Published in final edited form as:
Curr Pharm Des. 2009 ; 15(33): 3895–3903.

The VHL Tumor Suppressor: Master Regulator of HIF

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Abstract

Hypoxia-inducible factors (HIFs) are heterodimeric oxygen-sensitive basic helix-loop-helix transcription factors that play central roles in cellular adaptation to low oxygen environments. The von-Hippel Lindau tumor suppressor (pVHL) is the substrate recognition component of an E3 ubiquitin ligase and functions as a master regulator of HIF activity by targeting the hydroxylated HIF-alpha subunit for ubiquitylation and rapid proteasomal degradation under normoxic conditions. Mutations in pVHL can be found in familial and sporadic hemangioblastomas, clear cell carcinomas of the kidney, pheochromocytomas and inherited forms of erythrocytosis, illustrating the importance of disrupted molecular oxygen sensing in the pathogenesis of these diseases. Tissue-specific gene targeting of pVHL in mice has demonstrated that efficient execution of HIF proteolysis is critically important for normal tissue physiology, and has provided novel insights into the functional consequences of HIF activation on the cellular and tissue level. Here we focus on the contribution of individual HIF transcription factors to the development of VHL phenotypes and discuss how the pVHL/HIF axis could be exploited pharmacologically.

Keywords

von Hippel-Lindau (VHL) tumor suppressor; hypoxia-inducible factor (HIF); renal cell cancer; hemangioblastoma; erythropoietin; anemia; metabolism; kidney cysts; mouse model

INTRODUCTION

Patients with germ line mutations in the von Hippel-Lindau (VHL) tumor suppressor are affected by a rare familial tumor syndrome, which is characterized by the predisposition to develop highly vascularized tumors in multiple organs. These include hemangioblastomas of the retina and central nervous system, renal cancer of the clear cell type (CC-RCC) and pheochromocytomas [1]. VHL disease is grouped into 2 subtypes depending on the presence or absence of pheochromocytoma. Different clinical subtypes are associated with specific *VHL* mutations, which result in distinct functional and biochemical properties of the mutated *VHL* gene product, pVHL [2–4]. Biallelic *VHL* inactivation has been documented in the majority of sporadic CC-RCCs [5], but is less prominent in sporadic hemangioblastomas. pVHL has multiple functions. A major function is serving as the substrate recognition component of an E3 ubiquitin ligase, which ubiquitylates and targets the α-subunit of hypoxia-inducible factor (HIF) for oxygen-dependent proteolysis [6–18]

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AUTHOR STATEMENT
The author states that no conflict of interest exists.

(Fig. (1)). Therefore either loss of *VHL* expression as a result of gene deletion or promoter hypermethylation, or mutations in pVHL that affect its ability to capture and/or ubiquitylate HIF-α result in constitutive HIF stabilization and activation of HIF controlled transcriptional programs irrespective of oxygen levels. The pVHL/HIF-α interaction is highly conserved between species, underscoring its importance in molecular oxygen sensing. In order to examine the functional role of pVHL-mediated HIF proteolysis and to gain insights into the physiological consequences of constitutive HIF activation during embryonic development, and in the adult, we and other laboratories have used Cre-loxP recombination to generate mice with cell type- and tissue-specific *VHL* inactivation. Here, we discuss the contribution of individual HIF transcription factors to the development of the VHL phenotype and how the VHL/HIF axis could be exploited therapeutically.

pVHL: MASTER REGULATOR OF HIF

HIFs belong to the PAS (Per-ARNT-Sim) family of heterodimeric basic helix-loop-helix (bHLH) transcription factors. They regulate gene expression by binding to hypoxia-response elements (HREs), specific DNA recognition sequences located in hypoxia enhancer containing regulatory regions of HIF target genes, and consist of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, also known as the arylhydrocarbon receptor nuclear translocator (ARNT) or HIF-β [19, 20]. There are three known HIF α-subunits, HIF-1α, HIF-2α and HIF-3α, [18, 21], which are all are targeted by pVHL. Whereas HIF-1α and HIF-2α heterodimers function as transcriptional activators, the role of HIF-3α in transcriptional activation is less clear. Splice variants of HIF-3α have been shown inhibit HIF- dependent transcriptional activation [22, 23]. The spectrum of HIF-1 and HIF-2 regulated biological functions overlaps only partially. Anaerobic glycolysis, for example, appears to be predominantly controlled by HIF-1 [24], whereas HIF-2 appears to be the main regulator of erythropoietin (EPO) production and seems to have a distinct role in VHL-associated tumorigenesis [25–29].

