Published in final edited form as:

Semin Cell Dev Biol. 2005 ; 16(0): 564–574. doi:10.1016/j.semcdb.2005.03.006.

The VHL tumor suppressor in development and disease: Functional studies in mice by conditional gene targeting

Volker H. Haase*

Department of Medicine, University of Pennsylvania School of Medicine, 700 CRB, 415 Curie Boulevard, Philadelphia, Pennsylvania, PA 19104-6144, USA

Abstract

The von Hippel–Lindau tumor suppressor pVHL plays a critical role in the pathogenesis of familial and sporadic clear cell carcinomas of the kidney and hemangioblastomas of the retina and central nervous system. pVHL targets the oxygen sensitive alpha subunit of hypoxia-inducible factor (HIF) for proteasomal degradation, thus providing a direct link between tumorigenesis and molecular pathways critical for cellular adaptation to hypoxia. Cell type specific gene targeting of *VHL* in mice has demonstrated that proper pVHL mediated HIF proteolysis is fundamentally important for survival, proliferation and differentiation of many cell types and furthermore, that inactivation of pVHL may, unexpectedly, inhibit tumor growth under certain conditions. Mouse knock out studies have provided novel mechanistic insights into VHL associated tumorigenesis and established a central role for HIF in the development of the VHL phenotype.

Keywords

von Hippel–Lindau (VHL) tumor suppressor; Hypoxia-inducible factor (HIF); Conditional knock out mice; Renal cell cancer; Hemangioma

1. Introduction

The von Hippel–Lindau gene (*VHL*) was initially identified as the tumor suppressor gene that is mutated in patients with von Hippel–Lindau disease, a rare familial tumor syndrome characterized by the development of highly vascularized tumors in multiple organs. Typical clinical manifestations of VHL disease include hemangioblastomas of the retina and central nervous system, multifocal and bilateral renal cell cancer of the clear cell type often preceded by preneoplastic renal cysts, pancreatic cysts and tumors, pheochromocytomas and others [1]. More importantly, *VHL* was also found to be mutated in the majority of sporadic clear cell renal cell carcinomas (CC-RCC) indicating that loss of *VHL* function is a critical event during renal carcinogenesis [2]. This notion was supported by experiments in which reintroduction of wild type *VHL* into *VHL* deficient renal cancer cell lines resulted in suppression of tumor growth in vivo and in vitro [3–5]. The mechanisms by which the *VHL* gene product, pVHL, carried out its tumor suppressor function were initially obscure as homologies to known proteins did not exist when the gene was identified by positional cloning in 1993 [6]. Observations that oxygen dependent regulation of hypoxia inducible genes such as *vascular endothelial growth factor* (*VEGF*) and *glucose transporter 1* (*GLUT-1*) was lost in *VHL* deficient cell lines and could be restored after reintroduction of wild type pVHL suggested a role for pVHL in oxygen sensing [4,7,8]. After pVHL was

found to be associated with the elongins B and C, cullin2 and Rbx [9–14] in a complex that had E3 ubiquitin ligase activity [15,16], Maxwell et al. reported in a seminal paper that pVHL was critical for targeting the oxygen sensitive α -subunit of hypoxia-inducible factor (HIF) for oxygen dependent proteolysis [17], thus providing a direct link between VHL associated tumorigenesis and cellular adaptation to hypoxia via HIF. While it had already been reported that HIF- α was degraded by the ubiquitin-proteasome [18], pVHL mediated polyubiquitylation of HIF- α was subsequently established by several groups [19–22].

In order to understand the role of pVHL mediated HIF proteolysis in normal tissue physiology, tumorigenesis and development, we have used cell type specific gene targeting based on Cre/loxP mediated recombination. This review article will, after providing a brief synopsis of pVHL functions, summarize the findings from a variety of developmental and physiological studies with conditional VHL knock out mice and discuss their relevance for the successful generation of a VHL tumor model.

2. pVHL is part of a hypoxic switch

As a normal physiological response to hypoxia, HIF-1 and -2 facilitate both oxygen delivery and adaptation to oxygen deprivation by regulating genes that are involved in glucose uptake and metabolism, angiogenesis, erythropoiesis, cell proliferation and apoptosis [23,24]. HIFs belong to the PAS (Per-ARNT-Sim) family of basic helix-loop-helix (bHLH) transcription factors and bind DNA as heterodimers composed of an oxygen-sensitive α -subunit and a constitutively expressed β -subunit, also known as the arylhydrocarbon receptor nuclear translocator (ARNT) or HIF-1 [25]. HIF-1 is ubiquitously expressed [23,24,26], while expression of HIF-2 seems to be restricted to certain cell types, including endothelial cells, cardiomyocytes, hepatocytes, renal interstitial cells but not differentiated renal epithelial cells, type II pneumocytes and glial cells [27–29].

All three known HIF alpha subunits, HIF-1, -2 and -3 have been shown to interact with pVHL [17,30]. This highly conserved interaction requires hydroxylation of defined HIF-proline residues by HIF specific proline hydroxylases (PHDs) and is absolutely required for the execution of HIF proteolysis under normoxia [31–37]. Therefore, lack of pVHL results in maximal HIF- α stabilization, increased HIF transcriptional activity and up-regulation of HIF target genes such as *VEGF*, *GLUT-1* and *erythropoietin (EPO)* irrespective of oxygen levels, explaining some of the clinical features associated with *VHL* deficient tumors; e.g. the high degree of vascularity or erythrocytosis. A second hypoxic switch operates in the carboxy terminal transactivation domain of HIF- α with the hydroxylation of an asparagine residue; in hypoxia asparagine hydroxylation is blocked and CBP/p300 recruitment is facilitated [38,39].

3. HIF independent functions of pVHL

Although significant erythrocytosis is only seen in a relatively small fraction of patients with VHL disease [40,41], it is the clinical hallmark of patients with Chuvash disease, a congenital form of polycythemia mostly caused by homozygosity for a specific amino acid substitution at pVHL codon 200 (R200W) [42]. Interestingly this mutation, although it results in increased HIF transcriptional activity, does not result in a predisposition to classic VHL associated tumors [43], suggesting that additional HIF independent mechanisms must be important in VHL associated tumorigenesis, in particular renal carcinogenesis. This notion is also supported by VHL genotype–phenotype correlations, as in the case of type 2C VHL disease, where patients develop pheochromocytomas despite the fact that mutated pVHL retains the ability to polyubiquitylate HIF- α [44,45].

Besides regulating the degradation of HIF- subunits, pVHL has been shown to play a role in fibronectin extracellular matrix assembly and matrix turnover [46–49], microtubule stability [50], and in regulating the stability and/or activity of other proteins such as plant homeodomain protein Jade-1 [51,52] and atypical protein kinase C isoforms [53–57]. Other pVHL targets have been described, most notably transcription factor Sp1 [58], a KRAB-A domain protein, VHLak, repressing HIF transcriptional activity [59], de-ubiquitinating enzymes [60], the large subunit of RNA polymerase II [61] and the RNA-binding protein hnRNP A2 [62]. It is unclear at present, how other pVHL functions and recently discovered protein interactions contribute to the development of the VHL phenotype and to renal carcinogenesis, certainly, further investigations are needed.

