Novel functions of the von Hippel-Lindau tumor suppressor

Dorus Adriana Mans

“Strength does not come from physical capacity. It comes from an indomitable will.” - Gandhi

“No! Try not. Do or do not. There is no try.” - Yoda, *Star Wars*

“To Start Press Any Key. Where’s the ANY key?” - Homer Simpson, *The Simpsons*

Cover: Highway transport, designed by Ruud Kouwenberg

ISBN: 978-90-393-4777-5

Printed by: PrintPartners Ipskamp

The printing of this thesis was financially supported by de Belangenvereniging von Hippel-Lindau, Carl Zeiss B.V., BD Biosciences, Novartis Oncology and the University Medical Center Utrecht.

Novel functions of the von Hippel-Lindau tumor suppressor

Nieuwe functies voor de von Hippel-Lindau tumor suppressor

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 8 april 2008 des middags te 12.45 uur

door

Dorus Adriana Mans

geboren op 4 maart 1978 te Best

Promotor: Prof. dr. E.E. Voest

Co-promotor: Dr. R.H. Giles

University Medical Center Utrecht
Department of Medical Oncology
Utrecht, the Netherlands

The research described in this thesis was financially supported by the Dutch Organization for Scientific Research (ZonMw/NWO).

Table of contents

Chapter 1

General Introduction

VHL disease

Inheritance of a mutant allele of the *von Hippel-Lindau* gene (*VHL*; OMIM# 608537; affecting approximately 1 in 36,000 live births [1]) predisposes to the development of clear-cell renal cell carcinoma (ccRCCs), hemangioblastoma of the central nervous system (CNS) and/or retina, endocrine neoplasia of the adrenal gland (pheochromocytoma), endolymphatic sac tumors of the inner ear, pancreatic tumors, epididymal and broad ligament cystadenomas, and vascular neoplasia in patients with VHL disease (OMIM# 193300) [2, 3]. The *VHL* gene fits the Knudson two-hit hypothesis [4] of a classical tumor suppressor gene, in that tumors that arise in patients with VHL disease exhibit loss, silencing or mutation of the second allele of *VHL*. In addition to the inherited risk for developing cancer, *VHL* allelic mutations result in development of multiple cysts in the kidney, pancreas and liver [5]. Biallelic inactivation of *VHL* by somatic mutations has been found in ~57% of sporadic RCCs [6]. Furthermore, silencing of *VHL* due to hypermethylation of the *VHL* promoter has been documented in some (~19%) clear-cell RCCs lacking mutations in *VHL* [7], implying that the function of *VHL* is especially important for curtailing neoplasia of the kidney. Mutations in virtually each of the 213 amino acid residues, encoded by *VHL*, have been reported (see *VHL* mutation databases).

Genotype/phenotype correlation

Different inherited mutations in *VHL* give rise to consistent disease manifestations, suggesting that some genotype/phenotype correlation exists. Illustrating this correlation, less than 10% of patients with a *VHL* deletion or truncation develop pheochromocytoma, while the risk is around 50% in patients with a *VHL* missense mutation. In many cases, knowing the *VHL* mutation in a family can help making informed predictions as to which tumors might develop. According to the classification of the National Cancer Institute (NCI), VHL patients are clinically categorized based on their predisposition to pheochromocytoma. Type 1 VHL disease patients have a low incidence of pheochromocytoma, as indicated in the example above, whereas type 2 VHL disease patients have a high incidence of pheochromocytoma [8]. Type 2 is further subclassified as types 2A, 2B and 2C (as shown in Table 1). While type 2A VHL disease patients have a small risk of developing clear-cell RCCs, they may develop pheochromocytoma, hemangioblastoma and other visceral complications associated with VHL disease, e.g. multiple renal/pancreatic cysts. Type 2B VHL disease patients are susceptible to pheochromocytoma, hemangioblastoma and clear-cell RCCs. Zbar *et al*. confirmed previous observations that germline *VHL* type 2B mutations at codon 167 (R167Q or R167W) convey a much higher risk for the development of pheochromocytoma and clear-cell RCCs than families without a mutation at codon 167 (64% vs. 7%) [9]. Type 2C VHL disease patients develop only pheochromocytoma. The vast majority of VHL patients have either type 1 or type 2B. In 2002, recessive mutations of *VHL* were discovered in some Chuvash families. This distinct congenital syndrome was characterized by a constitutive homozygous *VHL* missense mutation (R200W) [10]. VHL patients with polycythemia manifest thrombosis and vascular abnormalities, however are not predisposed to tumor formation [11]. This notion is also supported in mice homozygous for the R200W mutation. These mice develop polycythemia highly similar to the human disease, including increased hematocrits, red blood cell numbers, hemoglobin levels and elevated serum levels of both vascular endothelial growth factor (VEGF) and erythropoietin (EPO), but no tumors [12]. Since then, other homozygous missense mutations have been identified in

congenital polycythemia [13].

The von Hippel-Lindau protein: part of an E3 ubiquitin ligase complex

The *VHL* gene, localized on the short arm of chromosome 3 (3p26-p25) [14], spans three exons. *VHL* encodes a ubiquitously expressed full length protein (pVHL30) and a short isoform of pVHL (pVHL19), generated by an alternative translation initiation site at methionine-54 [15, 16]. *VHL* orthologs have been identified in a variety of other species, including *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Danio rerio* [17, 18]. pVHL30 is found primarily in the cytoplasm, whereas pVHL19 is equally distributed between the nucleus and cytoplasm in interphase cells [16, 19]. For simplicity the term "pVHL" is used when referring to both isoforms generically. A well-characterized function for pVHL is as a recognition subunit of an E3 ubiquitin ligase complex. The C-terminus (α-domain; specifically aa 157-171) of pVHL binds directly to Elongin C and indirectly to Elongin B, Cullin-2 (Cul2) and Rbx1 (also called ROC1 or Hrt1) [20-23]. The binding of pVHL to Elongin C is facilitated by cytosolic chaperonin containing TCP-1 (CCT), protecting pVHL from auto-ubiquitination [24-27]. The most important recognition targets to date for pVHL are the alpha subunits of the hypoxia inducible factor (HIF) transcription factors (HIF1α, 2α and 3α). In normoxia, HIFα is hydroxylated on conserved prolyl residues in the oxygen-dependent degradation domain (ODD) [28], via prolyl hydroxylases belonging to the EglN family (also called PHDs, HPHs), which in human cells comprise EglN1, EglN2 and EglN3 [29-31]. pVHL binds hydroxylated HIFα via its β-domain (specifically via W88, Y98, S111, H115 and W117) [32], which results in the poly-ubiquitination and proteolytic degradation of HIFα [33-36]. Hypoxia, which occurs frequently in tumors, prevents HIFα hydroxylation and results in the accumulation of nuclear HIFα. Subsequently, HIFα dimerizes with constitutively stable HIF1β (also called arylhydrocarbon nuclear translocator or ARNT1) forming a transcriptionally active complex. Mutations that lead to loss of pVHL function, by either inadequate binding to hydroxylated HIFα or to Elongin C, allows HIFα to escape proteolytic cleavage, thereby increasing its stability, and results in constitutive transactivity of HIFα, even in the presence of oxygen (Figure 1) [37, 38]. Loss of pVHL function and subsequent increased HIFα transcriptional activity induces expression of genes, many of which promote angiogenesis and tumor formation [39, 40], e.g. VEGF, glucose transporter-1 (GLUT-1), and platelet-derived

Table 1. Characteristics of VHL disease

Classification	*VHL* mutation	Molecular defect	Clinical manifestations
Type 1	Total or partial *VHL* loss Improper folding	HIFα stabilization	Hemangioblastoma High risk of renal cell carcinoma Low risk of pheochromocytoma
Type 2A	*VHL* missense mutation (only 2 mutations known)	Partial HIFα stabilization Inability to stabilize MTs	Hemangioblastoma Pheochromocytoma Low risk of renal cell carcinoma
Type 2B	*VHL* missense mutation	HIFα stabilization	Hemangioblastoma Pheochromocytoma High risk of renal cell carcinoma
Type 2C	*VHL* missense mutation	No HIFα stabilization Decreased FN matrix assembly Inability to regulate junB	Pheochromocytoma only
Congenital polycythemia	(Homozygous) *VHL* C-terminal missense mutation	HIFα stabilization Increased VEGF and EPO serum levels	Increased vertebral hemangiomas Peripheral thrombosis Polycythemia

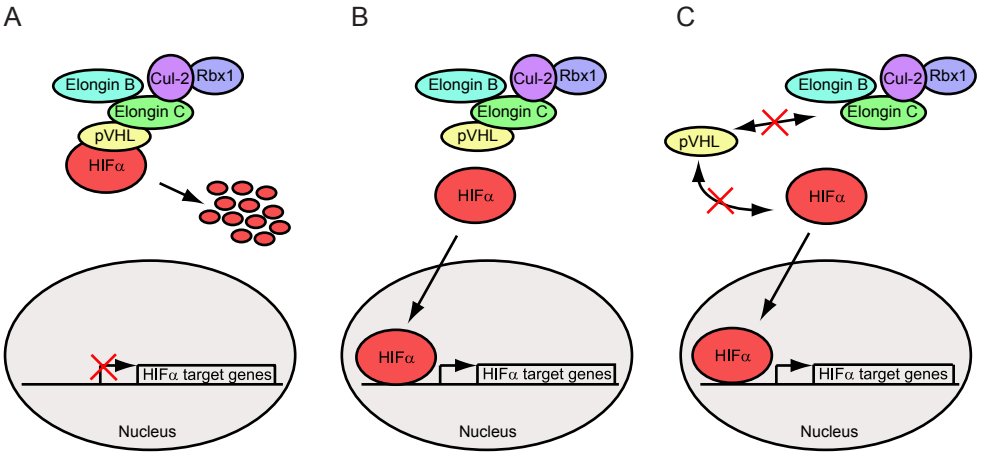


Figure 1: (A): Schematic overview of HIFα-recognition and subsequent degradation by the pVHL-E3 ubiquitin ligase complex in normoxia. (B): In hypoxia however, HIFα is no longer hydroxylated by prolylhydroxylases and is not recognized by the pVHL E3 ubiquitin ligase complex. This results in increased stability and nuclear accumulation of HIFα and transactivation of HIFα downstream target genes. (C): *VHL* mutations affecting binding of pVHL to either HIFα or Elongin C results in increased HIFα stability and activity, even in the presence of oxygen.

growth factor (PDGF)-β [41]. Interestingly, polycythemia patients with *VHL* mutations manifest elevated normoxic HIFα protein levels and increased expression of VEGF [11] but no tumor predisposition, suggesting that increased expression of both HIFα and VEGF are not sufficient for tumor formation [11].

Another known target of the VHL-ElonginC/B-Cul2-Rbx1 E3 ubiquitin ligase complex is (activated) atypical protein kinase C (aPKC). pVHL's β-domain binds directly to the regulatory domain of aPKC and results in aPKC poly-ubiquitination and subsequent proteasomal degradation [42, 43]. Furthermore, VHL-dependent ubiquitination has been described for de-ubiquitinating enzymes VDU1/USP33 and VDU2/USP20 [44, 45], and hyperphosphorylated Rbp1, a subunit of RNA polymerase II [46]. pVHL has also been reported to bind and stabilize the homeodomain-containing protein Jade-1 [47], and KRAB-A domain-containing protein (VHLaK), which may cooperate with pVHL to suppress HIFα transcriptional activity [48].

Mechanism(s) of HIFα independent tumor suppression by pVHL

Inheritance of VHL disease type 2C alleles, having completely unimpaired ability to target HIFα for oxygen-dependent proteolysis [49, 50], still predisposes affected individuals to develop adrenal tumors (pheochromocytoma), implying that pVHL has HIFα-independent tumor suppressing activities. One of these activities involves the pVHL/microtubule association. A region within the β-domain (aa 104-123) of pVHL has shown to be sufficient to inhibit renal tumor growth and invasion of renal cancer cells *in vitro* [51]. Interestingly, a similar region (aa 95-123) has shown to be essential in indirect microtubule-binding, controlling the orientation of microtubule growth [52], protecting microtubules from nocodazole-induced depolymerization [19] and increasing microtubule plus-end stability [53]. Phosphorylation of pVHL30 by glycogen synthase kinase 3β (GSK3β) on serine-68, with a priming phosphorylation at serine-72 by casein kinase 1 (CK1), negatively regulates pVHL's microtubule stabilization (but not binding) activity [54]. pVHL's microtubule regulatory function is believed to represent

a non-E3 ubiquitin ligase activity of this tumor suppressor [19, 53]. Interestingly, certain naturally occurring allelic mutations of *VHL* (e.g. Y98H, Y112H, F119S) display altered microtubule stabilization function, implying a link between this function and *VHL*-associated tumor suppressing activity [19].

Phosphorylation of pVHL30 by casein kinase 2 (CK2) at serines-33, 38 and 43 has shown to be essential for pVHL's tumor suppressor function in mouse xenograft experiments [55]. Interestingly, pVHL has shown to serve as an adaptor, promoting phosphorylation of Card9 (NF-KappaB agonist) by CK2, indicating that pVHL is an adaptor for both an ubiquitin conjugating enzyme and a kinase [56].

Pheochromocytoma in VHL type 2 patients have been proposed to result from failure of a pathway that regulates the developmental apoptosis of neural progenitor cells [57]. Persistence of these cells in the absence of pVHL activity is believed to contribute to the development of tumors. Loss of pVHL leads to increased JunB expression, reflecting both increased HIFα levels and aPKC activity. In turn, JunB antagonizes c-Jun mediated apoptosis. Whether such an activity of pVHL contributes to tumor suppression in other tissues is not known.

Yet another recently identified HIFα-independent activity of pVHL is as a positive regulator of p53. p53 directly associates with the α-domain of pVHL, competing for binding with Elongin C. pVHL also recruits ataxia telangiectasia mutated (ATM) and acetyltransferases (p300, pCAF), thereby blocking Mdm2-mediated ubiquitination and nuclear export of p53, resulting in subsequent increased p53 stability and transcriptional activity. In the absence of pVHL, p53 protein levels and transcriptional activity are reduced, and responses to DNA damage (e.g. G1 cell cycle arrest) are impaired [58] (as shown in Figure 2).

In addition to this publication, several reports have shown that pVHL affects cell cycle progression in a p53-independent manner. Early reports describe that loss of *VHL* enhances cyclin D1 expression [59] and downregulates expression of p27 in renal tumors [60]. Accordingly, reintroduction of pVHL into RCC cells was reported to increase expression of p27 [58, 61]. One other report contrasts this trend; Mack *et al.* (2005) reported that growth of *vhlh*$^{-/-}$ transformed mouse embryonic fibroblasts (MEFs) is retarded due to increased p21 and p27 expression [62]. This discrepancy might be attributable to different backgrounds between *VHL*$^{-/-}$ RCC cells and *vhlh*$^{-/-}$ transformed MEFs. Thus, *VHL* mutation may affect cell cycle progression, and compromise p53 function, leading to the accumulation of further genetic abnormalities and cancer progression.

Finally, pVHL plays an important role in maintaining the integrity and architecture of epithelial tissues. Loss of pVHL function induces many of the hallmarks of epithelial-mesenchymal transition (EMT), a process that is believed to be vital to the ability of tumor cells to invade and metastasize. *VHL*-deficient RCC cell lines display fibroblast-like morphology [63], exhibit HIFα-independent reduction in the number of adherens/tight junctions and loss of polarity [64], and HIFα-dependent loss of expression of E-cadherin [65-67]. pVHL is also required for the deposition of the extracellular matrix (ECM) components fibronectin and collagen IV [68-70]. The precise mechanism underlying this function is unknown, but probably involves binding of pVHL to the ECM component, although pVHL has also been shown to upregulate fibronectin expression [71]. Additionally, *VHL* mutant cells exhibit increased activities of matrix metalloproteinases MMP-2 and MMP-9 [72] and HIF2α induces the expression of membrane type-1 MMP [73, 74]. These enzymes degrade and remodel the ECM. Loss of pVHL function therefore causes defects in the ECM that may

promote angiogenesis, invasion and metasatasis of tumor cells. An overview of the various putative mechanisms of tumor suppression by pVHL is shown in Figure 2. Maintenance of the primary cilium might be an additional role for pVHL in tumor suppression, as discussed briefly later in this chapter and at length in chapters three and four of this thesis.

The other way around: inhibiting cell proliferation by HIF1α

One cellular response that may initially restrain tumor formation following loss of pVHL function may be HIF1α-induced cell cycle arrest. Tumor xenografts of embryonic stem (ES) cells or transformed MEFs harboring a deletion of the *vhlh* gene grew more poorly than their *vhlh*-wildtype counterparts [62, 75], suggesting that *vhlh* loss actually confers a growth disadvantage. This may partly be due to activation of HIF1α, which exerts a negative effect on cellular proliferation of MEFs in cell culture [76] and on human RCC cell lines grown as xenografts in mice [77]. HIF1α also has shown to induce pro-apoptotic genes, such as BCL2/adenovirus E1B 19-kDa interacting protein 3 (BniP3), BniP3-like (BniP3L), and DNA-damage-inducible transcript 4 (DDIT4) [78-80].

Different research groups have identified that HIF1α antagonizes the activity of the cell cycle promoting transcription factor c-Myc, through a mechanism that has been variously proposed to involve direct binding to c-Myc itself [81], competitive binding to the c-Myc co-factors Sp1 and Max [76], upregulation of the transcription of the c-Myc inhibitor Mxi-1, or promotion of proteosome-dependent degradation of c-Myc [82]. HIF1α may also prevent c-Myc activation indirectly by inhibiting the activity of β-catenin/TCF-4 transcription factor complex that normally promotes cell cycle progression by inducing c-Myc expression [83]. In summary, HIF1α activation results in inhibition of c-Myc mediated repression of the cyclin-dependent kinase inhibitors p21 and p27, resulting in their accumulation and cell cycle arrest.

Contrasting properties HIF1α and HIF2α

HIF1α and HIF2α induce largely overlapping, but partly distinct, transcriptional programs [84, 85]. One important functional difference between HIF1α and HIF2α lies in their respective effects on cellular proliferation. In contrast to the inhibitory effects of HIF1α, HIF2α promotes the proliferation of MEFs [76], promotes tumor formation in RCC xenografts [77, 86, 87] and enhances c-Myc activity [76], resulting in upregulation of cell cycle promoting genes such as cyclin D1 and cyclin D2 and promotion of cell cycle progression. Thus, it is likely that the relative balance of HIF1α and HIF2α activities may dictate the cell cycle response following loss of pVHL function. The molecular pathways that determine this balance remain largely unclear. However, it is interesting that HIF1α and HIF2α appear to be mutually suppressive in that increasing HIF1α protein abundance decreases HIF2α protein abundance and vice versa [77].

A watchtower preventing cell proliferation

The primary cilium is a microtubule-based structure that protrudes from the surface of most non-proliferating cell types and functions to sense molecular and mechanical signals in the extracellular environment [88]. The consequences of loss of primary cilia function on the kidney tubule epithelium are disastrous; cells proliferate uncontrollably and form large fluid filled cysts that eventually lead to kidney failure. Thus, the primary cilium plays a key role in negative regulation of cell proliferation. Recently, several groups have demonstrated that pVHL localizes to primary cilia *in*

vitro and *in vivo* and functions in the formation or maintenance of the primary cilium [52, 89-92].

Kidney cysts arise frequently in VHL patients and may, in at least a subset of patients, represent precursor lesions of clear-cell RCC [93, 94]. However, while kidneys of VHL patients contain many hundreds or thousands of cells with loss of pVHL function, cysts arise relatively infrequently [95], implying that loss of pVHL function alone does not impair the function of the primary cilium. Indeed, deletion of pVHL from primary cells did not affect ciliogenesis in culture. Rather, pVHL-loss sensitized cells to lose their cilia in response to signals that lead to inactivation of GSK3β, such as serum starvation. This cilia maintenance activity of pVHL that is uncovered by GSK3β inactivation is independent of its ability to control HIFα stability, but dependent on its ability to stabilize microtubules [54].

GSK3β has been shown to be inactivated by phosphorylation in *VHL*-negative cysts, but not in *VHL*-negative early lesions in human VHL patients [92]. This suggests that mutations or environmental alterations that lead to inactivation of GSK3β, for example through hyperactivation of protein kinase B (PKB) [96] or aPKC [97], may represent important secondary events in *VHL*-negative cells that result in failure to maintain the primary cilium, loss of control of cellular proliferation, the development of cysts and progression of cystic lesions towards clear-cell RCC. These studies illustrate the principle that development of kidney cysts in VHL disease are not solely the result of *VHL* mutation but may result from cooperation with alterations in other signaling pathways.

Finally, deregulation of other cellular processes that involve microtubules, such as polarity, migration and mitosis, have been implicated in tumor formation. It will be interesting to investigate whether these processes are also normally regulated by pVHL's microtubule-associated function and as such whether they may also represent tumor suppressor activities of pVHL.

Outline of this thesis

The von Hippel-Lindau tumor suppressor has proven to be a multi-functional tumor suppressor, as described earlier. In the research presented in this thesis we studied possible tumor suppressing activities of pVHL. In Chapter 2, we identified that expression and activity of the transcription factor E2F1 is negatively regulated by pVHL in a HIFα-independent manner. The majority (~90%) of clear-cell RCC samples from VHL patients show high nuclear E2F1 staining, suggesting that E2F1 might be involved in tumorigenesis in patients with VHL disease. The α-domain of pVHL seems to be essential for E2F1 regulation in RCC cells, however does not affect cell cycle progression of asynchronous RCC cells. In Chapter 3, we give an overview of recent literature linking ciliary components and signaling pathways with tumorigenesis. In Chapter 4, we focus on the role of pVHL in the regulation of primary cilia. We show that pVHL is able to maintain primary cilia and ciliary function of kidney epithelial cells, in a HIFα-independent fashion. In these cells we show axonemal localization of pVHL. However, *VHL* -type 2A- disease associated allele Y112H and pVHL lacking the microtubule-binding domain, both known for their inability to protect stabilized microtubules from nocodazole treatment [19], are no longer able to localize along the ciliary axoneme or to maintain functional primary cilia in cultured kidney epithelial cells. Surprisingly, pVHL19 was not observed to localize to the primary cilium nor could it restore cilia function as measured by calcium influx. In Chapter 5, we searched for the missing mechanism for pVHL's ciliary function. Applying yeast-

Figure 2: Summary of HIFα-dependent and HIFα-independent functions of pVHL that may contribute to the tumor suppressing activity of pVHL. ECM: extracellular matrix; EMT: epithelial to mesenchymal transition

two-hybrid assays, we identified an interaction between pVHL and KIF3A, a subunit of the anterograde kinesin-2 motorcomplex, shown to be important in correct intraflagellar transport (IFT) and ciliogenesis. We show that pVHL interacts endogenously with all members of kinesin-2 and we identify two domains to be important for binding kinesin-2. The N-terminal domain of pVHL binds KAP3 and the microtubule-binding domain binds KIF3A. Interestingly, *VHL* variant alleles displaying the inability to stabilize microtubules or to maintain primary cilia have a disrupted binding to kinesin-2, suggesting that kinesin-2 interaction is essential for pVHL to stabilize microtubules and/or to maintain primary cilia. In Chapter 6 we show that cytoplasmic mobility and subcellular localization of pVHL is dependent on the presence of ATP. Removing ATP does result in centrosomal localization of immobile pVHL. However, *VHL* variant alleles displaying a disrupted binding to kinesin-2 are less responsive to ATP-depletion. Furthermore, inhibiting pVHL-KIF3A interaction by means of a dominant negative form of KAP3 (ΔN-KAP3) or KIF3A RNAi increases pVHL mobility, indicating that KIF3A affects the mobility of pVHL. Finally, in Chapter 7 we give a summarizing discussion of the work described in this thesis and its implications.

References

1. **Maher, E.R. *et al.*** Von Hippel-Lindau disease: a genetic study. *J Med Genet* 28, 443-447 (1991).
2. **Lonser, R.R. *et al.*** von Hippel-Lindau disease. *Lancet* 361, 2059-2067 (2003).
3. **Kim, W.Y. & Kaelin, W.G.** Role of VHL gene mutation in human cancer. *J Clin Oncol* 22, 4991-5004 (2004).
4. **Knudson, A.G., Jr.** Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 68, 820-823 (1971).
5. **Kaelin, W.G., Jr.** Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer* 2, 673-682 (2002).
6. **Gnarra, J.R. *et al.*** Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet* 7, 85-90 (1994).
7. **Herman, J.G. *et al.*** Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A* 91, 9700-9704 (1994).
8. **Neumann, H.P. & Wiestler, O.D.** Clustering of features of von Hippel-Lindau syndrome: evidence for a complex genetic locus. *Lancet* 337, 1052-1054 (1991).
9. **Zbar, B. *et al.*** Germline mutations in the Von Hippel-Lindau disease (VHL) gene in families from North America, Europe, and Japan. *Hum Mutat* 8, 348-357 (1996).
10. **Ang, S.O. *et al.*** Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nat Genet* 32, 614-621 (2002).
11. **Gordeuk, V.R. *et al.*** Congenital disorder of oxygen sensing: association of the homozygous Chuvash polycythemia VHL mutation with thrombosis and vascular abnormalities but not tumors. *Blood* 103, 3924-3932 (2004).
12. **Hickey, M.M., Lam, J.C., Bezman, N.A., Rathmell, W.K. & Simon, M.C.** von Hippel-Lindau mutation in mice recapitulates Chuvash polycythemia via hypoxia-inducible factor-2alpha signaling and splenic erythropoiesis. *J Clin Invest* 117, 3879-3889 (2007).
13. **Pastore, Y. *et al.*** Mutations of von Hippel-Lindau tumor-suppressor gene and congenital polycythemia. *Am J Hum Genet* 73, 412-419 (2003).
14. **Stolle, C. *et al.*** Improved detection of germline mutations in the von Hippel-Lindau disease tumor suppressor gene. *Hum Mutat* 12, 417-423 (1998).
15. **Schoenfeld, A., Davidowitz, E.J. & Burk, R.D.** 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 U S A* 95, 8817-8822 (1998).
16. **Iliopoulos, O., Ohh, M. & Kaelin, W.G., Jr.** pVHL19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. *Proc Natl Acad Sci U S A* 95, 11661-11666 (1998).
17. **Woodward, E.R. *et al.*** Comparative sequence analysis of the VHL tumor suppressor gene. *Genomics* 65, 253-265 (2000).
18. **Adryan, B., Decker, H.J., Papas, T.S. & Hsu, T.** Tracheal development and the von Hippel-Lindau tumor suppressor homolog in Drosophila. *Oncogene* 19, 2803-2811 (2000).
19. **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* 5, 64-70 (2003).

20. **Duan, D.R. *et al.*** Inhibition of transcription elongation by the VHL tumor suppressor protein. *Science* 269, 1402-1406 (1995).
21. **Kamura, T. *et al.*** Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science* 284, 657-661 (1999).
22. **Kibel, A., Iliopoulos, O., DeCaprio, J.A. & Kaelin, W.G., Jr.** Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. *Science* 269, 1444-1446 (1995).
23. **Pause, A. *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 U S A* 94, 2156-2161 (1997).
24. **Feldman, D.E., Thulasiraman, V., Ferreyra, R.G. & Frydman, J.** Formation of the VHL-elongin BC tumor suppressor complex is mediated by the chaperonin TRiC. *Mol Cell* 4, 1051-1061 (1999).
25. **Hansen, W.J. *et al.*** Diverse effects of mutations in exon II of the von Hippel-Lindau (VHL) tumor suppressor gene on the interaction of pVHL with the cytosolic chaperonin and pVHL-dependent ubiquitin ligase activity. *Mol Cell Biol* 22, 1947-1960 (2002).
26. **Kamura, T., Brower, C.S., Conaway, R.C. & Conaway, J.W.** A molecular basis for stabilization of the von Hippel-Lindau (VHL) tumor suppressor protein by components of the VHL ubiquitin ligase. *J Biol Chem* 277, 30388-30393 (2002).
27. **Schoenfeld, A.R., Davidowitz, E.J. & Burk, R.D.** Elongin BC complex prevents degradation of von Hippel-Lindau tumor suppressor gene products. *Proc Natl Acad Sci U S A* 97, 8507-8512 (2000).
28. **Jaakkola, P. *et al.*** Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* 292, 468-472 (2001).
29. **Bruick, R.K. & McKnight, S.L.** A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294, 1337-1340 (2001).
30. **Epstein, A.C. *et al.*** C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107, 43-54 (2001).
31. **Ivan, M. *et al.*** Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. *Proc Natl Acad Sci U S A* 99, 13459-13464 (2002).
32. **Min, J.H. *et al.*** Structure of an HIF-1alpha -pVHL complex: hydroxyproline recognition in signaling. *Science* 296, 1886-1889 (2002).
33. **Cockman, M.E. *et al.*** Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem* 275, 25733-25741 (2000).
34. **Kamura, T. *et al.*** Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. *Proc Natl Acad Sci U S A* 97, 10430-10435 (2000).
35. **Ohh, M. *et al.*** Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol* 2, 423-427 (2000).
36. **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* 19, 4298-4309 (2000).