pVHL-mediated polyubiquitylation of HIF-α requires hydroxylation on specific proline residues (Pro402 and Pro564 in human HIF-1α; Pro405 and Pro531 in human HIF-2α) within its oxygen-dependent degradation domain (ODD) [30–36] and is necessary for its rapid proteasomal degradation under normoxic conditions. Hydroxylation of HIF-α is carried out by 2-oxoglutarate-dependent dioxygenases (prolyl-4-hydroxylase domain (PHD) proteins) and requires molecular oxygen, ferrous iron and ascorbate [37]. A defect in the ability to ubiquitylate HIF-α, i.e. as a consequence of mutated pVHL or loss of *VHL* expression, results in HIF-α stabilization, increased HIF transcriptional activity and up-regulation of HIF target genes such as *vascular endothelial growth factor (VEGF)*, *glucose transporter 1* (*GLUT-1)* and *EPO* irrespective of oxygen levels. In addition to heterodimerization with HIF-β, HIF-α modulates cellular signaling pathways through functional interaction with non-PAS domain proteins. These include, among others, tumor suppressor protein p53, the c-Myc proto-oncogene and the Notch intracellular domain [38–41]. These and other HIF-α/non-PAS domain protein interactions have to be considered when interpreting phenotypes associated with *VHL* inactivation. A second hypoxic switch operates in the carboxy-terminal transactivation domain of HIF-α with oxygen dependent asparagine hydroxylation by factor-inhibiting-HIF (FIH) blocking CBP/p300 recruitment. Inhibition of FIH under hypoxic conditions facilitates CBP/p300 recruitment, resulting in increased HIF target gene expression in *VHL*-deficient cell lines or under pronounced hypoxia [42–45].

Certain mutations in the *VHL* gene result in the development of familial polycythemia from excessive EPO production [46], but not in tumorigenesis. Chuvash polycythemia, a rare autosomal-recessive disease, which is endemic in central European Russia, is mostly

associated with a mutation in *VHL* codon 200 (C598T ⇒ R200W). Affected individuals, who are usually homozygous for this hypomorphic mutation (compound heterozygotes with other mutations have also been reported), are not predisposed to the development of tumors that are typically associated with VHL disease [47]. When expressed in murine ES cells (R166W) or in *VHL*-defective renal carcinoma cells the pVHL R200W mutant retained the ability to target HIF-α (HIF-1α > HIF-2α) for proteasomal degradation, but at reduced efficiency [48], whereas *VHL* alleles (e.g. *VHL* type 1 mutations) that are highly associated with the development of renal cell cancers have completely lost their ability to capture HIF-α for degradation [3, 4].

HIF-INDEPENDENT FUNCTIONS OF pVHL

The identification of HIF-independent pVHL functions and discovery of novel pVHL interacting proteins has provided important molecular insights into the pathogenesis of VHL-associated clinical phenotypes and represents an important step towards a complete understanding VHL-associated tumorigenesis. pVHL has been shown to regulate microtubule stability and is important for cilia maintenance [49–52]. It furthermore controls the stability of plant homeodomain protein Jade-1 [53, 54]. These observations provide a molecular basis for the pathogenesis of VHL-associated cysts, which are prominent visceral manifestations of VHL disease in the kidney and pancreas. Cystogenesis has been associated with the loss of the primary cilium, a microtubule based organelle, which functions as a luminal flow- and potential chemosensor on epithelial cells, and is also found on many other cell types including neurons, photoreceptors and fibroblasts [55]. Recent studies indicated that pVHL cooperates with glycogen synthase kinase (GSK)-3β in an interlinked signaling pathway that maintains the primary cilium [50]. This notion is supported by findings in knockout mice where inhibition of the PTEN tumor suppressor, which activates PI3K-Akt signaling thereby inhibiting GSK-3 activity, promotes urogenital cyst formation in a pVHL defective background [56, 57]. While these studies suggested HIF independence, Esteban *et al.* proposed a role for HIF in cilium maintenance [58]. Jade-1 is a recently identified pVHL interacting protein that links pVHL to the cysto- and oncogenic β-catenin signaling pathway (pVHL suppresses β-catenin signaling through Jade-1) [59]. Other pVHL modulated cellular processes or signaling pathways that are likely to be important for VHL-associated tumorigenesis and tumor progression include extra-cellular matrix (ECM) assembly and turnover [60–65], the formation of intracellular junctions [66], signaling through atypical protein kinase C isoforms [67–71] and NF-κB [72], c-Met receptor responsiveness to hepatocyte growth factor (HGF) [63, 73], and signaling through the regulation of p53 transcriptional activity [74]. In addition, pVHL has been shown to interact with KRAB-A domain protein, VHLak, repressing HIF transcriptional activity [75], deubiquitylating enzymes [76], the large subunit of RNA polymerase II [77] and the RNA-binding protein hnRNP A2 [78]. The role of these proteins in the pathogenesis of VHL-associated tumors, however, is unclear and awaits further study.