4. pVHL is essential for normal embryonic development

Mouse embryos with homozygous deletion of *Vhlh* (gene symbol for murine *VHL*) die during midgestation between embryonic day (E) 10.5 and 12.5 and are characterized by a lack of placental vasculogenesis. In a study by Gnarr et al. [63], a neomycin selection cassette was used to replace most of the *Vhlh* coding sequence in 129 J-1 ES cells, thus generating a complete null allele. Although the placenta and embryos of *Vhlh* null mice appeared to develop normally until E9.5, a severe disruption and loss of normal placental labyrinth architecture (absent development of syncytiotrophoblasts) associated with hemorrhage was noted by E11.5 to 12.5, suggesting that abnormal placental development had led to the demise of *Vhlh*^{−/−} embryos. Whether this phenotype is mediated by HIF has not been studied, but could be investigated by a direct phenotypic comparison of *Vhlh*^{−/−} and *PHD*^{−/−} knock out mice.

Independent of this important role in placental vascular development, conditional disruption of *Vhlh* in neuro-epithelial progenitor cells alone resulted in abnormal neuronal differentiation and embryonic lethality during late gestation (Haase et al., unpublished data and see Table 1), suggesting that proper pVHL function is not only required for vascular development of the placenta, but also for normal development of other tissues.

5. Mice which lack one copy of *Vhlh* are predisposed to the development of vascular tumors

While Gnarr et al. did not observe a phenotype in *Vhlh*^{+/−} mice bred in a mixed C57BL/6-129 genetic background, we observed that *Vhlh* heterozygotes in a BALB/c-129 background were predisposed to the development of cavernous liver hemangiomas with high phenotypic penetrance by 15 months of age [64]. By contrast hepatic vascular tumors are rare manifestations of VHL disease in humans [65,66]. The discrepancy in mouse phenotypes implies a strong dependence on genetic background. In deed, Ma et al. showed that the incidence of hepatic cavernous hemangiomas in a BALB/c background was 88% but only 18% in a C57BL/6 background, possibly a result of polymorphic differences in certain modifier genes as has been shown for other knock out mice [67].

Although the molecular mechanism for the liver phenotype in *Vhlh*^{+/−} mice has not been systematically investigated, it is most likely that, following Knudson's two-hit hypothesis, inactivation of the remaining *Vhlh* wild type allele in hepatocytes by, for example, loss of heterozygosity or promoter methylation, resulted in the formation of cavernous hemangiomas through increased HIF dependent vascular growth factor production. Hepatocyte specific inactivation of *Vhlh* (Fig. 1) resulted in the same phenotype, thus providing evidence that hepatic cavernous hemangiomas in *Vhlh* heterozygotes are the result of pVHL loss in this cell type and not in other liver cell types, such as endothelial cells. Endothelial cells in this model do however respond to uncontrolled, constitutive production

of vascular growth factors by hepatocytes and proliferate while expressing functional pVHL. This is mechanistically similar to human VHL hemangioblastomas, in which “stromal cells” and not endothelial cells have lost pVHL and represent the neoplastic component of this tumor [68]. The histogenesis of stromal cells is uncertain; however, there is evidence in the literature that these cells may be of angiogenic origin [69,70].

Other more classic VHL associated lesions such as renal cysts were only found at very low frequency in *Vhlh* heterozygotes; frequency of 1/30 in a study by Haase et al. [64] and 1/28 as reported by Kleymenova et al. [71]. In these reports renal cell carcinomas were not observed, nor were central nervous system hemangioblastomas or retinal angiomas. Treatment with streptozotocin did not result in increased susceptibility to renal carcinogenesis in *Vhlh* heterozygotes, but instead, in increased susceptibility to hemangiomas and hemangiosarcomas in the liver and other organs such as the uterus and ovaries [71].

6. Conditional inactivation of pVHL by Cre/loxP mediated recombination

In order to study pVHL in adult mice we have generated a conditional allele that allows tissue specific inactivation of *Vhlh* by Cre/loxP mediated recombination [64]. In this allele loxP sites flank the *Vhlh* promoter and exon 1 which are deleted upon Cre-mediated recombination, thus generating a *Vhlh* null allele (Fig. 2). Gene targeting by Cre/loxP mediated recombination allows inactivation of genes in a temporally and tissue restricted fashion, as opposed to conventional gene knock out protocols where the gene of interest is always disrupted in the germline [72,73]. This technology exploits the Cre/loxP system, a bacteriophage P1-derived site-specific recombination system, in which Cre-recombinase (Cre) catalyzes the recombination between two loxP sites which flank a genomic DNA sequence of interest, resulting in its deletion [74]. Genomic sequences or genes flanked by loxP sites are also referred to as “floxed” genes or 2-lox alleles and mice with tissue-specific gene inactivation can be generated by breeding the floxed allele to Cre-transgenic mice which express Cre-recombinase under the control of a tissue specific promoter. Similar to our approach Ma et al. generated a conditional allele in which *Vhlh* exons 2 and 3 were floxed [67].

7. Phenotypes generated by conditional inactivation of pVHL and their dependence on HIF

We have used a variety of Cre transgenic lines to inactivate pVHL in different tissues. Table 1 summarizes the phenotypes that were observed. In most tissues inactivation of pVHL resulted in abnormal differentiation, which was associated with either a decrease in proliferation or an increase in apoptosis (see Table 1).

In order to determine the role of the different HIF transcription factors in the development of the VHL phenotype in mice, we have generated mice that are either deficient for both *Vhlh* and *Hif-1*, or double deficient for *Vhlh* and *Hif-1* (*Arnt*). Using this double knock out approach, Biju et al. was able to show that a caspase-8 dependent pro-apoptotic phenotype in *Vhlh*^{-/-} thymocytes was completely Hif-1 mediated [75]. Hif-2, on the other hand, although expressed in thymocytes, was found to be non-functional. Inactivation of Hif-1 alone was sufficient to completely suppress the increase of *VEGF* mRNA levels and vascularity in *Vhlh*^{-/-} thymi [75]. Using the same approach, Rankin et al. [76] was able to determine that the development of vascular tumors in *Vhlh* deficient livers was entirely dependent on Hif. While inactivation of Hif-1 in the liver did not affect vascular tumorigenesis, inactivation of Hif-1 (*Arnt*), the binding partner for both Hif-1 and -2,

was sufficient to prevent the development of cavernous hemangiomas. It is important to note here that Hif-2 is fully functional in hepatocytes.

8. pVHL inactivation in apoptosis, cystogenesis and tumorigenesis: a central role for HIF

Biju et al. have investigated pVHL's role in the regulation of apoptosis and cell viability in thymocytes, a cell type that is normally not affected by VHL disease [75]. This study showed that tissue-specific inactivation of *Vhlh* in thymocytes resulted in highly vascularized thymi and in increased apoptosis rates through a Hif-1 dependent mechanism involving caspase-8. Although the development of a hypervascular phenotype was not surprising, as it is a general hallmark of VHL associated neoplasms, the finding of impaired cell survival was unexpected. In another study by Pfander et al. chondrocyte specific inactivation of *Vhlh* in the growth plate resulted in a reduction of proliferation through a probably Hif-1 dependent increase in cell cycle inhibitor p57^{kip2} [78]. In contrast, Hif-1 inactivation in chondrocytes resulted in a lack of p57^{kip2} mediated growth arrest followed by massive apoptosis [82].

Although pVHL's function as a tumor suppressor in CC-RCC and other VHL associated neoplasms has been well established [3–5,83], the unexpected finding that loss of pVHL can negatively affect cell survival and proliferation, raises interesting questions in regard to pVHL's role in tumor suppression. In CC-RCC, re-introduction of wt pVHL protected *VHL* deficient CC-RCC cells from apoptosis under certain conditions via upregulation of BCL-2 [84,85]. Of note, BCL-2 knock out mice are prone to develop renal cysts [86], and altered regulation of BCL-2 expression may play a role in the development of VHL associated renal cysts.

