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Complex cellular functions of the von Hippel–Lindau tumor suppressor gene: insights from model organisms

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Abstract

The von Hippel–Lindau tumor suppressor gene (*VHL*) has attracted intensive interest not only because its mutations predispose carriers to devastating tumors, but also because it is involved in oxygen sensing under physiological conditions. *VHL* loss-of-function mutations result in organ-specific tumors, such as hemangioblastoma of the central nervous system and renal cell carcinoma, both untreatable with conventional chemotherapies. The VHL protein is best known as an E3 ubiquitin ligase that targets hypoxia-inducible factor-α (HIF-α), but many diverse, non-canonical cellular functions have also been assigned to *VHL*, mainly based on studies in cell culture systems. As such, although the HIF-dependent role of *VHL* is critical, the full spectrum of pathophysiological functions of *VHL* is still unresolved. Such understanding requires careful cross-referencing with physiologically relevant experimental models. Studies in model systems, such as *Caenorhabditis elegans*, *Drosophila*, zebrafish and mouse have provided critical *in vivo* confirmation of the VHL–HIF pathway, and verification of potentially important cellular functions including microtubule stabilization and epithelial morphogenesis. More recently, animal models have also suggested systemic roles of *VHL* in hematopoiesis, metabolic homeostasis and inflammation. In this review, the studies performed in model organisms will be summarized and placed in context with existing clinical and *in vitro* data.

Keywords

VHL; HIF; model organisms

Introduction

The von Hippel–Lindau tumor suppressor gene (*VHL)* mutations were the genetic defects in the familial VHL disease (Latif *et al*., 1993), which manifests in a limited number of organ-specific tumors (Maher *et al*., 2011), predominantly in the kidney (clear-cell renal cell carcinoma, ccRCC) and central nervous system (hemangioblastoma). Less frequent VHL tumors include those in pancreas (pancreatic cysts, serous cystadenoma and pancreatic neuroendocrine tumors), adrenal gland (pheochromocytoma) and testes (epididymal cystadenomas). The presence of these diverse but specific VHL tumor types of widely different tissue origins is just one of the intriguing facts about the *VHL* gene. Among these 'VHL tumors', ccRCC is the main cause of disease-related death. Up to 70% of the carriers of germ-line *VHL* mutations eventually develop ccRCC (Lonser *et al*., 2003). In addition, loss of *VHL* function, including somatic mutations and epigenetic defects, is found in 70–

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90% of the sporadic ccRCC (Herman *et al.*, 1994; Kim and Kaelin, 2004; Banks *et al.*, 2006). The pathophysiological mechanism for such strong association is currently unknown.

VHL has proved a unique and enigmatic tumor suppressor gene. Most of the tumor suppressor genes associated with familial cancer syndromes can be assigned specific cellular functions; for example, Rb is a cell cycle inhibitor, WT1 and TP53 are transcription factors, adenomatous polyposis coli is a Wnt signaling suppressor, and NF1 and NF2 are GTPase-activating protein regulator and cytoskeletal protein merlin, respectively. The role of *VHL* in tumorigenesis is more complex. The protein encoded by the *VHL* gene (pVHL) is best known as the substrate-binding subunit of a SCF (Skp1-Cdc53/Cul-1-F-box protein) type E3 ubiquitin ligase containing, besides pVHL, Cullin-2, elongin B and C and Rbx-1 (Figure 1; Pause *et al.*, 1997; Lonergan *et al.*, 1998; Kamura *et al.*, 1999; Lisztwan *et al.*, 1999; Stebbins *et al.*, 1999). The best-known degradation target of VHL-containing E3 ligase is the α-subunit of hypoxia-inducible factor (HIF-α) in normal physiological conditions (Kaelin, 2005). At normal oxygen level, HIF-α is hydroxylated at the proline residues within an oxygen-dependent degradation domain. The prolyl-hydroxylated HIF-α is recognized by pVHL, leading to poly-ubiquitination and degradation. The hydroxylation reaction is mediated by the prolyl-hydroxylase domain proteins (PHDs; Berra *et al.*, 2003). In hypoxic conditions, the prolyl hydroxylases are inactive and HIF-α-subunit is stabilized. HIF-α then dimerizes with the β-subunit (HIF-β), also termed aryl hydrocarbon receptor nuclear translocator (ARNT), which is constitutively expressed. The HIF heterodimer translocates to the nucleus where it functions as a transcription factor. Its best-known target genes encode proteins involved in glycolysis (phosphoglycerate kinase), glucose transport (Glut-1), angiogenesis (vascular endothelial growth factor (VEGF)) and erythropoiesis (erythropoietin); that is, proteins that mediate the cellular response and adaptation to hypoxic conditions. In addition, chemokine receptor CXCR4 was also identified as a HIF target (Staller *et al.*, 2003), which indicates that HIF activation may contribute to the metastatic potential of cancer cells. These functions support a critical role of pVHL in regulating tumor progression, especially in hypervascularized tumors such as ccRCC. However, accumulated evidences have indicated that many HIF-independent activities of pVHL also exist (Figure 1), including regulation of extracellular matrix (Ohh *et al.*, 1998; Tang *et al.*, 2006; Feijoo-Cuaresma *et al.*, 2008; Kurban *et al.*, 2008), senescence (Young *et al.*, 2008), apoptosis (Guo *et al.*, 2009), phosphorylation enhancer (Yang *et al.*, 2007), microtubule stability and cilia formation (Hergovich *et al.*, 2003; Esteban *et al.*, 2006; Schermer *et al.*, 2006; Thoma *et al.*, 2007, 2009), RNA stability (Datta *et al.*, 2005; Danilin *et al.*, 2009), endocytosis (Hsu *et al.*, 2006), gene transcription (Mikhaylova *et al.*, 2008) and many others (Frew and Krek, 2007, 2008). Interestingly, many of these HIFindependent functions of pVHL are mediated through stabilization of its binding proteins, contrary to its known E3 ligase activity. It therefore appears that pVHL is an adaptor protein that, depending on the interacting partners, can promote protein degradation or serve as a chaperon. Some of these diverse activities likely also contribute significantly to the tumor suppressor and other physiological functions.