pVHL IS REQUIRED FOR NORMAL EMBRYONIC DEVELOPMENT

Germ line inactivation of pVHL in mice results in embryonic death during midgestation primarily from abnormal placental vascularization. Placentae from *VHL*-deficient embryos lack properly developed syncytiotrophoblasts and labyrinths and show evidence of hemorrhage by embryonic day 11.5 to 12.5 [79]. This phenotype appears to be largely HIF-mediated, as germ line inactivation of PHD2 result in similar, but not identical pathology [80], which may indicate HIF independent functions of either pVHL and/or PHD2 during placental development. pVHL is not only essential for normal placental development, but plays a critical role in the development, growth and differentiation of many other tissues. For example, tissue-specific inactivation of pVHL in neuro-epithelial progenitor cells results

in abnormal neuronal differentiation and embryonic lethality during late gestation (V.H. Haase, unpublished data), and chondrocyte specific inactivation of pVHL in the growth plate causes stunted bone growth, most likely a result of a HIF dependent increase in cell cycle inhibitor $p57^{kip2}$ [81].

HIF AND THE VHL PHENOTYPE

VHL haploinsufficincy (*VHL +/−*) in the murine germ line predisposes mice to the development of cavernous liver hemangiomas [82], which is strongly dependent on genetic background [83], and represents a rare manifestation of VHL disease in human patients [84, 85]. Since hepatocyte-specific deletion of *VHL*, phenocopied the liver pathology found in heterozygous mice, it is most likely that, following Knudson's two-hit hypothesis, inactivation of the remaining *VHL* wild-type allele resulted in the formation of cavernous hemangiomas through uncontrolled and HIF-dependent vascular growth factor production in hepatocytes and not endothelial cells (Fig. (2)). This is mechanistically similar to human VHL-associated hemangioblastomas, in which "stromal cells" and not endothelial cells are *VHL*-deficient and represent the neoplastic component of these tumors [86]. Inactivation of HIF signaling (pVHL/ARNT double mutants) resulted in a rescue of the liver phenotype and restored normal hepatic gene expression [26], indicating that the effects of *VHL* inactivation in hepatocytes are entirely HIF dependent. In keeping with this notion, forced expression of non-degradable HIF-1α and HIF-2α phenocopied VHL phenotypes in liver and skin [87].

To determine the role of individual HIF transcription factors (HIF-1 versus HIF-2) in the development of VHL-associated liver hemangiomas, we performed genetic studies with *VHL* knockout mice, which also lacked HIF-1α, HIF-2α or both in hepatocytes. Analysis of these mice demonstrated that inactivation of HIF-2α was sufficient to prevent the development of cavernous hemangiomas, whereas deletion of HIF-1α did not affect vascular tumorigenesis [29]. This HIF-2-dependent vascular tumor phenotype correlated with a HIF-2-dependent increase in angiogenic gene expression [27, 29], and is in contrast to other *VHL*-deficient organs, where vascular phenotypes (increased vascular density, but not tumors) were found to be HIF-1-dependent [88], indicating that the ability of HIF-1 and HIF-2 to regulate common target genes is tissue- and context-dependent [89]. Whether preferential recruitment of HIF-1 or HIF-2 to hypoxia enhancer elements requires tissue-specific transcriptional co-activators, certain DNA or protein modifications, absence of a recently postulated titratable repressor or other signaling events awaits further investigation [90]. The findings from the genetic analysis of mouse hemangioma development are in line with clinical studies in human patients, which have demonstrated a correlation between HIF-2α expression and the development of VHL-associated angiogenic lesions [91], and identify HIF-2 as a potential therapeutic target for the treatment of these tumors.