37. **Sutter, C.H., Laughner, E. & Semenza, G.L.** Hypoxia-inducible factor 1alpha protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutations. *Proc Natl Acad Sci U S A* 97, 4748-4753 (2000).
38. **Lonergan, K.M. *et al.*** Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. *Mol Cell Biol* 18, 732-741 (1998).
39. **Carmeliet, P. *et al.*** Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394, 485-490 (1998).
40. **Ryan, H.E., Lo, J. & Johnson, R.S.** HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *Embo J* 17, 3005-3015 (1998).
41. **Iliopoulos, O., Levy, A.P., Jiang, C., Kaelin, W.G., Jr. & Goldberg, M.A.** Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proc Natl Acad Sci U S A* 93, 10595-10599 (1996).
42. **Okuda, H. *et al.*** Direct interaction of the beta-domain of VHL tumor suppressor protein with the regulatory domain of atypical PKC isotypes. *Biochem Biophys Res Commun* 263, 491-497 (1999).
43. **Okuda, H. *et al.*** The von Hippel-Lindau tumor suppressor protein mediates ubiquitination of activated atypical protein kinase C. *J Biol Chem* 276, 43611-43617 (2001).
44. **Li, Z. *et al.*** Ubiquitination of a novel deubiquitinating enzyme requires direct binding to von Hippel-Lindau tumor suppressor protein. *J Biol Chem* 277, 4656-4662 (2002).
45. **Li, Z. *et al.*** Identification of a deubiquitinating enzyme subfamily as substrates of the von Hippel-Lindau tumor suppressor. *Biochem Biophys Res Commun* 294, 700-709 (2002).
46. **Kuznetsova, A.V. *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 U S A* 100, 2706-2711 (2003).
47. **Zhou, M.I. *et al.*** The von Hippel-Lindau tumor suppressor stabilizes novel plant homeodomain protein Jade-1. *J Biol Chem* 277, 39887-39898 (2002).
48. **Li, Z. *et al.*** The VHL protein recruits a novel KRAB-A domain protein to repress HIF-1alpha transcriptional activity. *Embo J* 22, 1857-1867 (2003).
49. **Clifford, S.C. *et al.*** Contrasting effects on HIF-1alpha regulation by disease-causing pVHL mutations correlate with patterns of tumourigenesis in von Hippel-Lindau disease. *Hum Mol Genet* 10, 1029-1038 (2001).
50. **Hoffman, M.A. *et al.*** von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. *Hum Mol Genet* 10, 1019-1027 (2001).
51. **Datta, K., Sundberg, C., Karumanchi, S.A. & 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* 61, 1768-1775 (2001).
52. **Schermer, B. *et al.*** The von Hippel-Lindau tumor suppressor protein controls ciliogenesis by orienting microtubule growth. *J Cell Biol* 175, 547-554 (2006).

53. **Lolkema, M.P. *et al.*** The von Hippel-Lindau tumor suppressor protein influences microtubule dynamics at the cell periphery. *Exp Cell Res* 301, 139-146 (2004).
54. **Hergovich, A. *et al.*** Priming-dependent phosphorylation and regulation of the tumor suppressor pVHL by glycogen synthase kinase 3. *Mol Cell Biol* 26, 5784-5796 (2006).
55. **Lolkema, M.P. *et al.*** Tumor suppression by the von Hippel-Lindau protein requires phosphorylation of the acidic domain. *J Biol Chem* 280, 22205-22211 (2005).
56. **Yang, H. *et al.*** pVHL Acts as an adaptor to promote the inhibitory phosphorylation of the NF-kappaB agonist Card9 by CK2. *Mol Cell* 28, 15-27 (2007).
57. **Lee, S. *et al.*** Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. *Cancer Cell* 8, 155-167 (2005).
58. **Roe, J.S. *et al.*** p53 stabilization and transactivation by a von Hippel-Lindau protein. *Mol Cell* 22, 395-405 (2006).
59. **Bindra, R.S., Vasselli, J.R., Stearman, R., Linehan, W.M. & Klausner, R.D.** VHL-mediated hypoxia regulation of cyclin D1 in renal carcinoma cells. *Cancer Res* 62, 3014-3019 (2002).
60. **Osipov, V., Keating, J.T., Faul, P.N., Loda, M. & Datta, M.W.** Expression of p27 and VHL in renal tumors. *Appl Immunohistochem Mol Morphol* 10, 344-350 (2002).
61. **Kim, M. *et al.*** Recombinant adenovirus expressing Von Hippel-Lindau-mediated cell cycle arrest is associated with the induction of cyclin-dependent kinase inhibitor p27Kip1. *Biochem Biophys Res Commun* 253, 672-677 (1998).
62. **Mack, F.A., Patel, J.H., Biju, M.P., Haase, V.H. & Simon, M.C.** Decreased growth of Vhl-/- fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. *Mol Cell Biol* 25, 4565-4578 (2005).
63. **Davidowitz, E.J., Schoenfeld, A.R. & Burk, R.D.** VHL induces renal cell differentiation and growth arrest through integration of cell-cell and cell-extracellular matrix signaling. *Mol Cell Biol* 21, 865-874 (2001).
64. **Calzada, M.J. *et al.*** von Hippel-Lindau tumor suppressor protein regulates the assembly of intercellular junctions in renal cancer cells through hypoxia-inducible factor-independent mechanisms. *Cancer Res* 66, 1553-1560 (2006).
65. **Esteban, M.A. *et al.*** Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res* 66, 3567-3575 (2006).
66. **Evans, A.J. *et al.*** VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail. *Mol Cell Biol* 27, 157-169 (2007).
67. **Krishnamachary, B. *et al.*** Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Res* 66, 2725-2731 (2006).
68. **Grosfeld, A. *et al.*** Interaction of hydroxylated collagen IV with the von hippel-lindau tumor suppressor. *J Biol Chem* 282, 13264-13269 (2007).
69. **Kurban, G. *et al.*** Collagen matrix assembly is driven by the interaction of

von Hippel-Lindau tumor suppressor protein with hydroxylated collagen IV alpha 2. *Oncogene* (2007).

70. **Ohh, M. *et al.*** The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol Cell* 1, 959-968 (1998).
71. **Bluyssen, H.A. *et al.*** Fibronectin is a hypoxia-independent target of the tumor suppressor VHL. *FEBS Lett* 556, 137-142 (2004).
72. **Koochekpour, S. *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* 19, 5902-5912 (1999).
73. **Petrella, B.L. & Brinckerhoff, C.E.** Tumor cell invasion of von Hippel Lindau renal cell carcinoma cells is mediated by membrane type-1 matrix metalloproteinase. *Mol Cancer* 5, 66 (2006).
74. **Petrella, B.L., Lohi, J. & Brinckerhoff, C.E.** Identification of membrane type-1 matrix metalloproteinase as a target of hypoxia-inducible factor-2 alpha in von Hippel-Lindau renal cell carcinoma. *Oncogene* 24, 1043-1052 (2005).
75. **Mack, F.A. *et al.*** Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. *Cancer Cell* 3, 75-88 (2003).
76. **Gordan, J.D., Bertout, J.A., Hu, C.J., Diehl, J.A. & Simon, M.C.** HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. *Cancer Cell* 11, 335-347 (2007).
77. **Raval, R.R. *et al.*** Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Mol Cell Biol* 25, 5675-5686 (2005).
78. **Bruick, R.K.** Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc Natl Acad Sci U S A* 97, 9082-9087 (2000).
79. **Shoshani, T. *et al.*** Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. *Mol Cell Biol* 22, 2283-2293 (2002).
80. **Sowter, H.M., Ratcliffe, P.J., Watson, P., Greenberg, A.H. & Harris, A.L.** HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res* 61, 6669-6673 (2001).
81. **Koshiji, M. *et al.*** HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *Embo J* 23, 1949-1956 (2004).
82. **Zhang, H. *et al.*** HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. *Cancer Cell* 11, 407-420 (2007).
83. **Kaidi, A., Williams, A.C. & Paraskeva, C.** Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nat Cell Biol* 9, 210-217 (2007).
84. **Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B. & Simon, M.C.** Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* 23, 9361-9374 (2003).
85. **Kim, W.Y. *et al.*** Failure to prolyl hydroxylate hypoxia-inducible factor alpha phenocopies VHL inactivation in vivo. *Embo J* 25, 4650-4662 (2006).
86. **Kondo, K., Kim, W.Y., Lechpammer, M. & Kaelin, W.G., Jr.** Inhibition of

HIF2alpha is sufficient to suppress pVHL-defective tumor growth. *PLoS Biol* 1, E83 (2003).

87. **Zimmer, M., Doucette, D., Siddiqui, N. & Iliopoulos, O.** Inhibition of hypoxia-inducible factor is sufficient for growth suppression of VHL-/- tumors. *Mol Cancer Res* 2, 89-95 (2004).
88. **Fliegauf, M., Benzing, T. & Omran, H.** When cilia go bad: cilia defects and ciliopathies. *Nat Rev Mol Cell Biol* 8, 880-893 (2007).
89. **Esteban, M.A., Harten, S.K., Tran, M.G. & Maxwell, P.H.** Formation of primary cilia in the renal epithelium is regulated by the von Hippel-Lindau tumor suppressor protein. *J Am Soc Nephrol* 17, 1801-1806 (2006).
90. **Lolkema, M.P. *et al.*** Allele-specific regulation of primary cilia function by the von Hippel-Lindau tumor suppressor. *Eur J Hum Genet* 16, 73-78 (2008).
91. **Lutz, M.S. & Burk, R.D.** Primary cilium formation requires von hippel-lindau gene function in renal-derived cells. *Cancer Res* 66, 6903-6907 (2006).
92. **Thoma, C.R. *et al.*** pVHL and GSK3beta are components of a primary cilium-maintenance signalling network. *Nat Cell Biol* 9, 588-595 (2007).
93. **Choyke, P.L. *et al.*** The natural history of renal lesions in von Hippel-Lindau disease: a serial CT study in 28 patients. *AJR Am J Roentgenol* 159, 1229-1234 (1992).
94. **Solomon, D. & Schwartz, A.** Renal pathology in von Hippel-Lindau disease. *Hum Pathol* 19, 1072-1079 (1988).
95. **Mandriota, S.J. *et al.*** HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron. *Cancer Cell* 1, 459-468 (2002).
96. **Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M. & Hemmings, B.A.** Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785-789 (1995).
97. **Lavoie, L., Band, C.J., Kong, M., Bergeron, J.J. & Posner, B.I.** Regulation of glycogen synthase in rat hepatocytes. Evidence for multiple signaling pathways. *J Biol Chem* 274, 28279-28285 (1999).

Chapter 2

The von Hippel-Lindau tumor suppressor regulates E2F1

Dorus A. Mans, Bart G. Weijts, Joost S. Vermaat, Laura G. Daenen,
Ellen van Rooijen, Alain de Bruin, Rob B. van der Luijt,
Emile E. Voest and Rachel H. Giles

Manuscript in preparation

Abstract

Biallelic mutations in the *von Hippel-Lindau* (*VHL*) gene are the most common cause of sporadic and inherited renal cell carcinoma (RCC). Inactivation of the VHL protein (pVHL) has been reported to result in deregulation of cell cycle and apoptosis related proteins. We show here that increased nuclear expression of E2F1 is observed in almost all clear-cell RCCs, derived from patients with known *VHL* missense mutations/truncations which is significantly higher than nuclear E2F1 expression in 30 sporadic clear-cell RCCs cases with unknown *VHL* status. Real-time PCR determined that E2F1 mRNA levels are also increased in 4/4 sporadic kidney tumors tested as compared to matched normal kidney tissue from the same patient. To examine the molecular relationship between pVHL and E2F1, we exogenously expressed pVHL in several RCC cell lines as well as mouse embryonic fibroblasts and observed marked and dose-dependent reduction of E2F1 mRNA, protein expression, and activity; however, no differences in cell cycle profiles were observed. From a panel of stable RCC cell lines reconstituted with disease-associated allelic variants of *VHL*, we demonstrate that alleles deficient in HIFα regulation can still downregulate E2F1 expression. Accordingly, knockdown of HIFα does not alter E2F1 levels, indicating that E2F1 regulation is a novel HIFα-independent function for pVHL.

Introduction

Biallelic inactivating mutations of the *von Hippel-Lindau* tumor suppressor gene (*VHL*) result in multiple cysts and tumor formation in patients [1]. These tumors often display aberrant stabilization of two parologs of the hypoxia-inducible factor alpha subunits (HIF1/2α) resulting in increased transactivation of HIF1/2α downstream effectors involved in angiogenesis, cell cycle progression and transformation. Many lines of evidence support a role for pVHL in an E3 ubiquitin ligase complex together with Elongin B/C, Cullin-2, and Rbx-1 [2-5]. Two targets poly-ubiquitinated by this complex are HIFα [6-9] and aPKC isoforms [10, 11] thereby targeting these proteins to the 26S proteasome. Besides being an active E3 ligase component, pVHL has other potentially tumor suppressing activities independent of HIFα, such as assembling of the extracelllular matrix [12, 13] primary cilia function [14], stability of peripheral microtubules [15, 16] and growth control [17, 18]. Loss of the wildtype *VHL* gene results in a specific cellular defect in serum-dependent growth control, which is correlated with decreased p27 expression in RCC cells [19, 20] and renal tumors [21], and increased cyclin D1 levels [22, 23]. Many, of the identified cell cycle components differentially expressed in *VHL*-deficient cells can be attributed to increased stability of HIF1/2α [22, 24, 25], however there is clear evidence that pVHL regulates cell proliferation via HIFα-independent mechanisms, e.g. p53 regulation, thereby blocking G1/S cell cycle transition and increasing the G1 population in the cell cycle [26].

Also involved in the transition from G1 to S is the transcription factor E2F1. The founding member of a highly related family of transcription factors, E2F1, controls the cell cycle and action of tumor suppressor proteins and is also a target of the transforming proteins of small DNA tumor viruses [27-30]. E2F1 is expressed in late G1 and is responsible for the transactivation of downstream components important for G1/S transition including minichromosome maintenance proteins (MCMs) [31-33], which are components of the DNA-replication initiation complex [34]. DNA damage or stress triggers an E2F1 transcriptional program, resulting in the transactivation of proteins

involved in the response to DNA damage (e.g. BRCA1, Rad51) or apoptosis (e.g. p73, Apaf-1) [35]. The best documented link between E2F1 and cancer lies in its regulation by the retinoblastoma tumor suppressor pRB. Hypophosphorylated pRB binds and inhibits transcriptional activity of E2F1, resulting in subsequent cell cycle arrest [36]. However, when pRB is hyperphosphorylated due to cyclin dependent kinases (cdks), E2F1 is released from this complex and is able to transactivate genes required for the G1/S transition. Increased expression of E2F1 has been observed in a variety of cancers (as reviewed in [37]).

In this study, we analyzed nuclear E2F1 and HIF1α expression in eight VHL disease associated clear-cell RCCs. High nuclear E2F1 and HIF1α expression are observed in almost all tumors. Strikingly, re-introduction of pVHL into *VHL*-deficient RCC cells results in a marked downregulation of (nuclear) E2F1 expression and activity in a dose dependent manner; however, no differences in cell cycle profiles were observed in these RCC cells. Together, these data suggest that E2F1 regulation is a novel HIFα-independent function for pVHL.

Materials & Methods

RCC collection and immunohistochemistry

Nephrectomy and biopsy specimens from eight VHL patients with clear-cell RCCs and 30 sporadic RCCs dating from January 1994 to September 2006 were collected from the University Medical Center Utrecht Pathology archives. All tumors were reviewed to confirm their histological type. Patient matched normal adjacent kidney tissue was also included in the analyses, allowing for comparative analyses of tumor versus matched normal kidney. 4 μm sections of each paraffin block were mounted on silane-coated glass slides and used for immunohistochemistry as previously described [38]. Stainings were performed using anti-E2F1 (KH95; 1:100; Santa Cruz Biotechnology) and anti-HIF1α (1:50; BD) [38]. An experienced pathologist blindly scored three fields per tumor containing approximately 2000-5000 cells each.

Plasmids

All VHL expression constructs were cloned via BamH1/EcoR1 into pBabe-puro containing an N-terminal HA-tag. Vsv-pVHL30 has been described before [39]. All plasmids used have been sequence-verified.

Cell culture and transfections

Cell lines RCC10 and RCC10 stably transfected with full length pVHL (clone 90 [40]) were a kind gift from Dr. Patrick Maxwell (Imperial College, London). RCC10 stably transfected with HA-pVHL30 (clone 3D10), HA-pVHL19, HA-pVHL-F119S, HA-pVHL-ΔF76 or pBabe-HA alone (Mock), and 786-0 stably transfected with either Vsv-pVHL30 or pcDNA3-Vsv alone (Mock) were generated by standard electroporation and puromycin (2 μg/ml) selection. Stable clones of 786-0 and RCC10 cells, *E2F7/8* double knock-out MEFs [42] and HEK293T cells were cultured in DMEM supplemented with antibiotics and 10% FCS. Custom siRNAs targeting HIF1/2α [41] and USP30 were purchased and transfected according to the manufacturer's protocol (Dharmafect, Dharmacon). All siRNA experiments were performed for 48 hours before the start of experiments. Transfections in *E2F7/8* double knock-out MEFs were done using Superfect (Qiagen), using the manufacturer's protocol. HEK293T

cells were transfected using polyethelenimine (PEI; Polysciences, USA). MG132 (5 μM; Sigma-Aldrich, USA) was used for 6 hours to inhibit proteasomal degradation and cycloheximide (CHX; 10 μg/ml, Sigma) was used for 1, 2 or 4 hours to inhibit protein synthesis. For serum starvation experiments, RCC cells were incubated for 24 hours in serum-free DMEM medium before the start of the experiment. To study the regulation of pVHL in hypoxia, RCC cells were incubated in a hypoxia workstation (1% O_2) for 24 hours.

Western blotting

Standard western blots were incubated with α-E2F1 (KH95, 1:500; Santa Cruz Biotechnology), α-HIF1α (1:500; Novus Biologicals), α-VHL (Ig32, 1:500;), α-β-catenin (1:500; BD-Biosciences) and α-β-actin (1:10000, Abcam, UK).

RT-PCR

Total RNA was isolated using Trizol (Invitrogen). cDNA was made using 2 μg of total RNA and reverse transcribed using oligo dT (Invitrogen) and Superscript RT II (Invitrogen). The following primers were used for the semi-quantitative RT-PCR (shown in Figure 2F): MCM3 F: 5'-TTCCTCAGCTGTGTGGTCTG-3' and MCM3 R: 5'- CAAGGGGATTGTTCTCCTCA-3'; 18S forward and reverse primer have been described earlier [38].

Real-time PCRs were performed using a BioRad iCycler and reactions were performed in duplo. Relative amounts of cDNA were normalized to GAPDH. The following primers have been used:
E2F1 F: 5'-GCCCTTGACTATCACTTTGGTCTC-3'
E2F1 R: 5'-CCTTCCCATTTTGGTCTGCTC-3'
E2F2 F: 5'-TAGGGAGATGTGGAGGATTCGG-3'
E2F2 R: 5'-AACTCAGGGTGGACAAACAAACAC-3'
E2F3a F: 5'-GCCTCTACACCACGCCACAAG-3'
E2F3a R: 5'-TCGCCCAGTTCCAGCCTTC-3'
E2F3b F: 5'-CGGAAATGCCCTTACAGC-3'
E2F3b R: 5'-CTCAGTCACTTCTTTGGACAG-3'
E2F7 F: 5'-CCCCCGAGATCCACACCTAC-3'
E2F7 R: 5'-CAGAGCCAGGCTGGTCAGAA-3'
E2F8 F: 5'GCCCAGAAATCAGCCCAAAC-3'
E2F8 R: 5'-GGGAGCGGAACTGATCT-3'
GAPDH F: 5'-CGGTGTGAACGGATTTGGC-3'
GAPDH R: 5'-TTTGATGTTAGTGGGGTCTCGC-3'

E2F1 luciferase reporter

E2F1 reporter assays were performed in triplicate using 200 ng *E2F1*-luc reporter, 20 ng renilla with 0, 100, 500 or 1000 ng Vsv-pVHL30 in *E2F7/8* knock-out MEFs [42] using the DUAL luciferase system (Promega), as previously described [38].

Cell cycle analysis

Cells were washed in PBS and fixed overnight in 70% ethanol. Fixed cells were washed in 1% BSA/PBS, treated with RNase (1:100; Sigma-Aldrich) and stained with propidiumiodide (1:100; Sigma-Aldrich). Cell cycle analysis was performed on the FACS Calibur (BD-Biosciences). All experiments were performed at least three times independently.

Results

E2F1 is upregulated in *VHL*-associated renal cell carcinoma

Development of clear-cell RCC in patients with germline inactivating *VHL* mutations has been closely associated with loss of the second, wild-type allele [43]. To investigate a relationship between E2F1 nuclear expression and loss of *VHL*, we analyzed E2F1 and HIF1α expression in paraffin-embedded tumor and normal renal tissue from 38 RCC patients, eight of which are VHL patients known by our hospital to have germline mutations in *VHL* (Figure 1A). For both HIF1α and E2F1, a sample was determined to be histologically positive when more than 25% of all nuclei were scored to have unambiguous staining. In normal kidney samples, some E2F1 staining is observed in the glomeruli. Proximal tubules often show a light cytoplasmic E2F1 staining, while distal tubules do not generally stain for E2F1 (Figure 1B, lower panels). We never observed more than 25% nuclear positivity for E2F1 or HIF1α in any of the normal tissues from all 38 patients examined. In primary tumor tissue of almost all VHL patients, we observed high nuclear HIF1α and E2F1 staining (Figure 1B). Chi-square statistical analysis of nuclear E2F1 expression (>25%) between clear-cell RCCs derived from VHL patients and thirty sporadic clear-cell RCCs with unknown *VHL* status revealed that E2F1 is significantly higher expressed in *VHL*-associated RCCs ($p=0.04$), compared to sporadic RCCs. No significance was found in a similar analysis of HIF1α expression.

In our VHL patient set, we had four individuals from a single family and two individuals from a different family, which allows analysis into genotype-phenotype correlation. Although the numbers are small it is pleasing that all patients with the same germline mutation exhibited the same phenotype with regard to HIF1α and E2F1 nuclear expression. Interestingly, one patient (#5; Figure 1A) showed high nuclear E2F1 expression, while nuclear HIF1α expression is low, suggesting that nuclear HIF1α and E2F1 expression are both highly expressed in *VHL*-associated clear-cell RCC however, do not always correlate.

Nuclear E2F1 expression is increased in sporadic renal cancers

In four kidney tumors matched with normal tissue from the same patient, freshly acquired post-operation material was used to quantitate the differences in E2F1 mRNA levels by real-time PCR. All four patient tumors tested exhibited upregulation of E2F1 compared to their normal kidney tissue (Figure 1C); however, E2F1 expression was statistically significant increased in only two samples, using a two-tailed Student's *t*-test (sample 046: $p=0.007$; sample 061: $p=0.03$). It is interesting to note that sample 039 did not demonstrate a significant difference in E2F1 mRNA levels. Accordingly, this tumor was also included in the 30 sporadic tumors screened by immunohistochemistry and was scored as negative for E2F1 staining.

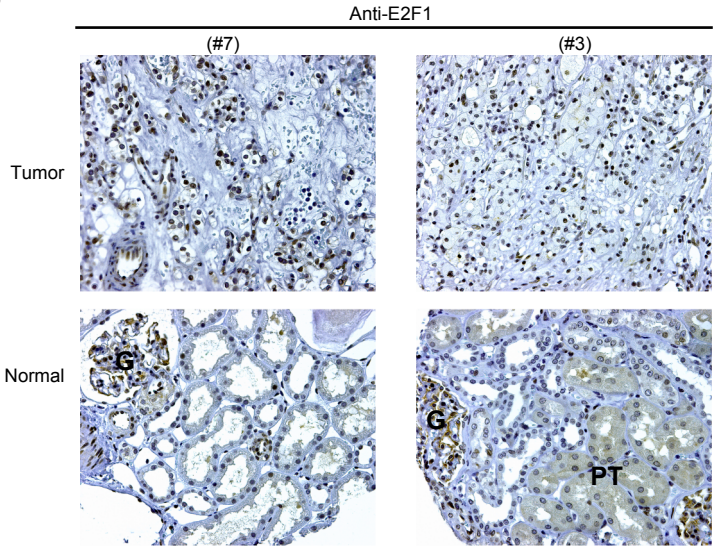
pVHL suppresses E2F1 expression

Because our subset of RCCs known to harbor inactivating *VHL* mutations exhibit a greater tendency to express high levels of nuclear E2F1, we hypothesized that this relationship may be causal. To test this notion *in vitro*, we re-introduced full-length pVHL into two different *VHL*-deficient RCC cell lines (RCC10 and 786-0), and examined the response of E2F1 expression. We observed decreased E2F1 protein levels in both cell lines when reconstituted with full-length pVHL (Figure 2A). To determine whether the differences in E2F1 levels are attributable to mRNA transcript

A

#	*VHL* mutation	Comment	Nuclear E2F1	Nuclear HIF1α
1	c.509T>A; p. Val170Asp	Missense mutation	90%	65%
2	c.509T>A; p. Val170Asp	Missense mutation	35%	100%
3	c.509T>A; p. Val170Asp	Missense mutation	90%	90%
4	c.500G>A; p. Arg167Gln	Missense mutation	90%	50%
5	c.340+1G>A	Splice site mutation, previously known as IVS1+1G>A	50%	10%
6	c.509T>A; p. Val170Asp	Missense mutation	50%	90%
7	c.1-?_463+?	Genomic deletions of exons 1 and 2, exact breakpoints unknown	50%	90%
8	c.1-?_463+?	Genomic deletions of exons 1 and 2, exact breakpoints unknown	35%	100%

B



C

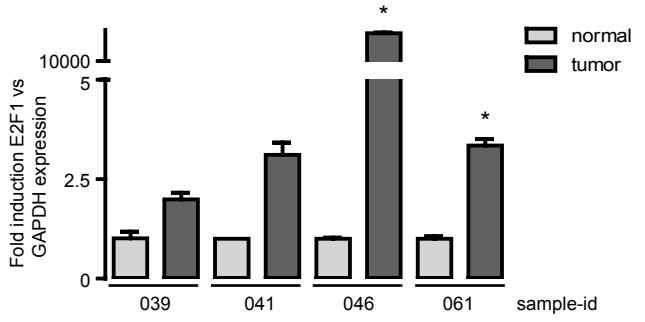


Figure 1: (A): Inherited *VHL* mutations of eight VHL patients with clear-cell RCCs, combined with the results of immunohistochemical staining of the associated RCCs for E2F1 and HIF1α. (B): Photos of paraffin-embedded tumor/normal material of the kidney from two patients with VHL disease, stained for E2F1. G, glomerulus; PT, proximal tubules. (C): Real-time PCR for E2F1 in four sporadic renal tumors, matched with normal kidney tissue for each patient. E2F1 expression normalized vs. GAPDH. A two-tailed Student's *t*-test was used to determine statistical significance. Asterikses (*) indicate statistical significance.

levels or to protein turnover, we isolated total RNA from these cell lines and performed real-time PCRs (in triplicate) for E2F1, E2F2, E2F3, E2F7, E2F8 and GAPDH. This experiment determined that re-introduction of pVHL decreased the expression of E2F1 mRNA in both RCC cell lines Figure 2B), while this difference was not observed for the other E2F family members tested (data not shown).

To investigate whether protein turnover of E2F1 is also affected by pVHL, perhaps as a target of the pVHL-Elongin B/C-Cul-2 E3 ligase complex, we transfected HEK293T cells with either HA-E2F1 in combination with empty vector or Vsv-pVHL30, and used MG132 to block proteasomal degradation. Concordant with our previous experiments, exogenous full-length pVHL decreased E2F1 protein levels in cells untreated with MG132 (Figure 2C, upper panel, lane 3). Exposure to MG132 for 6 hours resulted in the well-characterized accumulation of endogenous HIF1α (Figure 2C, bottom panel, lanes 2 and 4) [44]. We were unable to detect an increased pool of (poly)-ubiquitinated E2F1 in these cell lines irregardless of whether they expressed exogenous pVHL30 (Figure 2C, upper panel, lanes 2 and 4). To investigate, whether E2F1 protein synthesis is affected by pVHL30, we treated in RCC10 and RCC10 + pVHL cells with cycloheximide (CHX) for 1, 2 and 4 hours. E2F1 is reduced in both *VHL*-deficient and -proficient RCC cells, suggesting that E2F1 is degraded in a pVHL-independent fashion (Figure 2D).

pVHL decreases E2F1 transactivation

Next, we asked whether pVHL-associated drops in E2F1 levels would have a functional consequence on E2F1 transactivation. One of E2F1's more sensitive targets is its own *E2F1* promoter [45]. To this end we performed reporter assays with a luciferase construct under the control of the *E2F1* promoter [45] in mouse embryonic fibroblasts lacking both *E2F7* and *E2F8*, since they express high endogenous levels of E2F1 [42]. Transfecting increasing amounts of Vsv-pVHL resulted in a significant dose-dependent decrease in E2F1 activity, using a two-tailed Student's *t*-test (e.g. p=0.007, in 100 ng Vsv-pVHL; Figure 2E). Other well-characterized downstream targets of E2F1 are the minichromosome maintenance proteins (MCMs) involved in DNA-replication [34]. Using a semi-quantitative RT-PCR approach in two human RCC cell lines, stably transfected with either empty vector or pVHL30, we observed that the expression of MCM3 was less in *VHL*-proficient RCC10 and 786-0 cells (Figure 2F), indicating that pVHL not only decreases E2F1 expression, but function as well.

E2F1 regulation by pVHL does not affect cell proliferation in RCC cells

Because upregulated E2F1 levels upon *VHL* inactivation may simply reflect altered cell cycle profiles, we analyzed the cell cycle status of asynchronous 786-0 and RCC10 cells, with or without pVHL cultured subconfluently in the presence of serum (Figure 3). We did not observe any significant change in cell cycle profiles (Figure 3). Re-introduction of pVHL has shown to exert an effect on cell cycle proliferation in serum-starved subconfluent 786-0 RCC cells by increasing the G0/G1 content over time [20]. Unfortunately, we were not able to detect any significant effect of pVHL on cell cycle proliferation or apoptosis in these RCC cells, suggesting that the regulation of E2F1 by pVHL does not affect cell cycle proliferation in RCC cells *in vitro*, but does not exclude any role for pVHL in controlling cell cycle proliferation of renal cells *in vivo*.