Clinical observations support this view. Human mutations of PHD2, the major prolyl hydroxylase responsible for HIF-α degradation in normoxia, resulted in elevated expression of HIF-α in multiple tissues and a rare form of familial polycythemia, likely due to overexpression of erythropoietin, but without increased incidence of tumors (Berra *et al.*, 2003). Also, the human Chuvash disease is caused by a specific homozygous *VHL* mutation (R200W) and elevated HIF activity (Gordeuk *et al.*, 2004). The disease exhibits polycythemia without increased tumor incidence. These observations suggest that HIF activity is necessary but not sufficient for tumor formation. However, it is at times difficult to identify the most physiologically relevant functions of pVHL, particularly when the myriad of *VHL* functions is likely tissue and context dependent. To this end, the studies

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using model organisms have provided valuable insights. This review attempts to summarize these findings and place them in context with relevant data obtained from clinical and *in vitro* studies.

Caenorhabditis elegans

The first report of *C. elegans VHL* focused on evolutionary sequence conservation (Woodward *et al.*, 2000). Because *C. elegans VHL* was the first non-mammalian *VHL* cloned, it provided valuable insights into the critical protein domains and amino-acid residues. For example, some of the disease-related mutation hot spots were suspected of being due to 'founder effect' within the patient population, not necessarily indicating a proportionally high degree of functional importance. The founder effect is the result of a large number of descendents from an ancestral carrier of a specific mutation. In a relatively rare genetic disease, a disproportionately large number of descendants from a single mutational event can bias the perception of functional importance (Zlotogora, 1994). Some of these hot spots, however, may indeed be critical for *VHL* function because they are conserved in *C. elegans*, including the 'Black Forest mutation' at Y98, which has been suspected of being a founder-effect outlier because almost all of the affected families can be traced to the Black Forest region of Germany (Brauch *et al.*, 1995). This mutation was later shown to affect a critical amino-acid residue that confers a non-canonical function of VHL in promoting microtubule stability (Hergovich *et al.*, 2003; more in the Drosophila section below). In addition to the specific hot spots, 60% of the human pVHL amino-acid residues that were predicted to be on the binding surface (Stebbins *et al.*, 1999), and by extension important for protein–protein interaction, are conserved in *C. elegans*, thus supporting the molecular model.

The most significant early finding on pVHL function is the demonstration of its role in targeting prolyl-hydroxylated HIF-α (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). The VHL–HIF-prolyl hydroxylase triad was soon validated in *C. elegans* (Epstein *et al.*, 2001). This study provided genetic and biochemical proof that prolyl-hydroxylated HIF-α is regulated by pVHL. More importantly, the study identified the nematode prolyl hydroxylase (encoded by the 2-oxoglutarate-dependent oxygenase gene *egl-9*) as the mediator of HIF-α degradation, as well as the mammalian ortholog EGLN/PHD (*egl-9* homolog/prolyl hydroxylase domain). This cross-species validation was critical for the rapid acceptance of the model. Subsequent *C. elegans* studies of note came from whole-organism analysis of gene expression profiling. The *C. elegans* genome encodes a single *VHL* (*vhl-1*) and a single *HIF-α* (*hif-1*) gene. Interestingly, both *vhl-1* and *hif-1* loss-of-function mutants as well as double mutants are viable. The viability of these mutants made it possible to compare altered gene expression patterns in *vhl-1*-defective animals in the otherwise wild-type or *hif-1* mutant backgrounds (Bishop *et al.*, 2004). Thus, *hif*-dependent and independent *vhl*-responsive genes could be identified. Most of the *hif*-dependent genes upregulated in *vhl-1* mutant are also upregulated by hypoxia. These include *egl-9* that targets HIF-α during normoxia, indicating a possible feedback control. This mechanism is evolutionarily conserved because the mammalian ortholog of *egl-9*, the EGLN/PHD family of genes, was also upregulated in *VHL* mutant ccRCC cells (Harten *et al.*, 2009). Another prolyl hydroxylase (encoded by *phy-2*) that modifies collagen is upregulated in both *vhl-1* mutant and hypoxia. Curiously, no other familiar mammalian VHL–HIF target genes were revealed, such as homologs of *VEGF*, *IGFBP3*, *Glut-1* and *CXCR4*, suggesting a different requirement for coping with hypoxia in nematodes as compared with mammals. Interestingly, among the *vhl*-dependent but *hif*-independent targets, one encodes an aldose 1-epimerase that is involved in gluconeogenesis, and three encode the solute carrier (slc) family of proteins that may be involved in sugar and lactate transport. These *Vhl*-dependent

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genes imply that the ancestral *Vhl* function may be involved in modulation of metabolic homeostasis.

A second whole-genome analysis focused on the hypoxia response (Shen *et al.*, 2005). The analysis was designed to identify early response genes in acute hypoxia (4 h at 21°C in 0.1% oxygen). The analysis also compared three genotypes, wild type, *hif-1* mutant and *vhl-1* mutant, to identify *hif*-dependent and independent hypoxia-responsive genes. Among the hypoxia-inducible genes, only slightly more than half (63 out of 110) are *hif*-dependent. Most of the *hif*-dependent hypoxia-inducible genes are also *vhl* regulated, that is, they are upregulated in *vhl-1* mutant and cannot be further induced by hypoxia. This indicates that in *C. elegans*, *vhl* is the major regulator of *hif* in normoxic cells and that hypoxia is the main activator of *hif* function. Prominent among the hypoxia-induced genes encode components of energy metabolism. For example, pyruvate carboxylase *(pyc-1)* is induced in a *hif-1*-independent (and *vhl*-dependent) manner. The enzyme provides oxaloacetate precursor for gluconeogenesis. *gei-7* is induced in a *hif-1*- and *vhl*-dependent manner. It encodes isocitrate lyase that coverts isocitrate to succinate and glyoxylate in the glyoxylate cycle, a carbon-conserving pathway alternative to the carbon-consuming tricarboxylic acid cycle that converts isocitrate to succinyl-CoA. It is interesting to note that these gene products either promote energy store (glucose production) or conserve carbon sources, instead of the glycolysis switch observed in mammalian *Vhl* mutant (or hypoxic) cells. This may reflect the fact that there is no thermal homeostasis in nematodes, therefore, emergency energy production is not a priority. As in *vhl-1* mutant gene profiling (Bishop *et al.*, 2004), both *egl-9* and *phy-2* were upregulated in hypoxia in a *hif-1*- and *vhl-1*-dependent manner. Interestingly, *phy-2* mutation shows <10% survival (to adults) in 0.5% oxygen. Thus, modification of extracellular matrix may be important for maintaining cellular function during hypoxic stress. Taken together, these studies demonstrated that the VHL–HIF axis is evolutionarily conserved to serve as a hypoxia responder, although the exact hypoxic response at the molecular level is specific to individual species.