Inactivation of pVHL in hepatocytes furthermore results in the development of HIF-2-dependent steatosis (accumulation of neutral fat in hepatocytes) and erythrocytosis (Fig. (2)). Lipid accumulation is also found in stromal cells, the neoplastic components of VHL-associated hemangioblastomas [92], and in CC-RCCs, which are distinguished histologically from other types of renal cancer by the presence of 'clear' cytoplasm resulting from the washout of lipids during tissue processing.

Renal cysts, a major visceral manifestation of VHL disease in human patients [1, 93], were only found at very low frequency in *VHL +/−* mice (<5%) [82]. VHL-associated renal cysts can be malignant or benign, and should be viewed as pre-neoplastic lesions. Mice with tissue-specific inactivation of pVHL in the proximal renal tubule develop renal cysts at a frequency of ~20%, however CC-RCCs were not observed in these studies [82, 83, 94] (Fig. (3)). Renal cystogenesis was found to be HIF-, but not HIF-1-dependent, suggesting that

HIF-2 may act as a cystogenic transcription factor [94]. The notion of HIF having cystogenic properties is supported by *in vivo* experiments with CC-RCC cell lines where re-introduction of wild-type pVHL restored primary cilia in a HIF dependent manner [58], and by a mouse model of hereditary leiomyomatosis and renal cancer syndrome (HLRCC), where a genetic defect in fumarate hydratase, which results in HIF-α stabilization (fumarate inhibits HIF prolyl-hydroxylation, see Fig. (1)), led to kidney cyst development in aged mice [95]. These findings, however, would have to be reconciled with reports that suggest HIF-independent mechanisms by which pVHL suppresses cyst formation [50–52].

The absence of CC-RCC development in *VHL*-deficient mice in conjunction with genetic data from human patients indicates that transformation of cystic epithelium into CC-RCC requires additional genetic events, such as mutations in other tumor suppressor genes or oncogenes. Loss of pVHL function and stabilization of HIF-α, however, represent the earliest detectable molecular events in renal tumorigenesis [96], resulting in cellular alterations, which ultimately facilitate transformation to and progression of CC-RCC. These include HIF-dependent and HIF-independent loss of intercellular junctions and epithelial de-differentiation resulting from E-cadherin suppression [66, 97–99], HIF-dependent and HIF-independent alterations in p53, c-Myc or NF-κB activity [38, 39, 72, 74, 100], HGF signaling [63, 73, 101], increased susceptibility to transforming growth factor (TGF)-α/ epidermal growth factor (EGF) signaling [89, 102–106], as well as modifications in ECM turnover and re-modeling [60–65].

The importance of HIF activation in CC-RCC pathogenesis and growth is underscored by reports, which demonstrated that inhibition of HIF-α translation correlated with reduced tumor growth [107] and that the expression of certain HIF target genes, such as CXC chemokine receptor-4 (CXCR4) was associated with disease progression [108]. Since a bias towards HIF-2α expression was found in clinical CC-RCC samples with confirmed *VHL* defect and in CC-RCC cell lines [18, 109], VHL-associated tumor development may depend on de novo expression of HIF-2α or a shift in the ratio of HIF-1α versus HIF-2α levels towards an increase in HIF-2α (HIF-2α is not detectable in non-transformed renal epithelial cells following hypoxia/ ischemic injury [110]). In support of this notion are studies in CC-RCC cell lines, which indicate that HIF-2 is oncogenic and is able to override pVHL's tumor suppressor function by regulating molecular pathways that are critical for renal cell growth, such as signaling through the TGF-α/EGF receptor pathway, cyclin D1 and the c-Myc proto-oncogene [89, 100, 103–106, 111–115]. Taken together, there is substantial evidence that HIF-1 and HIF-2 have diverse functions with regard to VHL-associated renal tumorigenesis, which offers potential for therapeutic exploitation.

