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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<sup>−/−</sup>) 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: 000–000.

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). Targets 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 NFKB 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 MARS-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/vh1) 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 mavs 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-α-tubulin (EPR1333; Epitomics), anti-MAVS (166583; Santa Cruz). VHL (ABclonal). Zebrafish Mavs Ab (anti-DeMavS) was developed by injecting rabbits with GST-tagged partial zebrafish Mavs.

**Confoal microscopy**

The Cos-7 cells were transfected with RFP-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'-CAACGCTCCTGTCTGTCTG-3', the right target sequence is 5'-CCA-CAGGGGCGTGTCACG-3'. Vhl-TALENs were constructed by FastTALE TALEN Assembly kit (Sidansai Biotechnology, Shanghai, China) and the mRNAs of vhl-TALENs were synthesized by Amplipac Sp6 High Yield Message Maker kit (Cell Script).

**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 pTK-Renilla as an internal control. The cells were transferred for 18–24 h, the superantigen 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 viruses were purchased from 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-TACCTGAAGGCCAAGGA-3' and 5'-CAATTGTCAGCCTCCAGG3-3'; human IFN-stimulated gene 15 (ISG15): 5'-ATGGCTGGACCTGGACG-3' and 5'-TTGACCTCAGCGCCGCAAC-3'; human GAPDH (internal control): 5'-AGGCCCACTGGTCAAGACAC-3' and 5'-GCCAACTAGACCAAAATCC-3'; zebrafish ifn1: 5'-AAGTTTCTGCTCAGAATGTAAC-3' and 5'-TCCCATGTCCAAGGATTCAAG-3'; zebrafish ifn3: 5'-TCTGCTTTGTCAGGTTTG-3' and 5'-GGTATAGAAAAGCGGTCTGCTC-3'; zebrafish efa1a (internal control): 5'-CTTTCAGAGGCTACGTGGC-3' and 5'-CCCGTT-AGCATTACCTCC-3' (25).

**Immunoprecipitation and Western blotting**

Comunoprecipitation 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-illuminescent 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.

**Semiquantitative 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 Whole Tissue RNA Isolation System (Promega). The cDNA synthesis was carried out using a first-strand cDNA synthesis kit (Fermentas).

**Results**

pVHL negatively regulates MARS 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 MARS. Upon viral infection, RIG-1–like helicases bind to viral RNA and activate MARS, 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, the activity of an IRF-E reporter was upregulated significantly (Fig. 1B–F). However, coexpression of pVHL together with RIG-1 or MDA5 resulted in a mild reduction of 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 protein levels was independent of pVHL

**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 MAVS in a dose-dependent manner 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 VHL (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 Smurfl 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 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 1 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.
K420R mutant (2 μM) was added to the culture medium; DMSO was used as a carrier control. (B) pVHL catalyzes MAVS to form polyubiquitin chains on WT MAVS (6 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), K325R/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). 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). However, SeV infection caused translocation of pVHL to mitochondria completely (Fig. 5G, white arrows).

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⁺/+; Supplemental Fig. 2E). All embryos used for viral infection assays, including the homozygous null embryos (vhl⁻/⁻) and their WT siblings (vhl⁺/+), were regenotyped by sequencing.

Similar to that observed for mammalian VHL, zebrafish Vhl could still induce zebrafish Mavs degradation when overexpressed in HEK293T cells (Fig. 6A). Consistently, the protein level of Mavs in the homozygous embryos (vhl⁻/⁻; mutant 1) was higher than that of WT embryos (vhl⁺/+; Fig. 6B, 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, right two lanes). 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⁺/+; mutant 1) had the 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 vhl⁺/+; mutant 2). Interestingly, SVCV infection caused ifn1 and ifn3 to increase much more dramatically in vhl-deficient zebrafish embryos (vhl⁻/⁻; mutant 1) than in WT vhl⁺/+; mutant 2).

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. 2D). All embryos used for viral infection assays, including the homozygous null embryos (vhl⁻/⁻) and their WT siblings (vhl⁺/+), were regenotyped by sequencing.

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embryos (vhl<sup>++</sup>; 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...
Card 9, which links pVHL to control of NF-κB activity and tumorigenesis (40). However, the role of pVHL in the cellular antiviral response has not been defined. In this study, we have found that pVHL negatively regulates IRF3 activity in response to viral infection. Using additional assays, we have identified MAVS as a major target of pVHL for ubiquitination and proteasomal degradation. Intriguingly, after viral infection, pVHL colocalized to mitochondria onto which MAVS were anchored. Furthermore, in vhl-deficient zebrafish embryos, the protein level of zebrafish Mavs rose much higher after viral infection, compared with its level in the WT zebrafish embryos. In addition, vhl-deficient zebrafish embryos exhibited a greatly exaggerated antiviral response. Collectively, these data suggest that pVHL plays an important role in innate antiviral immunity. However, this negative regulatory role correlates with its tumor suppressive function is still unknown. Investigation of the correlations between pVHL’s tumor suppressive function and its negative regulation of antiviral immunity should uncover new avenues for understanding the relationship between tumor development and innate immunity.

Notably, during the identification of pVHL targets in the cellular antiviral innate immunity signaling pathway (Mda5/RIG-1—MAVS—TBK1—IRF3), we found that in addition to MAVS, RIG-1 and MDA5 also appear to be the targets of pVHL. Overexpression of pVHL together with RIG-1 not only suppressed the activity of the IRF-E reporter enhanced by RIG-1 (Fig. 1B), but also induced protein degradation of RIG-1 (Fig. 2A, Supplemental Fig. 1A), even though their degradation rate by pVHL was not as dramatic as that of MAVS. In addition, pVHL could also interact with RIG-1 and catalyze RIG-1 to form polyubiquitin chains, further suggesting that RIG-1 might be another target of pVHL in the cellular antiviral innate immunity. For MDA5, it appeared that pVHL could not mediate its protein degradation, but pVHL could still interact with MDA5 and suppressed MDA5-activated IRF-E reporter activity. Thus, MDA5 might also be a target of pVHL in the cellular antiviral innate immunity. pVHL may affect MDA5 function through a different mechanism other than inducing MDA5 degradation. Further investigation of the effect of pVHL on the RIG-1 and MDA5 will help to better understand the function of pVHL in cellular innate antiviral immunity.

The behavior of pVHL in cellular antiviral signaling is quite similar to that of E3 ligase RNF125, which can ubiquitinate and trigger degradation of MDA5, RIG-1, and MAVS simultaneously to suppress the cellular antiviral response (18). However, MAVS might play a greater role as a pVHL target than RIG-1 and MDA5, as indicated by the observations: the inhibitory ability of pVHL on MAVS-enhanced IRF3 activity was higher and the degradation rate of MAVS by pVHL was higher. However, because of the multiple steps and multiple regulation points of cellular RIG-1-like-helicase-mediated antiviral signaling, we still cannot rule out...
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

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