Perhaps the most intriguing aspect of the *C. elegans* array analyses of *VHL*-responsive genes is what they did not show. Most of the *VHL* target genes in *C. elegans* are not prominent *VHL* targets found in the human ccRCC cell system. No growth factors/cytokines are identified and no obvious cell-cycle regulators are revealed. In fact, it appears that the VHL pathway in *C. elegans* regulates specific cell-autonomous functions that deal with environmental and metabolic stresses. These may be more closely aligned with the ancestral or the normal physiological functions of *VHL*. Interestingly, *vhl-1* loss-of-function has recently been linked to *hif*-independent increased life span in *C. elegans* (Muller *et al.*, 2009). It is intriguing to consider that stress responses may function at the expense of underlying health of the organism or vitality of individual cells. It is also instructive to consider that an ancestral gene that modulates stress responses can function as a tumor suppressor in complex organisms such as human. Whether the tumor suppressor function of *VHL* in human is acquired during evolution or is the manifestation of its ancestral function deserves careful investigation. Such understanding may influence our thinking of treating and preventing VHL tumors.

Drosophila

The *Drosophila VHL* (*dVHL*) was identified by amino-acid sequence homology query against the newly deposited fly genomic sequence in the late 1990s (Adryan *et al.*, 2000). The dVHL protein was shown to interact with *Drosophila* elongin C *in vitro*. A similar strategy was used and the same *dVHL* sequence was identified independently at about the same time (Aso *et al.*, 2000). The latter study showed that dVHL protein could form complex with the human Cul-2, human elongin B and C and mouse Rbx-1, but not with the

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human elongin A. Thus, the ubiquitin ligase function of pVHL is conserved in insects. Later, Arquier *et al.* (2006) showed that *dVHL* is involved in degradation of hydroxylated oxygen-dependent degradation domain of either the human HIF-1α or the *Drosophila* homolog Sima in normoxia, and that the oxygen-dependent degradation–GFP fusion protein is stabilized in hypoxia *in vivo*.

The first phenotypic analyses (Adryan *et al.*, 2000) utilized the then new but promising RNA interference technology. *dVHL* was in fact one of the first *Drosophila* genes studied using siRNA-mediated knockdown. *Drosophila* contains a vascular system, the trachea, for transporting oxygen, which is capable of oxygen sensing (Jarecki *et al.*, 1999; Wingrove and O'Farrell, 1999). On the other hand, the Malpighian tubes perform excretory and osmoregulatory functions similar to those of the vertebrate kidneys. There was therefore much speculation as to which of these systems will be affected in *dVHL* mutant. As it turned out, injection of *dVHL*-specific siRNA into pre-blastoderm embryos resulted in ectopic looping and branching in the trachea, but not in the Malpighian tubes (Adryan *et al.*, 2000). Importantly, injection of either *dVHL* or human *VHL* sense RNA into wild-type embryos blocked tracheal branch migration. These reciprocal experiments demonstrated that *VHL* could negatively regulate vascular cell motility.

The function of *VHL* was further explored in the *Drosophila* system by analyzing the genomic knockout of the endogenous *dVHL* gene (Hsouna *et al.*, 2010). Homozygous *dVHL* mutant is lethal at the early larval stage and exhibits profound tracheal phenotypes, consistent with the earlier RNA interference study. Surprisingly, the lethality could be rescued by re-expressing *dVHL* specifically in the trachea, indicating that modulation of vascular development is the main developmental function of *dVHL*. This notion is consistent with the earlier mouse knockout phenotype (Gnarra *et al.*, 1997) and was later confirmed in zebrafish (van Rooijen *et al.*, 2010). In both flies and vertebrates, the key defining process in vasculogenesis is the outgrowth of branches, regulated by growth factor signaling pathways. In *Drosophila* embryo, the fibroblast growth factor (FGF) signaling pathway is utilized (Ghabrial *et al.*, 2003; Uv *et al.*, 2003; Akis and Madaio, 2004) to pattern the trachea in a stereotyped fashion. The tracheal phenotype in *dVHL* mutant resembled that resulting from over-active FGF receptor (FGFR) signaling (Dammai *et al.*, 2003). Indeed, FGFR-ERK-Ets1 signaling pathway is over-activated in *dVHL* mutant tracheal cells, which is the result of accumulated FGFR on the mutant tracheal cell surface (Hsouna *et al.*, 2010). Genetic epistasis analysis indicates that the surface accumulation of FGFR is the result of defective endocytic pathway and that dVHL functionally interacts with and stabilizes Abnormal Wing Discs (AWD), the homolog of human anti-metastasis factor Nm23, which has been shown to regulate endocytosis (Dammai *et al.*, 2003; Hsu *et al.*, 2006; Nallamothu *et al.*, 2008, 2009; Woolworth *et al.*, 2009) in part by stabilizing Rab5 (Woolworth *et al.*, 2009). The detailed phenotypic analysis also revealed that the endocytic function of *dVHL* not only regulates cell motility but also controls the size and length of the tubule lumen (Hsouna *et al.*, 2010). Formation of the tracheal lumen, and likely of other tubule systems in mammals, is controlled by secretion (exocytosis) of matrix material and, at the maturation phase, reabsorption through endocytosis of these structural materials (Behr *et al.*, 2007; Tsarouhas *et al.*, 2007). Mutations in *dVHL* result in defective endocytosis of the lumenal chitin and consequently, enlarged and tortuous lumen of the trachea. This phenotype can be reduced or exacerbated by expression of constitutively active Rab5 or dominant-negative Rab5, respectively. Interestingly, ectopic branching and dilated tubule phenotypes were also observed in the mouse *Vhl* knockout kidney tubules (Hsouna *et al.*, 2010).