THE VHL/HIF AXIS AS A THERAPEUTIC TARGET

Pharmacological targeting of the VHL/HIF/PHD axis offers enormous opportunities for the treatment of anemia and ischemic disorders, since HIF activation induces EPO and appears to mediate the tissue protective effects of ischemic preconditioning [116]. HIF hydroxylation is a prerequisite for binding to the pVHL-E3 ubiquitin ligase complex and proteasomal degradation in the presence of oxygen. Stabilization of HIF-α under normoxia results from the inability to hydroxylate HIF-α or to efficiently ubiquitylate hydroxylated HIF-α, which can be inherited or somatically acquired as is the case in HLRCC, VHL disease and in sporadic CC-RCCs, or it can result from pharmacological inhibition. Analogs of 2-oxoglutarate, which is the substrate for PHD enzymes (PHD1, PHD2, PHD3 are the three major HIF prolyl-4-hydroxylases) and FIH, have been successfully used for the stimulation of endogenous EPO production *in vivo*, and furthermore have the potential to improve the clinical outcome of acute ischemic injuries as demonstrated in animal models [117–119]. Some compounds have entered clinical trials for the treatment of renal anemia

(patients with chronic kidney disease loose the ability to produce adequate amounts of EPO) and are awaiting safety and efficacy evaluations.

In theory, it is conceivable that small molecule compounds could also be used to disrupt the VHL/HIF-α interaction, resulting in temporary HIF-α stabilization, while HIF-independent tumor suppressor functions of pVHL would be left intact. Hydroxylated HIF-α interacts with the β-domain of pVHL, which spans amino acid residues 64–154. This strategy was successfully used to stabilize HIF-α in cell lines using an engineered protein that contained the HIF-ODD fused to green fluorescent protein and resulted in disruption of the pVHL interaction with endogenous HIF-α [112].

In mouse models, our laboratory has shown that genetic inactivation of pVHL in hepatocytes results in HIF-2-dependent erythrocytosis from constitutive EPO production (a 2- to 3-fold rise in serum EPO levels is sufficient to produce erythrocytosis) [27]. This finding indicated that the VHL/HIF axis could be exploited therapeutically in clinical situations, where the ability of the kidney to generate adequate amounts of EPO is impaired, i.e. anemia of chronic kidney disease (the kidney is the main physiologic source of EPO in adults). Since constitutive activation of hepatic HIF can result in severe organ pathology, such as steatohepatitis and hemangioma development, pharmacological targeting of the VHL/HIF-α interaction would have to be intermittent in order to suppress these non-desirable on-target effects. Central questions in the design of strategies that aim at harnessing the VHL/HIF/PHD pathway for therapeutic purposes relate to whether intermittent normoxic HIF stabilization and activation of HIF controlled transcriptional programs is oncogenic or has other adverse clinical effects on the human body, such as alterations in glucose and fat metabolism. Both, inhibiting HIF prolyl-4-hydroxylation with 2-oxoglutarate analogs and disruption of the pVHL/ HIF-α interaction face the same clinical challenges and questions as far as HIF activation and patient safety is concerned. Aside from safety issues relating to HIF, disruption of the pVHL/HIF-α interaction has the potential to affect HIF-independent tumor suppressor functions of pVHL, which would have to be evaluated carefully before this strategy to activate HIF can be considered as an alternative to PHD inhibition.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Over the last 10 years major advances have been made in understanding molecular functions of the VHL tumor suppressor as they relate to tumorigenesis and normal physiology. Although targeting of HIF-α for proteolysis appears to be the dominant biological function of pVHL, there is growing evidence for pVHL regulated cellular processes that are critical for tumorigenesis and do not involve HIF signaling. It is likely that a direct comparison of PHD and pVHL deficiency phenotypes in the absence or presence of HIF will help to identify additional HIF-independent biological functions of either pVHL or PHDs.