In a non-RCC tumor model, Mack et al. found significant growth retardation in embryonic stem (ES) cell derived *Vhlh*^{-/-} teratocarcinomas [87]. Interestingly, an increase in apoptosis rates was not reported. Inactivation of *Vhlh* may therefore have differential effects on proliferation and apoptosis in cell types of diverse histogenetic origin and genetic backgrounds. This notion is furthermore supported by the fact that *VHL* is widely expressed [6,88,89], yet the spectrum of VHL associated tumors is limited [83].

The role of HIF in cell death and tumor progression has been intensely studied (for a recent review see [90]) and there is substantial evidence that HIF is important for VHL tumorigenesis and metastasis. In regard to HIF-1 and -2 function in VHL renal carcinogenesis, it is of interest that a significant number of *VHL* defective CC-RCC cell lines do not express HIF-1, but do express HIF-2 [17]. A bias towards HIF-2 expression was also found in clinical CC-RCC samples (94% versus 69% express HIF-2 in CC-RCC with confirmed *VHL* defect) [91]. Thus, VHL associated tumor development may depend on a shift in the ratio of HIF-1 versus HIF-2 expression towards HIF-2. VHL reconstituted 786-O CC-RCC cells, for example, transfected with a non-degradable form of HIF-2 were still able to form tumors in nude mice thereby overriding pVHL's tumor suppressor function [92], while, the expression of non-degradable HIF-1 in a similar experimental setting did not produce tumors [93]. Consistent with these data is the finding that inactivation of HIF-2 by RNA interference in a VHL deficient background suppressed tumor formation [94,95]. Taken together these reports suggest that HIF-1 and -2 have diverse functions in regard to VHL associated renal tumorigenesis. Whether differential effects on signaling pathways critical for renal epithelial cell growth may explain the functional differences between the two HIFs remains to be investigated. For example, the expression of transforming growth factor- β (TGF- β), a potent renal epithelial mitogen, is HIF dependent [96,97] and HIF-2 dependent activation of the TGF- β /epidermal growth factor receptor

pathway may be important in renal cystogenesis and carcinogenesis [98,99]. The exact role of chemokine receptor CXCR4, a direct HIF target, in renal cystogenesis and tumorigenesis is unclear, but high levels of expression correlate with poor survival and the ability to metastasize [100]. Another direct HIF target is EPO, which has been shown to stimulate renal tumor cell growth [101,102]. It is of interest in this context that EPO appears to be a preferential HIF-2 target, at least in hepatocytes [103].

Taken together, it is possible that reduced growth of *Vhlh*^{-/-} teratocarcinomas as reported by Mack et al. [87], may have been consequence of constitutively active Hif-1 in conjunction with nonfunctional Hif-2 (Celeste Simon, personal communication). Koshiji et al. have shown that increased levels of HIF-1 in colon cancer cells can result in a suppression of Myc transcriptional activity through direct protein to protein interaction, thus inhibiting cell cycle progression by up-regulating p21^{cip1} [104]. These findings are in concordance with observations made by Carmeliet et al. in a teratocarcinoma model derived from Hif-1 deficient ES cells. In this model, tumors that lacked Hif-1 had a growth advantage [105]. Whether increased HIF-1 in a pVHL deficient background in the presence or absence of functional HIF-2 has different effects on cell cycle regulation remains to be investigated. However, growth suppression in *Vhlh* deficient teratocarcinomas may not be entirely dependent on Hif-1. In a follow-up study to Mack et al. [87], Rathmell et al. showed that introduction of a mutated pVHL species (Y112H) into a *Vhlh*^{-/-} background remained growth suppressive despite a restoration of Hif- regulation [106].

9. Conditional inactivation of pVHL in the kidney: towards the development of a mouse model for VHL renal carcinoma

Besides its role in VHL associated renal cancer, pVHL plays a major role in the pathogenesis of sporadic renal cell carcinoma of the clear cell type (CC-RCC), the most common form of kidney cancer [2]. VHL associated CC-RCCs are often preceded by pre-neoplastic renal cysts, which are multifocal and bilateral in patients with VHL disease [107], and are generally thought to arise from the proximal renal tubule (PRT) [1,107]. The worldwide mortality from renal cancer is expected to exceed 100,000 per year [108] and therapeutic options are limited; the generation of a mouse model for VHL associated renal cancer therefore would be invaluable.

To inactivate of *Vhlh* in the PRT, we have generated Cre transgenic mice using a mutated version of the rat *phosphoenolpyruvate carboxykinase* (PEPCK) promoter (Rankin et al., unpublished). Analysis of Cre expression with ROSA26-*lacZ* Cre-reporter mice [109] demonstrated that PEPCK-Cre is expressed in all S3- and most S1- and S2-segments (>80%) of the PRT (Rankin et al., unpublished observation). PEPCK-Cre mutant mice developed renal cysts (Fig. 3) at low frequency (4/25), but renal tumors were not observed by 18 months of age (Rankin et al., unpublished observation). Whether renal cyst formation in these mutants is a direct result of *Vhlh* gene inactivation in the PRT remains to be investigated. Similar to our findings, renal tumors were not found by Ma et al. when α -actin promoter driven Cre-recombinase was used to generate mice which lacked pVHL in a mosaic pattern [67].

There are several potential explanations for this observation: (a) additional mutations in other tumor suppressor genes or oncogenes are required before tumors develop; (b) increased activity of Hif-1 may prevent the development of VHL associated renal lesions and loss or diminished activity of Hif-1 is necessary (see discussion in Section 8); (c) a shift towards high levels of Hif-2 expression may be required before cysts or tumors develop but has not occurred (as discussed in Section 8); (d) a specific gain of function mutation in

pVHL may be required; (e) the genetic background of mice may play a role; and (f) VHL associated renal lesions arise from nephron segments that were not targeted (Fig. 4).

Although it is widely believed that VHL associated renal tumors originate from the proximal nephron [1,107], it has been shown that CC-RCCs can express markers of both, the distal and the proximal nephron [110–113], implying a multipotential capacity of neoplastic renal cells to synthesize antigens that can be associated with different tubular segments and different stages of nephrogenesis. More recently Maxwell's group reported that in kidneys from VHL patients "early" multicellular lesions appeared to be more frequently derived from Tamm–Horsfall protein (THP) expressing tubular segments than from the proximal tubule. Tamm–Horsfall glycoprotein is expressed in the medullary thick ascending loop of Henle (mTAL) and in the early distal renal tubule (for a recent review see [115]). On the other hand, single cell foci of HIF-target gene expression were found more frequently in the proximal tubular cells [114], suggesting a site-specific tumor suppressor function for pVHL in the kidney. This finding supports the notion that VHL associated renal tumors may be of a different histogenetic origin than commonly believed and arise from more distal nephron segments (Fig. 4). Systematic targeting of *Vhlh* in different nephron segments using site specific promoters may therefore be necessary in order to reproduce classic VHL associated renal lesions in mice. Fig. 4 provides an overview of pVHL's and HIF's role in renal cysto- and carcinogenesis.