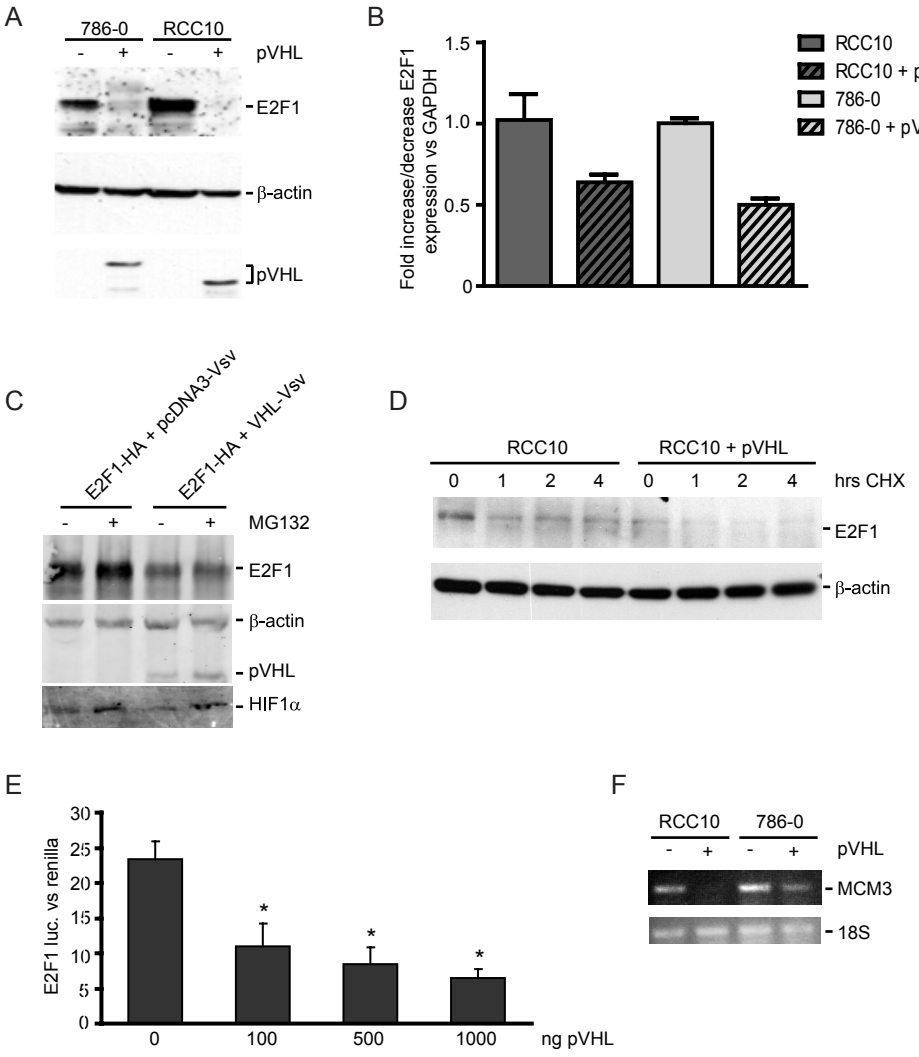


Figure 2: (A): Lysates of asynchronous RCC cells with or without pVHL expression analyzed for E2F1 expression. β-actin used as loading control. (B): Analysis of E2F1 mRNA expression in RCC cells with or without pVHL expression via real-time PCR. Each measurement is done in triplicate. mRNA expression of E2F1 normalized versus GAPDH. (C): Western blot analysis of E2F1 expression in HEK293T cells with or without exogenous pVHL. Cells were exposed to MG132 for 6 hours before harvesting to block proteasomal degradation. β-actin used as loading control. (D): Western blot analysis of E2F1 expression in RCC10 cells with or without pVHL. Cells were treated with cycloheximide (CHX) for 1, 2 and 4 hours before harvesting to block protein production. β-actin, loading control. (E): Analysis of exogenous pVHL on the activity of E2F1 using a *E2F1* promoter luciferase (luc.) assay in *E2F7/8* double knockout MEFs. A two-tailed Student's *t*-test was used to determine statistical significance. Asterikses (*) indicate statistical significance. (F): Semi-quantitative RT-PCR in *VHL*-deficient and -proficient RCC cells, analyzing for MCM3 expression. 18S measured as input control.

HIFα-independent regulation of E2F1 expression

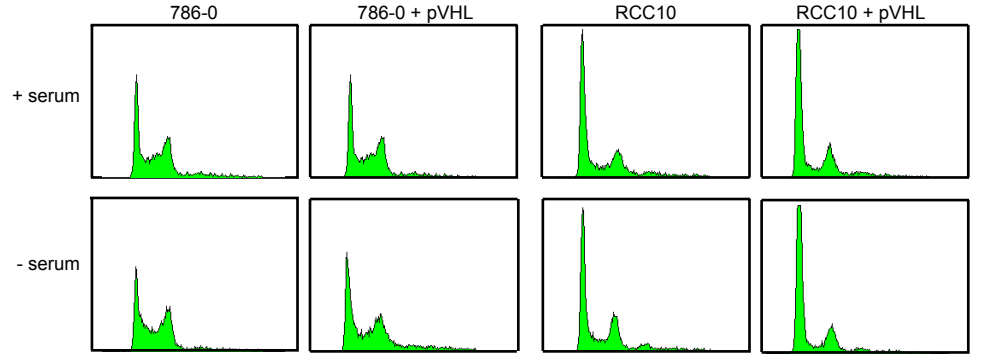
pVHL's best characterized function is as an E3 ubiquitin ligase, regulating HIF1/2α proteasomal degradation in normoxic conditions [46]. In hypoxic conditions however, these transcription factors can no longer be recognized by pVHL which results in an increased stability of (nuclear) HIF1/2α and subsequent increase of

downstream targets of HIF1/2α. Since E2F1 expression has shown by others to be increased as a result of hypoxia [47], we wondered whether upregulation of E2F1 upon pVHL inactivation is mediated through HIF1/2α. We began by incubating the previously used RCC10 and 786-0 cells with or without pVHL30 for 24 hours in hypoxia (1% O_2). Indeed, E2F1 is upregulated in hypoxic RCC cells lacking pVHL. However, reintroduction of pVHL is still able to decrease E2F1 expression in hypoxic cells (Figure 4A), suggesting that the regulation of E2F1 expression by pVHL is independent on HIFα.

We further tested the involvement of HIF1/2α by depleting cellular levels of either or both of these proteins in RCC10 cells using custom siRNAs targeting HIF1/2α [41] and used a siRNA targeting USP30, as control in this experiment. While effective in downregulating the expression of the HIF1α, the RNAi knock-downs did not result in aberrant E2F1 protein expression (Figure 4B). We conclude that the observed downregulation of E2F1 upon re-introduction of full-length pVHL in cell lines is not mediated by HIFα.

Pinpointing the region(s) in pVHL necessary for E2F1 regulation

We examined E2F1 expression in subconfluent RCC10 clones stably expressing a panel of *VHL*-variant alleles. pVHL19, a normally occurring wild-type pVHL isoform originating from an alternative translation start site and capable of downregulating HIFα [48, 49], decreased E2F1 expression, like full-length pVHL30 (Figure 4C). Interestingly, disease-associated pVHL-ΔF76, a common *VHL* type 1 allele no longer capable of regulating HIFα levels [50-52] (data not shown), was also able to downregulate E2F1 expression (Figure 4C). Another disease-allele -pVHL-F119S, associated with pheochromocytoma only in VHL disease [53]- destabilizes HIFα and downregulates E2F1 efficiently (Figure 4C). These data link *VHL* inactivation causally to the upregulation of E2F1 in kidney tumors and demonstrate that this event is independent of HIFα.



RCC cell line	+ serum		- serum	
	2n	4n	2n	4n
786-0	58%	42%	64%	36%
786-0 + pVHL	56%	44%	62%	38%
RCC10	65%	35%	70%	30%
RCC10 + pVHL	78%	22%	73%	27%

Figure 3: Cell cycle profiles of *VHL*-deficient and -proficient RCC cells in the presence or absence of serum. Cells were serum deprivated for 24 hours, where indicated, before fixation. Cells were analyzed for their 2n and 4n count using propidiumiodide. Below, percentages of cell populations in 2n/4n corresponding with FACS profiles above.

Discussion

We observed that E2F1 expression is increased in renal tumors, corroborating and extending recent data describing E2F1 expression in renal cell carcinoma [54]. Moreover, we identified that clear-cell RCCs with known inherited *VHL* mutations display even significantly higher nuclear E2F1 expression than sporadic tumors of unknown *VHL* status, suggesting that *VHL* loss increases E2F1 expression *in vivo*. To further test this regulation we used two different *VHL*$^{-/-}$ RCC cells and re-introduced pVHL in this system. Re-introduction of pVHL decreases E2F1 mRNA, protein expression, activity and to a small extent synthesis. Further studies will definitively reveal if the E3 ubiquitin ligase function of pVHL is involved in this regulation. Furthermore, transactivation of E2F1 target MCM3 is decreased upon re-introduction of pVHL into RCC cells, supporting microarray expression studies in *C. elegans*, showing increased expression of MCMs, resulting from *vhl1* loss [55].

E2F1 and MCMs are known to be essential for the G1/S cell cycle transition [56]. However, we were unable to detect an increased G1 content in *VHL*-proficient RCC cells, indicating that the difference observed in E2F1 and MCM3 expression is not related to cell cycle proliferation in these cells. Since *VHL*-negative RCC cells display a highly proliferative background, we cannot exclude a role for the pVHL-E2F1 connection in cell cycle progression of primary renal cells, or renal cells *in vivo*.

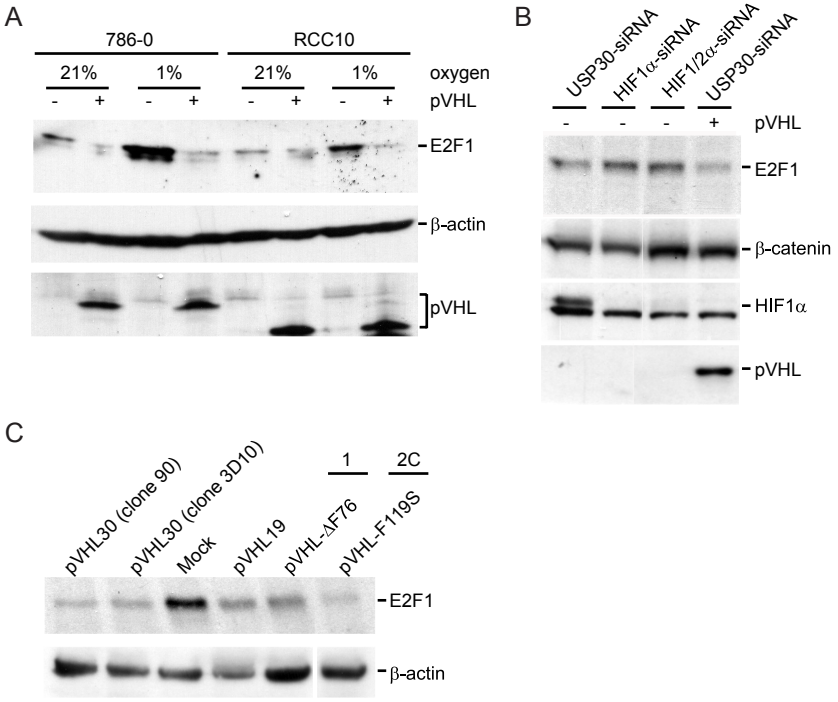


Figure 4: (A): RCC cell lines 786-0 and RCC10 with or without pVHL30 expression were incubated for 24 hours in normoxia (21% O_2) or hypoxia (1% O_2), where indicated. Lysates were analyzed on western blot for E2F1 expression. β-actin used as loading control. (B): RCC10 cells with or without pVHL30 were transfected with siRNAs targeting USP30 (negative control), HIF1α, or HIF1α together with HIF2α respectively. Lysates were made 48 hours after transfection and analyzed for E2F1, HIF1α and pVHL. β-catenin used as loading control. (C): Subconfluent RCC cells, stably transfected with pVHL variants, were analyzed for E2F1 protein expression on western blot. β-actin was used in this experiment as loading control.

Our work suggests that E2F1 regulation is independent of HIFα. Using siRNA approaches and hypoxia, we did not see any effect of HIFα on the regulation of E2F1 expression by pVHL, making it unlikely that HIFα is responsible for the increased levels of E2F1 in hypoxic conditions (Figure 4A and [47]). However, we do not know by which mechanism pVHL regulates E2F1 expression. We have previously described HIFα-independent transcriptional regulation [57], it is therefore conceivable that E2F1 repression is mediated by a pVHL target in a similar manner. We attempted to pinpoint the domain(s) of pVHL important for regulating E2F1, by testing a panel of *VHL* disease-associated alleles, including type 1 and 2C mutations. We discovered that E2F1 was downregulated by all *VHL* alleles tested. Since all of these pVHL variants have an intact C-terminal α-domain, a region to bind either Elongin C or p53 [26], future studies using *VHL* variant alleles with mutations in their α-domain, e.g. R167Q (the most frequent tumor-associated *VHL* mutation), will reveal if this domain is essential to regulate E2F1 expression. Nevertheless, eight VHL patient RCCs manifested increased E2F1 levels. This might reflect the nature of the secondary somatic mutation, or might reflect a differential response *in vivo* to *VHL* loss. Only future studies will clarify these questions.

Acknowledgements

We would like to thank Petra van der Groep, Maryam Saghafiam, Sabrina Elshof, Sander Basten, Susanne van Schelven and Cristel Snijckers for technical assistance, Maartje Los and Paul van Diest for collecting and scoring of renal cell carcinoma tissue, Benjamin Rowland and René Medema for helpful discussions. This work is supported by the Dutch Cancer Society (KWF UU 2006-3565 to E.V.) and the Netherlands Organization for Scientific Research (VIDI award 016.066.354 to R.G.).

References

1. **Kaelin, W.G.** Von hippel-lindau disease. *Annu Rev Pathol* 2, 145-173 (2007).
2. **Duan, D.R. *et al.*** Inhibition of transcription elongation by the VHL tumor suppressor protein. *Science (New York, N.Y* 269, 1402-1406 (1995).
3. **Kamura, T. *et al.*** Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science (New York, N.Y* 284, 657-661 (1999).
4. **Kibel, A., Iliopoulos, O., DeCaprio, J.A. & Kaelin, W.G., Jr.** Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. *Science (New York, N.Y* 269, 1444-1446 (1995).
5. **Pause, A. *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 U S A* 94, 2156-2161 (1997).
6. **Cockman, M.E. *et al.*** Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem* 275, 25733-25741 (2000).
7. **Kamura, T. *et al.*** Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. *Proc Natl Acad Sci U S A* 97, 10430-10435 (2000).
8. **Ohh, M. *et al.*** Ubiquitination of hypoxia-inducible factor requires direct

binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol* 2, 423-427 (2000).

9. **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* 19, 4298-4309 (2000).
10. **Okuda, H. *et al.*** Direct interaction of the beta-domain of VHL tumor suppressor protein with the regulatory domain of atypical PKC isotypes. *Biochem Biophys Res Commun* 263, 491-497 (1999).
11. **Okuda, H. *et al.*** The von Hippel-Lindau tumor suppressor protein mediates ubiquitination of activated atypical protein kinase C. *J Biol Chem* 276, 43611-43617 (2001).
12. **Kurban, G., Hudon, V., Duplan, E., Ohh, M. & Pause, A.** Characterization of a von Hippel Lindau pathway involved in extracellular matrix remodeling, cell invasion, and angiogenesis. *Cancer Res* 66, 1313-1319 (2006).
13. **Tang, N., Mack, F., Haase, V.H., Simon, M.C. & Johnson, R.S.** pVHL function is essential for endothelial extracellular matrix deposition. *Molecular and cellular biology* 26, 2519-2530 (2006).
14. **Lolkema, M.P. *et al.*** Allele-specific regulation of primary cilia function by the von Hippel-Lindau tumor suppressor. *Eur J Hum Genet* 16, 73-78 (2008).
15. **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* 5, 64-70 (2003).
16. **Lolkema, M.P. *et al.*** The von Hippel-Lindau tumor suppressor protein influences microtubule dynamics at the cell periphery. *Exp Cell Res* 301, 139-146 (2004).
17. **Datta, K., Sundberg, C., Karumanchi, S.A. & 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* 61, 1768-1775 (2001).
18. **Lieubeau-Teillet, B. *et al.*** von Hippel-Lindau gene-mediated growth suppression and induction of differentiation in renal cell carcinoma cells grown as multicellular tumor spheroids. *Cancer Res* 58, 4957-4962 (1998).
19. **Kim, M. *et al.*** Recombinant adenovirus expressing Von Hippel-Lindau-mediated cell cycle arrest is associated with the induction of cyclin-dependent kinase inhibitor p27Kip1. *Biochem Biophys Res Commun* 253, 672-677 (1998).
20. **Pause, A., Lee, S., Lonergan, K.M. & Klausner, R.D.** The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum withdrawal. *Proc Natl Acad Sci U S A* 95, 993-998 (1998).
21. **Osipov, V., Keating, J.T., Faul, P.N., Loda, M. & Datta, M.W.** Expression of p27 and VHL in renal tumors. *Appl Immunohistochem Mol Morphol* 10, 344-350 (2002).
22. **Baba, M. *et al.*** Loss of von Hippel-Lindau protein causes cell density dependent deregulation of CyclinD1 expression through hypoxia-inducible factor. *Oncogene* 22, 2728-2738 (2003).
23. **Bindra, R.S., Vasselli, J.R., Stearman, R., Linehan, W.M. & Klausner, R.D.** VHL-mediated hypoxia regulation of cyclin D1 in renal carcinoma cells. *Cancer Res* 62, 3014-3019 (2002).
24. **Carmeliet, P. *et al.*** Role of HIF-1alpha in hypoxia-mediated apoptosis, cell

proliferation and tumour angiogenesis. *Nature* 394, 485-490 (1998).
25. **Goda, N., Dozier, S.J. & Johnson, R.S.** HIF-1 in cell cycle regulation, apoptosis, and tumor progression. *Antioxid Redox Signal* 5, 467-473 (2003).
26. **Roe, J.S. *et al.*** p53 stabilization and transactivation by a von Hippel-Lindau protein. *Mol Cell* 22, 395-405 (2006).
27. **Cao, L. *et al.*** Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. *Nature* 355, 176-179 (1992).
28. **Huang, P.S. *et al.*** Protein domains governing interactions between E2F, the retinoblastoma gene product, and human papillomavirus type 16 E7 protein. *Molecular and cellular biology* 13, 953-960 (1993).
29. **Nevins, J.R.** E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science (New York, N.Y* 258, 424-429 (1992).
30. **Reichel, R., Kovesdi, I. & Nevins, J.R.** Developmental control of a promoter-specific factor that is also regulated by the E1A gene product. *Cell* 48, 501-506 (1987).
31. **Tsuruga, H. *et al.*** HsMCM6: a new member of the human MCM/P1 family encodes a protein homologous to fission yeast Mis5. *Genes Cells* 2, 381-399 (1997).
32. **Leone, G. *et al.*** E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev* 12, 2120-2130 (1998).
33. **Ohtani, K. *et al.*** Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. *Oncogene* 18, 2299-2309 (1999).
34. **Maiorano, D., Lutzmann, M. & Mechali, M.** MCM proteins and DNA replication. *Curr Opin Cell Biol* 18, 130-136 (2006).
35. **Tsantoulis, P.K. & Gorgoulis, V.G.** Involvement of E2F transcription factor family in cancer. *Eur J Cancer* 41, 2403-2414 (2005).
36. **Weinberg, R.A.** The retinoblastoma protein and cell cycle control. *Cell* 81, 323-330 (1995).
37. **Johnson, D.G. & Degregori, J.** Putting the Oncogenic and Tumor Suppressive Activities of E2F into Context. *Curr Mol Med* 6, 731-738 (2006).
38. **Giles, R.H. *et al.*** Interplay between VHL/HIF1alpha and Wnt/beta-catenin pathways during colorectal tumorigenesis. *Oncogene* 25, 3065-3070 (2006).
39. **Lolkema, M.P. *et al.*** The von Hippel-Lindau tumour suppressor interacts with microtubules through kinesin-2. *FEBS Lett* 581, 4571-4576 (2007).
40. **Krieg, M. *et al.*** Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. *Oncogene* 19, 5435-5443 (2000).
41. **Sowter, H.M., Raval, R.R., Moore, J.W., Ratcliffe, P.J. & Harris, A.L.** Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. *Cancer Res* 63, 6130-6134 (2003).
42. **Li, J. *et al.*** Synergistic function of E2F7 and E2F8 is essential for cell survival and embryonic development. *Developmental cell* 14, 62-75 (2008).
43. **Kim, W.Y. & Kaelin, W.G.** Role of VHL gene mutation in human cancer. *J Clin Oncol* 22, 4991-5004 (2004).

44. **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* 272, 22642-22647 (1997).
45. **Araki, K., Nakajima, Y., Eto, K. & Ikeda, M.A.** Distinct recruitment of E2F family members to specific E2F-binding sites mediates activation and repression of the E2F1 promoter. *Oncogene* 22, 7632-7641 (2003).
46. **Maxwell, P.H. *et al.*** The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399, 271-275 (1999).
47. **O'Connor, D.J. & Lu, X.** Stress signals induce transcriptionally inactive E2F-1 independently of p53 and Rb. *Oncogene* 19, 2369-2376 (2000).
48. **Iliopoulos, O., Ohh, M. & Kaelin, W.G., Jr.** pVHL19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. *Proc Natl Acad Sci U S A* 95, 11661-11666 (1998).
49. **Schoenfeld, A., Davidowitz, E.J. & Burk, R.D.** 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 U S A* 95, 8817-8822 (1998).
50. **Chen, F. *et al.*** Germline mutations in the von Hippel-Lindau disease tumor suppressor gene: correlations with phenotype. *Hum Mutat* 5, 66-75 (1995).
51. **Kishida, T., Stackhouse, T.M., Chen, F., Lerman, M.I. & Zbar, B.** Cellular proteins that bind the von Hippel-Lindau disease gene product: mapping of binding domains and the effect of missense mutations. *Cancer Res* 55, 4544-4548 (1995).
52. **Zbar, B. *et al.*** Germline mutations in the Von Hippel-Lindau disease (VHL) gene in families from North America, Europe, and Japan. *Hum Mutat* 8, 348-357 (1996).
53. **Eng, C. *et al.*** Mutations in the RET proto-oncogene and the von Hippel-Lindau disease tumour suppressor gene in sporadic and syndromic phaeochromocytomas. *J Med Genet* 32, 934-937 (1995).
54. **Turowska, O. *et al.*** Overexpression of E2F1 in Clear Cell Renal Cell Carcinoma: A Potential Impact of Erroneous Regulation by Thyroid Hormone Nuclear Receptors. *Thyroid* 17, 1039-1048 (2007).
55. **Bishop, T. *et al.*** Genetic analysis of pathways regulated by the von Hippel-Lindau tumor suppressor in Caenorhabditis elegans. *PLoS Biol* 2, e289 (2004).
56. **Ohtani, K.** Implication of transcription factor E2F in regulation of DNA replication. *Front Biosci* 4, D793-804 (1999).
57. **Bluyssen, H.A. *et al.*** Fibronectin is a hypoxia-independent target of the tumor suppressor VHL. *FEBS Lett* 556, 137-142 (2004).

Chapter 3

All along the watchtower: is the cilium a tumor suppressor organelle?

Dorus A. Mans, Emile E. Voest and Rachel H. Giles

Biochimica et Biophysica Acta - Reviews on Cancer (in press)

Abstract

Cilia or flagella have been around since almost the beginning of life, and have now developed specialized cell-type specific functions from locomotion to acting as environmental sensors participating in cell signalling. Genetic defects affecting cilia result in a myriad of pathological instances, including infertility, obesity, blindness, deafness, skeletal malformations, and lung problems. However, the consistency in which the common kidney cyst is coupled with cilia dysfunction has raised interest in the possibility that ciliary dysfunction might contribute to other neoplasms as well. A suite of recent papers convincingly linking cilia to hedgehog signalling, platelet derived growth factor signalling, WNT signalling and the von Hippel-Lindau tumor suppressor protein has rapidly expanded the knowledge base connecting cilia to cancer. We propose that these data support the notion of the cilium as a cellular Watchtower, whose absence can be an initiating event in neoplastic growth. Furthermore, we predict that we are just now seeing the tip of the iceberg, and that the list of cancers associated with altered ciliary signalling will grow exponentially in the next few years.

Introduction

Linnaeus is remembered for writing “Natura maxime miranda in minimis”, or “nature is greatest in little things”. Ciliary structures (and flagellary) activity is a perfect example of this maxim. Whip-like beating movements cleaning airways, transporting plankton through water, defining our left from our right, and regulating our fertility, cilia are present on virtually all of our polarized cells. As an organelle, cilia are old, but three theories suggest quite different origins. The first suggests that cilia -much like mitochondria- are derived from ancestrally ingested bacterial spirochetes, which over time became a permanent union. This theory is based on structural similarities observed in microtubule composition between the ciliary axoneme and the spirochete body [1]. More evidence supports the view that cilia arose from the duplication and adaptation of existing cellular processes. Homology between proteins regulating ciliary transport (intraflagellar transport complex or IFT proteins) to components of coat protein I and clathrin-coated vesicles, suggest that membrane-bound IFT evolved as a specialized form of coated vesicle transport [1, 2]. A third theory proposes ciliary origin to be a self-assembly RNA enveloped virus that becomes the centriole and basal body, which would account for the self-assembly and self-replicative properties of these organelles [3].

Introduction to cilia

All those tiny hairs on unicellular ciliated protozoa seen under highschool microscopes and textbooks probably share a common internal organization with many of the cilia on your cells: nine microtubule fibers surrounding two central microtubule fibers, collectively called a “9+2” axoneme (see Figure 1) [4]. Depending upon how some of the fibers contract, the cilium or flagellum bends one way or the other, either in a rigid power stroke, or a relaxed recovery stroke. Examples of these types of cilia can be found on cells in the inner ear, the lining of your lungs, or wherever fluid or particles need to be pushed along. Particularly in vertebrates, however, axonemal structures display some variability [5]. For example, cilia not involved in motility tend to lack the two central microtubule fibers (“9+0” axoneme, Figure 1). Because

there is only one of these cilia per cell, this type is usually referred to as a primary cilium (or monocilium). In general, primary cilia function as environmental sensors, translating extracellular signals into internal cellular adaptations. Examples of cells with primary cilia are found in the epithelium lining kidney, pancreas, and liver ducts. Like weather vanes, cells use primary cilia to sense their immediate environment by protruding these hair-like outgrowths into the lumen of fluid-filled tubules (e.g. urine in kidney tubules). Almost all eukaryotic cells (except bone marrow-derived cells and intercalated cells of the collecting ducts) can display primary cilia (http://www.bowserlab.org/primarycilia/cilialist.html). Cilia have been observed to extend as long as 30 μM, although the average length is usually about 6-10 μM [6-8].

The axoneme emerges from the apical side of the cell from the basal body. Like cilia, basal bodies are also made of microtubules; however, they contain an array of nine sets of tubulin triplets with no doublet in the center. How the triplets in the basal body turn into the ciliary doublet is unknown. A basal body is actually just a specialized centriole, which has acquired a transitional zone contiguous with the axoneme. After exiting the cell cycle, the mature mother centriole is able to nucleate ciliogenesis [4, 9].

Because protein synthesis cannot occur in the cilium, all structural and signalling components of the cilium must be actively transported by IFT proteins into the cilium (Figure 2). Bi-directional transport of IFT particles along the ciliary axoneme is established by the action of IFT motor proteins [10]. Movement from the base of the cilium to the tip (anterograde transport) is mediated by kinesin-2, a heterotrimeric protein that consists of two motor units (KIF3A and KIF3B) and one non-motor unit (KAP3, kinesin-associated protein 3) [11]. Alternatively, retrograde transport from the

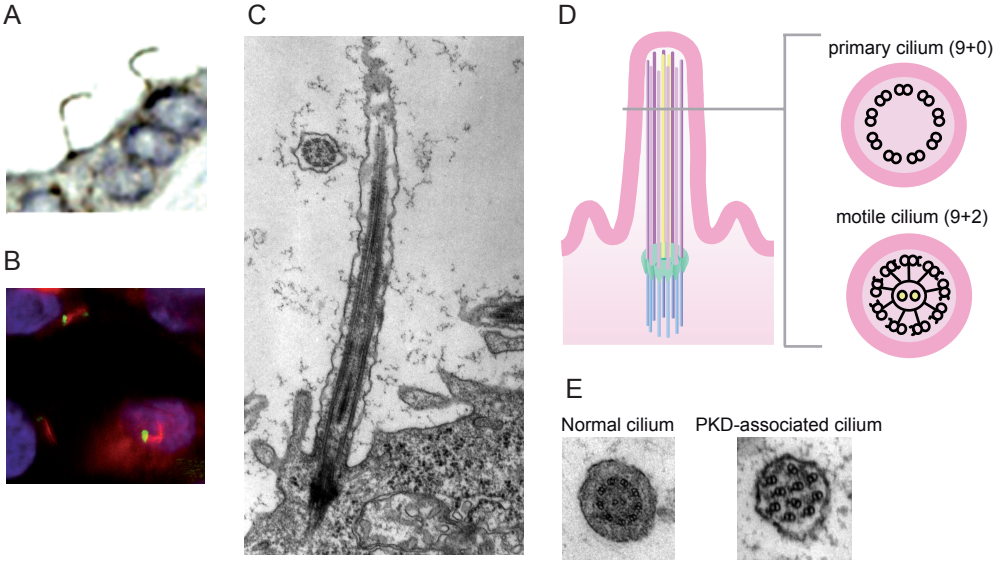


Figure 1: (A): Immunohistochemistry of kidney tubules showing the presence of cilia (photo courtesy of M. Tran, M. Esteban and P. Maxwell). (B): Immunofluorescense microscopy of kidney epithelial cells showing basal bodies (green) and ciliary axonemes (red). Nuclei are shown in blue. (C): Electron microscopy picture of a cilium in zebrafish (photo courtesy of E. van Rooijen and J. Griffith). (D): Cartoon illustration of primary and motile ciliary architecture. (E): Electron microgram of axonemal cross-sections taken from of a wildtype zebrafish vs. a zebrafish with polycystic kidney disease (photo courtesy of E. van Rooijen and J. Griffith).

tip of the cilium back to the basal body is dependent on cytoplasmic dynein 1b [12]. It is thought that ciliary precursor proteins and IFT-dynein are transported to the tip of the cilium by anterograde transport, while turnover products and kinesin-2 motor proteins are transported back to the cell body by retrograde IFT transport [10].