Mouse models, clinical and *in vitro* studies have been used to define the role of individual HIF transcription factors in the development of VHL phenotypes, and studies with pVHL knockout mice have helped to identify novel HIF regulated cellular processes and target genes. These investigations have established that HIF-1 and HIF-2, although structurally closely related, have distinct cell type- and context-dependent biological functions with potential for therapeutic exploitation. In the non-oncologic setting, pharmacological targeting of the VHL/HIF/PHD axis with the goal to activate HIF under normoxic conditions, may be useful in the treatment of anemia and ischemic disorders in the short term and is currently undergoing clinical evaluation. Whether long-term treatment with HIF stabilizing compounds is safe will have to be carefully established.

Acknowledgments

VHH is supported by the Krick-Brooks chair in Nephrology and by grants from the National Cancer Institute (NCI) and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

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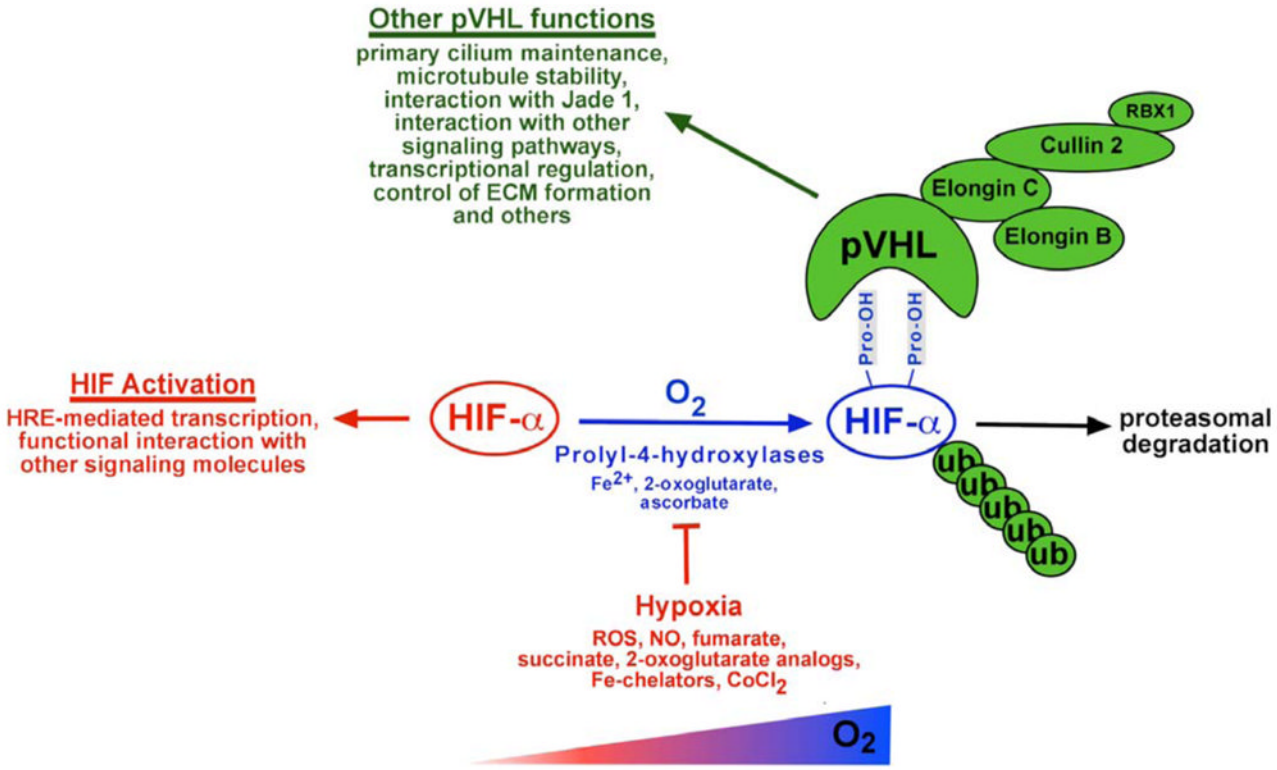

Fig. 1.

pVHL: master regulator of HIF. Schematic overview of pVHL functions. Aside from targeting HIF-α for proteasomal degradation, pVHL has multiple other functions. These include maintenance of the primary cilium, regulation of microtubule stability and interactions with several other signaling pathways. Binding to hydroxylated HIF-α occurs at the β-domain of pVHL, which spans amino acid residues 64 – 154. The C-terminal α-domain links pVHL *via* elongin C to the E3 ubiquitin ligase. Indicated are also conditions and molecules, which inhibit HIF prolyl-hydroxylation. Abb.: $CoCl_2$, cobalt chloride; NO, nitric oxide; ROS, reactive oxygen species; ub, ubiquitin.