10. Concluding remarks and future directions

In this review I have summarized findings from the analysis of VHL knock out mice and have discussed the role of HIF in the context of VHL associated tumorigenesis. Proper targeting of HIF- subunits for proteolysis by the pVHL E3-ubiquitin ligase complex is critical for normal embryogenesis and tissue physiology. In the context of VHL tumorigenesis HIF plays a central role. While both HIF-1 and -2 seem to be capable of mediating the highly vascular nature of VHL deficient tissues, they differ substantially in regard to their effects on apoptosis and proliferation, especially in the context of CC-RCC tumorigenesis. The negative effects of pVHL inactivation on cell survival and proliferation in thymocytes, chondrocytes and ES derived teratocarcinomas were largely unexpected for a protein with tumor suppressor function. They most likely emphasize tissue specific differences in HIF-1 and -2 activity levels and target gene expression, as well as tissue specific aspects of HIF-independent pVHL functions. Clearly, for a more comprehensive understanding of pVHL's role in normal physiology and tumor suppression, further knock out and transgenic studies are needed that investigate cell type specific functions of individual HIF transcription factors in wild type and VHL deficient backgrounds, as well as the role of specific *VHL* mutations.

Acknowledgments

The author is supported by grants from the National Cancer Institute (NCI), the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the American Heart Association (AHA). The author apologizes to those colleagues whose work could not be cited because of space limitations. The author would like to thank Michael P. Madaio and Erinn B. Rankin for their helpful comments during preparation of this manuscript.

Abbreviations

VHL	von Hippel–Lindau
<i>Vhlh</i>	murine gene symbol for the <i>VHL</i> gene
pVHL	von Hippel–Lindau protein

HIF	hypoxia-inducible factor
HIF	refers to human or HIF in general
Hif	refers to murine HIF
ARNT	arylhydrocarbon receptor nuclear translocator
ES cell	embryonic stem cell
Cre	Cre-recombinase
PHD	HIF specific prolyl hydroxylase
VEGF	vascular endothelial growth factor
GLUT-1	glucose transporter 1
EPO	erythropoietin
CC-RCC	renal cell carcinoma of the clear cell type
PRT	proximal renal tubule
PEPCK	phosphoenolpyruvate-carboxykinase
THP	Tamm–Horsfall protein
wt	wild type

References

1. Maher ER, Kaelin WG Jr. von Hippel–Lindau disease. *Medicine (Baltimore)*. 1997; 76:381–91. [PubMed: 9413424]
2. Gnarr JR, Tory K, Weng Y, Schmidt L, Wei MH, Li H, et al. Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet*. 1994; 7:85–90. [PubMed: 7915601]
3. Iliopoulos O, Kibel A, Gray S, Kaelin WG Jr. Tumour suppression by the human von Hippel–Lindau gene product. *Nat Med*. 1995; 1:822–6. [PubMed: 7585187]
4. Gnarr JR, Zhou S, Merrill MJ, Wagner JR, Krumm A, Papavassiliou E, et al. Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. *Proc Natl Acad Sci USA*. 1996; 93:10589–94. [PubMed: 8855222]
5. Schoenfeld A, Davidowitz EJ, Burk RD. A second major native von Hippel–Lindau gene product, initiated from an internal translation start site, functions as a tumor suppressor. *Proc Natl Acad Sci USA*. 1998; 95:8817–22. [PubMed: 9671762]
6. Latif F, Tory K, Gnarr J, Yao M, Duh FM, Orcutt ML, et al. Identification of the von Hippel–Lindau disease tumor suppressor gene. *Science*. 1993; 260:1317–20. [PubMed: 8493574]
7. Iliopoulos O, Levy AP, Jiang C, Kaelin WG Jr, Goldberg MA. Negative regulation of hypoxia-inducible genes by the von Hippel–Lindau protein. *Proc Natl Acad Sci USA*. 1996; 93:10595–9. [PubMed: 8855223]
8. Siemeister G, Weindel K, Mohrs K, Barleon B, Martiny-Baron G, Marme D. Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel–Lindau tumor suppressor protein. *Cancer Res*. 1996; 56:2299–301. [PubMed: 8625303]
9. Duan DR, Pause A, Burgess WH, Aso T, Chen DY, Garrett KP, et al. Inhibition of transcription elongation by the VHL tumor suppressor protein [see comments]. *Science*. 1995; 269:1402–6. [PubMed: 7660122]
10. Kibel A, Iliopoulos O, DeCaprio JA, Kaelin WG Jr. Binding of the von Hippel–Lindau tumor suppressor protein to elongin B and C [see comments]. *Science*. 1995; 269:1444–6. [PubMed: 7660130]

11. Pause A, Lee S, Worrell RA, Chen DY, Burgess WH, Linehan WM, et al. The von Hippel–Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. *Proc Natl Acad Sci USA*. 1997; 94:2156–61. [PubMed: 9122164]
12. Kamura T, Koepp DM, Conrad MN, Skowrya D, Moreland RJ, Iliopoulos O, et al. Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase [see comments]. *Science*. 1999; 284:657–61. [PubMed: 10213691]
13. Stebbins CE, Kaelin WG Jr, Pavletich NP. Structure of the VHL–elongin C–elongin B complex: implications for VHL tumor suppressor function. *Science*. 1999; 284:455–61. [PubMed: 10205047]
14. Feldman DE, Thulasiraman V, Ferreyra RG, Frydman J. Formation of the VHL–elongin BC tumor suppressor complex is mediated by the chaperonin TRiC. *Mol Cell*. 1999; 4:1051–61. [PubMed: 10635329]
15. Iwai K, Yamanaka K, Kamura T, Minato N, Conaway RC, Conaway JW, et al. Identification of the von Hippel–Lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proc Natl Acad Sci USA*. 1999; 96:12436–41. [PubMed: 10535940]
16. Lisztwan J, Imbert G, Wirbelauer C, Gstaiger M, Krek W. The von Hippel–Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. *Genes Dev*. 1999; 13:1822–33. [PubMed: 10421634]
17. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis [see comments]. *Nature*. 1999; 399:271–5. [PubMed: 10353251]
18. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem*. 1997; 272:22642–7. [PubMed: 9278421]
19. Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, et al. Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel–Lindau tumor suppressor protein. *J Biol Chem*. 2000; 275:25733–41. [PubMed: 10823831]
20. Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel–Lindau tumor suppressor protein. *EMBO J*. 2000; 19:4298–309. [PubMed: 10944113]
21. Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, et al. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel–Lindau protein. *Nat Cell Biol*. 2000; 2:423–7. [PubMed: 10878807]
22. Kamura T, Sato S, Iwai K, Czyzyk-Krzeska M, Conaway RC, Conaway JW. Activation of HIF1alpha ubiquitination by a reconstituted von Hippel–Lindau (VHL) tumor suppressor complex. *Proc Natl Acad Sci USA*. 2000; 97:10430–5. [PubMed: 10973499]
23. Semenza GL. HIF-1 and mechanisms of hypoxia sensing. *Curr Opin Cell Biol*. 2001; 13:167–71. [PubMed: 11248550]
24. Wenger RH. Cellular adaptation to hypoxia: O₂-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O₂-regulated gene expression. *FASEB J*. 2002; 16:1151–62. [PubMed: 12153983]
25. Kewley RJ, Whitelaw ML, Chapman-Smith A. The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int J Biochem Cell Biol*. 2004; 36:189–204. [PubMed: 14643885]
26. Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, et al. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB J*. 2001; 15:2445–53. [PubMed: 11689469]
27. Kietzmann T, Cornesse Y, Brechtel K, Modaressi S, Jungermann K. Perivenous expression of the mRNA of the three hypoxia-inducible factor alpha-subunits, HIF1alpha, HIF2alpha and HIF3alpha, in rat liver. *Biochem J*. 2001; 354:531–7. [PubMed: 11237857]
28. Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, et al. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. *FASEB J*. 2003; 17:271–3. [PubMed: 12490539]

29. Compennolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, et al. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat Med.* 2002; 8:702–10. [PubMed: 12053176]
30. Maynard MA, Qi H, Chung J, Lee EH, Kondo Y, Hara S, et al. Multiple splice variants of the human HIF-3alpha locus are targets of the VHL E3 ubiquitin ligase complex. *J Biol Chem.* 2003; 278:21211–21. [PubMed: 12531761]
31. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF-1alpha to the von Hippel–Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science.* 2001; 292:468–72. [PubMed: 11292861]
32. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, et al. HIF1alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science.* 2001; 292:464–8. [PubMed: 11292862]
33. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, et al. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell.* 2001; 107:43–54. [PubMed: 11595184]
34. Hon WC, Wilson MI, Harlos K, Claridge TD, Schofield CJ, Pugh CW, et al. Structural basis for the recognition of hydroxyproline in HIF-1 alpha by pVHL. *Nature.* 2002; 417:975–8. [PubMed: 12050673]
35. Masson N, Willam C, Maxwell PH, Pugh CW, Ratcliffe PJ. Independent function of two destruction domains in hypoxia-inducible factor-1alpha chains activated by prolyl hydroxylation. *EMBO J.* 2001; 20:5197–206. [PubMed: 11566883]
36. Yu F, White SB, Zhao Q, Lee FS. HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proc Natl Acad Sci USA.* 2001; 98:9630–5. [PubMed: 11504942]
37. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science.* 2001; 294:1337–40. [PubMed: 11598268]
38. Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev.* 2001; 15:2675–86. [PubMed: 11641274]
39. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev.* 2002; 16:1466–71. [PubMed: 12080085]
40. Da Silva JL, Lacombe C, Bruneval P, Casadevall N, Leporrier M, Camilleri JP, et al. Tumor cells are the site of erythropoietin synthesis in human renal cancers associated with polycythemia. *Blood.* 1990; 75:577–82. [PubMed: 2297568]
41. Krieg M, Marti HH, Plate KH. Coexpression of erythropoietin and vascular endothelial growth factor in nervous system tumors associated with von Hippel–Lindau tumor suppressor gene loss of function. *Blood.* 1998; 92:3388–93. [PubMed: 9787178]
42. Ang SO, Chen H, Hirota K, Gordeuk VR, Jelinek J, Guan Y, et al. Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nat Genet.* 2002; 32:614–21. [PubMed: 12415268]
43. Gordeuk VR, Sergueeva AI, Miasnikova GY, Okhotin D, Voloshin Y, Choyke PL, et al. Congenital disorder of oxygen sensing: association of the homozygous Chuvash polycythemia VHL mutation with thrombosis and vascular abnormalities but not tumors. *Blood.* 2004; 103:3924–32. [PubMed: 14726398]
44. Hoffman MA, Ohh M, Yang H, Kaelin JM, Ivan M, Kaelin WG Jr. von Hippel–Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. *Hum Mol Genet.* 2001; 10:1019–27. [PubMed: 11331612]
45. Clifford SC, Cockman ME, Smallwood AC, Mole DR, Woodward ER, Maxwell PH, et al. Contrasting effects on HIF-1alpha regulation by disease-causing pVHL mutations correlate with patterns of tumorigenesis in von Hippel–Lindau disease. *Hum Mol Genet.* 2001; 10:1029–38. [PubMed: 11331613]
46. Ohh M, Yauch RL, Lonergan KM, Whaley JM, Stemmer-Rachamimov AO, Louis DN, et al. The von Hippel–Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol Cell.* 1998; 1:959–68. [PubMed: 9651579]

47. Koochekpour S, Jeffers M, Wang PH, Gong C, Taylor GA, Roessler LM, et al. The von Hippel–Lindau tumor suppressor gene inhibits hepatocyte growth factor/scatter factor-induced invasion and branching morphogenesis in renal carcinoma cells. *Mol Cell Biol.* 1999; 19:5902–12. [PubMed: 10454537]
48. Davidowitz EJ, Schoenfeld AR, Burk RD. VHL induces renal cell differentiation and growth arrest through integration of cell–cell and cell–extracellular matrix signaling. *Mol Cell Biol.* 2001; 21:865–74. [PubMed: 11154273]
49. Bishop T, Lau KW, Epstein AC, Kim SK, Jiang M, O'Rourke D, et al. Genetic analysis of pathways regulated by the von Hippel–Lindau tumor suppressor in *Caenorhabditis elegans*. *PLoS Biol.* 2004; 2:e289. [PubMed: 15361934]
50. Hergovich A, Lisztwan J, Barry R, Ballschmieter P, Krek W. Regulation of microtubule stability by the von Hippel–Lindau tumour suppressor protein pVHL. *Nat Cell Biol.* 2003; 5:64–70. [PubMed: 12510195]
51. Zhou MI, Wang H, Foy RL, Ross JJ, Cohen HT. Tumor suppressor von Hippel–Lindau (VHL) stabilization of Jade-1 protein occurs through plant homeodomains and is VHL mutation dependent. *Cancer Res.* 2004; 64:1278–86. [PubMed: 14973063]
52. Zhou MI, Wang H, Ross JJ, Kuzmin I, Xu C, Cohen HT. The von Hippel–Lindau tumor suppressor stabilizes novel plant homeodomain protein Jade-1. *J Biol Chem.* 2002; 277:39887–98. [PubMed: 12169691]
53. Pal S, Claffey KP, Dvorak HF, Mukhopadhyay D. The von Hippel–Lindau gene product inhibits vascular permeability factor/vascular endothelial growth factor expression in renal cell carcinoma by blocking protein kinase C pathways. *J Biol Chem.* 1997; 272:27509–12. [PubMed: 9346879]
54. Pal S, Claffey KP, Cohen HT, Mukhopadhyay D. Activation of Sp1-mediated vascular permeability factor/vascular endothelial growth factor transcription requires specific interaction with protein kinase C zeta. *J Biol Chem.* 1998; 273:26277–80. [PubMed: 9756852]
55. Datta K, Nambudripad R, Pal S, Zhou M, Cohen HT, Mukhopadhyay D. Inhibition of insulin-like growth factor-I-mediated cell signaling by the von Hippel–Lindau gene product in renal cancer. *J Biol Chem.* 2000; 275:20700–6. [PubMed: 10748176]
56. Datta K, Sundberg C, Karumanchi SA, Mukhopadhyay D. The 104–123 amino acid sequence of the beta-domain of von Hippel–Lindau gene product is sufficient to inhibit renal tumor growth and invasion. *Cancer Res.* 2001; 61:1768–75. [PubMed: 11280720]
57. Okuda H, Saitoh K, Hirai S, Iwai K, Takaki Y, Baba M, et al. The von Hippel–Lindau tumor suppressor protein mediates ubiquitination of activated atypical protein kinase C. *J Biol Chem.* 2001; 276:43611–7. [PubMed: 11574546]
58. Mukhopadhyay D, Knebelmann B, Cohen HT, Ananth S, Sukhatme VP. The von Hippel–Lindau tumor suppressor gene product interacts with Sp1 to repress vascular endothelial growth factor promoter activity. *Mol Cell Biol.* 1997; 17:5629–39. [PubMed: 9271438]
59. Li Z, Wang D, Na X, Schoen SR, Messing EM, Wu G. The VHL protein recruits a novel KRAB-A domain protein to repress HIF-1alpha transcriptional activity. *EMBO J.* 2003; 22:1857–67. [PubMed: 12682018]
60. Li Z, Na X, Wang D, Schoen SR, Messing EM, Wu G. Ubiquitination of a novel deubiquitinating enzyme requires direct binding to von Hippel–Lindau tumor suppressor protein. *J Biol Chem.* 2002; 277:4656–62. [PubMed: 11739384]
61. Kuznetsova AV, Meller J, Schnell PO, Nash JA, Ignacak ML, Sanchez Y, et al. von Hippel–Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination. *Proc Natl Acad Sci USA.* 2003; 100:2706–11. [PubMed: 12604794]
62. Pioli PA, Rigby WF. The von Hippel–Lindau protein interacts with heteronuclear ribonucleoprotein a2 and regulates its expression. *J Biol Chem.* 2001; 276:40346–52. [PubMed: 11517223]
63. Gnarr JR, Ward JM, Porter FD, Wagner JR, Devor DE, Grinberg A, et al. Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. *Proc Natl Acad Sci USA.* 1997; 94:9102–7. [PubMed: 9256442]