Primary cilia are sensory organelles that can detect a wide variety of stimuli including mechanic, osmotic, photonic, hormonal or olfactory signals [13]. They are usually present on differentiated cells in G0 and early G1 stages of the cell cycle. In kidney cysts it has been proposed that the primary cilia on kidney epithelial cells are unable to mechanically sense urine flow through the renal tube. Normally pushed over by fluid flow, the bending of the primary cilium has been observed to increase in intracellular Ca^{2+} levels in renal cells in vitro presumably by opening cilia-specific calcium channels. Acting as a second messenger, Ca^{2+} subsequently switches on numerous signalling activities inside the cell including non-canonical Wnt signalling and other signals regulating cell division (Figure 2) [7, 13]. Additionally, cilia are thought to serve as restricted subcellular compartments containing localized, concentrated proteins that function in downstream signalling from the tip of the cilium to the cell interior [10]. Cilium-based signalling is thought to be directed back to the cytoplasm of the cell bodies and to the nucleus by retrograde transport particles [10].

Ciliopathies

A diverse range of human disorders has been linked to ciliary dysfunction including sporadic renal cystic disease, left-right asymmetry, infertility, hydrocephalus, retinal degeneration, obesity, and skeletal defects, as well as specific congenital conditions such as Nephronophthisis (Senior-Loken), Alström syndrome, Oral-facial-digital syndrome type I, Bardet-Biedl-syndrome (BBS), Meckel-Gruber syndrome, and von Hippel-Lindau (VHL) disease. While the understanding of these pathological conditions -collectively known as ciliopathies- is profoundly incomplete, it is recognized that many genes mutated in these diseases generate protein products that localize and function in the ciliary axoneme or basal body [4]. Several recent reviews explore these congenital diseases, with emphasis on the developmental disturbances caused by cilia dysfunction [4, 13, 14]. However, inactivating mutations in cilia-related proteins have also been implicated in neoplasms such as basal cell carcinoma, medulloblastoma, (non)-small cell lung cancer, glioma, gastrointestinal tumors, renal cell carcinoma, hemangioblastoma, pheochromocytoma, rhabdomyosarcoma, adenocarcinoma, and myeloma (Table 1). Here we will focus only on the conditions in which cilia loss supports a role for this organelle in cell proliferation defects, in tumor initiation or cancer progression, with particular attention to renal neoplasms.

The most frequent genetic lethal disease in humans, polycystic kidney disease (PKD), is a failure of mechanotransduction by the renal primary cilium, underscoring the consequences of disturbing this delicate structure [15]. Autosomal dominant PKD (ADPKD) is the most common inherited form of PKD and is caused by mutations in *PKD1* or *PKD2* (Table 1). Autosomal recessive PKD (ARPKD) is caused by mutations in *PKHD1* (Table 1). Mutations in tens of different genes involved in renal cilia assembly, maintenance or function have now been implicated with the development of cystic kidneys in humans, mice, rats, and zebrafish [16, 17], including components of kinesin-2 (Table 1).

Kinesin-2 is a heterotrimeric motor protein that transports vesicles and molecules to the plus-ends of microtubules. The two ATP-dependent motor elements KIF3A and KIF3B bind tightly to each other and are bound at their tails by KAP3.

Studies in sea urchins [18], *Chlamydomonas* [19], and *Tetrahymena* [20] indicate that kinesin-2 is responsible for transporting IFT particles from the cell body to the tip of the cilium; these same studies showed that mutationally blocking the action of kinesin-2 function completely prevents ciliary assembly in all organisms tested. Null mutations of the genes encoding either *KIF3A* or *KIF3B* in mice impede the formation of cilia in the embryonic node and produce left-right asymmetry defects [21, 22]. Left-right asymmetry defects have been long associated with ciliary defects in humans [23]. Targeted deletion of murine *KIF3A* in renal epithelium results in polycystic disease characterized by lack of primary cilia [24]. Murine KAP3 has been suggested to function as a tumor suppressor in neural cells [25].

VHL and primary cilia

The von Hippel-Lindau (VHL) disease is an autosomal dominant disorder in which affected individuals have a propensity for developing both benign and malignant tumors in many organ systems. VHL patients develop vascular neoplasia, angiomas of the central nervous system and/or retina, renal clear-cell carcinomas (RCCs), endocrine neoplasia of the adrenal gland (pheochromocytomas), and low-grade adenocarcinomas of the temporal bone. Tumors arising in VHL patients are often excessively vascularized.

The malignant degeneration of renal cysts is commonly believed to be the most serious VHL manifestation. Approximately 75% of patients demonstrate multiple renal cysts [26], which is almost invariably correlated with the “second hit” inactivation of the wild-type *VHL* gene [27]. Renal cysts are common in the general population and are rarely clinically significant; however, in VHL patients they have a predilection to degenerate to conventional clear-cell renal cell carcinoma (RCC), the leading cause of death in patients with VHL disease (35-75% prevalence in one autopsy series) [28]. In VHL patients, there is considered to be progression from simple cysts with a single layer of tubular epithelium, to atypical cysts demonstrating diffuse epithelial hyperplasia with stratification to three or more cell layers, to either cystic or solid RCCs [26] (Figure 3). These renal pathologies are numerous in the kidneys of VHL patients, with more than 1000 simple or atypical cysts and several hundred carcinomas in the typical VHL kidney [29]. RCC is the presenting feature in only 10% of VHL patients, but the risk of developing RCC by the age of 60 years is approximately 70% [30].

Malignant transformation is a multistage process involving initiation and promotion. *VHL*-loss appears to be necessary for RCC initiation. In addition to renal lesions in VHL patients, the vast majority (>75%) of conventional RCCs carry inactivating somatic mutations of *VHL* [31-33]. In nude mouse xenograft assays, subcutaneous injection of RCC cells lacking functional *VHL* results in tumor growth, whereas re-introduction of wild-type pVHL in these cells significantly suppresses tumor growth [34]. These data collectively support a critical role for pVHL in the maintenance of normal kidney physiology [31, 35-37], and suggest that *VHL*-loss is a virtual prerequisite for human RCC development.

The *VHL* gene product regulates the degradation of hypoxia inducible factor alpha (HIFα), an important transcriptional activator of the genetic program triggered by hypoxia, through ubiquitination [38]. Inactivating mutations of *VHL*, thus, mimic a cell’s response to hypoxia, artificially stabilizing HIFα and resulting in the overexpression of angiogenic factors. Although these findings explain the high vascularization of *VHL*-related tumors, upregulation of HIFα alone does not explain the developmentof tumors in VHL disease. For example, a subset of VHL cancer patients have been

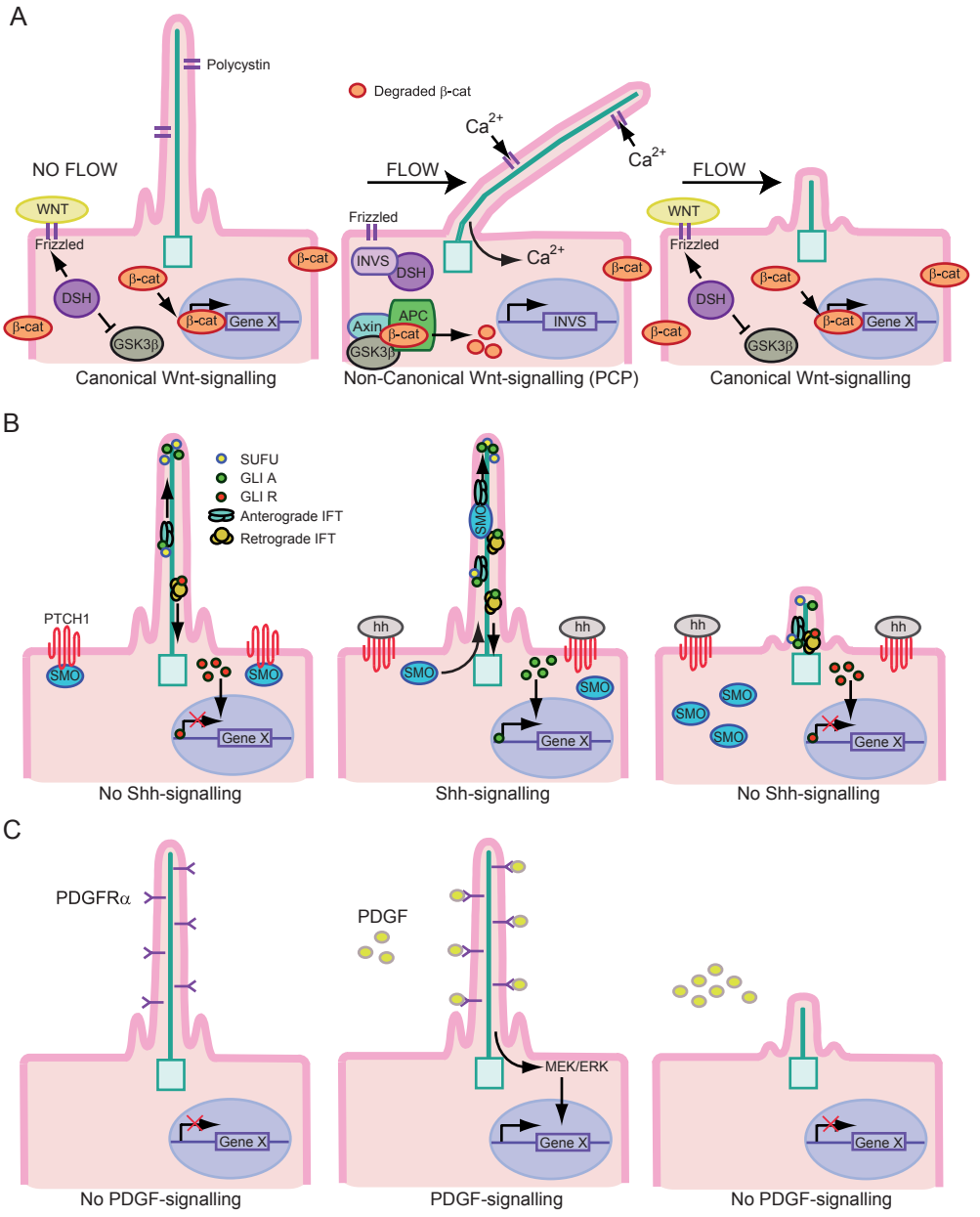


Figure 2: (A): Cilia-mediated signalling acts as a switch between canonical and non-canonical Wnt signalling. In the absence of flow, canonical Wnt signalling can occur when WNT binds the frizzled receptor, thereby recruiting dishevelled (DSH) and inactivating glycogen synthase kinase-3β (GSK3β). β-catenin (β-cat) becomes subsequently stabilized and translocates to the nucleus where it acts as transcriptional co-activator and induces transcription of WNT target genes (left panel). Upon mechanosensation by fluid flow, intracellular Ca^{2+} release increases inversin (INVS) expression. Non-canonical Wnt-signalling sequesters components of Wnt signalling, favoring β-catenin degradation by axin, ademomatous polyposis coli (APC) and GSK3β (middle panel). Fluid flow not causing mechanosensation in cells due to disturbed cilia function switch to a predominant canonical Wnt-signalling pathway (right panel). (B): Upon hedgehog (hh) ligand binding to the receptor patched-1 (PTCH1), smoothened (SMO) is released and transported via IFT particles to the ciliary tip, where it turns off GLI repressive processing by binding suppressor of fused (SUFU). The activated form of GLI is transported back to the cell body, translocates to the nucleus and transactivates genes involved in embryonic patterning and cell growth regulation (middle panel). Cells without hh binding

to PTCH1 or cells lacking functional cilia, do not transport SMO into the cilium, which prevents the hh signalling to occur. (C): Receptors such a platelet-derived growth factor receptor α (PDGFRα) are located within the ciliary axoneme. Binding of PDGF to its ciliary receptor induces cellular responses through downstream signalling pathways such as the MEK/ERK pathway (middle panel). The ligand PDGF and the ciliary axoneme are necessary for the PDGFRα signalling (left and right panel, respectively).

shown to carry germline mutations of *VHL* which do not fully abrogate the protein's ability to regulate HIFα levels, yet these patients develop tumors. Furthermore, naturally occurring stabilizing mutations of any of the three *HIFα* gene parologs have never been reported in tumor or other tissues. Most convincingly, however, is the existence of a distinct VHL congenital syndrome: the autosomal recessive Chuvash polycythemia, constitutively hypomorphic with respect to HIF [39]. These patients exhibit elevated red blood cell counts, thrombosis and vascular abnormalities, but are not predisposed to cancer [40]. Mouse models chronically expressing stabilized HIFα are likewise not particularly predisposed to tumor formation [41, 42]. Thus, upregulation of HIFα alone does not appear to be oncogenic in humans, suggesting an alternate HIF-independent tumor suppressor function for pVHL.

Alternate functions for pVHL have been described that might be of service here. Firstly, pVHL binds and targets atypical protein kinase C (aPKC) for proteolytic degradation [43]. Secondly, the Krek lab showed that pVHL could stabilize microtubules in a HIF-independent manner [44]. A recent study used *in vivo* immunoprecipitation experiments coupled with microtubule cosedimentation assays to prove that pVHL association with microtubules is indirect, through kinesin-2 [45]. pVHL binds to kinesin-2 subunits KIF3A and KAP3 directly, and KIF3B/C indirectly, and co-localization of pVHL and KIF3A in the primary cilium is observed [45, 46]. Furthermore, Lolkema *et al*. demonstrate that disease-associated alleles of pVHL unable to bind either KAP3 or KIF3A no longer generate cilia capable of mechanotransduction, similar to retroviral expression of a dominant-negative construct in wild-type cells that interrupts pVHL-KIF3A binding [8], suggesting a physiological relevance for this interaction. Importantly, a subset of *VHL* mutant alleles fails to bind kinesin-2, and HIF activation was unaffected by pVHL-kinesin-2 association. A follow-up study demonstrated that ATP-dependent mobility of pVHL is abrogated by disrupting kinesin-2 binding in interphase cells [46]. These data link *VHL* loss to cyst formation in the kidney, known to be regulated by kinesin-2 and cilia function.

Given the cystic phenotype presented in VHL patients, many groups set out to establish a link between pVHL and cilia. Esteban *et al.* demonstrated that RCC cell lines reconstituted with pVHL were able to restore cilia on the apical surfaces of confluent cells. Using RNAi knock down technology, this study demonstrated that the function of VHL on primary cilia is mediated via HIFα [47]. Using different cell systems, four other groups independently established a role for pVHL in either the presence or maintenance of primary cilia in kidney cells [8, 48-50]. These four subsequent studies however all excluded a role of HIF in the cilia function ascribed to pVHL. It is difficult to reconcile this one major discrepancy, but certainly future efforts will resolve it. Various other aspects of pVHL's cilia regulation were teased out in these subsequent studies as well. Lutz and Burk observed that whereas ectopic expression of wild-type *VHL* isoforms and a *VHL* mutant not associated with RCC development could reconstitute ciliogenesis in RCC cells, expression of RCC-associated *VHL* mutants could not [48]. The Krek lab used primary kidney cells and a large collection of *VHL* mutant alleles to refine the model: their work elegantly demonstrates that ciliary maintenance, not genesis, is regulated by pVHL through phosphorylation by the kinase GSK3β [49].

Table 1. Basal body/ciliary proteins involved in cyst/neoplasm formation

Ciliopathy	Gene (human)	Protein (human)	Localization	Ref
ADPKD (human)	*PKD1*	Polycystin-1 precursor	Basal body/ cilium	124-126
ADPKD (human)	*PKD2*	Polycystin-2	Cilium	125-128
ARPKD (human)	*PKHD1*	Polycystic kidney and hepatic disease 1 precursor	Basal body/ cilium	129-133
PKD (mouse)	*CYS1*	Cystin-1	Cilium	125, 134
PKD (mouse)	*NEK1*	Serine/threonine-protein kinase Nek1	Basal body/ cilium	135, 136
PKD (mouse)	*NEK8*	Serine/threonine-protein kinase Nek8	Basal body/ cilium	136, 137
Polycystic kidneys (mouse)	*KIF3A*	Kinesin-like protein KIF3A	Cilium	18, 21, 24
Polycystic kidneys (mouse)	*IFT88*	Intraflagellar transport 88 homolog	Basal body/ cilium	125, 138-141
Polycystic kidneys (zebrafish)	*LRRC6*	Leucine-rich repeat-containing protein 6	Basal body	142, 143
Polycystic kidneys (zebrafish)	*DNAH9*	Ciliary dynein heavy chain 9	Cilium	84, 144, 145
Polycystic kidneys (zebrafish)	*ARL13B*	ADP-ribosylation factor-like protein 13B	Cilium	142, 146
Polycystic kidneys (zebrafish)	*IFT172*	IFT172 protein	Cilium	60, 142, 147
Polycystic kidneys (zebrafish)	*IFT57*	Intraflagellar transport 57 homolog	Cilium	142
Polycystic kidneys (zebrafish)	*IFT81*	Intraflagellar transport 81	Cilium	142
Polycystic kidneys (zebrafish)	*CLUAP1*	Clusterin-associated protein 1	Cilium	142, 148, 149
Nephronophthisis/ Senior-Løken type I (human)	*NPHP1*	Nephrocystin-1	Cilium	150-153
Nephronophthisis/ Senior-Løken type II (human)	*INVS*	Inversin	Basal body/ cilium	79, 111, 154
Nephronophthisis/ Senior-Løken type III (human)	*NPHP3*	Nephrocystin-3	Cilium	155
Nephronophthisis/ Senior-Løken type IV (human)	*NPHP4*	Nephrocystin-4	Basal body/ cilium	152, 153, 156-158
Nephronophthisis/ Senior-Løken type V (human)	*IQCB1*	IQ calmodulin-binding motif-containing protein 1	Cilium	159
Nephronophthisis/ Senior-Løken type VI/ Meckel-Gruber syndrome (human)	*CEP290*	Centrosomal protein Cep290	Basal body	160, 161
Alström's syndrome (human)	*ALMS1*	Alstrom syndrome protein 1	Basal body	162-164
Oral-facial-digital syndrome I (human)	*OFD1*	Oral-facial-digital syndrome 1 protein	Basal body	158, 165-168

Bardet-Biedl syndrome (human)	*BBS1*	Bardet-Biedl syndrome 1 protein	Basal body/ cilium	169, 170
Bardet-Biedl syndrome (human)	*BBS2*	Bardet-Biedl syndrome 2 protein	Basal body/ cilium	171, 172
Bardet-Biedl syndrome (human)	*ARL6*	ADP-ribosylation factor-like protein 6	Basal body/ cilium	173
Bardet-Biedl syndrome (human)	*BBS4*	Bardet-Biedl syndrome 4 protein	Basal body/ cilium	170, 174-176
Bardet-Biedl syndrome (human)	*BBS5*	Bardet-Biedl syndrome 5 protein	Basal body/ cilium	177
Bardet-Biedl syndrome (human)	*MKKS/ BBS6*	McKusick-Kaufman/ Bardet-Biedl syndromes putative chaperonin	Basal body/ cilium	178-180
Bardet-Biedl syndrome (human)	*BBS7*	Bardet-Biedl syndrome 7 protein	Basal body/ cilium	181, 182
Bardet-Biedl syndrome (human)	*TTC8*	Tetratricopeptide repeat protein 8	Basal body/ cilium	182, 183
Bardet-Biedl syndrome/ Wilms' tumor (human)	*BBS9*	Protein PTHB1	Basal body	176, 184, 185
VHL disease (human)	*VHL*	Von Hippel-Lindau disease tumor suppressor	Cilium	8, 47-50
Meckel-Gruber syndrome (human)	*MKS1*	Meckel syndrome type 1 protein	Basal body	158, 186, 187
Meckel-Gruber syndrome (human)	*TMEM67*	Meckelin	Cilium	187, 188
Meckel-Gruber syndrome (human)	*RPGRIP1L*	Protein fantom	Basal body	189
Glioma and gastrointestinal stromal tumors (human)	*PDGFRA*	Alpha-type platelet-derived growth factor receptor precursor	Cilium	120, 190, 191
Non small cell lung cancer (human)	*EGFR*	Epidermal growth factor receptor precursor	Cilium	192, 193
Medulloblastoma (human)	*SUFU*	Suppressor of fused homolog	Cilium	61, 194
Different tumor types* (human)	*SMO*	Smoothened homolog precursor	Cilium	57, 195, 196
Different tumor types* (human)	*GLI2*	Zinc finger protein GLI2	Cilium	61, 197

Lolkema *et al.* performed the first localization and functional assays measuring *VHL*-allele specific ciliary mechanotransduction in cultured RCC cells [8]. Schermer *et al.* took into account that cilia contain organized longitudinal strands of microtubules and examined the role of pVHL in orienting microtubules [50]. In addition to this study, two studies interrogated the contribution of pVHL's effect on microtubule stability on ciliogenesis. Using patient alleles that retain residual HIFα regulation ability, but are unable to stabilize microtubules (type 2A), these three studies concordantly support the notion that pVHL's role in primary cilia function might overlap with its role in microtubule stabilization [8, 49, 50]. Schermer *et al*. also formally demonstrated that pVHL interacts with the cell polarity complex Par3-Par6-aPKC [50]. The Par3-Par6-aPKC is targeted to the cilium by the kinesin-2 motor complex, raising the distinct possibility that this process is pVHL-mediated [45, 51].

However, conditional gene targeting of mouse *VHL* ortholog *vhlh* in a kidney-specific manner did not result in RCC; although a higher incidence of renal cysts was observed [52]. One could argue that this mouse model supports the idea that in humans all early *VHL* mutant lesions are also cystic in nature, but have some innate unique characteristic allowing a small subset to transform into cancer. Thoma *et al.* argue that *VHL*-associated cyst formation requires secondary genetic lesions which lead to the inactivation of GSK3β, resulting in loss of cilia maintenance [49]. Renal cysts derived from other genetic mutations have never been reported to associate with renal cancer, so this would not be purely a cilia-dependent phenomenon. Alternatively, it is at least as likely that renal cells with bi-allelic *VHL* mutations have the potential to become typical benign cysts, which only in rare events immediately achieve malignant transformation. In this last scenario, RCC would not be derived from the cystic phase and cancer initiation may not necessarily involve loss of cilia signalling (Figure 3). Furthermore, it is entirely unclear if RCC develops in a similar manner in the vast majority of spontaneous cases.

Hedgehog signalling and cilia

Hedgehog (Hh) signalling plays an important role in a wide range of processes during embryonic development, including regulating growth patterning, cell fate specification, cell proliferation, and cell survival [53]. Constitutive activation of Hh signalling through the GLI family of transcription factors has been described in several tumor types, including basal cell carcinoma, medulloblastoma, breast carcinoma, lung, esophageal and biliary cancer, as well as pancreatic and prostate cancer [54]. Inductive Hh signalling begins when the inhibitory association between two membrane proteins -Patched (Ptc), encoded by a tumor suppressor gene, and Smoothened (Smo), encoded by a proto-oncogene-, is disrupted by secreted Hh morphogen binding to Ptc [55, 56] (Figure 2B). How exactly Ptc inhibits Smo in the absence of Hh remains unclear, but Smo localization is vital for Hh signalling; a mutant in Smo that is unable to localize to the cell membrane can not induce Hh pathway activity [57]. In the absence of Hh ligand Gli2 and Gli3 are proteolytically processed into transcriptional repressors of Hh target genes [58, 59]. Upon Smo translocation and relay of a signal to the Gli/Cos-2/Sufu/Fu-complex, proteolytic cleavage of Gli3 is disrupted, repression of Hh target genes is relieved and transcription of Hh targets by Gli1 and Gli2 is induced. Therefore Hh binding to Ptc, as well as mutations in the pathway activating it inappropriately, result in the activation of all three Gli transcription factors.

A novel link between Hh signalling and cilia was initially noticed when mouse mutants of the IFT protein polaris (IFT88) manifested phenotypic overlap with mice with dysfunctional Hh signalling, including loss of left-right asymmetry, dorsalization of the CNS, a great reduction in digit number, and renal dysplasia including disorganization of structures within the renal parenchyma and cystic renal tubules [60-62]. The link was further supported when a genetic screen to identify new genes implicated in the generation of Hh-dependent neural cells ended up identifying four genes required for cilia formation, including Kif3a [63], a protein that binds the pVHL- and the adenomatous polyposis coli (APC)- tumor suppressor proteins [45, 64]. Subcellular localization studies of Hh signalling components revealed that all three Gli full-length proteins (but not processed Gli3 repressor) as well as endogenous Sufu, localize to the distal tip of the primary cilium, as well as to the nucleus [61]. Moreover, Hh signalling or activating mutations in Smo promote ciliary localization of Smo, suggesting that Smo acts in the primary cilium to transduce Hh signalling [57]. Another Hh mediator that was shown to

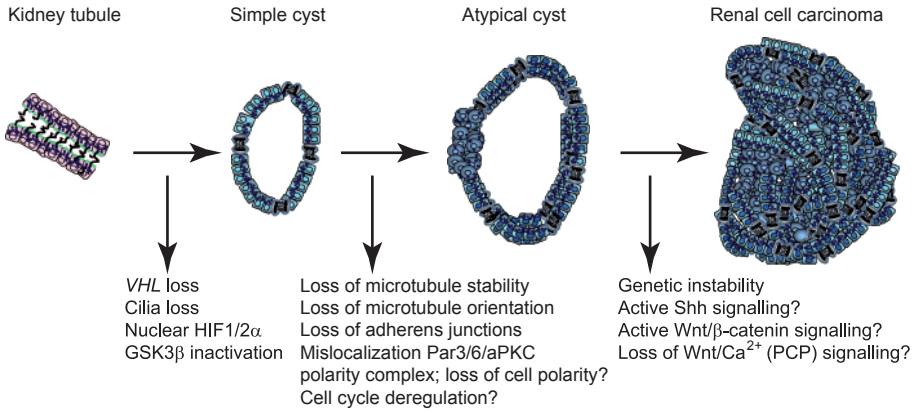


Figure 3: Schematic model for progression of *VHL*-mediated tumorigenesis in the kidney. A number of steps are probably required for renal cell carcinoma to develop, several of which might be induced by cilia loss. Only the events occurring between healthy tubular tissue and a simple cyst are well based on experimental evidence. The rest of the model is speculative in nature, based on data discussed in this review.

be concentrated to the cilia tip is β-arrestin 2, a known Smo interactor [57, 65]. Thus, ciliary IF transport regulates Hh signalling by transporting Hh components Smo, Sufu, β-arrestin 2, and all three unprocessed Gli transcription factors from the cell body to the tip of the cilium via kinesin-2 anterograde transport (Figure 2B). At the tip of the cilium Smo encounters Gli proteins and accessory proteins like Sufu to regulate the proteolytic processing of Gli2 and Gli3. The relevance of this interaction is illustrated by the identification of inactivating mutations in *Sufu* in both mouse and humans tumors [66, 67]. Retrograde transport directs Gli proteins from the tip of the cilium back to the basal body and ultimately to the nucleus to serve as transcriptional activator or repressor [10]. When cilia are disrupted the ciliary compartment required for generation of Gli repressor forms is disrupted, and as is evident from the various *IFT* knockout mouse models, Hh signalling is illegitimately activated.

Hh and pVHL signalling: speculating on potential pathway crosstalk in *VHL*-associated tumors

It is interesting to speculate that if cells in *VHL*-associated RCC no longer maintain primary cilia, that perhaps some underlying Hh signature characterizes RCCs. Indeed, evaluating published RCC microarray datasets, some incidental evidence for Hh upregulation in this tumor type was observed. For example, Hh target PAX2 functions to repress apoptosis during embryonic kidney development, before being downregulated to barely detectable levels once nephrogenesis is complete [68, 69]. Mice engineered to express PAX2 in their adult kidneys develop renal cysts [70]. In support of this hypothesis, PAX2 overexpression has been reported in 73% of *VHL*-associated RCCs [69, 71], and is associated with resistance to platinum-based therapies. Another potential example of cryptic Hh signalling in RCC could be the overexpression of Hh target gene SNAIL, repressor of E-cadherin [72]. Two groups have shown that HIF upregulation in *VHL*$^{-/-}$ renal cells resulted in higher levels *SNAIL* and subsequent downregulation of tumor suppressor E-cadherin [73, 74]. Loss of *E-cadherin* causes epithelial to mesenchymal transition, a hallmark of cancer that is associated with increased invasion and metastasis [75].

The cell cycle: who's in control?

There has been a fair amount of unsubstantiated speculation about the cilia regulating the cell cycle. Perhaps because the hypothesis is older, the scales currently weigh towards the cell cycle regulating the cilium. That there is a tight link is undeniable [76-78]: the basal body lays claim to the mother centriole, therefore the cilium must be disassembled before the mitotic spindle pole can function in cell division. However a few more recent studies suggest that more than just physical sequestration of the mother centriole regulates the cell cycle with respect to ciliogenesis. For instance, inversin can localize to the primary cilium, the basal body and the centrosome, which is particularly interesting given its short half-life and cell-cycle dependent expression. The added fact that the anaphase promoting complex protein 2 has been shown to regulate inversin's protein stability strengthens this hypothesis to a certain extent [79]. Further studies supporting an upstream role for cilia involve two IFT proteins. Robert and colleagues used RNAi to deplete IFT88, mutations in which are associated with kidney cysts (Table 1), and observed an accelerated cell cycle through the retinoblastoma tumor suppressor pathway [80]. Conversely, overexpression of IFT188 caused a cell cycle arrest at the G1-S transition. Reduction of Rab-like GTPase IFT27 in the algae *Chlamydomonas reinhardtii*, on the other hand, resulted in a growth arrest due to cytokinesis defects [81].

Recent experiments with kinesin-2 subunits have also supplied arguments for an upstream role for cilia. Overexpressing the tail domain of KIF3B resulted in cytokinesis defects and subsequent multinucleated cells [82]. Likewise, overexpressing a form of KIF3B unable to bind KAP3 resulted in chromosomal aneuploidy [83]. Additional evidence for the role of kinesin-2 subunit participation in cell cycle is derived from a study by Teng and colleagues, who show that murine neural progenitor cells lacking *KAP3* undergo malignant transformation [25]. Another axoneme associated protein, the plus-end binding protein EB1 [84-87], binds the most commonly mutated gene in colorectal cancers, *APC* [88], possibly participating in Wnt signalling. Interfering with EB1 function perturbs chromosomal stability [89] and cytokinesis [90, 91], suggestive of a role in cell cycle related processes. Defects in cilia leading to aberrant chromosomal stability and/or cytokinesis might also occur through many of the overlapping proteins regulating microtubule dynamics (e.g. EB1), by affecting spindle-microtubule function. While this hypothesis seems attractive, mutations in *KIF3B*, *KAP3*, *IFT27* and *IFT88* are not known to affect microtubule dynamics, but do affect cilia.