We also showed that specific regulation of FGFR internalization is evolutionarily conserved. In *VHL* mutant human ccRCC cells and primary human endothelial cells knocked down with *VHL*-specific RNA duplex, FGFR over-accumulates on the cell surface, leading to

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increased FGFR, ERK and Ets1 transcription factor activation, elevated cell motility and increased angiogenic potential *in vitro* (Hsu *et al.*, 2006; Champion *et al.*, 2008). In addition, heterozygous *Vhl* knockout mouse shows increased angiogenic response toward bFGF *in vivo* (Champion *et al.*, 2008). Importantly, this endocytic function is at least partly independent of HIF function, as *Hif-α* knockdown could not rescue the cell motility phenotypes in multiple cell systems. However, pVHL has recently been shown to regulate endocytosis of EGF receptor via HIF-dependent suppression of a Rab5 effector, rabaptin-5, in ccRCC cell lines (Wang *et al.*, 2009). It is not known whether these different mechanisms are specific for different surface receptors.

In a separate study (Mortimer and Moberg, 2009), the tracheal branching phenotype in *dVHL* knockdown flies (mediated by trachea-specific expression of *dVHL* shRNA) was shown to be partly the result of over-expression of *sima*, the *Drosophila* homolog of *HIF-1α*. *sima* in turn stimulates the transcription of *btl* (*FGFR*), leading to ectopic branching. *sima*-dependent *btl* transcription has also been demonstrated in terminal branching in the larval trachea, which is induced by localized hypoxia (Centanin *et al.*, 2008). Thus, both canonical and non-canonical functions of *dVHL* are involved in the tubule formation in *Drosophila*.

The role of *dVHL* in regulating motile epithelial cells was further studied in *Drosophila* border cells (Doronkin *et al.*, 2010), which have been considered an *in vivo* model for epithelial cell invasion and epithelial–to–mesenchymal transition (Duchek *et al.*, 2001; McDonald *et al.*, 2003). During oogenesis, a specialized group of follicular epithelial cells (border cells) delaminates from the epithelium and invades through the germ cell complex until they reach the anterior end of the oocyte (Rorth, 2002; Montell, 2003). The precise movement of the border cells is guided by the *Drosophila* platelet-derived growth factor/VEGF signaling pathway. Interestingly, Doronkin *et al.* (2010) showed that the *dVHL-sima* system is involved in border cell migration. However, the exact correlation between border cell migration and *dVHL* function appears to be complicated, because the extents of hypoxia and the expression levels of *sima* or *dVHL* could cause either slowed or accelerated migration. Nonetheless, this study presented the first *in vivo* demonstration of the role of pVHL in regulating epithelial cell invasion.

Because *VHL* mutant ccRCC involves pathological transformation of epithelial tissues, whether and how *VHL* regulates epithelial morphogenesis has been of great interest. One useful epithelial model is again the *Drosophila* follicular epithelium in the egg chamber (Duchi *et al.*, 2010). Epithelial cells are characterized by asymmetrical specification of membrane domains. One crucial step in establishing epithelial polarity is the specification of the apical domain, which is defined by the localized accumulation of a complex containing atypical PKC (aPKC), Bazooka (Baz; mammalian and worm PAR-3) and PAR-6 (Suzuki and Ohno, 2006). The PAR complex is required for subsequent localization of the basolateral complex that consists of known tumor suppressors Discs Large, Lethal Giant Larvae and Scribble (Bilder *et al.*, 2003; Betschinger *et al.*, 2005), and the adherens junction (Yamanaka *et al.*, 2003). In mutant *dVHL* clones of follicle cells, microtubule bundles are disrupted and aPKC is mislocalized, leading to adenoma-like piling up of the epithelium (Duchi *et al.*, 2010). Consistent with the known human VHL activity (Hergovich *et al.*, 2003), wild-type dVHL can bind to microtubules. The direct influence of microtubule stability by dVHL leading to aPKC localization is supported by the *ex vivo* culture of *dVHL* mutant egg chambers, in which the aPKC mislocalization and epithelial phenotypes could be rescued by treatment with microtubule stabilizing agent paclitaxel, whereas treatment of wild-type egg chambers with microtubule destabilizing agent nocodazole could recapitulate the *dVHL* mutant phenotypes (Duchi *et al.*, 2010). Furthermore, although wild-type *dVHL* could rescue the phenotypes, the type 2A mutant Y98H (Y51H in *Drosophila*), which has

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been shown to lose the microtubule-stabilizing function but retain partly the HIF-degradation function (Hergovich *et al.*, 2003), could not. Therefore, the initial apical localization of aPKC requires intact microtubule bundles, which is maintained by dVHL protein (Figure 2). The *Drosophila* study is the first demonstration of physiological and developmental significance of the microtubule-stabilizing function of VHL *in vivo*.

Taken together, the *Drosophila* system has confirmed the HIF regulatory pathway, but unbiased genetic studies also revealed novel and important HIF-independent functions in endocytosis and microtubule-stabilizing functions in morphogenesis of both epithelial tubule system and polarized epithelial tissue. Also important, the *Drosophila* system demonstrated clearly tissue-specific functions of *VHL*; that is, the endocytic function of *dVHL* is required in tubule epithelial cells whereas the microtubule-stabilizing activity is functional in organcovering epithelium.

Zebrafish

Zebrafish genome encodes a *vhl* ortholog and a *vhl*-like gene; the latter is fish-specific (van Rooijen *et al.*, 2009). Two lines of *zvhl* nonsense mutants were isolated using the Targeting Induced Local Lesions in Genomes method (van Rooijen *et al.*, 2009). The mutant embryos develop to term and survive up to a week at the larval stage. These mutants exhibit behavioral and physiological hypoxic response in non-hypoxic conditions, including hyperventilation and increased cardiophysiological responses such as elevated heart rate (50% higher) and cardiac output (15-fold). As a result, the mutant larvae display cardiomegaly and stretched cardiomyocytes. They eventually develop edema and die. These symptoms are similar to but more severe than those in the familial Chuvash polycythemia patients, who carry a homozygous point mutation in the *VHL* gene (R200W; Gordeuk *et al.*, 2004). Molecularly, whole-embryo gene expression profiling using 7-d.p.f. (days post fertilization) animals showed increased expression of genes involved in anaerobic metabolism, oxygen sensing and angiogenesis, including *glut1*, *PHD3*, *Epo*, *Epo receptor*, *transferrin* and *vegf-a*. In *zvhl* mutant most of these genes are hypoxia-inducible and are HIF targets. Therefore, the main physiological defects in *zvhl* mutant are mediated by the canonical VHL–HIF axis. Significantly, as in nematodes and mammals, homologs of *egl-9* and *phy-2* are both prominently overexpressed in z*vhl* mutant. In z*vhl* mutant fish as in Chuvash disease patients, the polycythemic phenotype is mainly attributable to the increased HIF-α activity. The pseudo-hypoxic response in *zvhl* mutant is later confirmed, because the phenotypes of cardiovascular flexibility and oxygen consumption in *zvhl* mutant in normoxia could be reproduced by placing wild-type animal in extreme hypoxic conditions (Yaqoob and Schwerte, 2010).