64. Haase VH, Glickman JN, Socolovsky M, Jaenisch R. Vascular tumors in livers with targeted inactivation of the von Hippel–Lindau tumor suppressor. *Proc Natl Acad Sci USA*. 2001; 98:1583–8. [PubMed: 11171994]
65. Rojiani AM, Owen DA, Berry K, Woodhurst B, Anderson FH, Scudamore CH, et al. Hepatic hemangioblastoma. An unusual presentation in a patient with von Hippel–Lindau disease. *Am J Surg Pathol*. 1991; 15:81–6. [PubMed: 1898683]
66. McGrath FP, Gibney RG, Morris DC, Owen DA, Erb SR. Case report: multiple hepatic and pulmonary haemangioblastomas—a new manifestation of von Hippel–Lindau disease. *Clin Radiol*. 1992; 45:37–9. [PubMed: 1740034]
67. Ma W, Tessarollo L, Hong SB, Baba M, Southon E, Back TC, et al. Hepatic vascular tumors, angiectasis in multiple organs, and impaired spermatogenesis in mice with conditional inactivation of the VHL gene. *Cancer Res*. 2003; 63:5320–8. [PubMed: 14500363]
68. Vortmeyer AO, Gnarr JR, Emmert-Buck MR, Katz D, Linehan WM, Oldfield EH, et al. von Hippel–Lindau gene deletion detected in the stromal cell component of a cerebellar hemangioblastoma associated with von Hippel–Lindau disease. *Hum Pathol*. 1997; 28:540–3. [PubMed: 9158701]
69. Lach B, Gregor A, Rippstein P, Omulecka A. Angiogenic histogenesis of stromal cells in hemangioblastoma: ultrastructural and immunohistochemical study. *Ultrastruct Pathol*. 1999; 23:299–310. [PubMed: 10582267]
70. Vortmeyer AO, Frank S, Jeong SY, Yuan K, Ikejiri B, Lee YS, et al. Developmental arrest of angioblastic lineage initiates tumorigenesis in von Hippel–Lindau disease. *Cancer Res*. 2003; 63:7051–5. [PubMed: 14612494]
71. Kleymanova E, Everitt JI, Pluta L, Portis M, Gnarr JR, Walker CL. Susceptibility to vascular neoplasms but no increased susceptibility to renal carcinogenesis in Vhl knock out mice. *Carcinogenesis*. 2004; 25:309–15. [PubMed: 14604887]
72. Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science*. 1994; 265:103–6. [PubMed: 8016642]
73. Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, et al. Subregion- and cell type-restricted gene knock out in mouse brain. *Cell*. 1996; 87:1317–26. [PubMed: 8980237]
74. Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA*. 1988; 85:5166–70. [PubMed: 2839833]
75. Biju MP, Neumann AK, Bensinger SJ, Johnson RS, Turka LA, Haase VH. Vhlh gene deletion induces Hif-1-mediated cell death in thymocytes. *Mol Cell Biol*. 2004; 24:9038–47. [PubMed: 15456877]
76. Rankin EB, Higgins DF, Walisser JA, Johnson RS, Bradfield CA, Haase VH. Inactivation of the arylhydrocarbon receptor nuclear translocator (Arnt) suppresses von Hippel–Lindau disease associated vascular tumors in mice. *Mol Cell Biol*. 2005; 25:3163–72. [PubMed: 15798202]
77. Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Colgan SP, Haase VH. Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *J Clin Invest*. 2004; 114:1098–106. [PubMed: 15489957]
78. Pfander D, Kobayashi T, Knight MC, Zelzer E, Chan DA, Olsen BR, et al. Deletion of Vhlh in chondrocytes reduces cell proliferation and increases matrix deposition during growth plate development. *Development*. 2004; 131:2497–508. [PubMed: 15128677]
79. Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, et al. HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell*. 2003; 112:645–57. [PubMed: 12628185]
80. Boutin A, Haase VH, Johnson RS. Biology of hypoxia: the role of oxygen sensing in development, normal function and disease, *Keystone Symposia*. Colorado: Steamboat Springs; 2004. Response to oxygenation in differentiation, function and pathology of the epidermis; p. 114
81. Seagroves TN, Liao D, Cardiff R, Haase VH, Johnson RS. Biology of hypoxia: the role of oxygen sensing in development, normal function and disease, *Keystone Symposia*. Colorado: Steamboat Springs; 2004. Deletion of vhl impairs normal mammary gland development; p. 100

82. Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Johnson RS. Hypoxia in cartilage: HIF-1 α is essential for chondrocyte growth arrest and survival. *Genes Dev.* 2001; 15:2865–76. [PubMed: 11691837]
83. Kaelin WG Jr. Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer.* 2002; 2:673–82. [PubMed: 12209156]
84. Schoenfeld AR, Parris T, Eisenberger A, Davidowitz EJ, De Leon M, Talasazan F, et al. The von Hippel–Lindau tumor suppressor gene protects cells from UV-mediated apoptosis. *Oncogene.* 2000; 19:5851–7. [PubMed: 11127815]
85. Devarajan P, De Leon M, Talasazan F, Schoenfeld AR, Davidowitz EJ, Burk RD. The von Hippel–Lindau gene product inhibits renal cell apoptosis via Bcl-2-dependent pathways. *J Biol Chem.* 2001; 20:20.
86. Sorenson CM, Padanilam BJ, Hammerman MR. Abnormal postpartum renal development and cystogenesis in the bcl-2(–/–) mouse. *Am J Physiol.* 1996; 271:F184–93. [PubMed: 8760259]
87. Mack FA, Rathmell WK, Arsham AM, Gnarr J, Keith B, Simon MC. Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. *Cancer Cell.* 2003; 3:75–88. [PubMed: 12559177]
88. Kessler PM, Vasavada SP, Rackley RR, Stackhouse T, Duh FM, Latif F, et al. Expression of the Von Hippel–Lindau tumor suppressor gene, VHL, in human fetal kidney and during mouse embryogenesis. *Mol Med.* 1995; 1:457–66. [PubMed: 8521303]
89. Los M, Jansen GH, Kaelin WG, Lips CJ, Blijham GH, Voest EE. Expression pattern of the von Hippel–Lindau protein in human tissues. *Lab Invest.* 1996; 75:231–8. [PubMed: 8765323]
90. Harris AL. Hypoxia—a key regulatory factor in tumour growth. *Nat Rev Cancer.* 2002; 2:38–47. [PubMed: 11902584]
91. Turner KJ, Moore JW, Jones A, Taylor CF, Cuthbert-Heavens D, Han C, et al. Expression of hypoxia-inducible factors in human renal cancer: relationship to angiogenesis and to the von Hippel–Lindau gene mutation. *Cancer Res.* 2002; 62:2957–61. [PubMed: 12019178]
92. Kondo K, Klcio JM, Nakamura E, Lechpammer M, Kaelin WG. Inhibition of HIF is necessary for tumor suppression by the von Hippel–Lindau protein. *Cancer Cell.* 2002; 1:237–46. [PubMed: 12086860]
93. Maranchi JK, Vasselli JR, Riss J, Bonifacio JS, Linehan WM, Klausner RD. The contribution of VHL substrate binding and HIF-1 α to the phenotype of vhl loss in renal cell carcinoma. *Cancer Cell.* 2002; 1:247–53. [PubMed: 12086861]
94. Zimmer M, Doucette D, Siddiqui N, Iliopoulos O. Inhibition of hypoxia-inducible factor is sufficient for growth suppression of VHL–/– tumors. *Mol Cancer Res.* 2004; 2:89–95. [PubMed: 14985465]
95. Kondo K, Kim WY, Lechpammer M, Kaelin WG Jr. Inhibition of HIF2 α is sufficient to suppress pVHL-defective tumor growth. *PLoS Biol.* 2003; 1:E83. [PubMed: 14691554]
96. Gunaratnam L, Morley M, Franovic A, de Paulsen N, Mekhail K, Parolin DA, et al. Hypoxia inducible factor activates the transforming growth factor- α /epidermal growth factor receptor growth stimulatory pathway in VHL(–/–) renal cell carcinoma cells. *J Biol Chem.* 2003; 278:44966–74. [PubMed: 12944410]
97. de Paulsen N, Brychzy A, Fournier MC, Klausner RD, Gnarr JR, Pause A, et al. Role of transforming growth factor- α in von Hippel–Lindau (VHL)(–/–) clear cell renal carcinoma cell proliferation: a possible mechanism coupling VHL tumor suppressor inactivation and tumorigenesis. *Proc Natl Acad Sci USA.* 2001; 98:1387–92. [PubMed: 11171960]
98. Gunaratnam, L.; Morley, M.; Smith, K.; Lee, S. Biology of hypoxia: the role of oxygen sensing in development, normal function and disease, *Keystone Symposia. Colorado: Steamboat Springs;* 2004. Mechanisms of Hif-2 α -dependent tumorigenesis; p. 78
99. Lowden DA, Lindemann GW, Merlino G, Barash BD, Calvet JP, Gattone VH 2nd. Renal cysts in transgenic mice expressing transforming growth factor- α . *J Lab Clin Med.* 1994; 124:386–94. [PubMed: 8083581]
100. Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W. Chemokine receptor CXCR4 downregulated by von Hippel–Lindau tumour suppressor pVHL. *Nature.* 2003; 425:307–11. [PubMed: 13679920]

101. Westenfelder C, Baranowski RL. Erythropoietin stimulates proliferation of human renal carcinoma cells. *Kidney Int.* 2000; 58:647–57. [PubMed: 10916088]
102. Westenfelder C. Unexpected renal actions of erythropoietin. *Exp Nephrol.* 2002; 10:294–8. [PubMed: 12381912]
103. Warnecke C, Zaborowska Z, Kurreck J, Erdmann VA, Frei U, Wiesener M, et al. Differentiating the functional role of hypoxia-inducible factor (HIF)-1 α and HIF-2 α (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2 α target gene in Hep3B and Kelly cells. *FASEB J.* 2004; 18:1462–4. [PubMed: 15240563]
104. Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, Huang LE. HIF-1 α induces cell cycle arrest by functionally counteracting Myc. *EMBO J.* 2004; 23:1949–56. [PubMed: 15071503]
105. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, et al. Role of HIF-1 α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature.* 1998; 394:485–90. [PubMed: 9697772]
106. Rathmell WK, Hickey MM, Bezman NA, Chmielecki CA, Carraway NC, Simon MC. In vitro and in vivo models analyzing von Hippel–Lindau disease-specific mutations. *Cancer Res.* 2004; 64:8595–603. [PubMed: 15574766]
107. Neumann HP, Zbar B. Renal cysts, renal cancer and von Hippel–Lindau disease [editorial]. *Kidney Int.* 1997; 51:16–26. [PubMed: 8995713]
108. Motzer RJ, Bander NH, Nanus DM. Renal-cell carcinoma. *N Engl J Med.* 1996; 335:865–75. [PubMed: 8778606]
109. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet.* 1999; 21:70–1. [PubMed: 9916792]
110. Cohen C, McCue PA, Derosé PB. Histogenesis of renal cell carcinoma and renal oncocytoma. An immunohistochemical study. *Cancer.* 1988; 62:1946–51. [PubMed: 2458824]
111. Hughson MD, Johnson LD, Silva FG, Kovacs G. Nonpapillary and papillary renal cell carcinoma: a cytogenetic and phenotypic study. *Mod Pathol.* 1993; 6:449–56. [PubMed: 8415591]
112. Droz D, Zachar D, Charbit L, Gogusev J, Chretien Y, Iris L. Expression of the human nephron differentiation molecules in renal cell carcinomas. *Am J Pathol.* 1990; 137:895–905. [PubMed: 1699423]
113. Ulrich W, Horvat R, Krisch K. Lectin histochemistry of kidney tumours and its pathomorphological relevance. *Histopathology.* 1985; 9:1037–50. [PubMed: 2417930]
114. Mandriota SJ, Turner KJ, Davies DR, Murray PG, Morgan NV, Sowter HM, et al. HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron. *Cancer Cell.* 2002; 1:459–68. [PubMed: 12124175]
115. Serafini-Cessi F, Malagolini N, Cavallone D. Tamm–Horsfall glycoprotein: biology and clinical relevance. *Am J Kidney Dis.* 2003; 42:658–76. [PubMed: 14520616]
116. Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc Natl Acad Sci USA.* 2000; 97:9082–7. [PubMed: 10922063]
117. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res.* 2001; 61:6669–73. [PubMed: 11559532]
118. Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, et al. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol.* 2000; 20:5454–68. [PubMed: 10891486]
119. Bindra RS, Vasselli JR, Stearman R, Linehan WM, Klausner RD. VHL-mediated hypoxia regulation of cyclin D1 in renal carcinoma cells. *Cancer Res.* 2002; 62:3014–9. [PubMed: 12036906]
120. Zatyka M, da Silva NF, Clifford SC, Morris MR, Wiesener MS, Eckardt KU, et al. Identification of cyclin D1 and other novel targets for the von Hippel–Lindau tumor suppressor gene by expression array analysis and investigation of cyclin D1 genotype as a modifier in von Hippel–Lindau disease. *Cancer Res.* 2002; 62:3803–11. [PubMed: 12097293]