An unexpected link came from studies of the Aurora A, a centrosomal kinase that regulates mitotic entry through activation of cdk1-cyclin B (organizes mitotic spindle) [92]. In a superb paper from the Golemis lab, they interrogated the role of kinase Aurora A in activation of ciliary resorption, finding that basal body co-localization of Aurora A and prometastatic scaffolding protein HEF1 (also called NEDD9) was necessary for cilia resorption prior to mitotic entry [93]. The molecular mechanism of this event was pinned to the temperospatial phosphorylation and activation of histone deacetylase 6 (HDAC6), which in turn destabilizes/disassembles ciliary tubulin. Suggestively, this cellular understanding fits well with the pathological data available that Aurora A is highly overexpressed in a variety of tumors characterized by centrosomal amplification and genomic stability [94-96]. Small molecule inhibitors of Aurora A and HDAC6 reduce the regulates disassembly of cilia [93] which may predict bright futures for the use of these drugs in ciliopathy treatments.

Wnt signalling

There is recent and exciting evidence emerging from mouse/zebrafish models and cell-based systems that cilia function might influence both canonical and non-canonical Wnt signalling, and vice versa [97, 98]. In differentiated cells, β-catenin is associated with adherens junctions at the cell membrane. A destruction complex consisting of axin, glycogen synthase kinase-3β (GSK-3β), and APC protein polices accumulation of β-catenin in the cytoplasm though proteasomal degradation [99]. Stabilization of β-catenin, either through activating mutations of *β-catenin* itself or inactivating mutations of *APC* cause the most common cause of cancer deaths, colorectal cancer [99]. Transgenic mice overexpressing β-catenin develop renal cysts [100]; likewise, the renal cystic epithelium from mice mutant for *xylosyltransferase 2* [101] and *Apc* [102] also display unregulated β-catenin. Remarkably, these kidney-specific *Apc* hypomorphs also develop renal cell carcinomas, constituting the first genetic mouse model for kidney cancer [102]. However, this model is quite limited in its resemblance to the human RCC genetic signature. Human patients heterozygous for *APC* mutations are not predisposed to an increased chance of developing RCC, nor are sporadic RCCs associated with *APC* mutations [103].

The non-canonical Wnt/Ca^{2+} pathway (also called planar cell polarity pathway, or PCP for short) is important for dictating the plane of the epithelium to be perpendicular to the cells' apical-basal axis and the orientation of the mitotic spindle [104]. When Wnt ligand binds its receptor, an early event involving a protein called dishevelled (Dsh) determines whether canonical (e.g. Wnt3a) or non-canonical Wnt (e.g. Wnt 5a) signalling is initiated (Figure 2A). For example, phosphorylation of Dsh by either casein kinase I or by protein kinase C (PKC) can determine whether canonical or Wnt/Ca^{2+} signalling is transduced, respectively [105-107]. Evidence that Wnt/Ca^{2+} signalling is directly involved in cancer first became convincing when a gene expression profiling study correlated overexpression of Wnt5a and concomitant increase in PKC activity with human melanoma progression in the absence of nuclear β-catenin [108]. Additional connections have been recently reviewed elsewhere [109].

Tubulular fluid flow present in the kidney has been demonstrated to upregulate inversin, which in turn switches off the canonical Wnt/β-catenin pathway by targeting Dsh for destruction, thereby forcing the tubular epithelium down the non-canonical Wnt/Ca^{2+} pathway [110]. Mutations in the human inversin ortholog (*NPHP2*) cause nephronophthisis (the most common autosomal recessive renal disease) which is characterized by interstitial cell infiltration, with fibrosis, and duct proliferation with cyst development [111].

Bardet-Biedl syndrome (BBS) is a significant cause of chronic renal failure in children, as well as causing obesity, cognitive impairments, and limb deformities [112]. When mouse mutants for BBS proteins 1, 4, and 6 displayed a strikingly similar array of developmental defects as mutants in the Wnt/Ca^{2+} signalling pathway, a study was initiated to define the genetic common denominator. When *BBS4* heterozygous healthy mice were crossed with Wnt/Ca^{2+} signalling component *van gogh like-2* (*VANGL2*) heterozygous mice, the pups heterozygous for both genes displayed the severe homozygous Wnt/Ca^{2+} phenotype [113]. Furthermore, in an independent study using zebrafish, suppression of bbs4 in *vangl2* mutant embryos similarly resulted in a phenotype typically caused by defective Wnt/Ca^{2+} signalling [114]. Together, these *in vivo* data strongly argue for a functional link between Wnt/Ca^{2+} signalling and cilia function. Accordingly VANGL2 was observed to co-localize with BBS proteins at the basal body and axoneme of ciliated cells [113]. Whether this interaction has implications

for cystogenesis was addressed by Fischer and colleagues, who measured the mitotic orientation of kidney tubule cells taken from a rat model of polycystic kidney disease and found them to be significantly disorientated [115]. They attributed this to altered expression of PKHD1, a ciliary membrane protein mutated in ARPKD, and one of the most common sporadically mutated genes in colorectal cancer [116] (Table 2). As mitotic spindle orientation is regulated by the Wnt/Ca^{2+} pathway [104], aberrations caused by BBS protein dysfunction could cause similar mitotic defects in BBS kidneys.

In their landmark paper, the Katsanis lab fleshed out how Wnt signalling might be influenced by the cilium and basal body [117]. Using zebrafish morpholinos to knockdown BBS1, 4 and 6 orthologs, they determined that suppression of any of these proteins produced dose-dependent disturbances of gastrulation movements characteristic of non-canonical Wnt signalling defects. Given the reciprocal relationship between the non-canonical and canonical arms of Wnt signal at the level of Dsh, the authors confirmed upregulation of canonical Wnt signalling in embryos whose basal body and ciliary function was perturbed due to depletion of bbs1, 4, or 6, which could be rescued by forced expression of Dsh at the membrane in these zebrafish embryos. Furthermore, BBS4 influences the canonical-non-canonical balance in human kidney cell lines as well; upregulation of nuclear β-catenin was observed upon BBS4 suppression which could be reversed when the cells were exposed to WNT5A, a ligand that only activates the Wnt/Ca^{2+} pathway. These results could be partly attributable to proteasomal defects affecting β-catenin stabilization following basal body disruption and demonstrating that defective Wnt signalling may be causally related to a number of phenotypes associated with ciliopathies [117]. The collective results of these various studies sketch the beginning of scenario in cilia/basal body dysfunction where the canonical Wnt pathway stimulates hyperplasia which is complicated with concomitant defects in planar cell polarization.

Cilia are biochemical sensors

Although many labs have focussed on the mechanotransduction aspect of ciliary signalling that can be silenced by cilia loss, losing chemosensing functions that some types of cilia possess might contribute to neoplastic growth. Many cilia bear receptors specific to the specialized function of their surrounding tissues. For example, neuronal cilia display the 5-HT_6 serotonin receptor [118] and somatostatin receptor 3 [119]. These particular receptors -while not particularly relevant in cancer biology- exemplify the complexity of tissue-specific homeostasis signalling that would be abrogated upon cilia loss. In short, there is a tremendous amount of room for understanding in this field.

One example of localized receptor signalling implicated in cancer has provided insight into cilia tumor suppression. It is been well established that signalling through the two platelet-derived growth factor receptors (PDGFRs) stimulate cell proliferation, survival and migration. PDGFRs are receptor tyrosine kinases that initiate signal transduction by phosphorylating tyrosine residues in their downstream target proteins. PDGFs secreted by tumors contribute to the induction of angiogenesis and lymphogenesis. These processes provide routes for cancer cells that have detached from the tumor to metastasize.

Schneider *et al.* demonstrated that an isoform of the PDGFR family, PDGFRα, is localized in the primary cilium of quiescent primary fibroblasts as well as cultured NIH3T3 cells [120]. The localization of PDGFRα in the ciliary membrane regulates its ability to respond to ligand activation by activating the PKB and MEK/ERK pathways

(Figure 2C). MEK1/2 is phosphorylated in the cilium or at the basal body [120], thereby contributing to mitogenic control. Fibroblasts derived from polycystic kidney model *Tg737* (*orpk*) mice fail to form normal cilia and to upregulate the level of PDGFRα, and the Mek/Erk pathway is not activated.

Cancer Genomic Landscapes: are cilia on the horizon?

Recent attempts to define the genomic landscape of breast and colorectal cancer by sequencing more than half a genome per patient (>90% of the protein-coding genes), recent attempts to define the cancer genomic landscape of breast and colorectal cancer suggest that a few cilia-related genes are frequently mutated in these tumors (Table 2) [116, 121]. A list of candidate cancer (CAN) genes was assembled, defined as a gene that harbors at least one nonsynonymous mutation through all the screens and if the total number of mutations per nucleotide sequenced exceeds a minimum threshold [121]. Three CAN-genes from breast cancer (*GLI1*, *RPGRIP1* and *DNAH9*) and two CAN-genes from the colorectal cancer screen (*GLI3* and *PKHD1*) have known ciliary localization and/or function (Table 2). Mutations in *PKHD1*, *DNAH9* and *RPGRIP1* have been associated with ARPKD (Table 1), polycystic kidney development (Table 1) and Leber congenital amaurosis [122] respectively but never with tumorigenesis, unlike *GLI1* and *GLI3* (Table 1). These sorts of studies suggest that we are just beginning to grasp the importance of cilia function in tumorigenesis and mark the beginning of personalized cancer genomics allowing tailored therapies focusing on the alterations identified in a particular patient's cancer.

Ciliotherapeutics and concluding remarks

Defining the cellular problems involved in cancer is only the beginning. The challenges inherent in drug design notwithstanding, the first question to tackle when thinking about cilioreplacement or ciliosubstitution therapy is which signal to rescue? The genetic data discussed here point to three decent candidates. In kidney tumors, the tumor suppressor pVHL's function in stabilizing and orienting microtubules at the cell periphery points suggest that new generation microtubule stabilizing drugs and/or GSK3β mimetics might have beneficial effects in combination with current therapies [44, 49, 50]. Given the correlative evidence that Hh signalling might be illegitimately activated in *VHL*-associated renal cell carcinomas, these patients might also benefit from inhibitors of this pathway. Secondly, the Aurora A-HEF1-HDAC relationship regulating ciliary disassembly is a highly attractive target. Tumor cells often manifest upregulated Aurora A and HEF1, which might be regulated with chemotherapeutic agents inhibiting Aurora A, which are now in clinical trials. Furthermore, inhibitors of HDACs are broad-spectrum, well tolerated, and have shown some efficacy in certain

Table 2. Cilia-related CAN-genes commonly mutated in sporadic cancers

Tumor	Gene	CAN-protein	Localization	Ref
Breast	*GLI1*	Zinc finger protein GLI1	cilium	61, 116, 121
Breast	*RPGRIP1*	X-linked retinitis pigmentosa GTPase regulator-interacting protein 1	basal body	121, 198-200
Breast	*DNAH9*	Ciliary dynein heavy chain 9	cilium	116, 145
Colorectal	*GLI3*	Zinc finger protein GLI3	cilium	61, 121
Colorectal	*PKHD1*	Polycystic kidney and hepatic disease 1 precursor	Basal body/ cilium	116, 132

tumor types. Thirdly, we have discussed here that the delicate balance between the two arms of Wnt signalling is perturbed upon cilia loss, favoring the oncogenic canonical Wnt pathway. Many industrial and academic efforts are fully dedicated to interrupting this pathway in patients. Neoplasms associated with cilia loss are likely to benefit from these studies. Lastly, although direct genetic evidence is lacking, there is evidence from humans and mouse models suggesting that inhibitors of the mammalian target of rapamycin (mTOR) might be effective in ciliopathologies. Shillingford *et al.* described that mTOR is inappropriately activated not only in human *PKD*-defective cysts but also in several polycystic mouse models [123]. Moreover, treatment of two different polycystic mouse models with rapamycin stopped kidney growth, while not affecting the kidneys of normal mice [123]. It is tempting to speculate from these data that mTOR activation may be a common consequence of the loss of cilia function.

In summary, data accumulated from many model systems in the last decade robustly support the notion that presence and function of cilia protect against the initiation of hyperplastic growth. Like the classical "gatekeeper" or "caretaker" tumor suppressor proteins, the cilium functions as an organelle to maintain signalling homeostasis in tissues. Given the discrete spatial compartment of the cilium, we have dubbed this the "watchtower" function of cilia.

Acknowledgements

We thank Susanne van Schelven, Patrick Maxwell, Ellen van Rooijen and Janice Griffith for unpublished figures, Babet van der Vaart and all the members of the Giles and Voest labs for helpful discussion. We apologize to all the researchers whose outstanding contributions to the field we were unable to discuss or cite here. Funding provided by Dutch Organization for Scientific Research (NWO-VIDI to RHG), and the Dutch Cancer Society (KWF to EEV).

References

1. **Mitchell, D.R.** The evolution of eukaryotic cilia and flagella as motile and sensory organelles. *Adv Exp Med Biol* 607, 130-140 (2007).
2. **Jekely, G. & Arendt, D.** Evolution of intraflagellar transport from coated vesicles and autogenous origin of the eukaryotic cilium. *Bioessays* 28, 191-198 (2006).
3. **Satir, P., Guerra, C. & Bell, A.J.** Evolution and persistence of the cilium. *Cell Motil Cytoskeleton* 64, 906-913 (2007).
4. **Fliegauf, M., Benzing, T. & Omran, H.** When cilia go bad: cilia defects and ciliopathies. *Nat Rev Mol Cell Biol* 8, 880-893 (2007).
5. **Feistel, K. & Blum, M.** Three types of cilia including a novel 9+4 axoneme on the notochordal plate of the rabbit embryo. *Dev Dyn* 235, 3348-3358 (2006).
6. **Hildebrandt, F. & Otto, E.** Cilia and centrosomes: a unifying pathogenic concept for cystic kidney disease? *Nat Rev Genet* 6, 928-940 (2005).
7. **Praetorius, H.A. & Spring, K.R.** A physiological view of the primary cilium. *Annu Rev Physiol* 67, 515-529 (2005).
8. **Lolkema, M.P. *et al.*** Allele-specific regulation of primary cilia function by the von Hippel-Lindau tumor suppressor. *Eur J Hum Genet* (2007).
9. **Benzing, T. & Walz, G.** Cilium-generated signaling: a cellular GPS? *Curr Opin Nephrol Hypertens* 15, 245-249 (2006).

10. **Scholey, J.M. & Anderson, K.V.** Intraflagellar transport and cilium-based signaling. *Cell* 125, 439-442 (2006).
11. **Yamazaki, H., Nakata, T., Okada, Y. & Hirokawa, N.** Cloning and characterization of KAP3: a novel kinesin superfamily-associated protein of KIF3A/3B. *Proc Natl Acad Sci U S A* 93, 8443-8448 (1996).
12. **Signor, D. *et al.*** Role of a class DHC1b dynein in retrograde transport of IFT motors and IFT raft particles along cilia, but not dendrites, in chemosensory neurons of living Caenorhabditis elegans. *J Cell Biol* 147, 519-530 (1999).
13. **Christensen, S.T., Pedersen, L.B., Schneider, L. & Satir, P.** Sensory cilia and integration of signal transduction in human health and disease. *Traffic* 8, 97-109 (2007).
14. **Bisgrove, B.W. & Yost, H.J.** The roles of cilia in developmental disorders and disease. *Development* 133, 4131-4143 (2006).
15. **Nauli, S.M. *et al.*** Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet* 33, 129-137 (2003).
16. **Pazour, G.J.** Intraflagellar transport and cilia-dependent renal disease: the ciliary hypothesis of polycystic kidney disease. *J Am Soc Nephrol* 15, 2528-2536 (2004).
17. **van Rooijen, E. *et al.*** LRRC50, a conserved ciliary protein implicated in polycystic kidney disease. *J Am Soc Nephrol* (in press) (2008).
18. **Morris, R.L. & Scholey, J.M.** Heterotrimeric kinesin-II is required for the assembly of motile 9+2 ciliary axonemes on sea urchin embryos. *J Cell Biol* 138, 1009-1022 (1997).
19. **Kozminski, K.G., Beech, P.L. & Rosenbaum, J.L.** The Chlamydomonas kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. *J Cell Biol* 131, 1517-1527 (1995).
20. **Brown, J.M., Marsala, C., Kosoy, R. & Gaertig, J.** Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in Tetrahymena. *Mol Biol Cell* 10, 3081-3096 (1999).
21. **Marszalek, J.R., Ruiz-Lozano, P., Roberts, E., Chien, K.R. & Goldstein, L.S.** Situs inversus and embryonic ciliary morphogenesis defects in mouse mutants lacking the KIF3A subunit of kinesin-II. *Proc Natl Acad Sci U S A* 96, 5043-5048. (1999).
22. **Nonaka, S. *et al.*** Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell* 95, 829-837 (1998).
23. **Afzelius, B.A.** Cilia-related diseases. *J Pathol* 204, 470-477 (2004).
24. **Lin, F. *et al.*** Kidney-specific inactivation of the KIF3A subunit of kinesin-II inhibits renal ciliogenesis and produces polycystic kidney disease. *Proc Natl Acad Sci U S A* 100, 5286-5291 (2003).
25. **Teng, J. *et al.*** The KIF3 motor transports N-cadherin and organizes the developing neuroepithelium. *Nat Cell Biol* 7, 474-482 (2005).
26. **Van Poppel, H. *et al.*** Precancerous lesions in the kidney. *Scand J Urol Nephrol Suppl* 205, 136-165 (2000).
27. **Lubensky, I.A. *et al.*** Allelic deletions of the VHL gene detected in multiple microscopic clear cell renal lesions in von Hippel-Lindau disease patients. *Am J Pathol* 149, 2089-2094 (1996).
28. **Evans, J.P. & Skrzynia, C.** (2002).
29. **Walther, M.M., Lubensky, I.A., Venzon, D., Zbar, B. & Linehan, W.M.**

Prevalence of microscopic lesions in grossly normal renal parenchyma from patients with von Hippel-Lindau disease, sporadic renal cell carcinoma and no renal disease: clinical implications. *J Urol* 154, 2010-2014; discussion 2014-2015 (1995).

30. **Maher, E.R. *et al.*** Clinical features and natural history of von Hippel-Lindau disease. *Q J Med* 77, 1151-1163 (1990).
31. **Gnarra, J.R. *et al.*** Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet* 7, 85-90. (1994).
32. **Latif, F. *et al.*** Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 260, 1317-1320. (1993).
33. **Herman, J.G. *et al.*** Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A* 91, 9700-9704. (1994).
34. **Iliopoulos, O., Kibel, A., Gray, S. & Kaelin, W.G., Jr.** Tumour suppression by the human von Hippel-Lindau gene product. *Nat Med* 1, 822-826. (1995).
35. **Kaelin, W.G., Jr.** Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer* 2, 673-682. (2002).
36. **Foster, K. *et al.*** Somatic mutations of the von Hippel-Lindau disease tumour suppressor gene in non-familial clear cell renal carcinoma. *Hum Mol Genet* 3, 2169-2173. (1994).
37. **Kaelin, W.G., Jr.** The von Hippel-Lindau tumor suppressor gene and kidney cancer. *Clin Cancer Res* 10, 6290S-6295S (2004).
38. **Lonser, R.R. *et al.*** von Hippel-Lindau disease. *Lancet* 361, 2059-2067 (2003).
39. **Ang, S.O. *et al.*** Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nat Genet* 32, 614-621 (2002).
40. **Gordeuk, V.R. *et al.*** Congenital disorder of oxygen sensing: association of the homozygous Chuvash polycythemia VHL mutation with thrombosis and vascular abnormalities but not tumors. *Blood* 103, 3924-3932 (2004).
41. **Kim, W.Y. *et al.*** Failure to prolyl hydroxylate hypoxia-inducible factor alpha phenocopies VHL inactivation in vivo. *Embo J* 25, 4650-4662 (2006).
42. **Hickey, M.M., Lam, J.C., Bezman, N.A., Rathmell, W.K. & Simon, M.C.** von Hippel-Lindau mutation in mice recapitulates Chuvash polycythemia via hypoxia-inducible factor-2alpha signaling and splenic erythropoiesis. *J Clin Invest* (2007).
43. **Okuda, H. *et al.*** Direct interaction of the beta-domain of VHL tumor suppressor protein with the regulatory domain of atypical PKC isotypes. *Biochem Biophys Res Commun* 263, 491-497 (1999).
44. **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* 5, 64-70. (2003).
45. **Lolkema, M.P. *et al.*** The von Hippel-Lindau tumour suppressor interacts with microtubules through kinesin-2. *FEBS Lett* 581, 4571-4576 (2007).
46. **Mans, D.A. *et al.*** Mobility of the von Hippel-Lindau tumour suppressor protein is regulated by kinesin-2 *Exp Cell Res* (in press) (2008).
47. **Esteban, M.A., Harten, S.K., Tran, M.G. & Maxwell, P.H.** Formation of Primary Cilia in the Renal Epithelium Is Regulated by the von Hippel-Lindau Tumor Suppressor Protein. *J Am Soc Nephrol* (2006).
48. **Lutz, M.S. & Burk, R.D.** Primary cilium formation requires von hippel-lindau

gene function in renal-derived cells. *Cancer Res* 66, 6903-6907 (2006).
49. **Thoma, C.R. *et al.*** pVHL and GSK3beta are components of a primary cilium-maintenance signalling network. *Nat Cell Biol* (2007).
50. **Schermer, B. *et al.*** The von Hippel-Lindau tumor suppressor protein controls ciliogenesis by orienting microtubule growth. *J Cell Biol* 175, 547-554 (2006).
51. **Fan, S. *et al.*** Polarity proteins control ciliogenesis via kinesin motor interactions. *Curr Biol* 14, 1451-1461 (2004).
52. **Rankin, E.B., Tomaszewski, J.E. & Haase, V.H.** Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. *Cancer Res* 66, 2576-2583 (2006).
53. **Wang, Y., McMahon, A.P. & Allen, B.L.** Shifting paradigms in Hedgehog signaling. *Curr Opin Cell Biol* 19, 159-165 (2007).
54. **Ruiz i Altaba, A., Mas, C. & Stecca, B.** The Gli code: an information nexus regulating cell fate, stemness and cancer. *Trends Cell Biol* 17, 438-447 (2007).
55. **Rohatgi, R., Milenkovic, L. & Scott, M.P.** Patched1 regulates hedgehog signaling at the primary cilium. *Science* 317, 372-376 (2007).
56. **Rohatgi, R. & Scott, M.P.** Patching the gaps in Hedgehog signalling. *Nat Cell Biol* 9, 1005-1009 (2007).
57. **Corbit, K.C. *et al.*** Vertebrate Smoothened functions at the primary cilium. *Nature* 437, 1018-1021 (2005).
58. **Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M. & Kondoh, H.** Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* 126, 3915-3924 (1999).
59. **Osterlund, T. & Kogerman, P.** Hedgehog signalling: how to get from Smo to Ci and Gli. *Trends Cell Biol* 16, 176-180 (2006).
60. **Huangfu, D. *et al.*** Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature* 426, 83-87 (2003).
61. **Haycraft, C.J. *et al.*** Gli2 and gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. *PLoS Genet* 1, e53 (2005).
62. **Liu, A., Wang, B. & Niswander, L.A.** Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. *Development* 132, 3103-3111 (2005).
63. **Huangfu, D. & Anderson, K.V.** Cilia and Hedgehog responsiveness in the mouse. *Proc Natl Acad Sci U S A* 102, 11325-11330 (2005).
64. **Jimbo, T. *et al.*** Identification of a link between the tumour suppressor APC and the kinesin superfamily. *Nat Cell Biol* 4, 323-327. (2002).
65. **Chen, W. *et al.*** Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. *Science* 306, 2257-2260 (2004).
66. **Lee, Y. *et al.*** Loss of suppressor-of-fused function promotes tumorigenesis. *Oncogene* 26, 6442-6447 (2007).
67. **Reifenberger, J. *et al.*** Somatic mutations in the PTCH, SMOH, SUFUH and TP53 genes in sporadic basal cell carcinomas. *Br J Dermatol* 152, 43-51 (2005).
68. **Hu, M.C. *et al.*** GLI3-dependent transcriptional repression of Gli1, Gli2 and kidney patterning genes disrupts renal morphogenesis. *Development* 133,

569-578 (2006).
69. **Gnarra, J.R. & Dressler, G.R.** Expression of Pax-2 in human renal cell carcinoma and growth inhibition by antisense oligonucleotides. *Cancer Res* 55, 4092-4098 (1995).
70. **Stayner, C. *et al.*** Pax2 gene dosage influences cystogenesis in autosomal dominant polycystic kidney disease. *Hum Mol Genet* 15, 3520-3528 (2006).
71. **Hueber, P.A., Waters, P., Clark, P., Eccles, M. & Goodyer, P.** PAX2 inactivation enhances cisplatin-induced apoptosis in renal carcinoma cells. *Kidney Int* 69, 1139-1145 (2006).
72. **Batlle, E. *et al.*** The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* 2, 84-89 (2000).
73. **Esteban, M.A. *et al.*** Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res* 66, 3567-3575 (2006).
74. **Evans, A.J. *et al.*** VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail. *Mol Cell Biol* 27, 157-169 (2007).
75. **Conacci-Sorrell, M. *et al.*** Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK. *J Cell Biol* 163, 847-857 (2003).
76. **Pan, J. & Snell, W.** The primary cilium: keeper of the key to cell division. *Cell* 129, 1255-1257 (2007).
77. **Wheatley, D.N., Wang, A.M. & Strugnell, G.E.** Expression of primary cilia in mammalian cells. *Cell Biol Int* 20, 73-81 (1996).
78. **Quarmby, L.M. & Parker, J.D.** Cilia and the cell cycle? *J Cell Biol* 169, 707-710 (2005).
79. **Morgan, D. *et al.*** Expression analyses and interaction with the anaphase promoting complex protein Apc2 suggest a role for inversin in primary cilia and involvement in the cell cycle. *Hum Mol Genet* 11, 3345-3350 (2002).
80. **Robert, A. *et al.*** The intraflagellar transport component IFT88/polaris is a centrosomal protein regulating G1-S transition in non-ciliated cells. *J Cell Sci* 120, 628-637 (2007).
81. **Qin, H., Wang, Z., Diener, D. & Rosenbaum, J.** Intraflagellar transport protein 27 is a small G protein involved in cell-cycle control. *Curr Biol* 17, 193-202 (2007).
82. **Fan, J. & Beck, K.A.** A role for the spectrin superfamily member Syne-1 and kinesin II in cytokinesis. *J Cell Sci* 117, 619-629 (2004).
83. **Haraguchi, K., Hayashi, T., Jimbo, T., Yamamoto, T. & Akiyama, T.** Role of the kinesin-2 family protein, KIF3, during mitosis. *J Biol Chem* 281, 4094-4099 (2006).
84. **Pazour, G.J., Agrin, N., Leszyk, J. & Witman, G.B.** Proteomic analysis of a eukaryotic cilium. *J Cell Biol* 170, 103-113 (2005).
85. **Pedersen, L.B., Geimer, S., Sloboda, R.D. & Rosenbaum, J.L.** The Microtubule plus end-tracking protein EB1 is localized to the flagellar tip and basal bodies in Chlamydomonas reinhardtii. *Curr Biol* 13, 1969-1974 (2003).
86. **Schroder, J.M., Schneider, L., Christensen, S.T. & Pedersen, L.B.** EB1 is required for primary cilia assembly in fibroblasts. *Curr Biol* 17, 1134-1139 (2007).
87. **Sloboda, R.D. & Howard, L.** Localization of EB1, IFT polypeptides, and

kinesin-2 in Chlamydomonas flagellar axonemes via immunogold scanning electron microscopy. *Cell Motil Cytoskeleton* 64, 446-460 (2007).

88. **Su, L.K. *et al.*** APC binds to the novel protein EB1. *Cancer Res* 55, 2972-2977 (1995).
89. **Tirnauer, J.S. & Bierer, B.E.** EB1 proteins regulate microtubule dynamics, cell polarity, and chromosome stability. *J Cell Biol* 149, 761-766 (2000).
90. **Strickland, L.I., Donnelly, E.J. & Burgess, D.R.** Induction of cytokinesis is independent of precisely regulated microtubule dynamics. *Mol Biol Cell* 16, 4485-4494 (2005).
91. **Strickland, L.I., Wen, Y., Gundersen, G.G. & Burgess, D.R.** Interaction between EB1 and p150glued is required for anaphase astral microtubule elongation and stimulation of cytokinesis. *Curr Biol* 15, 2249-2255 (2005).
92. **Bischoff, J.R. *et al.*** A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. *Embo J* 17, 3052-3065 (1998).
93. **Pugacheva, E.N., Jablonski, S.A., Hartman, T.R., Henske, E.P. & Golemis, E.A.** HEF1-dependent Aurora A activation induces disassembly of the primary cilium. *Cell* 129, 1351-1363 (2007).
94. **Anand, S., Penrhyn-Lowe, S. & Venkitaraman, A.R.** AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell* 3, 51-62 (2003).
95. **Goepfert, T.M. *et al.*** Centrosome amplification and overexpression of aurora A are early events in rat mammary carcinogenesis. *Cancer Res* 62, 4115-4122 (2002).
96. **Gritsko, T.M. *et al.*** Activation and overexpression of centrosome kinase BTAK/Aurora-A in human ovarian cancer. *Clin Cancer Res* 9, 1420-1426 (2003).
97. **Benzing, T., Simons, M. & Walz, G.** Wnt signaling in polycystic kidney disease. *J Am Soc Nephrol* 18, 1389-1398 (2007).
98. **Giles, R.H. *et al.*** Interplay between VHL/HIF1alpha and Wnt/beta-catenin pathways during colorectal tumorigenesis. *Oncogene* 25, 3065-3070 (2006).
99. **Giles, R.H., van Es, J.H. & Clevers, H.** Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta* 1653, 1-24 (2003).
100. **Saadi-Kheddouci, S. *et al.*** Early development of polycystic kidney disease in transgenic mice expressing an activated mutant of the beta-catenin gene. *Oncogene* 20, 5972-5981 (2001).
101. **Condac, E. *et al.*** Polycystic disease caused by deficiency in xylosyltransferase 2, an initiating enzyme of glycosaminoglycan biosynthesis. *Proc Natl Acad Sci U S A* 104, 9416-9421 (2007).
102. **Qian, C.N. *et al.*** Cystic renal neoplasia following conditional inactivation of apc in mouse renal tubular epithelium. *J Biol Chem* 280, 3938-3945 (2005).
103. **Giles, R.H. & Voest, E.E.** Tumor suppressors APC and VHL: gatekeepers of the intestine and kidney. *Prog Mol Subcell Biol* 40, 151-181 (2005).
104. **Gong, Y., Mo, C. & Fraser, S.E.** Planar cell polarity signalling controls cell division orientation during zebrafish gastrulation. *Nature* 430, 689-693 (2004).
105. **Kuhl, M. *et al.*** Antagonistic regulation of convergent extension movements in Xenopus by Wnt/beta-catenin and Wnt/Ca2+ signaling. *Mech Dev* 106, 61-76 (2001).