More detailed phenotypic analyses revealed that the homozygous mutants display increased blood vessel formation (van Rooijen *et al.*, 2010). Significant increase in neovascularization was observed in the brain, eye and trunk. These abnormal vessels are leaky and can lead to macular edema and retinal detachment. Increased neovascularization is at least in part mediated by increased VEGF signaling, because treatment with VEGFR tyrosine kinase inhibitors could relieve the phenotypes. One important novel observation in the fish model is the increased activity of hematopoietic stem cells (HSCs), which not only increases the number of red blood cells, but also results in increased levels of endothelial progenitors in peripheral blood. This is highly interesting because in human hemangioblastoma, loss of heterozygosity of *VHL* occurs in stromal cells neighboring the proliferating blood vessels (Vortmeyer *et al.*, 1997) and these *VHL* mutant stromal cells display multipotent cell characteristics (Park *et al.*, 2007). The fish model may provide a clue to the origin of these mutant stromal cells.

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Another recent study of *zvhl* provided some important insights into potential cancer therapeutics. Inhibitors of mammalian target of rapamycin (mTOR) signaling have been used in clinical trials for treating ccRCC. This is based on the observation that mTOR can promote HIF-α translation (Thomas *et al.*, 2006; Wilhelm *et al.*, 2006; Cho *et al.*, 2007; Kaelin, 2009) and that mTOR signaling is upregulated in some cultured ccRCC cells because *VHL* mutant cells secret VEGF and platelet-derived growth factor-β, which activate PI3K-Akt-mTOR signaling (Wilhelm *et al.*, 2006; Clark, 2009). Therefore, inhibiting mTOR signaling may potentially reduce the expression level of HIF-α in *VHL* mutant cells. However, the efficacy of mTOR inhibitors has not proven satisfactory. The zebrafish model may provide a plausible answer to this discrepancy. TOR signaling is the main regulator of protein synthesis and is the primary target for control during energy stress (Ma and Blenis, 2009). One of the major energy stress response pathway is mediated by the liver kinase β1 (LKβ1) and AMP-activated protein kinase cascade (Alexander and Walker, 2011). A primary inhibitory target of AMP-activated protein kinase is TOR. Thus, during energy starvation AMP-activated protein kinase is activated by LKβ1 and it reduces protein synthesis by inhibiting TOR activity. Interestingly *zvhl* mutation can suppress the hypermetabolism phenotype of *lkβ* mutant, due to reduced TOR signaling in *zvhl* mutant (van der Velden *et al.*, 2011). This is presumably because persistent pseudo-hypoxic response in *zvhl* mutant cells leads to reduced TOR signaling via a yet unidentified alternative pathway. Therefore, if in physiological conditions, TOR signaling is already reduced in *VHL* mutant, additional TOR inhibitors is not likely to exert significant effects. This may explain the unsatisfactory efficacy of mTOR inhibitors in treating ccRCC.

Mouse

The mouse *Vhl* cDNA sequence was published in 1995 soon after the cloning of human *VHL* (Gao *et al.*, 1995). *In situ* RNA analysis showed that *Vhl* is expressed ubiquitously in embryogenesis but with enrichment in the epithelial tissues of the lung, kidney and eye (Kessler *et al.*, 1995). There are a number of mouse knockout models for *Vhl*, which have been described in an excellent review (Kapitsinou and Haase, 2008). Here I will highlight a few more recent attempts and discuss how they relate to the previous models. The first mouse knockout was generated as a constitutively null allele, which resulted in homozygous lethality at 10.5–12.5 days of gestation (Gnarra *et al.*, 1997). The lethality was attributed to defects in vasculogenesis in the placenta. This is the first indication that *VHL* is indeed a factor in vasculoangiogenesis, as predicted. One seemingly counter-intuitive nature of this phenotype is that the outcome is a lack of vasculature, instead of the expected increased vasculogenesis (due to over-expressed VEGF). This is likely because the *Vhl* mutant vascular cells in the placenta, as in *Drosophila* trachea (Adryan *et al.*, 2000), exhibit ectopic migratory activity, thus failing to interconnect and form vascular labyrinth.

The next knockout was a conditional allele (Haase *et al.*, 2001). Surprisingly, both heterozygous null (one *lox* allele knockout in embryonic stem cells) and hepatocyte-specific knockout (using *albumin* promoter-directed *Cre*) resulted in cavernous hemangiomas of the liver, which can be correlated with increased level of HIF-2α and increased expression of VEGF. In addition, hepatocyte-specific knockout mutant mice developed polycythemia due to increased erythropoietin production in the liver. Although no hyperplasia was induced in these early *Vhl* knockout models, they did provide the *in vivo* confirmation of the VHL–HIF pathway. Subsequently, the first kidney proximal tubule conditional knockout was generated using the *phosphoenolpyruvate carboxykinase* (*PEPCK*) driver. Proximal tubule-specific knockout was attempted because proximal tubules have long been considered the origin of ccRCC. Somewhat surprisingly, this model generated only modest phenotype of renal microcysts in about 25% of >12-month old mice but no tumors. This was nonetheless significant because cysts are believed to be the precursor of ccRCC. It is formally possible

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that other genetic factors are needed for tumorigenesis in *Vhl* mutants. On the other hand, a recent seminal study suggests that proximal tubule cells may not be the origin of ccRCC, because in human VHL patient samples, *VHL* mutant proximal tubule cells do not show capacity to proliferate (Mandriota *et al.*, 2002). More recently, *Ksp1.3-Cre* (expressed in distal tubules, ascending and descending loops of Henle and collecting ducts, but rarely in proximal tubules) was used to knockout *Vhl* outside of the proximal tubule domain (Frew *et al.*, 2008b). This conditional knockout alone yielded hydronephrosis but no abnormalities in the tubule epithelium. However, when combined with another tumor suppressor gene knockout *Pten*, the kidney displayed, in addition to hydronephrosis, hyperproliferation of the urothelium and high penetrance of enlarged kidneys due to multiple epithelial tubule cysts in the cortex and medulla. This model strengthened the notion that loss of *Vhl* alone is insufficient for tumor initiation. Similarly, *Vhl-Pten* double conditional knockout in male genital track generated cystadenoma and squamous metaplasia (Frew *et al.*, 2008a).