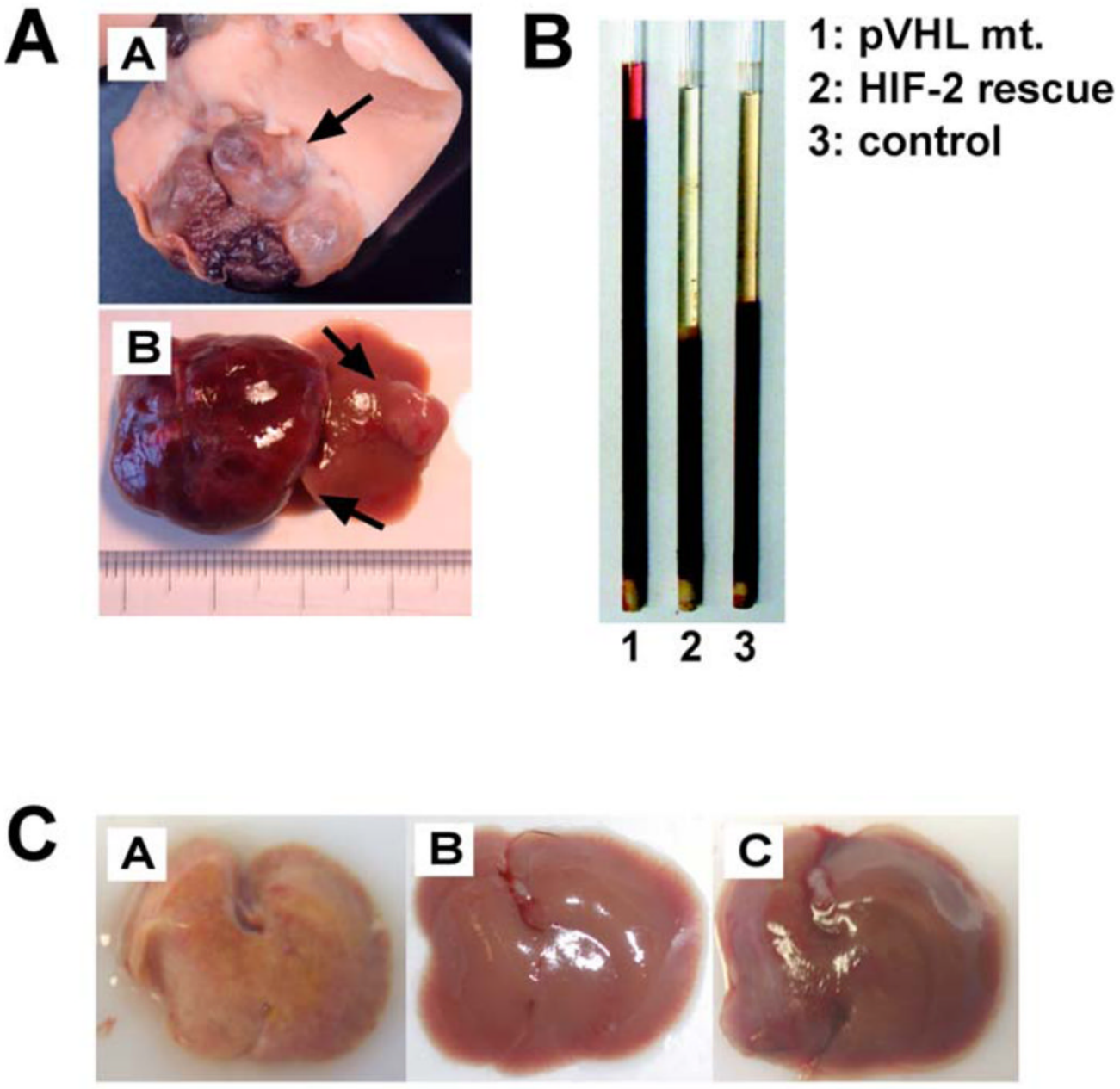

Fig. 2.