106. **Peters, J.M., McKay, R.M., McKay, J.P. & Graff, J.M.** Casein kinase I transduces Wnt signals. *Nature* 401, 345-350 (1999).
107. **McKay, R.M., Peters, J.M. & Graff, J.M.** The casein kinase I family in Wnt signaling. *Dev Biol* 235, 388-396 (2001).
108. **Weeraratna, A.T. *et al.*** Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer Cell* 1, 279-288 (2002).
109. **Katoh, M.** WNT/PCP signaling pathway and human cancer (review). *Oncol Rep* 14, 1583-1588 (2005).
110. **Simons, M. *et al.*** Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways. *Nat Genet* 37, 537-543 (2005).
111. **Otto, E.A. *et al.*** Mutations in INVS encoding inversin cause nephronophthisis type 2, linking renal cystic disease to the function of primary cilia and left-right axis determination. *Nat Genet* 34, 413-420 (2003).
112. **Tobin, J.L. & Beales, P.L.** Bardet-Biedl syndrome: beyond the cilium. *Pediatr Nephrol* 22, 926-936 (2007).
113. **Ross, A.J. *et al.*** Disruption of Bardet-Biedl syndrome ciliary proteins perturbs planar cell polarity in vertebrates. *Nat Genet* 37, 1135-1140 (2005).
114. **Badano, J.L. *et al.*** Dissection of epistasis in oligogenic Bardet-Biedl syndrome. *Nature* 439, 326-330 (2006).
115. **Fischer, E. *et al.*** Defective planar cell polarity in polycystic kidney disease. *Nat Genet* 38, 21-23 (2006).
116. **Sjoblom, T. *et al.*** The consensus coding sequences of human breast and colorectal cancers. *Science* 314, 268-274 (2006).
117. **Gerdes, J.M. *et al.*** Disruption of the basal body compromises proteasomal function and perturbs intracellular Wnt response. *Nat Genet* 39, 1350-1360 (2007).
118. **Brailov, I. *et al.*** Localization of 5-HT(6) receptors at the plasma membrane of neuronal cilia in the rat brain. *Brain Res* 872, 271-275 (2000).
119. **Handel, M. *et al.*** Selective targeting of somatostatin receptor 3 to neuronal cilia. *Neuroscience* 89, 909-926 (1999).
120. **Schneider, L. *et al.*** PDGFRalphaalpha signaling is regulated through the primary cilium in fibroblasts. *Curr Biol* 15, 1861-1866 (2005).
121. **Wood, L.D. *et al.*** The Genomic Landscapes of Human Breast and Colorectal Cancers. *Science* (2007).
122. **Dryja, T.P. *et al.*** Null RPGRIP1 alleles in patients with Leber congenital amaurosis. *Am J Hum Genet* 68, 1295-1298 (2001).
123. **Shillingford, J.M. *et al.*** The mTOR pathway is regulated by polycystin-1, and its inhibition reverses renal cystogenesis in polycystic kidney disease. *Proc Natl Acad Sci U S A* 103, 5466-5471 (2006).
124. The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. The European Polycystic Kidney Disease Consortium. *Cell* 78, 725 (1994).
125. **Yoder, B.K., Hou, X. & Guay-Woodford, L.M.** The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. *J Am Soc Nephrol* 13, 2508-2516 (2002).
126. **Barr, M.M. *et al.*** The Caenorhabditis elegans autosomal dominant polycystic kidney disease gene homologs lov-1 and pkd-2 act in the same pathway. *Curr Biol* 11, 1341-1346 (2001).

127. **Mochizuki, T. *et al.*** PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. *Science* 272, 1339-1342 (1996).
128. **Pazour, G.J., San Agustin, J.T., Follit, J.A., Rosenbaum, J.L. & Witman, G.B.** Polycystin-2 localizes to kidney cilia and the ciliary level is elevated in orpk mice with polycystic kidney disease. *Curr Biol* 12, R378-380 (2002).
129. **Onuchic, L.F. *et al.*** PKHD1, the polycystic kidney and hepatic disease 1 gene, encodes a novel large protein containing multiple immunoglobulin-like plexin-transcription-factor domains and parallel beta-helix 1 repeats. *Am J Hum Genet* 70, 1305-1317 (2002).
130. **Ward, C.J. *et al.*** The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein. *Nat Genet* 30, 259-269 (2002).
131. **Ward, C.J. *et al.*** Cellular and subcellular localization of the ARPKD protein; fibrocystin is expressed on primary cilia. *Hum Mol Genet* 12, 2703-2710 (2003).
132. **Wang, S., Luo, Y., Wilson, P.D., Witman, G.B. & Zhou, J.** The autosomal recessive polycystic kidney disease protein is localized to primary cilia, with concentration in the basal body area. *J Am Soc Nephrol* 15, 592-602 (2004).
133. **Zhang, M.Z. *et al.*** PKHD1 protein encoded by the gene for autosomal recessive polycystic kidney disease associates with basal bodies and primary cilia in renal epithelial cells. *Proc Natl Acad Sci U S A* 101, 2311-2316 (2004).
134. **Hou, X. *et al.*** Cystin, a novel cilia-associated protein, is disrupted in the cpk mouse model of polycystic kidney disease. *J Clin Invest* 109, 533-540 (2002).
135. **Upadhya, P., Birkenmeier, E.H., Birkenmeier, C.S. & Barker, J.E.** Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. *Proc Natl Acad Sci U S A* 97, 217-221 (2000).
136. **Mahjoub, M.R., Trapp, M.L. & Quarmby, L.M.** NIMA-related kinases defective in murine models of polycystic kidney diseases localize to primary cilia and centrosomes. *J Am Soc Nephrol* 16, 3485-3489 (2005).
137. **Liu, S. *et al.*** A defect in a novel Nek-family kinase causes cystic kidney disease in the mouse and in zebrafish. *Development* 129, 5839-5846 (2002).
138. **Pazour, G.J. *et al.*** Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene tg737, are required for assembly of cilia and flagella. *J Cell Biol* 151, 709-718 (2000).
139. **Qin, H., Rosenbaum, J.L. & Barr, M.M.** An autosomal recessive polycystic kidney disease gene homolog is involved in intraflagellar transport in C. elegans ciliated sensory neurons. *Curr Biol* 11, 457-461 (2001).
140. **Taulman, P.D., Haycraft, C.J., Balkovetz, D.F. & Yoder, B.K.** Polaris, a protein involved in left-right axis patterning, localizes to basal bodies and cilia. *Mol Biol Cell* 12, 589-599 (2001).
141. **Haycraft, C.J., Swoboda, P., Taulman, P.D., Thomas, J.H. & Yoder, B.K.** The C. elegans homolog of the murine cystic kidney disease gene Tg737 functions in a ciliogenic pathway and is disrupted in osm-5 mutant worms. *Development* 128, 1493-1505 (2001).

142. **Sun, Z. *et al.*** A genetic screen in zebrafish identifies cilia genes as a principal cause of cystic kidney. *Development* 131, 4085-4093 (2004).
143. **Morgan, G.W. *et al.*** An evolutionarily conserved coiled-coil protein implicated in polycystic kidney disease is involved in basal body duplication and flagellar biogenesis in Trypanosoma brucei. *Mol Cell Biol* 25, 3774-3783 (2005).
144. **Kramer-Zucker, A.G. *et al.*** Cilia-driven fluid flow in the zebrafish pronephros, brain and Kupffer's vesicle is required for normal organogenesis. *Development* 132, 1907-1921 (2005).
145. **Fliegauf, M. *et al.*** Mislocalization of DNAH5 and DNAH9 in respiratory cells from patients with primary ciliary dyskinesia. *Am J Respir Crit Care Med* 171, 1343-1349 (2005).
146. **Caspary, T., Larkins, C.E. & Anderson, K.V.** The graded response to Sonic Hedgehog depends on cilia architecture. *Dev Cell* 12, 767-778 (2007).
147. **Pedersen, L.B. *et al.*** Chlamydomonas IFT172 is encoded by FLA11, interacts with CrEB1, and regulates IFT at the flagellar tip. *Curr Biol* 15, 262-266 (2005).
148. **Marshall, W.F.** Human cilia proteome contains homolog of zebrafish polycystic kidney disease gene qilin. *Curr Biol* 14, R913-914 (2004).
149. **Ou, G., Qin, H., Rosenbaum, J.L. & Scholey, J.M.** The PKD protein qilin undergoes intraflagellar transport. *Curr Biol* 15, R410-411 (2005).
150. **Saunier, S. *et al.*** A novel gene that encodes a protein with a putative src homology 3 domain is a candidate gene for familial juvenile nephronophthisis. *Hum Mol Genet* 6, 2317-2323 (1997).
151. **Hildebrandt, F. *et al.*** A novel gene encoding an SH3 domain protein is mutated in nephronophthisis type 1. *Nat Genet* 17, 149-153 (1997).
152. **Jauregui, A.R. & Barr, M.M.** Functional characterization of the C. elegans nephrocystins NPHP-1 and NPHP-4 and their role in cilia and male sensory behaviors. *Exp Cell Res* 305, 333-342 (2005).
153. **Winkelbauer, M.E., Schafer, J.C., Haycraft, C.J., Swoboda, P. & Yoder, B.K.** The C. elegans homologs of nephrocystin-1 and nephrocystin-4 are cilia transition zone proteins involved in chemosensory perception. *J Cell Sci* 118, 5575-5587 (2005).
154. **Mochizuki, T. *et al.*** Cloning of inv, a gene that controls left/right asymmetry and kidney development. *Nature* 395, 177-181 (1998).
155. **Olbrich, H. *et al.*** Mutations in a novel gene, NPHP3, cause adolescent nephronophthisis, tapeto-retinal degeneration and hepatic fibrosis. *Nat Genet* 34, 455-459 (2003).
156. **Mollet, G. *et al.*** The gene mutated in juvenile nephronophthisis type 4 encodes a novel protein that interacts with nephrocystin. *Nat Genet* 32, 300-305 (2002).
157. **Mollet, G. *et al.*** Characterization of the nephrocystin/nephrocystin-4 complex and subcellular localization of nephrocystin-4 to primary cilia and centrosomes. *Hum Mol Genet* 14, 645-656 (2005).
158. **Keller, L.C., Romijn, E.P., Zamora, I., Yates, J.R., 3rd & Marshall, W.F.** Proteomic analysis of isolated chlamydomonas centrioles reveals orthologs of ciliary-disease genes. *Curr Biol* 15, 1090-1098 (2005).
159. **Otto, E.A. *et al.*** Nephrocystin-5, a ciliary IQ domain protein, is mutated in Senior-Loken syndrome and interacts with RPGR and calmodulin. *Nat Genet* 37, 282-288 (2005).

160. **Sayer, J.A. *et al.*** The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4. *Nat Genet* 38, 674-681 (2006).
161. **Baala, L. *et al.*** The Meckel-Gruber syndrome gene, MKS3, is mutated in Joubert syndrome. *Am J Hum Genet* 80, 186-194 (2007).
162. **Collin, G.B. *et al.*** Mutations in ALMS1 cause obesity, type 2 diabetes and neurosensory degeneration in Alstrom syndrome. *Nat Genet* 31, 74-78 (2002).
163. **Hearn, T. *et al.*** Subcellular localization of ALMS1 supports involvement of centrosome and basal body dysfunction in the pathogenesis of obesity, insulin resistance, and type 2 diabetes. *Diabetes* 54, 1581-1587 (2005).
164. **Li, G. *et al.*** A role for Alstrom syndrome protein, alms1, in kidney ciliogenesis and cellular quiescence. *PLoS Genet* 3, e8 (2007).
165. **Ferrante, M.I. *et al.*** Identification of the gene for oral-facial-digital type I syndrome. *Am J Hum Genet* 68, 569-576 (2001).
166. **Stolc, V., Samanta, M.P., Tongprasit, W. & Marshall, W.F.** Genome-wide transcriptional analysis of flagellar regeneration in Chlamydomonas reinhardtii identifies orthologs of ciliary disease genes. *Proc Natl Acad Sci U S A* 102, 3703-3707 (2005).
167. **Romio, L. *et al.*** OFD1 is a centrosomal/basal body protein expressed during mesenchymal-epithelial transition in human nephrogenesis. *J Am Soc Nephrol* 15, 2556-2568 (2004).
168. **Ferrante, M.I. *et al.*** Oral-facial-digital type I protein is required for primary cilia formation and left-right axis specification. *Nat Genet* 38, 112-117 (2006).
169. **Mykytyn, K. *et al.*** Identification of the gene (BBS1) most commonly involved in Bardet-Biedl syndrome, a complex human obesity syndrome. *Nat Genet* 31, 435-438 (2002).
170. **Kulaga, H.M. *et al.*** Loss of BBS proteins causes anosmia in humans and defects in olfactory cilia structure and function in the mouse. *Nat Genet* 36, 994-998 (2004).
171. **Nishimura, D.Y. *et al.*** Positional cloning of a novel gene on chromosome 16q causing Bardet-Biedl syndrome (BBS2). *Hum Mol Genet* 10, 865-874 (2001).
172. **Nishimura, D.Y. *et al.*** Bbs2-null mice have neurosensory deficits, a defect in social dominance, and retinopathy associated with mislocalization of rhodopsin. *Proc Natl Acad Sci U S A* 101, 16588-16593 (2004).
173. **Fan, Y. *et al.*** Mutations in a member of the Ras superfamily of small GTP-binding proteins causes Bardet-Biedl syndrome. *Nat Genet* 36, 989-993 (2004).
174. **Mykytyn, K. *et al.*** Identification of the gene that, when mutated, causes the human obesity syndrome BBS4. *Nat Genet* 28, 188-191 (2001).
175. **Kim, J.C. *et al.*** The Bardet-Biedl protein BBS4 targets cargo to the pericentriolar region and is required for microtubule anchoring and cell cycle progression. *Nat Genet* 36, 462-470 (2004).
176. **Nachury, M.V. *et al.*** A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. *Cell* 129, 1201-1213 (2007).
177. **Li, J.B. *et al.*** Comparative genomics identifies a flagellar and basal body proteome that includes the BBS5 human disease gene. *Cell* 117, 541-552

(2004).
178. **Slavotinek, A.M. *et al.*** Mutations in MKKS cause Bardet-Biedl syndrome. *Nat Genet* 26, 15-16 (2000).
179. **Katsanis, N. *et al.*** Mutations in MKKS cause obesity, retinal dystrophy and renal malformations associated with Bardet-Biedl syndrome. *Nat Genet* 26, 67-70 (2000).
180. **Kim, J.C. *et al.*** MKKS/BBS6, a divergent chaperonin-like protein linked to the obesity disorder Bardet-Biedl syndrome, is a novel centrosomal component required for cytokinesis. *J Cell Sci* 118, 1007-1020 (2005).
181. **Badano, J.L. *et al.*** Identification of a novel Bardet-Biedl syndrome protein, BBS7, that shares structural features with BBS1 and BBS2. *Am J Hum Genet* 72, 650-658 (2003).
182. **Blacque, O.E. *et al.*** Loss of C. elegans BBS-7 and BBS-8 protein function results in cilia defects and compromised intraflagellar transport. *Genes Dev* 18, 1630-1642 (2004).
183. **Ansley, S.J. *et al.*** Basal body dysfunction is a likely cause of pleiotropic Bardet-Biedl syndrome. *Nature* 425, 628-633 (2003).
184. **Nishimura, D.Y. *et al.*** Comparative genomics and gene expression analysis identifies BBS9, a new Bardet-Biedl syndrome gene. *Am J Hum Genet* 77, 1021-1033 (2005).
185. **Vernon, E.G. *et al.*** The parathyroid hormone-responsive B1 gene is interrupted by a t(1;7)(q42;p15) breakpoint associated with Wilms' tumour. *Oncogene* 22, 1371-1380 (2003).
186. **Kyttala, M. *et al.*** MKS1, encoding a component of the flagellar apparatus basal body proteome, is mutated in Meckel syndrome. *Nat Genet* 38, 155-157 (2006).
187. **Dawe, H.R. *et al.*** The Meckel-Gruber Syndrome proteins MKS1 and meckelin interact and are required for primary cilium formation. *Hum Mol Genet* 16, 173-186 (2007).
188. **Smith, U.M. *et al.*** The transmembrane protein meckelin (MKS3) is mutated in Meckel-Gruber syndrome and the wpk rat. *Nat Genet* 38, 191-196 (2006).
189. **Delous, M. *et al.*** The ciliary gene RPGRIP1L is mutated in cerebello-oculo-renal syndrome (Joubert syndrome type B) and Meckel syndrome. *Nat Genet* 39, 875-881 (2007).
190. **Heinrich, M.C. *et al.*** Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol* 21, 4342-4349 (2003).
191. **Yu, J., Liu, X.W. & Kim, H.R.** Platelet-derived growth factor (PDGF) receptor-alpha-activated c-Jun NH2-terminal kinase-1 is critical for PDGF-induced p21WAF1/CIP1 promoter activity independent of p53. *J Biol Chem* 278, 49582-49588 (2003).
192. **Paez, J.G. *et al.*** EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304, 1497-1500 (2004).
193. **Ma, R. *et al.*** PKD2 functions as an epidermal growth factor-activated plasma membrane channel. *Mol Cell Biol* 25, 8285-8298 (2005).
194. **Taylor, M.D. *et al.*** Mutations in SUFU predispose to medulloblastoma. *Nat Genet* 31, 306-310 (2002).
195. **Stone, D.M. *et al.*** The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* 384, 129-134 (1996).

196. **Xie, J. *et al.*** Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* 391, 90-92 (1998).
197. **Grachtchouk, M. *et al.*** Basal cell carcinomas in mice overexpressing Gli2 in skin. *Nat Genet* 24, 216-217 (2000).
198. **Roepman, R. *et al.*** The retinitis pigmentosa GTPase regulator (RPGR) interacts with novel transport-like proteins in the outer segments of rod photoreceptors. *Hum Mol Genet* 9, 2095-2105 (2000).
199. **Mavlyutov, T.A., Zhao, H. & Ferreira, P.A.** Species-specific subcellular localization of RPGR and RPGRIP isoforms: implications for the phenotypic variability of congenital retinopathies among species. *Hum Mol Genet* 11, 1899-1907 (2002).
200. **Shu, X. *et al.*** RPGR ORF15 isoform co-localizes with RPGRIP1 at centrioles and basal bodies and interacts with nucleophosmin. *Hum Mol Genet* 14, 1183-1197 (2005).

Chapter 4

Allele-specific regulation of primary cilia function by the von Hippel-Lindau tumor suppressor

Martijn P. Lolkema*, Dorus A. Mans*, Laurien H. Ulfman,
Stefano Volpi, Emile E. Voest§ and Rachel H. Giles§
*,§ these authors contributed equally

European Journal of Human Genetics 2008 Jan; 16(1):73-8

Abstract

Patients with von Hippel-Lindau (VHL) disease often develop *VHL*$^{-/-}$ kidney cysts, which possibly progress into clear-cell renal carcinomas (ccRCC). Recent data link the *VHL* gene product to formation of the primary cilium, an organelle that extends apically into the renal lumen. Exactly how pVHL induces ciliogenesis or function is unknown. Here we demonstrate that ciliary assembly and mechanotransduction is rapidly restored in *VHL*$^{-/-}$ ccRCC cells upon ectopic reconstitution of wild-type -but not variant alleles of- *VHL*. These data support and expand upon recent studies implicating a role for pVHL in the initiation of ciliogenesis. Furthermore, reduction of cellular levels of pVHL in this cell system was associated with fewer ciliated cells, suggesting a role for pVHL in ciliary maintenance.

Introduction

Heterozygous mutations in the *von Hippel-Lindau* gene (*VHL*, OMIM# 608537) predispose patients to a variety of tumors and cysts [1, 2]. Similarly, kidney-specific inactivation of *VHL* in conditional knock-out mice results in cyst development [3]. The role of *VHL* as a global gatekeeper of cellular growth in the kidney is further supported by the occurrence of biallelic somatic *VHL* mutations in 70% of conventional kidney cancer [2, 4].

Many lines of research have linked the *VHL* gene product to regulation of the Hypoxia Inducible Factor (HIF) [5]. We and others have also described new functions for pVHL in microtubule dynamics and regulation of the primary cilium [6-10]. Here, we provide the first evidence that cilia function in renal carcinoma cells is dependent on two domains in pVHL: residues 1-53, constituting an acidic domain, and residues 95-123, previously implicated in microtubule binding and tumor suppression [11, 12].

Materials and Methods

Cell culture

Primary mouse kidney cells were isolated from 4 day-old mice and cultured in DMEM supplemented with antibiotics and 20% fetal calf serum; all other cell lines were cultured in 5% fetal calf serum. KC12TR and KC12TR/VHL-TO cell lines were generated and induced with 1 μg/ml doxycycline (Sigma-Aldrich, St. Louis, MO) as previously described [10]. Transfections were performed using Fugene-6 (Boehringer Mannheim, Ingelheim, Germany) or by electroporation (KC12 cells: 270V, 1.0 mF).

Cilia detection

Cells were cultured to confluency, then received serum-free medium for an additional 3 days. Immunofluorescence was performed with: α-VHL (Ig32, 1:500; BD-Pharmingen, San Diego, CA), α-acetylated tubulin (1:20 000, Sigma-Aldrich, St.Louis, MO), α-γ-tubulin (1: 500, Sigma-Aldrich, St.Louis, MO), or α-Vsv (1:400, Abcam, Cambridge, UK). Secondary antibody was goat-anti-mouse Alexa568, goat anti-rabbit Alexa633 (1:400, Molecular probes, Eugene, OR, USA) or goat anti-rabbit 488, (1:400, DAKO, Glostrup, Denmark). Secondary antibody controls revealed no aspecific staining. Where indicated, VHL constructs were cloned into pEGFP-C1/2 (Clontech, Palo Alto, CA) and transfected into KC12 cells (*VHL*$^{-/-}$) before being seeded onto coverslips. One thousand interphase nuclei (as determined by DAPI)

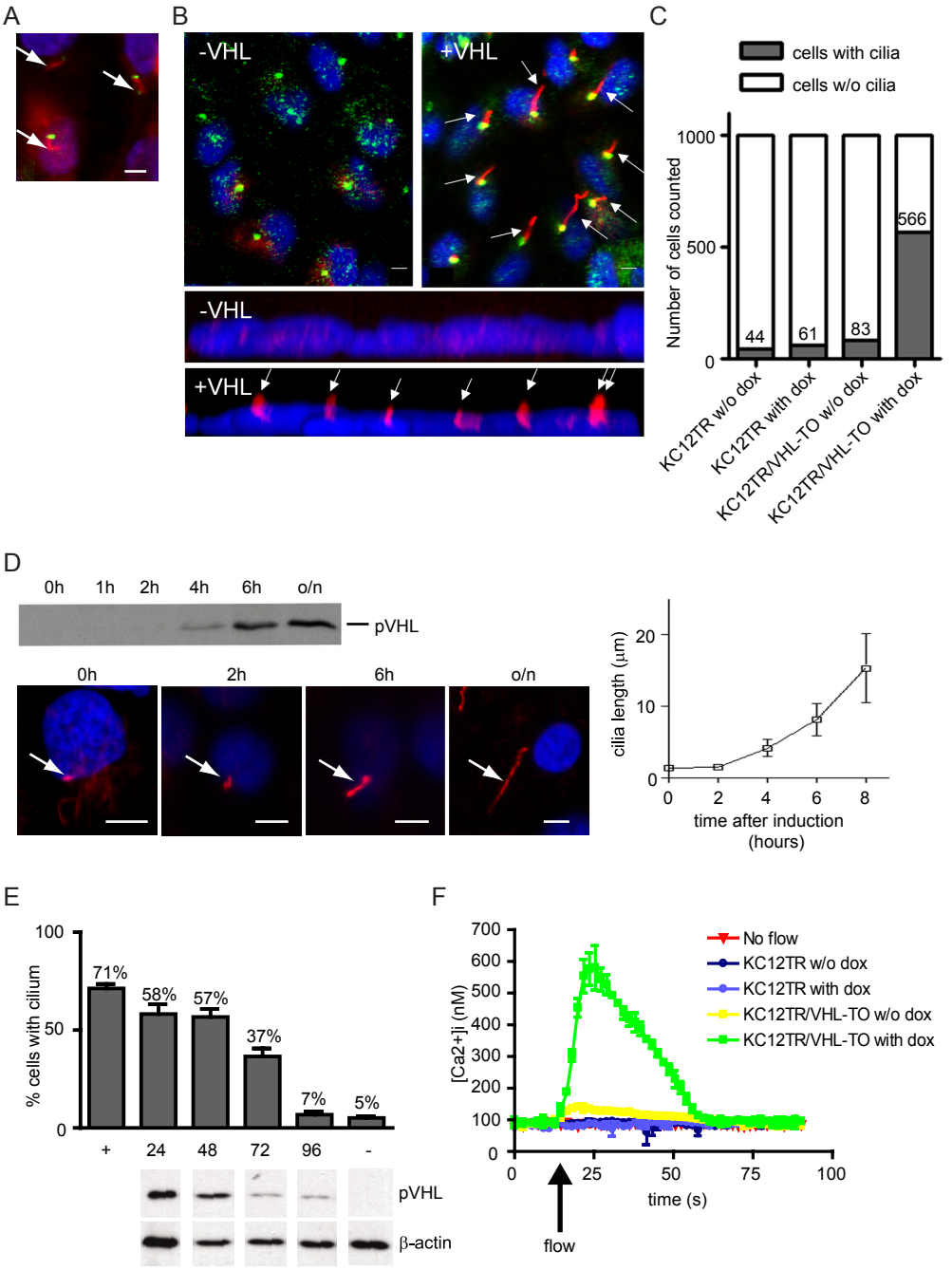


Figure 1: pVHL regulates renal cilia maintenance and function. (A): Endogenous pVHL localizes to the primary cilium of primary mouse kidney cells. Immunofluorescent γ-tubulin staining (green) indicates the position of the basal body at the base of the cilium. pVHL (red) is seen to be diffusely cytoplasmic with brighter staining along hair-like structures (arrows) extending from the basal bodies. Size bar, 5 μm. The nucleus is stained with DAPI (blue). (B): Re-expression of pVHL in KC12 renal cell carcinoma cells derived from a VHL patient restores cilia. KC12TR/VHL-TO cells were untreated (-VHL) or induced to express pVHL with doxycycline (+VHL), then immunostained for acetylated tubulin (red) and for γ-tubulin (green, upper panels only). Nuclei are DAPI stained (blue). Lower panels depict a z-axis 3D projection generated

from a confocal z-stack of a representative cell field at 63x. Arrows indicate cilia; size bar indicates 5 μm. (C): Frequency of cilia in 1000 KC12TR cells and KC12TR/VHL-TO cells untreated (w/o dox) or treated with doxycycline for 24 hrs. Y-axis shows absolute number of ciliated cells. (D): Dynamics of pVHL-induced ciliogenesis. KC12TR/VHL-TO cells were grown to confluency and pVHL expression was induced with doxycycline. The upper panel shows a western blot analysis of whole cell lysates analyzed for pVHL protein expression after 0, 2, 4, 6 hours, and overnight (o/n) induction of pVHL expression by doxycycline. The lower four panels show representative figures of cilia length at each time point. Arrows indicate cilia stained for acetylated tubulin (red); size bar, 5 μm. (E): pVHL regulates cilia maintenance. Withdrawal of doxycycline from ciliated KC12TR/VHL-TO cells for 0 (+), 24, 48, 72, 96 hours and uninduced control (-) were scored for ciliated cell frequency (>500 cells scored), doxycycline removal. Error bars represent standard deviation. Below each time point are western blots of pVHL and β-actin protein levels. (F): Re-expression of pVHL in $VHL^{-/-}$ cells restores ciliary mechanotransduction. KC12TR cells and KC12TR/VHL-TO cells were treated with or without (w/o) doxycycline (dox) and used to measure intracellular calcium ($[Ca^{2+}]_i$) concentrations in response to flow (turned on at arrow). Data shown are experiments performed in triplicate or quadruplicate from a single representative day; however, all elements of these data were repeated on multiple days with similar results. Error bars represent standard deviations.

were scored for the presence of cilia without knowledge of the sample identity. Z-stack images of every 20th cell validated the presence of cilia. Experiments were performed on at least two different days and the data combined. Staining was visualized on a Zeiss LSM510 confocal imaging unit (Jena, Germany). Cilia length was measured with the ruler function of LSM Image Browser (Zeiss).

Western blots

Standard western blots [13] were immunostained with either α-VHL (1:500, Ig32, BD-Pharmingen, San Diego, CA), or α-β-actin (1:10 000; Sigma-Aldrich, St.Louis, MO), followed by rabbit α-mouse HRP (1:20 000; Pierce).