The vascular function of *VHL* was verified using endothelial cell-specific (*Tie2-Cre*-driven) knockout (Tang *et al.*, 2006). The endothelial knockout also confirmed the defect in fibronectin deposition previously demonstrated in cell culture (Ohh *et al.*, 1998; Feijoo-Cuaresma *et al.*, 2008). Acute tamoxifen-induced mosaic inactivation of the *Vhl* gene at E10.5 resulted in embryonic lethality between E14.5 and E15.0 with extensive hemorrhage and necrosis (Hong *et al.*, 2006). Liver damage and placental abnormalities were also observed, which confirmed the phenotypes of the previous *Vhl* knockouts. A number of subsequent conditional knockouts in other organs have shown defects in spermatogenesis, thymus cell survival and bone development due to reduced cell proliferation and/ or increased cell death (Ma *et al.*, 2003; Biju *et al.*, 2004; Pfander *et al.*, 2004). More recently, *Vhl* has also been shown to influence the activity of HSCs (Takubo *et al.*, 2010), which reside in hypoxic niches in the bone marrow and maintain a quiescent state. In *Hif-1α*-conditional allele (driven by interferon-inducible *Mx1-Cre*) mice, the HSCs lost cell cycle quiescence and their numbers decreased during various stress settings including bone marrow transplantation. Over-stabilization of HIF-1α in conditional biallelic loss of *Vhl* induced cell cycle quiescence in HSCs and their progenitors, as expected, but unexpectedly also resulted in impairment in bone marrow transplantation capacity, probably due to over-quiescence. In contrast, heterozygous loss of *Vhl* induced cell cycle quiescence in HSCs but improved (over-wild type) bone marrow engraftment after transplantation. It is possible that *VHL* modulates HSC activity by both HIF-dependent and independent mechanisms, and that the precise levels of its target proteins are critical. In this regard, it will be important to take into account the heterozygosity of *VHL* in the VHL disease patients, especially concerning the hematopoietic and vascular components of the disease manifestations.

The metabolic imbalance in *VHL* mutant cells due to HIF-mediated glycolic switch has attracted intensive interest, because this may present a new therapeutic approach by forcing oxidative metabolism on the mutant cells. A recent knockout model provided the physiological proof of glycolic switch in *Vhl* mutant cells (Zehetner *et al.*, 2008). In this study, *Vhl* was conditionally knocked out in β cells of the pancreas. The mice developed impaired systemic glucose tolerance. This, intriguingly, can be attributed to the false sense of glucose shortage by the β cells because these *Vhl* mutant cells undergo glycolic switch and respond by increasing insulin secretion. In another study (Shen *et al.*, 2009), intending to model the pancreatic cystadenoma of VHL disease, *Vhl* knockout in either α or β cells did not result in cysts formation. However, when *Vhl* was knocked out in the pancreatic progenitor cells using *Pdx1-Cre*, highly vascularized microcystic adenomas and hyperplastic islets did develop, thus representing the success modeling of one VHL disease-associated tumor phenotype.

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One other intriguing disease model is the human polycythemic Chuvash disease. These patients inherit a homozygous *VHL* mutation that is incapable of down-regulate HIF-α, but they show no increased tumor incidences (Gordeuk *et al.*, 2004). A mouse model for the Chuvash disease was generated by knocking in the R200W (R166W in mouse) mutant *Vhl* allele (Hickey *et al.*, 2010). This mouse recapitulates the polycythemic phenotype of the human disease and exhibits pulmonary hypertension and lung fibrosis in a *Hif-2α*-dependent manner. The latter phenotypes also manifest in some Chuvash patients (Bushuev *et al.*, 2006; Smith *et al.*, 2006), which can be induced in hypoxic conditions (Stenmark *et al.*, 2006). The fibrotic phenotype recalled an earlier knockout strain specific for the podocyte in kidney, which exhibited glomerulomegaly and occasional glomerulosclerosis (Brukamp *et al.*, 2007). Interestingly, the earlier *PEPCK*-driven *Vhl* knockout could be induced to develop renal fibrosis after subtotal nephrectomy of one kidney and complete removal of the other (Kimura *et al.*, 2008). In addition, hypoxia and increased Hif-1α activity have been linked to kidney fibrosis in mouse (Higgins *et al.*, 2007). The connection between *Vhl* mutant cells and inflammation is worthy of more in-depth investigation, because prolonged inflammation can promote proliferation through the action of secreted cytokines, and importantly, can induce genetic changes in pre-cancerous cells by induction of reactive oxygen species or by oxidative inactivation of mismatch repair enzymes (Hussain *et al.*, 2003; Colotta *et al.*, 2009; Kraus and Arber, 2009; Grivennikov *et al.*, 2010).

The information obtained from the above model organisms relevant to the epithelial and vascular phenotypes in VHL disease patients is summarized in Figure 3.

Conclusion and perspective

The studies in model organisms are summarized in Table 1. They have provided physiological proof of the VHL–HIF regulatory pathway. The ancestral function of *C. elegans vhl* suggests a role as a metabolic regulator in normal physiological condition and in hypoxia. Also importantly, potentially significant *VHL* functions in microtubule stabilization and endocytosis found physiological relevance in morphogenesis of organ-covering and tubular epithelia in the *Drosophila* system. New insights such as a potential role in hematopoietic maintenance and metabolic homeostasis were revealed in zebrafish and mouse systems. *VHL* has proved a unique and enigmatic tumor suppressor gene. Research in the past 20 years consisting of over 2000 publications has revealed that pVHL is a multi-functional protein. Accumulated evidence also suggests that, although the apparent pleiotropic functions of pVHL may have been the hindrance to a full understanding of its role in tumorigenesis, it is likely the combined loss of some of these functions that underlie the initiation of VHL tumors. To fully resolve the VHL disease mechanism, a systemic view of the disease process is essential to understand such complex problems as the tissue specificity of VHL tumors, metabolic abnormality, hematopoietic over-activation, and inflammation. Undoubtedly model systems will continue to have a critical role in elucidating the functions of this challenging but fascinating tumor suppressor gene.