121. Baba M, Hirai S, Yamada-Okabe H, Hamada K, Tabuchi H, Kobayashi K, et al. Loss of von Hippel–Lindau protein causes cell density dependent deregulation of CyclinD1 expression through hypoxia-inducible factor. *Oncogene*. 2003; 22:2728–38. [PubMed: 12743597]
122. Wykoff CC, Sotiriou C, Cockman ME, Ratcliffe PJ, Maxwell P, Liu E, et al. Gene array of VHL mutation and hypoxia shows novel hypoxia-induced genes and that cyclin D1 is a VHL target gene. *Br J Cancer*. 2004; 90:1235–43. [PubMed: 15026807]
123. Foy, RL.; Chitalia, VC.; Panschenko, MV.; Boletta, A.; Qian, F.; Tsoikas, L., et al. Regulation of jadel by the polycystins. *J Am Soc Nephrol; American Society of Nephrology, Annual Meeting; St. Louis, Missouri*. 2004. p. P0689

Fig. 1. Hepatic vascular tumors in mice with hepatocyte specific inactivation of *Vhlh*. (A and B) Gross photography of formalin fixed livers from hepatocyte-specific *Vhlh* knock out mice. Arrows in (A) point to cavernous hemangiomas that appear deflated after resection of the liver. The gallbladder is labeled by a star. (B) shows a section of the same liver. Arrows point to blood filled cavities containing fibrin clots. (C and D) Paraffin sections of livers with cavernous hemangiomas stained with H&E. (C) Large endothelial cell lined, blood filled cavities are depicted by stars. Arrow depicts an area of hemorrhage. (D) Cavernous hemangiomas are typically associated with macro- and microvesicular hepatocellular steatosis, which results from the accumulation of neutral lipid [64], here depicted by arrows. Lipid accumulation is also a feature of stromal cells, the neoplastic component of VHL associated central nervous system hemangioblastomas [69,70]. Magnification in (C) is $\times 100$ and in (D) is $\times 400$.

Fig. 2.

Genomic structure of the *Vhlh* conditional allele. Shown are the genomic structures of *Vhlh* in wild type (wt) configuration, the *Vhlh* 2-lox allele and the recombined *Vhlh* 1-lox allele. LoxP sites are depicted as red triangles, *Vhlh* exons are shown as black boxes. Exon 1 and the *Vhlh* promoter are deleted by Cre-mediated recombination, deleted area is shown in red. This allele was generated in 129 J-1 ES cells by homologous recombination with a targeting vector containing a “floxed” hygromycin-thymidine-kinase selection cassette, which was removed after transient transfection with Cre-recombinase [64]. *Abbreviations:* H, *HindIII*; N, *NcoI*.

Fig. 3.

Inactivation of *Vhlh* in proximal renal tubule (PRT) cells results in renal cysts at low frequency. (A) Gross in situ photograph of the right kidney from a conditional knock out mouse with inactivation of *Vhlh* in the PRT. Arrows point to two superficial cysts. Evidence of renal cell cancer or epithelial dysplasia was not detected (Rankin et al., unpublished observation). (B) Paraffin section of kidney with renal cysts stained with H&E. Shown is a cluster of smaller cysts lined with renal epithelial cells (arrows). Magnification is ×200.

Fig. 4.

HIF plays a central role in VHL associated renal cystogenesis and tumorigenesis. Above: proposed sites of origin of VHL renal cysts and tumors. A schematic representation of a nephron is shown above. The area shaded in blue represents the proximal renal tubule, the area shaded in red represents the THP expressing medullary thick ascending limb of Henle and early distal tubule. Below: overview of selected hypoxia/HIF dependent and independent pVHL targets with potential importance for renal cystogenesis and tumorigenesis. Ovals identify transcriptionally regulated targets, rectangles identify targets, which are regulated by non-transcriptional mechanisms; vertical arrows indicate up- or down regulation. HIF-1 counteracts Myc activity through direct protein-protein interaction and has been shown to induce cell cycle arrest in colon cancer cells [104], what this interaction means for renal tumorigenesis is unclear. BNIP-3 is a pro-apoptotic factor and is directly up-regulated by HIF-1 [116–118]. BCL-2, an anti-apoptotic protein, has been shown to be HIF-1 dependent in ES cell derived teratomas [105]. Up-regulation of cyclin D1

(*CCND1*), an important regulator of cell cycle progression, was found in VHL-deficient renal cancer cell lines and shown to be hypoxia-dependent in a cell type specific manner [119–122]. Chemokine receptor 4 (CXCR4) is a direct HIF target and has been shown to correlate with poor survival and metastatic ability of CC-RCC [100]. Jade 1 is a novel pVHL interacting protein that may play a role in renal cytogenesis through interaction with polycystin-1 [123]. *Abbreviations:* gl, glomerulus; cd, collecting duct.

Table 1Summary of phenotypes observed in different tissue specific *Vhlh* knock out strains

Cre-transgenic (promoter)	Reference	Cell type targeted	Phenotype
Albumin-Cre	Haase et al. [64], Rankin et al. [76]	Hepatocytes	Angiectasis, hemangiomas, endothelial cell proliferation, severe hepatic steatosis (neutral fat accumulation in hepatocytes), erythrocytosis from increased liver erythropoietin production; phenotype is dependent on Hif; both Hif-1 and -2 are functional in hepatocytes and the development of the phenotype can be suppressed by inactivation of Arnt; Hif-2 appears to be the main mediator of this phenotype [76]
Lck-Cre (lymphocyte protein tyrosine kinase, proximal promoter)	Biju et al. [75]	Thymocytes	Small, highly vascularized thymi, increased apoptosis in CD4/CD8 double positive cells involving a caspase 8 dependent mechanism; phenotype is strongly Hif-1 dependent; Hif-2 is non-functional in thymocytes
Fabp-Cre (fatty acid binding protein)	Karhausen et al. [77]	Colonic epithelial cells	Increased expression of Hif regulated barrier protective factors such as intestinal trefoil factor; protection from inflammatory bowel disease clinically; accumulation of glycogen in luminal epithelial cells
-Actin-Cre and tamoxifen-inducible ER-Cre	Ma et al. [67]	Mosaic	Hepatic vascular tumors, angiectasis in heart, liver, pancreas, lung, kidney, infertility, abnormal spermatogenesis
ColIII-Cre (collagen II)	Pfander et al. [78]	Growth plate, chondrocytes	Severe dwarfism, severe decrease in chondrocyte proliferation, up-regulation of Hif-1 dependent p57 ^{kip2} , increased extracellular matrix deposition
LysM-Cre (lysozyme M)	Cramer et al. [79]	Myeloid cells	Increase in inflammatory response through Hif-1
K14-Cre (keratin 14)	Boutin et al. [80] ^a	Epidermis	Increased dermal vasculature, higher metabolic rate, low weight, early death
WAP-Cre (whey acidic protein)	Seagroves et al. [81] ^a	Mammary gland epithelium	Abnormal differentiation of gland epithelium during lactation in multiply bred mutants, collapsed alveoli, little or no milk production, enlarged blood vessels, no hyperplasia or tumors
Nestin-Cre	Haase et al. unpublished	Neuro-epithelial progenitor cells	Abnormal neuronal differentiation, embryonic lethality
PEPCK-Cre (phosphoenolpyruvate carboxykinase)	Rankin et al., unpublished	Renal proximal tubule, hepatocytes	Renal cysts at low frequency, liver hemangiomas, polycythemia; phenotype is Hif dependent

For clarification, the names of genes from which promoter elements were derived for Cre-transgene construction is shown in brackets.

^aDenotes meeting abstracts.