Ca^{2+} microfluorimetry

Cells were grown to full confluence and then cultured an additional three days in serum-free medium on uncoated 24x60 mm glass coverslips. KC12TR/VHL-TO cells were treated with doxycycline for 24 hours prior to some experiments. Where indicated, four million KC12 cells were transfected by electroporation with 10 μg appropriate plasmid and 1 μg pBABE-puro before seeding. 24 hrs after transfection, cells were selected on puromycin. The experimental setup for flow has previously been described in detail [14]. Briefly, we incubated cells for 30 min. with the Ca^{2+} sensitive probe Fura-2/AM (5 μM) at 37ºC. We then washed cells three times to remove excess Fura-2/AM and placed them in a perfusion chamber in 20 mM HEPES buffer (pH 7.4) containing 132 mM NaCl, 4.2 mM $NaHCO_3$, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, and supplemented with 5 mM glucose, 1 mM $CaCl_2$, and 0.5% Human Serum Albumin. We captured images every 4 seconds at excitation wavelengths of 340 nm and 380 nm and detected the signal emission wavelengths at 512 nm. After equilibration of the cells in media for at least 5 min., we applied a fluid shear stress of 0.75 dyne/cm^2 to the cells. Freshly diluted thrombin (1 μM) was used to validate the potential for $[Ca^{2+}]_i$ response after each flow experiment was concluded.

Results

pVHL-mediated cilia function

We performed immunofluorescence and confocal microscopy to confirm endogenous pVHL localization to ciliary structures in primary cells derived from

murine kidney tissue. In addition to diffuse cytoplasmic staining, pVHL clearly localized to cilia extending form basal bodies stained with γ-tubulin (Figure 1A). To confirm the effect of pVHL on ciliogenesis in our preferred cell system, we used a stable cell line derived from a VHL patient engineered to re-express empty vector (KC12TR) or VHL (KC12TR/VHL-TO) upon induction by doxycycline [10] and observed rapid assembly of cilia (Figures 1B-C). Because the kinetics of pVHL-dependent cilia growth are unknown, ciliary assembly/extension in induced KC12TR/VHL-TO cells were measured over time. We observed an 80-minute doubling time (Figure 1D), consistent with published growth rates [15]. We next asked what the effect might be of doxycycline withdrawal on ciliated KC12TR/VHL-TO. Cellular levels of pVHL dropped significantly by 72 hours after removal of doxycycline, which was followed by a drop in ciliated cell frequency (Figure 1E).

When cultured kidney cells are subjected to flow, they rapidly demonstrate a cilia-related calcium increase [16]. To explore whether the mechanotransduction events triggered by flow are sensitive to levels of pVHL, confluent cultures of KC12TR/VHL-TO cells treated with or without doxycycline for 30 hours were incubated with fluorescent Fura-2/AM and placed in a perfusion chamber under flow as previously described [14]. We observed that only KC12 cells re-expressing pVHL were capable of responding to flow by mounting a rapid increase in intracellular Ca^{2+} concentrations (max 539-612 nM) as compared to the same cells stably transfected with empty plasmids (KC12TR) and induced with doxycycline (max. 78-90 nM) or uninduced cells (max. 123-133 nM; 56 cells) (Figure 1F), demonstrating significant changes in the area under the curve (AUC, p=0.004). These data are the first to demonstrate that pVHL-induced cilia function normally in the mechanotransduction characteristic of primary cilia [17].

Allelic variants of *VHL* do not localize to the renal cilium

No one has yet determined whether *VHL* alleles unable to initiate ciliogenesis are capable of ciliary localization. To this end, we transfected GFP-tagged pVHL variants into HEK293T cells and then immunostained for cilia with anti-acetylated tubulin. Like endogenous pVHL, GFP-pVHL30 clearly localized to cilia. By contrast, GFP-pVHL19, a naturally occurring *VHL* isoform lacking the acidic domain, was not observed to be present in the cilium. Similarly, *VHL* variants such as VHL disease type 2A GFP-pVHL-Y112H, and GFP-pVHL-Δ95-123, both of which affect the microtubule-binding domain showed diffuse cytoplasmic staining and no particular overlap with anti-acetylated tubulin (Figure 2).

Natural and patient-associated alleles fail to form functional cilia

Whether these *VHL* allelic variants could reconstitute cilia in *VHL*-deficient KC12 cells was addressed by transfecting GFP-pVHL30, GFP-pVHL19, GFP-pVHL-Y112H or GFP-pVHL-Δ95-123 into KC12 cells (Figure 3A). Most of the KC12 cells transfected with GFP-pVHL30 produced cilia; however, GFP-pVHL19, GFP-pVHL-Δ95-123, and GFP-pVHL-Y112H were unable to recapitulate the efficacy of full-length pVHL (Figure 3A). To analyze whether *VHL* variants could stimulate cellular calcium influx characteristic of cilia bending, we reconstituted KC12 cells with pBABE-puro and Vsv-tagged *VHL* allelic variants before measuring the intracellular calcium response of these cells to fluid flow. Vsv-pVHL30 recapitulated 85% of the response observed in Figure 1F (41 cells). Measuring the AUCs, we observed that the level of response was significantly decreased in cells expressing Vsv-pVHL19 (p=0.004, 58 cells), Vsv-

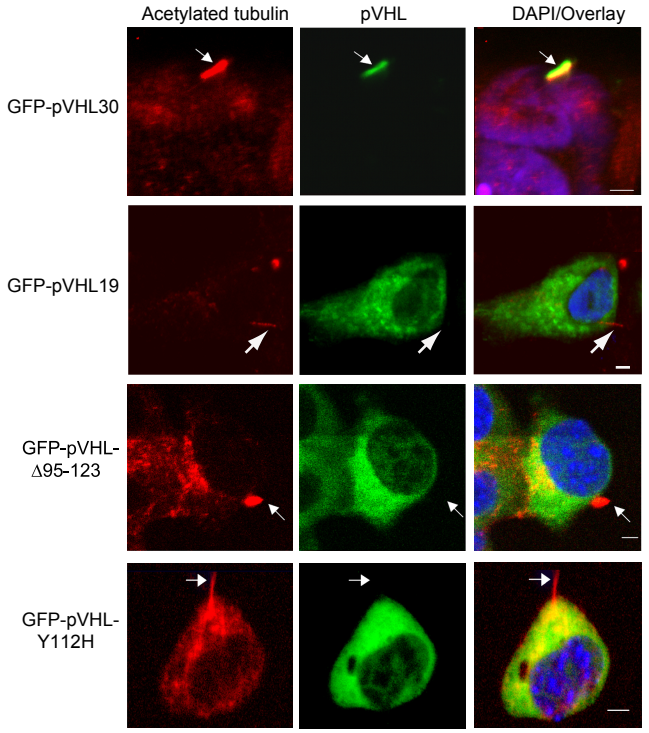


Figure 2: Variant alleles of *VHL* do not localize to the cilium (arrows). Confocal image projections of ciliated kidney cells expressing GFP-pVHL30 (green), GFP-pVHL19, GFP-pVHL-Δ95-123 and GFP-pVHL-Y112H, and counterstained for acetylated tubulin (red). Nuclei are stained with DAPI (blue). Cilia are indicated by arrows. Size bars in the overlay/DAPI column indicate 5 μm.

pVHL-Δ95-123 ($p<0.001$, 77 cells) or Vsv-pVHL-Y112H ($p<0.001$, 69 cells). After the experiment, we confirmed >90% transfection efficiency by α-Vsv immunostaining. Similarly, α-acetylated tubulin/γ-tubulin staining confirmed that the cilia frequency correlated with Figure 3A. We therefore conclude that the reduction of calcium influx in pVHL variants is probably a result of reduced cilia frequency.

Discussion

Our data support and expand upon recent findings establishing a role for pVHL in cilia regulation [6-9]. However, discrepancies between the five studies, including ours, are evident. For example, Esteban *et al*. claim HIF responsible for the development of cilia [6], whereas we and others fail to find this connection [7-9]. The KC12 cells we use here are devoid of HIF1/2α [10] and subsequently do not show upregulated mRNA levels of HIF targets VEGF and GLUT1 in RT-PCR experiments (unpublished data), arguing that cilia regulation is indeed a HIF-independent function of pVHL. Furthermore our data support the notion put forward by the Krek group [8] that regulating primary cilia requires residues 95-123, previously implicated in microtubule stability [12]. The Burk and Benzing labs do not observe this connection in their cell systems [7, 9]. In different cells, Lutz and Burk report cilia regulation by the murine short isoform of pVHL, *vhlh-p18* [7]; however, we observe no human pVHL19 localization in the cilium, and significantly reduced functioning of this organelle in our calcium-flow experiments. Thus, our data suggest that cilia function in renal carcinoma cells is dependent on two domains in pVHL, residues 1-53, known as the acidic domain, and residues 95-123, previously implicated in microtubule binding and tumor suppression.

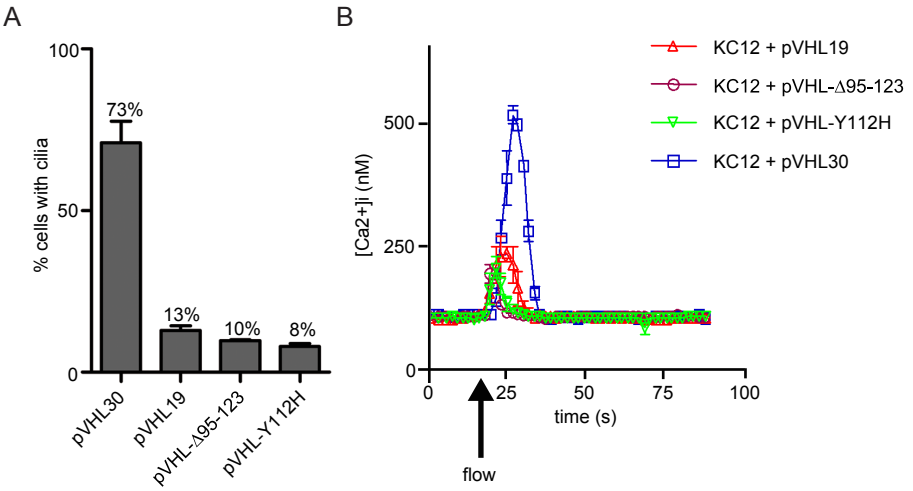


Figure 3: *VHL* mutant alleles fail to reconstitute functional cilia. (A): One thousand interphase nuclei were scored in three experiments to determine the frequency of cilia in GFP-expressing cells. Variant alleles of *VHL* all show a significantly decreased cilia frequency compared to GFP-pVHL30: GFP-pVHL19 (p=0.007), GFP-pVHL-Y112H (p=0.006), and GFP-pVHL-Δ95-123 (p=0.005). (B): Variant alleles of *VHL* reduce calcium influx in response to flow. Intracellular calcium concentrations ($[Ca^{2+}]_i$) were determined by influx assays on KC12 cells reconstituted with Vsv-pVHL30, Vsv-pVHL19, Vsv-pVHL-Δ95-123, or Vsv-pVHL-Y112H variants. Experiments were performed in quadruplicate on at least two independent days. Error bars represent standard deviation.

References

1. **Latif, F. *et al.*** Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 260, 1317-1320 (1993).
2. **Lonser, R.R. *et al.*** von Hippel-Lindau disease. *Lancet* 361, 2059-2067 (2003).
3. **Rankin, E.B., Tomaszewski, J.E. & Haase, V.H.** Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. *Cancer Res* 66, 2576-2583 (2006).
4. **Kim, W.Y. & Kaelin, W.G.** Role of VHL gene mutation in human cancer. *J Clin Oncol* 22, 4991-5004 (2004).
5. **Pugh, C.W. & Ratcliffe, P.J.** The von Hippel-Lindau tumor suppressor, hypoxia-inducible factor-1 (HIF-1) degradation, and cancer pathogenesis. *Semin Cancer Biol* 13, 83-89 (2003).
6. **Esteban, M.A., Harten, S.K., Tran, M.G. & Maxwell, P.H.** Formation of primary cilia in the renal epithelium is regulated by the von Hippel-Lindau tumor suppressor protein. *J Am Soc Nephrol* 17, 1801-1806 (2006).
7. **Lutz, M.S. & Burk, R.D.** Primary cilium formation requires von hippel-lindau gene function in renal-derived cells. *Cancer Res* 66, 6903-6907 (2006).
8. **Thoma, C.R. et al.** pVHL and GSK3beta are components of a primary cilium-maintenance signalling network. *Nat Cell Biol* 9, 588-595 (2007).
9. **Schermer, B. *et al.*** The von Hippel-Lindau tumor suppressor protein controls ciliogenesis by orienting microtubule growth. *J Cell Biol* 175, 547-554 (2006).
10. **Lolkema, M.P. *et al.*** The von Hippel-Lindau tumor suppressor protein

influences microtubule dynamics at the cell periphery. *Exp Cell Res* 301, 139-146 (2004).

11. **Datta, K., Sundberg, C., Karumanchi, S.A. & 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* 61, 1768-1775 (2001).
12. **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* 5, 64-70 (2003).
13. **Lolkema, M.P. *et al.*** Tumor suppression by the von Hippel-Lindau protein requires phosphorylation of the acidic domain. *J Biol Chem* 280, 22205-22211 (2005).
14. **Ulfman, L.H. *et al.*** IL-8 induces a transient arrest of rolling eosinophils on human endothelial cells. *J Immunol* 166, 588-595 (2001).
15. **Brown, J.M., Marsala, C., Kosoy, R. & Gaertig, J.** Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in Tetrahymena. *Mol Biol Cell* 10, 3081-3096 (1999).
16. **Praetorius, H.A. & Spring, K.R.** Bending the MDCK cell primary cilium increases intracellular calcium. *J Membr Biol* 184, 71-79 (2001).
17. **Nauli, S.M. *et al.*** Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet* 33, 129-137 (2003).

Chapter 5

The von Hippel-Lindau tumor suppressor interacts with microtubules through kinesin-2

Martijn P. Lolkema[#], Dorus A. Mans[#], Cristel M. Snijckers,
Mascha van Noort, Moniek van Beest, Emile E. Voest[*] and Rachel H. Giles[*]
[#],[*] these authors contributed equally

FEBS Letters 2007 Oct 2; 581(24): 4571-6

Abstract

Synthesis and maintenance of primary cilia are regulated by the von Hippel-Lindau (VHL) tumor suppressor protein. Recent studies indicate that this regulation is linked to microtubule-dependent functions of pVHL such as orienting microtubule growth and increasing plus-end microtubule stability, however little is known how this occurs. We have identified the kinesin-2 motor complex, known to regulate cilia, as a novel and endogenous pVHL binding partner. The interaction with kinesin-2 facilitates pVHL-binding to microtubules. These data suggest that microtubule-dependent functions of pVHL are influenced by kinesin-2.

Introduction

The rare hereditary VHL disease is caused by heterozygous germline mutations in the *VHL* gene [1], which predisposes patients to a variety of cysts and tumors. Multifocal renal cysts exhibit loss of *VHL* heterozygosity [2]; moreover, kidney-specific inactivation of *vhl* in mice results in the development of kidney cysts [3]. Both isoforms, pVHL30 and pVHL19, function as an ubiquitin E3 ligase, targeting hydroxylated HIFα for poly-ubiquitination and subsequent proteasomal degradation [1,4,5]. Hypoxia interferes with HIFα-hydroxylation, thereby enabling stabilized HIFα to transactivate genes necessary for adjusting to a hypoxic environment.

Recent studies support a function for pVHL in the primary cilium [6,7]. The axoneme core of cilia serves as tubulin scaffolding for the kinesin-2 complex, which transports all cargo into the cilium [8]. Accordingly, genetic disruption of any of the individual components of the kinesin-2 complex (KIF3A, KIF3B, and KAP3) invariably results in cilia dysfunction and renal cyst development [9]. Here, we report the identification of kinesin-2 as novel and endogenous pVHL binding partner, mediating binding to microtubules. Since pVHL and kinesin-2 have overlapping functions regarding primary cilia regulation, these data may provide novel mechanistic insight into microtubule (MT)-dependent regulation of primary cilia by pVHL.

Materials and Methods

Construction of plasmids

All expression constructs were generated using pcDNA3-derived vectors (Promega, USA) containing either N-terminal Myc- or Vsv-tag by standard cloning techniques. GFP-fusion constructs were generated by cloning into pEGFPc1/2 (Clontech, USA). KIF3A (gift from Dr. L. Goldstein), KIF3B, ΔN-KAP3 (gift from Dr. T. Akiyama), VHL-Δ95-123 and VHL-Y112H (gift from Dr. W. Krek) constructs were cloned into the appropriate vectors. pBABE-puro was a gift from Dr. G. Nolan.

Yeast-two-hybrid screen

A human fetal brain cDNA library (Matchmaker, Clontech, USA) was co-transfected with VHL-pMD4 into *Saccharomyces cerevisiae* strain Hf7c. Plasmids were recovered from His+/LacZ+ clones and tested for specificity with irrelevant baits and 2.5 mM or 25 mM of 3-aminotriazole (3AT).

Cell culture

HEK293T, MDCK, murine neuroblastoma N1E-115 cells, and murine C26

colorectal carcinoma cells were cultured in DMEM supplemented with antibiotics and 5-10% fetal calf serum. Primary kidney cells were isolated from 4 day-old mice by sequential trypsinisation steps and cultured in DMEM supplemented with antibiotics and 20% fetal calf serum to confluency. Cells cultured to produce cilia, then received serum-free medium for an additional 3 days after reaching confluency to stimulate cilia outgrowth. Transfections were performed using Fugene-6 (Boehringer Mannheim, Germany). MG132 (5 μM; Sigma-Aldrich, USA) was used for 8 hours to inhibit proteasomal degradation. Desferoxamine (100 μM; Sigma-Aldrich, USA) was used for 4 hours to stabilize HIFα.

Retroviral transductions

After 24 hours transfection with the indicated pBABE plasmids, Phoenix ecotropic packaging cells (gift from Dr. G. Nolan) were incubated at 32ºC for 24 hours before harvesting culture supernatants. N1E-115 or C26 cells were transduced with sterile-filtered culture supernatants 24-36 hours at 32ºC with 8 μg/ml polybrene. Fresh medium supplemented with puromycin (1 μg/ml; Sigma-Aldrich) replaced the supernatants for an additional 24 hours at 37ºC.

Immunoprecipitations

Approximately $1*10^6$ cells were lysed in 400 µl buffer (20 mM TRIS, 1% Triton-X-100, 140 mM NaCl, 10% glycerol, pH 8.0) with protease inhibitors (Roche, Switzerland), then centrifuged 10 min. at 13000 rpm. Mouse monoclonal α-VHL (1.5 μg Ig32; BD-Pharmingen, USA) coupled to protein A/G agarose beads (7.5 μl; Santa Cruz Biotechnology, USA), was added to 170 μl cleared lysate. For endogenous IPs, $1*10^7$ cells were lysed in 400 μl buffer of which 100 μl fresh cleared lysate was added to 25 μl protein A/G agarose beads pre-coupled to 7.5 μg mouse monoclonal α-VHL, 7.5 μg mouse monoclonal α-KIF3A (BD-Transduction Labs, USA), or 7.5 μg mouse monoclonal α-RPTPμ (clone 3D7) for specificity control. IPs were incubated 4 hours at RT, washed three times and analyzed by western blot. IVTT samples were generated according to manufacturer's protocol (Promega, USA).

Immunofluorescence staining

Fixed cells were incubated with α-KIF3A (1:500; BD-Transduction Labs), α-acetylated tubulin (1:20000; Sigma-Aldrich) or α-VHL (1:500; Ig32, BD-Pharmingen). Goat-anti-mouse Alexa568 (1:400, Molecular probes, USA) was used as secondary antibody, as previously described [10].

Western blotting

Standard western blots [11] were incubated with α-Myc (9E10, hybridoma supernatant 1:5), α-Vsv (P4D5, hybridoma supernatant 1:5), α-VHL (1:500, Ig32, BD-Pharmingen), α-KIF3A, α-KIF3B, α-KAP3 (1:500; BD-Transduction Labs) or α-MAPK (1:500, gift from Dr. O. Kranenburg).

Microtubule co-sedimentation assays

Transfected HEK293T cells were lysed in PTN buffer (10 mM Pipes, 30 mM TRIS, 50 mM NaCl, 1 mM EGTA, 1.25 mM EDTA, 1 mM DTT, 1% Triton-X-100, protease inhibitors, pH 6.3). IVTT samples were generated according to manufacturer's protocol (Promega). Lysates were pre-cleared by centrifugation at 100000 x *g* for 45 min. Co-sedimentation assays were performed using the MAP Spin-down Biochem

Kit (Cytoskeleton, USA) with the following adjustments: polymerized microtubules were centrifuged at 100000 x *g* for 30 min. before being resuspended in pre-cleared lysates containing 50 μM paclitaxel and AMP-PNP (Sigma-Aldrich; final concentration 10 mM) where indicated, and incubated for 30 min. Samples were loaded onto 100 μl of the kit's cushion buffer and centrifuged at 100000 x *g* for 40 min. Pre-cleared lysates, supernatant and pellet fractions were analyzed by western blot.

Reporter assays

HIF reporter assays were performed in duplicate with 0, 100, or 300 ng Myc-ΔN-KAP3 and/or 100 ng wild-type HIF1α as previously described [11].

Results

pVHL interacts with kinesin-2

We performed a yeast-two-hybrid screen of a human fetal brain cDNA library with full-length pVHL as bait. Eight clones bound pVHL robustly in this assay, including a known pVHL interactor, chaperonin CCTη [12]. Interestingly, one of the remaining clones has a strong link to cilia regulation: KIF3A, component of the kinesin-2 microtubule motor complex. We re-transformed yeast with KIF3A and four different pVHL bait constructs and determined that the first 136 residues of pVHL largely determine this interaction (Figure 1A). Co-immunoprecipitation (IP) assays using *in vitro* translated (IVTT) pVHL and KIF3A, validated the direct nature of this interaction (Figure 1B). Suggesting physiological relevance, immunoprecipitating endogenous pVHL readily pulled down KIF3A, KIF3B and KAP3 in ciliated primary mouse kidney cells (Figure 1C); likewise, α-KIF3A pulled down pVHL, while an irrelevant antibody (α-RPTPμ) did not retrieve either pVHL or KIF3A (Figure 1C). Confocal microscopy showed endogenous pVHL or GFP-pVHL30 translocation to multiple cilia in kidney cells partially overlapping with KIF3A localization (Figures 1D-E). Because pVHL functions as an E3 ubiquitin ligase, we blocked proteasomal degradation with MG132 and observed no increased cellular levels or poly-ubiquitination of KIF3A (Figure 1F). Taken together, these data suggest endogenous interaction between pVHL and kinesin-2 in primary kidney cells, not affecting KIF3A turnover.

Mutant *VHL* alleles do not bind kinesin-2

We characterized the binding of pVHL to kinesin-2 by testing pVHL30, pVHL19, pVHL-Δ95-123, implicated in defective regulation of MT stability [13] and pVHL-Y112H, a patient missense mutation. Retroviral reconstitution of these *VHL* allelic variants in *VHL*-deficient C26 cells was confirmed by α-VHL IP and analyzed for binding to endogenous KIF3A and KAP3 (Figure 2A). While transduced pVHL30 precipitated endogenous KIF3A and KAP3, pVHL19 was less effective at co-precipitating KIF3A and failed to co-precipitate KAP3. The opposite was true for the deletion variant pVHL-Δ95-123 and the Y112H patient mutation, which failed to co-precipitate KIF3A, but recovered some KAP3. These data indicate that the N-terminal acidic domain mediates binding of pVHL30 to KAP3 and that the MT-binding domain is required for KIF3A binding to pVHL (Figure 2B).

pVHL-KIF3A binding does not affect HIFα

To experiment with the pVHL-KIF3A interaction, we checked whether pVHL could bind a dominant-negative variant of the non-motor subunit KAP3 (missing

residues 1-184; ΔN-KAP3), which disrupts binding between tumor suppressor APC and kinesin-2 [14]. Indeed, exogenously expressed Vsv-pVHL30 could co-precipitate GFP-tagged ΔN-KAP3 (Figure 3A). To validate an effect of ΔN-KAP3 on pVHL30-KIF3A binding, α-VHL IPs were performed with N1E-115 cells either mock or ΔN-KAP3-transduced, and loss of endogenous binding between pVHL30 and KIF3A was observed (Figure 3B). ΔN-KAP3 however, does not affect HIF1α stabilization in response to desferoxamine (Figure 3C); nor are *HIF* responsive reporter assays affected by increasing amounts of ΔN-KAP3 (Figure 3D). Therefore, we concluded that the interaction between pVHL and KIF3A does not affect HIF1α regulation or function.

KIF3A increases pVHL binding to microtubules

To address the possibility that kinesin-2 mediates the previously reported binding of pVHL30 to MTs [13], we performed MT co-sedimentation assays with lysates of HEK293T cells transfected with Vsv-pVHL30 (Figure 4A). To determine whether this association of pVHL with MTs is direct, we repeated this assay using highly purified tubulin and *in vitro* translated full length pVHL; yet pVHL30 never bound MTs in this assay. These data suggest that endogenous cellular factors, e.g. kinesin-2, enhance binding of pVHL to MTs. Two groups have previously performed microtubule cosedimentation assays with VHL. Most recently, Schermer *et al.* [15] and Thoma *et al.* [7] show that cell lysates overexpressing pVHL copelleted with polymerized tubulin; however, endogenous factors such as kinesin-2 are also present in these cell lysates. Schermer *et al.* [15] also show that pVHL IPs tubulin, again in a lysate setting. The discrepancy between our data and Hergovich's published data using IVTT pVHL30 [13] might be explained by different protocols used in the two studies; we used a sucrose cushion in the co-sedimentation assay. This sucrose cushion serves as a density gradient separating the lysate from the pellet and increasing the specificity of the spun down fraction. To address the different outcomes, we directly compared the two methods with *in vitro* translated pVHL30 in the presence or absence of a sucrose cushion separation. In the absence of the sucrose cushion we found more pVHL in the pellet with microtubules than in the pellet without microtubules (Figure 4A). However, using the modified assay (with sucrose cushion) we found no direct interaction between microtubules and pVHL (Figure 4A). Because MT motor proteins such as KIF3A/3B require nucleotides for MT-binding, we repeated these assays in the presence of AMP-PNP, a non-hydrolyzable form of ATP. Treatment with AMP-PNP shifts more pVHL into the pellet fraction (Figure 4B) consistent with the involvement of a motor protein in MT-binding by pVHL.

To study the effect of KIF3A on MT-binding of pVHL, we performed MT co-sedimentation assays using lysates of HEK293T cells transfected with Vsv-pVHL30 and Myc-KIF3A or empty vector. Myc-KIF3A enhanced binding of Vsv-pVHL30 to MTs (Figure 4C). Interestingly, co-transfecting Vsv-pVHL30 with Myc-ΔN-KAP3 greatly reduced pVHL30 association with MTs (Figure 4D).

Discussion

Here we describe KIF3A as a novel pVHL binding partner and confirm direct binding between these proteins. Endogenous binding of pVHL to KIF3A and other subunits of the anterograde kinesin-2 microtubule motor is also observed, which can be compromised either by pVHL mutations or by overexpression of a dominant-negative

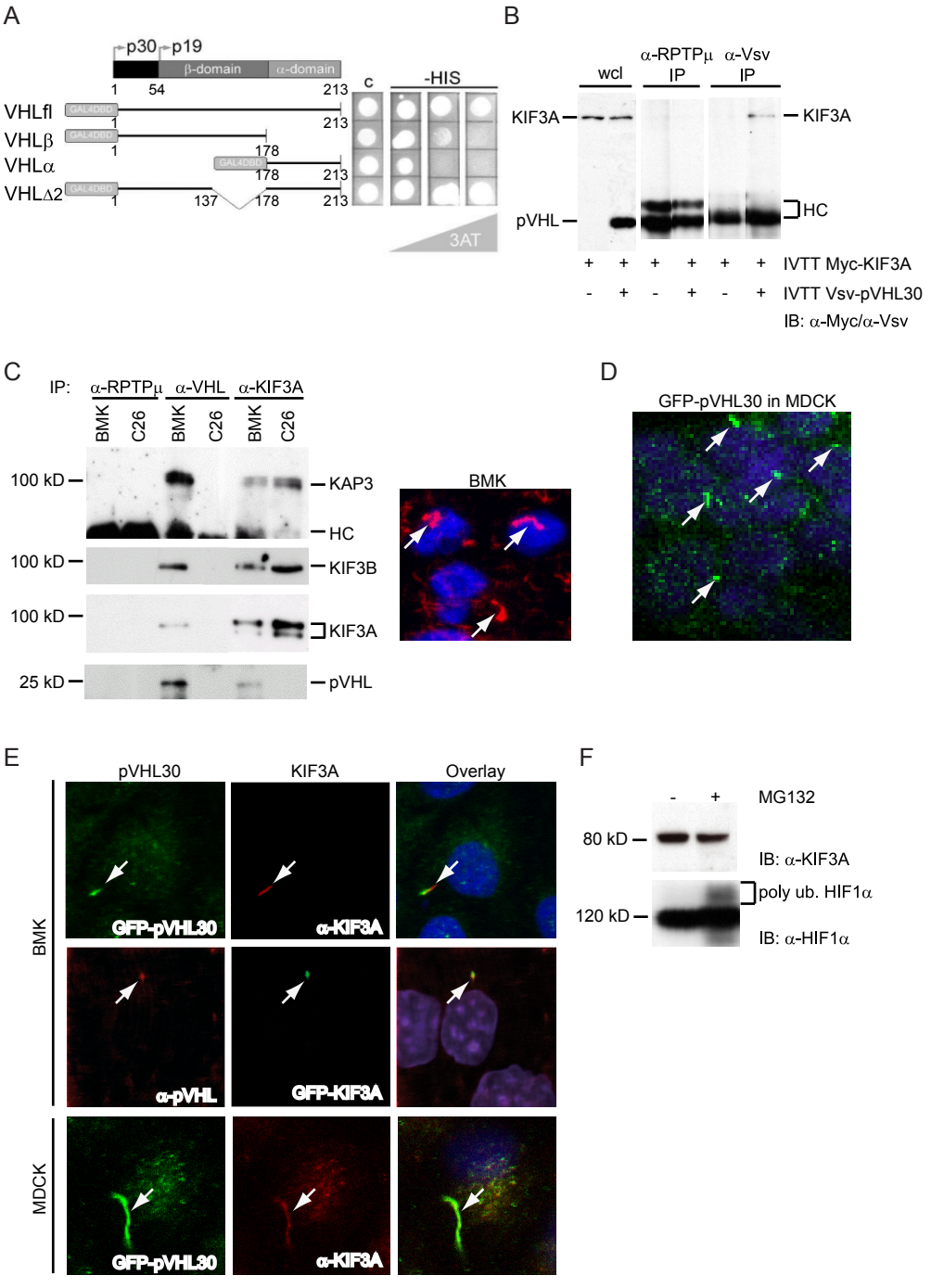


Figure 1: pVHL interacts with kinesin-2. (A): Yeast-two-hybrid assay with Gal4 DNA binding domain fused to: full-length pVHL (VHL_{fl}), the acidic and β-domain of pVHL (VHL_{β}), the α-domain of pVHL (VHL_{α}) or pVHL lacking exon 2 ($VHL_{\Delta 2}$) used as bait and KIF3A used as prey. Right panel: colony growth of transformed yeast, using increasing 3-aminotriazole (3AT; 0, 12.5 and 25 mM) without histidine (-HIS). Control colony (c) in the presence of histidine. (B): Western blot analysis of IVTT Myc-KIF3A binding to IVTT Vsv-pVHL30. α-RPTPμ serves as unrelated antibody control. wcl, whole cell lysates; HC, immunoglobin heavy chain. IB, immunoblot. (C): Western blot analysis of endogenous KAP3, KIF3B, KIF3A and pVHL recovery after

α-RPTPμ, α-VHL and α-KIF3A IPs in *VHL*-deficient C26 cells and ciliated baby mouse kidney (BMK). Right panel, BMK cells stained for acetylated tubulin (red), demonstrate cilia (arrows) when used for the IP. DAPI (blue) stains nucleus. (D): Translocalization of GFP-pVHL30 to multiple cilia in MDCK cells. (E): pVHL30 co-localizes with a subset of ciliary KIF3A in kidney cells. Confocal images of BMK and MDCK cells transfected with GFP-pVHL30 co-localizing with endogenous KIF3A and conversely, GFP-KIF3A co-localizes with endogenous pVHL. (F): Exogenous KIF3A does not accumulate or become poly-ubiquitinated like HIF1α after MG132 treatment.

form of KAP3, ΔN-KAP3. We identified two domains within pVHL to be necessary for binding kinesin-2: the N-terminal acidic domain (residues 1-54) facilitates KAP3 binding, and residues 95-123 bind KIF3A. How pVHL-ΔN-KAP3 binding prevents the pVHL-moiety from simultaneous interaction with intact kinesin-2 could be explained by steric hindrance. Because pVHL residues 95-123 have already been implicated in microtubule binding [13], we performed microtubule co-sedimentation assays and observed that a significant portion -if not all- of pVHL binding to microtubules is mediated through interaction with kinesin-2. These findings correlate well with the regions and mutations of pVHL described as being deficient in stabilizing microtubules [13] and deficient in maintaining primary cilia in kidney epithelial cells [7], suggesting that kinesin-2 binding mediates microtubule-related functions of pVHL.