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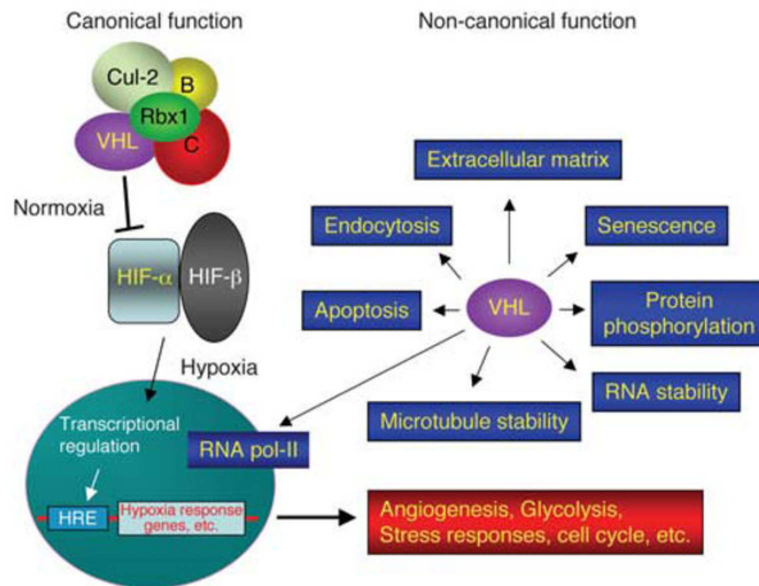

Figure 1.

Pleiotropic VHL functions. See text for detail. Canonical VHL functions denote the degradation of HIF-α in normoxia through the E3 ubiquitin ligase activity. The E3 ligase complex contains VHL, Rbx1, cullin-2 (Cul-2) and elongin B and C (B, C). In hypoxia, HIF-α is stabilized, form active transcription factor complex with HIF-β and regulates gene expression via the hypoxia-responsive element (HRE). Non-canonical VHL functions denote those independent of HIF activity. Some, but not all, of these non-canonical functions are listed (in blue boxes).

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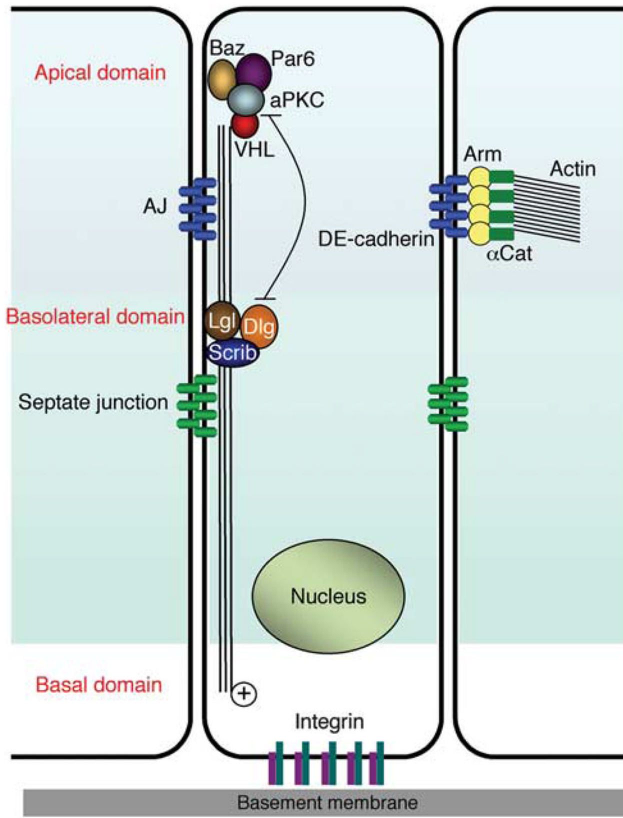

Figure 2.

Microtubule-stabilizing function of dVHL contributes to epithelial morphogenesis. See Duchi *et al.* (2010) for detailed experimental evidence. Schematics showing polarized follicle cells. In many organ-associated epithelial cells such as the follicle cells, microtubules are not centrosome anchored, but are instead organized in cortical bundles parallel to the apicobasal axis, with the plus-end localized basally (Bartolini and Gundersen, 2006). VHL interacts with microtubule and aPKC. Microtubule bundles are stabilized by VHL and are important for proper localization of the aPKC–Par6–Baz complex. aPKC–Par6–Baz complex prevents apical spreading of the Dlg–Lgl–Scrib complex, which in turn restricts aPKC–Par6–Baz complex to the apical region. The opposing actions of aPKC–Par6–Baz complex and Dlg–Lgl–Scrib complex help define the location of adherens junction (AJ). AJ, adherens junction; Arm, Armadillo (Drosophila homolog of β-catenin); α-Cat, α-catenin; Baz, Bazooka; Dlg, discs large; Lgl, lethal giant larvae; Scrib, scribble. Septate junction is an adhesion complex similar in function to the mammalian tight junction.