Functional consequences of pVHL inactivation in hepatocytes. Inactivation of pVHL in hepatocytes results in multiple phenotypes. A. VHL-associated hemangiomas. Mice with germ line haploinsufficiency for pVHL (*VHL +/−)* develop cavernous liver hemangiomas (A). Hemangioma development (arrows) also occurs when pVHL is inactivated conditionally using Cre-loxP recombination. 20–30 % of hepatocytes are targeted in the case shown in (B). B. Erythrocytosis. Hepatocytes are capable of producing EPO when HIF is stabilized. EPO production in the liver is HIF-2-dependent and inactivation of HIF-2 in pVHL deficient livers restores normal erythropoiesis. Shown are spun micro-hematocrits. 1: pVHL mutant mouse (20–30% of hepatocytes are targeted) with hematocrit > 90%; 2: pVHL mutant animal in which HIF-2 was inactivated simultaneously; 3: control mouse. C.

Steatohepatitis. Inactivation of pVHL in > 80% of hepatocytes results in severe HIF-2-dependent steatohepatitis, illustrated by the yellow discoloration of the liver. (A), pVHL mutant; (B), pVHL/HIF-2 double mutant liver; (C), control liver.

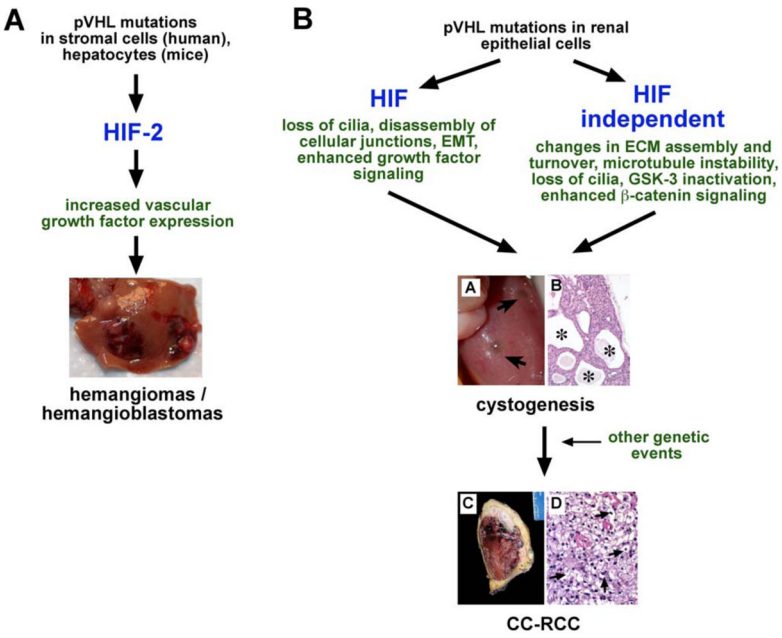

Fig. 3.

Proposed HIF-dependent and HIF-independent functions of pVHL in VHL-associated tumorigenesis. A clinical hallmark of VHL disease is the development of CNS hemangioblastomas and clear cell carcinoma of the kidney (CC-RCC). Panel A: Pathogenesis of VHL-associated hemangiomas/hemangioblastomas. Mice with germ line mutations in pVHL (*VHL+/−*) are predisposed to the development of liver hemangiomas (photograph). Genetic studies in mice demonstrated exclusive HIF-2-dependence, which is in line with HIF expression studies in human hemangioblastomas. Panel B: Pathogenesis of VHL-associated renal cancer. The pathogenesis of CC-RCC is more complex and most likely involves both, HIF-dependent and HIF independent functions of pVHL, as well as other genetic events that lead to malignant transformation. Shown is the macroscopic (A) and microscopic (B) appearance of large cortical renal cysts in mouse kidneys with pVHL inactivation (arrows and stars). (B), magnification x100. (C) and (D), human CC-RCC; (C), gross photography; (D), clear cell histology (arrows), magnification x400. Images (C) and (D) were kindly provided by Dr. John Tomaszewski, Department of Pathology, University of Pennsylvania, Philadelphia, PA.