well); after 24 h, VSV-eGFP virus was added to the culture medium (MOI = 10); 8 h later, the cells were checked and photographed under a Nikon-TE2000U inverted fluorescent microscope. (A and B) Data are presented based on three independent experiments. (C) Overexpression of pVHL suppresses IFN-β expression induced by SeV infection (MOI = 1), but not by HSV-1 infection (MOI = 1). HEK293T cells were transfected with Myc-VHL expression vector (4 μg/well) or empty vector control (4 μg/well); after 24 h, SeV or HSV-1 viruses were added to culture medium; 8 h later, the cells were harvested for total RNA extraction; IFN-β expression was detected by semiquantitative RT-PCR assays. (D) Knockdown of pVHL by pSUPER-VHL1 and pSUPER-VHL2 enhances IFN-β expression induced by VSV infection in HEK293T cells. HEK293T cells were transfected with pSUPER-VHL1 or pSUPER-VHL2 (6 μg/well), pSUPER empty vector control (6 μg/well); after 24 h, VSV viruses (MOI = 1) were added to the culture medium; 8 h later, the cells were harvested for total RNA extraction; IFN-β expression was detected by semiquantitative RT-PCR assays. (E) Overexpression of pVHL suppresses ISG15 expression induced by SeV infection. HEK293T cells were transfected with Myc-VHL expression vector (4 μg/well) or empty vector control (4 μg/well); after 24 h, SeV viruses (MOI = 1) were added to culture medium; 8 h later, the cells were harvested for total RNA extraction; ISG15 expression was detected by semiquantitative RT-PCR assays. (F-G) Data are presented based on three independent experiments performed in triplicate. (F) Without SeV infection, pVHL does not localize in mitochondria; the mitochondria are marked by the white arrows. Cos-7 cells were transfected with RFP-tagged VHL (4 μg/well). (G) pVHL translocates into mitochondria after SeV infection; the mitochondria are marked by the white arrows. Cos-7 cells were transfected with RFP-tagged VHL expression vector (4 μg/well); after 24 h, SeV viruses were added to the culture medium (MOI = 10); 8 h later, the cells were stained with MitoTracker Green (1:5000 dilution) for 45 min and photographed under a Zeiss confocal microscope (LSM-710). (F and G) Data are presented based on three independent experiments. Original magnification ×4 in (A) and (B) and ×63 in (F) and (G).
FIGURE 6. Zebrafish Vhl (DrVhl) induces zebrafish Mavs degradation (DrMavs) and negatively regulates zebrafish antiviral response. (A) Zebrafish Vhl promotes zebrafish Mavs degradation. HEK293T cells were transfected with HA-DrMavs (2 μg/well) together with Myc-DrVhl expression vector (2 μg/well) after 18–24 h, the cells were harvested and examined by Western blot analysis. (B) Zebrafish Vhl-deficient embryos (vhl−/−; Mutant 1) exhibit higher endogenous Mavs protein compared with the WT zebrafish (vhl+/+) with or without SVCV infection. After 5 dpf, SVCV viruses (∼2 × 10^5 TCID₅₀/ml) in water were added to zebrafish vhl WT and null embryos and incubated for 24 h, and endogenous Mavs was detected by Western blot analysis using a polyclonal anti-DrMavs Ab. (C) Expression of ifn1 (Drinfl) in Vhl-deficient embryos (vhl−/−; mutant 1) is much higher than that in both Vhl heterozygous embryos (vhl+/−; p < 0.0001) and WT embryos (vhl+/+; p < 0.0001); expression of ifn1 (Drinfl) in Vhl heterozygous embryos (vhl+/−; mutant 1) is also higher than that in WT embryos (vhl+/+; p = 0.0002). (D) SVCV infection (∼2 × 10^5 TCID₅₀/ml) in zebrafish promotes upregulation of ifn1 (Drinfl) much more dramatically in vhl-null embryos (vhl−/−) (Mutant 1) compared with that in vhl WT embryos (vhl+/+; p < 0.0001). (E) SVCV infection (∼2 × 10^5 TCID₅₀/ml) in zebrafish promotes upregulation of ifn3 (Drinfl) much more dramatically in vhl-null embryos (vhl−/−; mutant 1) compared with that in vhl WT embryos (vhl+/+; p = 0.0097). After 5 dpf, SVCV viruses (∼2 × 10^5 TCID₅₀/ml) in water were added to zebrafish vhl WT and null embryos (mutant 1) and incubated for 24 h after which Drinfl and Drin3 expression levels were detected by semiquantitative RT-PCR analysis. Data are presented as mean ± SEM of three independent experiments performed in triplicate.
other targets of pVHL involved in this signaling pathway (17, 31, 41, 42).

As a well-known disorder, VHL disease has attracted much attention from clinicians and biologists (43–46), culminating in the collection of much clinical and epidemiological data. However, most data pertain to angiogenic tumors (43, 45, 46). So far, no epidemiological data describe the relationship between VHL diseases and viral infection. Elucidation of the correlation between VHL disease and viral infection should provide new clues for understanding the physiological function of pVHL.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Data

Supplemental Figure 1

**A** Overexpression of pVHL caused ectopically-expressed RIG-1 to be reduced in a dose-dependent manner in HEK293T cells. (B) Ectopically-expressed HA-VHL interacts with ectopically-expressed Myc-RIG-1. HEK293T cells were transfected with the indicated expression vectors, and coimmunoprecipitation was performed with anti-HA antibody. (C) pVHL catalyzes RIG-1 to form poly-ubiquitin chains. HEK293T cells were transfected with the indicated expression vectors; the cell lysates underwent affinity purification using Ni²⁺-NTA resin with anti-Myc antibody was used for detection. (D) Overexpression of pVHL did not caused ectopically-expressed RIG-1 to be reduced in HEK293T cells. (E) Ectopically-expressed HA-VHL interacts with ectopically-expressed Myc-MDA5. HEK293T cells were transfected with the indicated expression vectors, and coimmunoprecipitation was performed with anti-HA antibody.
Supplemental Figure 2

Sequence alignment of mouse, rat, human and zebrafish MAVS proteins. The evolutionarily conserved lysine residues are marked by red arrows and the positions are marked by blue numbers (designated to human MAVS protein).
Supplemental Figure 3 Generation of vhl"-" zebrafish by TALEN. (A) Targeting strategy for generating mutations in exon 1 of vhl locus by TALEN. The target sequences are underlined. (B) PCR analysis of the targeting locus from zebrafish embryos. (C) Sequence analysis for the locus from the wild type embryos and mutant embryos. (D) The representative morphology of wild type (vhl"+/") and vhl-null(vhl"-") embryos at 5 days post fertilization (5dpf). (E) The representative pictures of wild type and vhl-null embryos at 7 dpf after SVCV infection for 2 days (48 h).