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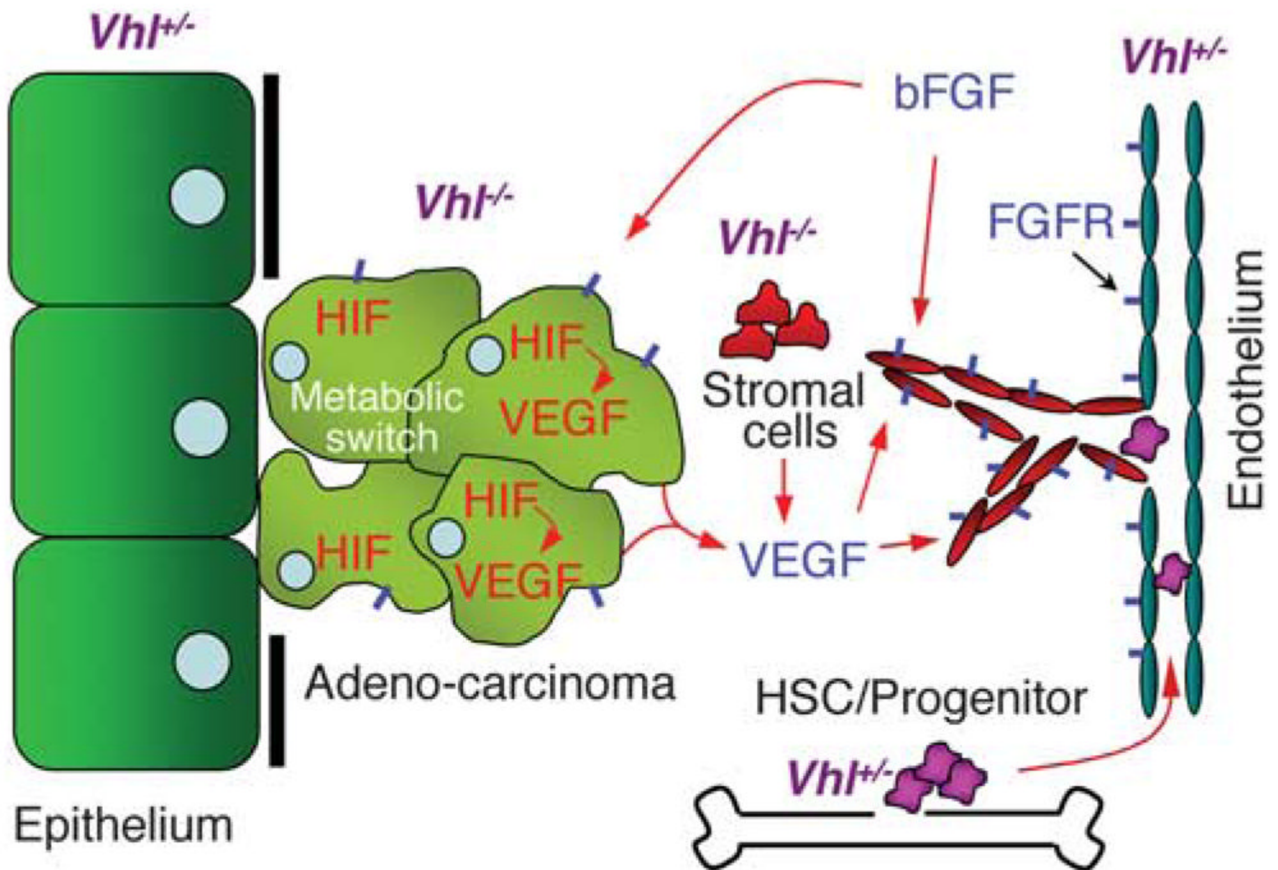

Figure 3.

Epithelial–stromal interaction in VHL disease. The model is based on *VHL* functions verified in the model organisms and is focused on the epithelial and vascular phenotypes. Homozygous *VHL* mutant epithelial cells are shown losing epithelial characteristics (Duchi *et al.*, 2010; Hsouna *et al.*, 2010). These cells as well as heterozygous *VHL* mutant endothelial cells overexpress FGFR (short blue bar) on the surface, contributing to their increased response to the chemotactic bFGF ligand (Dammai *et al.*, 2003; Champion *et al.*, 2008; Hsouna *et al.*, 2010). Mutant *VHL* cells overexpress HIF-α, leading to overproduction of VEGF, which in turn induces angiogenesis (van Rooijen *et al.*, 2009, 2010) as well as metabolic switch that mimic hypoxic response (Bishop *et al.*, 2004; Shen *et al.*, 2005; Zehetner *et al.*, 2008; van Rooijen *et al.*, 2009). Homozygous *VHL* mutant 'stromal cells' neighboring the blood vessels, although not yet identified in model organisms, have been shown in human patients, presumably inducing angiogenesis via increased VEGF secretion (Vortmeyer *et al.*, 1997; Park *et al.*, 2007). Heterozygosity of *VHL* in the HSCs and resultant endothelial progenitor cells exhibit increased activity (Takubo *et al.*, 2010; van Rooijen *et al.*, 2010) and potentially can contribute to neovasculogenesis.

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Table 1

Summary of VHL functions revealed in model organisms

Species	Functions	References
Caenorhabditis elegans	Evolutionary conservation	(Woodward *et al.*, 2000)
	Identification of HIF prolyl hydroxylase	(Epstein *et al.*, 2001)
	Regulation of hypoxic response	(Bishop *et al.*, 2004; Shen *et al.*, 2005)
	Regulation of energy-conserving metabolic pathways	(Bishop *et al.*, 2004; Shen *et al.*, 2005)
Drosophila	VHL-elongin B/C complex formation	(Adryan *et al.*, 2000; Aso *et al.*, 2000)
	Regulation of vasculogenesis	(Adryan *et al.*, 2000; Centanin *et al.*, 2008; Mortimer and Moberg, 2009; Hsouna *et al.*, 2010)
	Regulation of hypoxic response	(Arquier *et al.*, 2006; Centanin *et al.*, 2008; Mortimer and Moberg, 2009)
	Regulation of endocytosis	(Hsouna *et al.*, 2010)
	Regulation of epithelial cell motility	(Doronkin *et al.*, 2010; Hsouna *et al.*, 2010)
	Microtubule stabilization in epithelial morphogenesis	(Duchi *et al.*, 2010)
Zebrafish	Regulation of vasculo/angiogenesis	(van Rooijen *et al.*, 2009, 2010)
	Regulation of hypoxic response	(van Rooijen *et al.*, 2009, 2010; Yaqoob and Schwerte, 2010)
	Polycythemic phenotype in mutant	(van Rooijen *et al.*, 2009)
	Regulation of hematopoietic stem cell activity	(van Rooijen *et al.*, 2009, 2010)
	Reduced TOR signaling in mutant	(van der Velden *et al.*, 2011)
Mouse	Regulation of vasculo/angiogenesis	(Gnarra *et al.*, 1997; Hong *et al.*, 2006; Tang *et al.*, 2006)
	Regulation of fibronectin deposition	(Tang *et al.*, 2006)
	Cystic/adenoma growth in mutant	(Rankin *et al.*, 2006; Frew *et al.*, 2008a, b; Shen *et al.*, 2009)
	Metabolic homeostasis	(Zehetner *et al.*, 2008)
	Polycythemic phenotype	(Haase *et al.*, 2001; Hickey *et al.*, 2010)
	Regulation of hematopoietic stem cell activity	(Takubo *et al.*, 2010)
	Inflammation/fibrosis phenotypes	(Brukamp *et al.*, 2007; Kimura *et al.*, 2008; Hickey *et al.*, 2010)

Abbreviations: HIF, hypoxia-inducible factor; TOR, target of rapamycin; VHL, von Hippel–Lindau tumor suppressor gene.