Notably, KAP3 is the first protein reported to bind the acidic domain of pVHL. Phosphorylation of the acidic domain has shown to be important for the full tumor suppressor function of pVHL in mouse xenografts [16]. KAP3 has also been shown to exert tumor suppressor function, as illustrated by the malignant transformation of neural progenitor cells by targeted deletion of *KAP3* [17]. Whether these two tumor suppressors influence each others' function has yet to be determined.

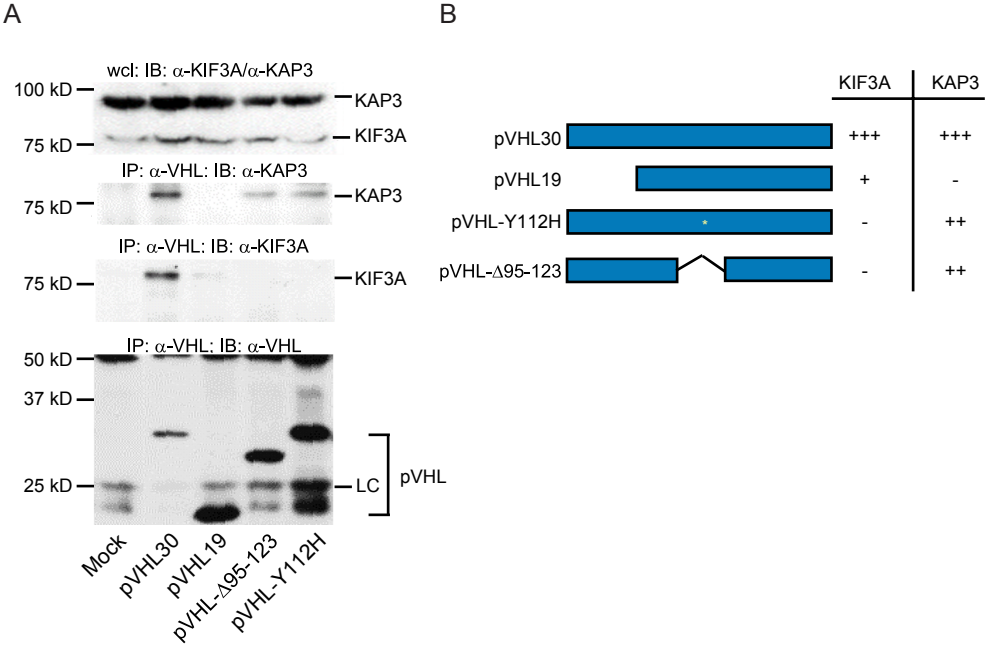


Figure 2: Characterizing pVHL-kinesin-2 interaction. (A): α-VHL IPs of retrovirally reconstituted pVHL variants in C26 cells. Upper panel, input endogenous KIF3A and KAP3. Second and third panels, co-precipitation of endogenous KAP3 and KIF3A respectively. Lower panel, pVHL recovery. LC, immunoglobin light chains. (B): Schematic representation of pVHL variants and their ability to bind KIF3A and KAP3.

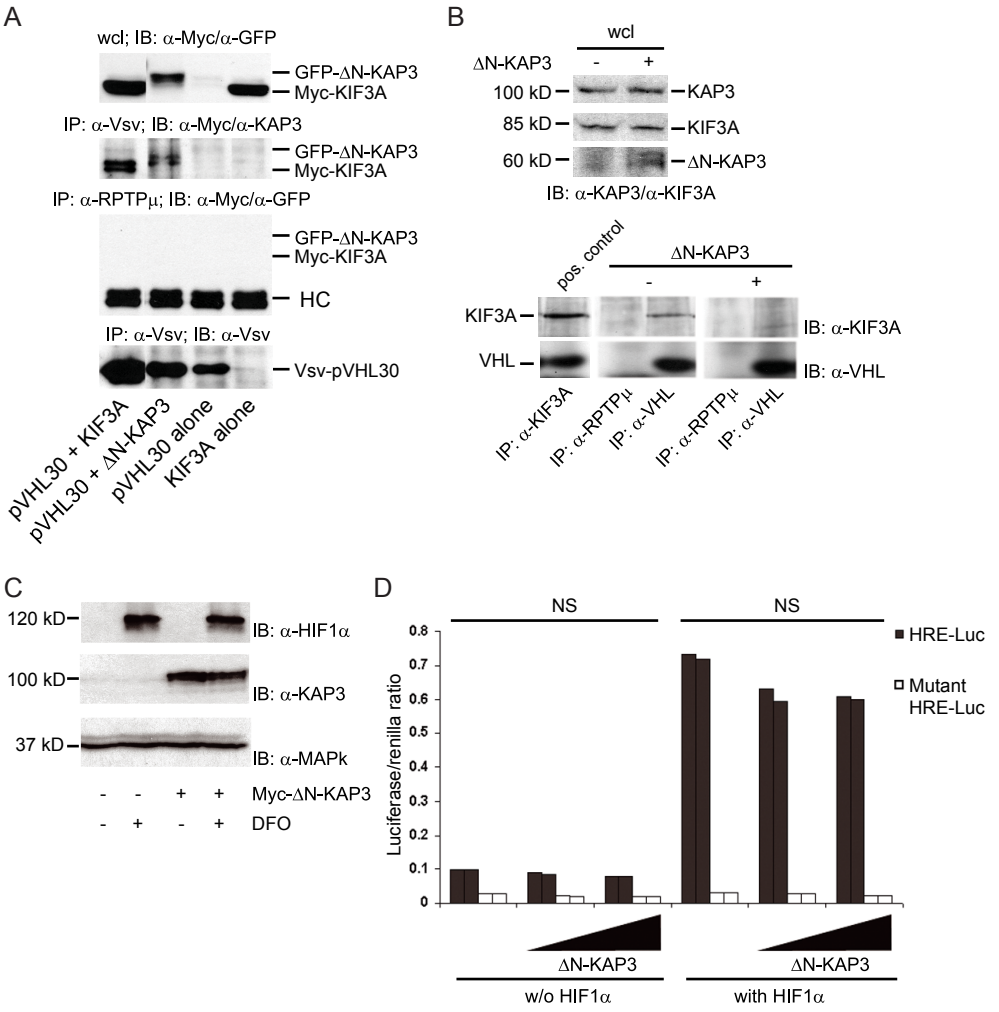


Figure 3: ΔN-KAP3 disrupts pVHL-KIF3A binding. (A): α-Vsv IPs of transfected Vsv-pVHL30 with Myc-KIF3A or GFP-ΔN-KAP3. Upper panel, transfection efficiency for Myc-KIF3A and GFP-ΔN-KAP3. Second panel, Myc-KIF3A and GFP-ΔN-KAP3 recovery in α-Vsv IP fractions. Third panel, aspecific protein recovery in α-RPTPμ IPs. Bottom panel, Vsv-pVHL30 recovery. (B): ΔN-KAP3 disrupts endogenous pVHL-KIF3A interaction. Upper panel, endogenous KAP3 and KIF3A. An IP of KIF3A shows specific recovery of KIF3A (positive control). Retrovirally transduced pBABE-puro with either no insert (-) or ΔN-KAP3 (+) specifically affects KIF3A recovery after α-VHL IPs. α-RPTPμ, unrelated antibody control. All IPs are from the same blot, same exposure, but lanes have been rearranged to improve presentation. (C): HIF1α response to DFO not affected by ΔN-KAP3. Upper panel shows endogenous HIF1α expression in HEK293T; Lanes treated with DFO or transfected with empty vector/Myc-ΔN-KAP3, as indicated. Middle panel, expression Myc-ΔN-KAP3. Lower panel, MAPK loading control (D): Hypoxia Response Element reporters (HRE-Luc; filled bars) or mutated HRE (Mutant HRE-Luc; empty bars) with 0, 100 or 300 ng Myc-ΔN-KAP3 with or without 100 ng HIF1α. Response to 100 ng HIF1α was statistically significant (p=0.0004). NS, not significant.

Recent studies describe pVHL interaction with the cell polarity complex Par3-Par6-aPKCζ [6]. Like pVHL, this complex also regulates ciliogenesis, however Par3-Par6-aPKCζ -mediated ciliogenesis has been established to operate through kinesin-2 [18]. A similar mechanism for pVHL-driven ciliogenesis has been hypothesized [6], however kinesin-2 binding to pVHL could not be detected in their cell system. Our data adds an unique element to ongoing studies concerning the role of pVHL in cilia regulation.

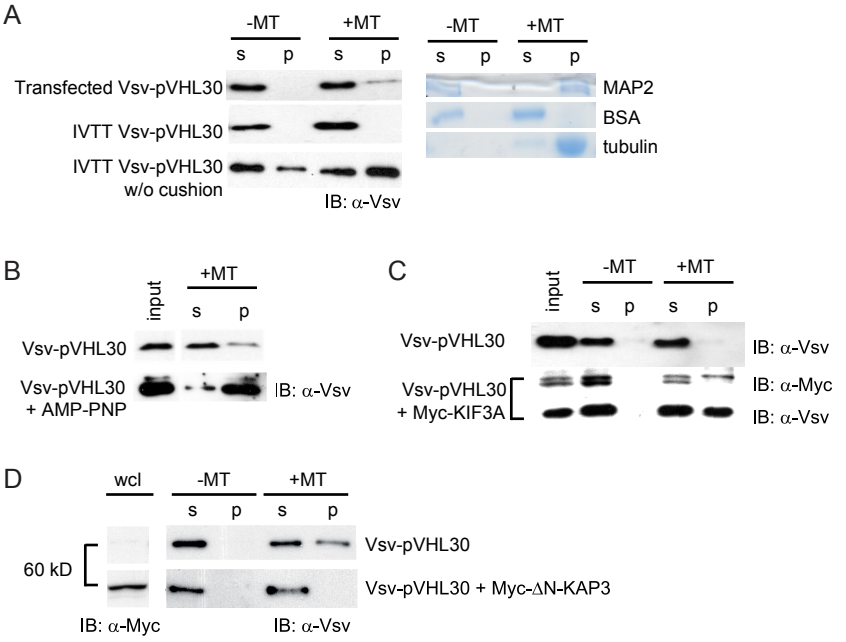


Figure 4: KIF3A mediates binding of pVHL to microtubules. (A): Left panels, transfected vs. IVTT Vsv-pVHL30 recovered from supernatant (s) or pellet (p) in a microtubule co-sedimentation assay. MT, microtubules. Lower panel, same assay repeated without the recommended cushion buffer using IVTT Vsv-pVHL30. Right panels, Coomassie Blue stainings of MT co-sedimentation controls tubulin, Microtubule-Associated Protein 2 (MAP2; serves as a positive control) and Bovine Serum albumin (BSA; serves as a negative control). (B): Recovery of transfected Vsv-pVHL30 in supernatant and pellet of microtubule spin down fractions without (upper panel) or with AMP-PNP (lower panel). (C): Upper panel, recovery of transfected Vsv-pVHL30 from supernatant and pellet with or without MTs. Middle and lower panel, recovery of pVHL and KIF3A using HEK293T transfected with Vsv-pVHL30 and Myc-KIF3A. (D): Upper panel MT co-sedimentation of Vsv-pVHL30 transfected with empty vector or with Myc-ΔN-KAP3 (60 kD). Expression Myc-ΔN-KAP3 shown on the left.

Acknowledgements

We would like to thank all lab members and especially Dr. R. Medema, Dr. M. Gebbink and Dr. N. Galjart for helpful discussions and reagents. This work is supported by the Dutch Cancer Society (Grants UU 1999-1879/2114 to E.V.) and the Netherlands Organization for Scientific Research (AGIKO grant 920-03-179 to M.L. and VIDI/Aspasia award 016.066.354 to R.G.).

References

1. **Kaelin, W.G., Jr.** The von Hippel-Lindau tumor suppressor gene and kidney cancer. *Clin Cancer Res* 10, 6290S-5S (2004).
2. **Lubensky, I.A.. *et al.*** Allelic deletions of the VHL gene detected in multiple microscopic clear cell renal lesions in von Hippel-Lindau disease patients. *Am J Pathol* 149, 2089-94 (1996).
3. **Rankin, E.B., Tomaszewski, J.E. & Haase, V.H.** Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. *Cancer Res* 66, 2576-83 (2006).
4. **Schoenfeld, A., Davidowitz, E.J. & Burk, R.D.** 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 U S A* 95, 8817-22 (1998).

5. **Iliopoulos, O., Ohh, M. & Kaelin, W.G., Jr.** pVHL19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. *Proc Natl Acad Sci U S A* 95, 11661-6 (1998).
6. **Kuehn, E.W., Walz, G. & Benzing, T.** Von hippel-lindau: a tumor suppressor links microtubules to ciliogenesis and cancer development. *Cancer Res* 67, 4537-40 (2007).
7. **Thoma, C.R. *et al.*** pVHL and GSK3beta are components of a primary cilium-maintenance signalling network. *Nat Cell Biol* 9, 588-95 (2007).
8. **Yamazaki, H., Nakata, T., Okada, Y. & Hirokawa, N.** KIF3A/B: a heterodimeric kinesin superfamily protein that works as a microtubule plus end-directed motor for membrane organelle transport. *J Cell Biol* 130, 1387-99 (1995).
9. **Davenport, J.R. & Yoder, B.K.** An incredible decade for the primary cilium: a look at a once-forgotten organelle. *Am J Physiol Renal Physiol* 289, F1159-69 (2005).
10. **Lolkema, M.P. *et al.*** The von Hippel-Lindau tumor suppressor protein influences microtubule dynamics at the cell periphery. *Exp Cell Res* 301, 139-46 (2004).
11. **Giles, R.H. *et al.*** Interplay between VHL/HIF1alpha and Wnt/beta-catenin pathways during colorectal tumorigenesis. *Oncogene* 25, 3065-70 (2006).
12. **Feldman, D.E., Thulasiraman, V., Ferreyra, R.G. & Frydman, J.** Formation of the VHL-elongin BC tumor suppressor complex is mediated by the chaperonin TRiC. *Mol Cell* 4, 1051-61 (1999).
13. **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* 5, 64-70 (2003).
14. **Jimbo, T. *et al.*** Identification of a link between the tumour suppressor APC and the kinesin superfamily. *Nat Cell Biol* 4, 323-7 (2002).
15. **Schermer, B. *et al.*** The von Hippel-Lindau tumor suppressor protein controls ciliogenesis by orienting microtubule growth. *J Cell Biol* 175, 547-54 (2006).
16. **Lolkema, M.P. *et al.*** Tumor suppression by the von Hippel-Lindau protein requires phosphorylation of the acidic domain. *J Biol Chem* 280, 22205-11 (2005).
17. **Teng, J. *et al.*** The KIF3 motor transports N-cadherin and organizes the developing neuroepithelium. *Nat Cell Biol* 7, 474-82 (2005).
18. **Fan, S. *et al.*** Polarity proteins control ciliogenesis via kinesin motor interactions. *Curr Biol* 14, 1451-61 (2004).

Chapter 6

Mobility of the von Hippel-Lindau tumor suppressor protein is regulated by kinesin-2

Dorus A. Mans*, Martijn P. Lolkema*, Moniek van Beest,
Laura G. Daenen, Emile E. Voest§ and Rachel H. Giles§
*,§ these authors contributed equally

Experimental Cell Research (in press)

Abstract

The von Hippel-Lindau tumor suppressor protein (pVHL) participates in many cellular processes including oxygen sensing, microtubule stability and primary cilia regulation. Recently, we identified ATP-dependent motor complex kinesin-2 to endogenously bind the full-length variant of VHL (pVHL30) in primary kidney cells, and mediate its association to microtubules. Here we show that pVHL also endogenously binds the neuronal kinesin-2 complex, which slightly differs from renal kinesin-2. To investigate the role of kinesin-2 in pVHL mobility, we performed fluorescence recovery after photobleaching (FRAP) experiments in neuroblastoma cells. We observe that pVHL30 is a highly mobile cytoplasmic protein, which becomes an immobile centrosomal protein after ATP-depletion in living cells. This response to ATP-depletion is independent of GSK3β-dependent phosphorylation of pVHL30. Furthermore, *VHL* variant alleles with reduced binding to kinesin-2 fail to respond to ATP-depletion. Accordingly, interfering with pVHL30-KIF3A interaction by either overexpressing a dominant negative construct or by reducing endogenous cellular levels of KIF3A by RNAi abolishes pVHL's response to ATP-depletion. From these data we suggest that mobility of a subcellular pool of pVHL is regulated by the ATP-dependent kinesin-2 motor. Kinesin-2 driven mobility of cytoplasmic pVHL might enable pVHL to function as a tumor suppressor.

Introduction

Sporadic renal cell carcinoma (RCC) and central nervous system (CNS) hemangioblastoma are often caused by inactivating mutations/deletions of the *von Hippel-Lindau* (*VHL*) gene encoding the pVHL tumor suppressor protein [1]. In the central nervous system, pVHL is expressed in neurons and drives neural differentiation of central nervous system progenitor cells [2]. Furthermore, neuroblastoma cells transduced with exogenous pVHL transform into functional neuron-like cells [3].

pVHL localizes predominantly to the cytoplasm [4-6], with a small pool shuttling between the nucleus and cytoplasm [7-11]. One report demonstrates that pVHL is also present in mitochondria [12]. Recently pVHL has been shown to decorate microtubules, and that an intact microtubule network is critical for the proper localization of cytoplasmic pVHL [13]. The interaction of pVHL with microtubules results in increased microtubule stability [5, 13] and subsequent primary cilia regulation [14]. Endogenous pVHL in the cilium of primary kidney cells has also been described [15].

Recently we found that full-length pVHL (pVHL30) endogenously interacts with -but is not involved in the degradation of- the anterograde ATP-dependent kinesin-2 microtubule motor subunits KIF3A, KIF3B and KAP3 in primary kidney cells [16]. In the present study we confirm pVHL association with neuronal kinesin-2, which differs from renal kinesin-2 by a single subunit. Because binding of pVHL to kinesin-2 suggests ATP-driven transport in a cell, we applied FRAP-analysis to examine pVHL movement. Similar to Mekhail *et al*. [17] we observe that GFP-labelled pVHL30 is a highly mobile protein under standard conditions. However, we describe altered kinetics of cytoplasmic pVHL movement in conditions where kinesin-2 transport is affected. Our data suggest that a significant portion of pVHL is transported by kinesin-2 in neural cells.

Materials & Methods

Construction of plasmids

GFP-pVHL30, GFP-pVHL19, GFP-pVHL-Y112H, GFP-pVHL-Δ95-123 have been described before [18]. GFP-pVHL-S111N and GFP-pVHL-R167Q were cloned via BglII/EcoR1 into pEGFPc1/2 (Clontech, USA). CFP-pVHL30 was generated by KpnI/ApaI cloning pVHL30 out of pEGFPc1 into pECFPc1 (Clontech). HA-pVHL30 and Myc-HIF1α have been described before [19, 20]. GFPc1-pVHL-S68/72A was a gift from Dr. W. Krek. GFP-KIF3A was constructed by cloning KIF3A out of Myc-KIF3A [16] into pEGFPc1 via EcoR1/XhoI. DsRed-γ-tubulin was a gift from Dr. J. Ellenberg. Myc-ΔN-KAP3 has been described before [16]. Murine KIF3A RNAi target sequence (5'-GGTGGTGGTTAGGTGCCGG-3') was constructed in pTER (gift from Dr. H. Clevers) via BglII/HindIII cloning. All plasmids used have been sequence-verified.

Cell culture

HEK293T and murine neuroblastoma N1E-115 cells were cultured in DMEM supplemented with antibiotics and 5-10% fetal calf serum. Cells were plated 18 hours prior to transfection by polyethelenimine (PEI; Polysciences, USA) or standard calcium phosphate transfection, and left for 48 hours before the start of experiments. For immunofluorescence and live cell imaging, N1E-115 cells were seeded on gelatin-coated cover slips and incubated in maturation medium (DMEM, 1 μM cAMP, 0.5% FCS, 1% DMSO) 16-24 hours prior to imaging. ATP-depletion was obtained by washing the cover slips twice with Dulbecco's phosphate-buffered saline (PBS) and then incubating the cells in PBS containing 5 mM sodium azide (Sigma-Aldrich, USA) and 1 mM 2-deoxy-D-glucose (Merck, Germany) at 37°C for 30-60 minutes. ATP-reconstitution of ATP-depleted cells was performed by incubating the cells for one hour in maturation medium at 37°C. Stable lines of N1E-115 cells were generated to inducibly express RNAi for KIF3A (KIF3A-pTER construct; clone M7) using the T-Rex protocol (Invitrogen, USA). Positive clones were selected using zeocin (100 μg/ml; Invitrogen) and blasticidin (1 μg/ml; Invitrogen) and tested for inducible expression of the construct using doxycycline (Sigma-Aldrich).

Immunoprecipitations

To study endogenous pVHL-kinesin-2 binding in N1E-115 cells, approximately $1*10^7$ cells were lysed in 500 µl 1% Triton-X lysis buffer (20 mM TRIS pH 8.0, 1% Triton-X-100, 140 mM NaCl, 10% glycerol) containing 'complete' cocktail of protease inhibitors (Roche, Switzerland) at room temperature. Cell remnants were spun down by 10 min. centrifugation at room temperature after which cleared lysates were immediately used for immunoprecipitations (IPs). α-VHL (7.5 µg of Ig32; BD-Biosciences, USA) or α-HA (clone 12CA5, hybridoma supernatant; 200 µl) were coupled to protein A/G agarose beads (30 µl; Santa Cruz Biotechnology, USA) in the presence of 1% BSA (Roche) and added to 200-400 µl of cleared lysates. To study GFP-pVHL30 binding to exogenous KIF3A, approximately $1*10^6$ cells were lysed in 400 µl buffer (20 mM TRIS, 1% Triton-X-100, 140 mM NaCl, 10% glycerol, pH 8.0) with protease inhibitors (Roche), then centrifuged 10 min. at 13000 rpm. Mouse monoclonal α-VHL (1.5 μg Ig32; BD-Biosciences) coupled to protein A/G agarose beads (7.5 μl; Santa Cruz Biotechnology), was added to 170 μl cleared lysate. IP reactions were incubated for 4 hours at room temperature, washed four times with 1% Triton-X lysis buffer and analyzed by western blotting. The IPs were repeated in

at least two independent experiments.

Western blotting

Specific protein bands were visualized using the following antibodies: α-VHL (Ig32, 1:500; BD-Biosciences), α-KIF3A (1:500; BD-Biosciences), α-KAP3 (1:500; BD-Biosciences), α-KIF3C (1:500; BD-Biosciences), α-mitogen activated protein (MAP) kinase (1:5000; gift from Dr. O. Kranenburg), α-Myc (1:5; clone 9E10, hybridoma supernatant), α-GFP (1:500; Santa Cruz Biotechnology) and α-β-actin (1:10000; Abcam, UK). All antibodies were diluted in PBS containing 5% milk and 0.1% Tween-20. Rabbit α-mouse Ig conjugated to horseradish peroxidase (1:20000; Pierce, USA) or goat α-rabbit Ig conjugated to horseradish peroxidase (1:2000; DAKO, Denmark) were used as secondary antibody after which enhanced chemiluminescence (Roche) was used for detection.

Cell cycle analysis

Doxycycline treated and untreated asynchronous M7 and M34 cells were washed in PBS and fixed overnight in 70% ethanol. Fixed cells were washed in PBS, treated with RNase (1:100; Sigma-Aldrich) and stained with propidiumiodide (1:100; Sigma-Aldrich). Cell cycle analysis was performed on the FACS Calibur (BD-Biosciences).

Live cell imaging

Transfected N1E-115 neuroblastoma cells were visualized on a Zeiss LSM 510 confocal scanning microscope (Carl Zeiss, Germany) fitted with a climate control chamber maintaining humidified 37°C and 5% CO_2. A circular region of interest with 10 μm radius was photobleached with 80 pulses of approximately 15 mW laser intensity after which the cell was followed by time-lapse imaging (every 0.25 sec, total 400 frames). The pre-bleach fluorescence intensity (F_i) was defined as the mean of 15 images before photobleaching. Total cell fluorescence was measured over time and used to normalize the data as described before [5]. All values were calculated as percentage F_i. The recovery fluorescence (F_ω) was defined as the mean of the last 15 scans. The mobile fraction (Mf) was calculated using the following equation: $Mf=(F_\omega-F_0)/(F_i-F_0)$ as previously described [21]. All experiments were repeated independently on at least three separate days. Two-tailed student's *t*-test was applied to determine significance of both F_0 and Mf.

Reporter assays

HIF1α reporter assays were performed in duplicate with 200 ng GFP-pVHL30, GFP-pVHL19 or GFP-pVHL-Y112H transfected with 50 ng Myc-HIF1α in HEK293T cells as previously described [20].

Immunofluorescence

Methanol fixed cells were incubated with α-Myc (9E10; 1:1000, Covance, USA) or α-γ-tubulin (1:400, Sigma-Aldrich). Goat-anti-mouse Alexa568 (1:400, Molecular probes, USA) was used as secondary antibody as described previously [5]. Photos were taken using the Zeiss LSM 510 confocal scanning microscope (Carl Zeiss Inc.).

Results & Discussion

pVHL binds neuronal kinesin-2

Kinesin-2 has been described to be involved in transport of protein complexes within cilia and flagella [22-24] and anterograde transport of membrane-bound organelles in neurons and melanosomes [25-27]. Recent reports that pVHL regulates cilia and participates in the aPKC/Par3/Par6 complex [15], known to be transported by kinesin-2 [28, 29], led us to investigate the interaction of pVHL with kinesin-2 in neural cells. Unlike most epithelial cells, kinesin-2 subunit KIF3C is preferentially expressed instead of KIF3B in neuronal cells [30, 31]. We began by performing IPs of pVHL to validate the interaction between pVHL and neuronal kinesin-2 in murine N1E-115 neuroblastoma cells. Precipitations using an antibody against hemagglutinin (HA) were performed in parallel as antibody specificity control. As expected, pVHL precipitated itself efficiently (Figure 1A; lower panel). The antibody light chain was detected in these IPs, running slightly lower than the endogenous pVHL protein (Figure 1A; lower panel). Endogenous kinesin-2 components KAP3 (Figure 1A; upper panel), KIF3C (Figure 1A; second panel) and KIF3A (Figure 1A; third panel) were recovered in pVHL precipitations. Anti-HA IPs did not recover endogenous KAP3, KIF3C or KIF3A protein in this experiment. This is the first data demonstrating interaction between pVHL and kinesin-2 subunit KIF3C. Because kinesin-2 function is nucleotide-dependent, we repeated this IP in cells treated with 2-deoxyglucose and sodium azide to reduce cellular ATP-levels. This treatment did not disturb pVHL-kinesin-2 interaction (Figure 1A).

pVHL is a highly mobile protein

To examine localization and mobility of VHL in living cells, we constructed a green fluorescent protein (GFP) tagged expression construct of full length pVHL (GFP-pVHL30) and confirmed its binding to exogenous KIF3A in immunoprecipitation experiments (Supplemental Figure 1A). Examining methanol-fixed cover slips of GFP-pVHL30 expressing cells revealed perinuclear, vesicular structures as previously described [13]. However in living N1E-115 cells, we observed that GFP-pVHL30 localizes predominantly to the cytoplasm and has a seemingly random distribution (Figure 1B). Whereas pVHL30 has been previously described to be localized to the membrane and cytoplasm in fixed cells [32], we only observed pVHL30 to be cytoplasmic in this particular cell type in living (Figure 1B) and fixed cells (Supplemental Figure 3). The discrepancies between these data might be attributable to cell-specific qualities, fixation techniques, cell density, or cell culturing techniques. To study the dynamics of GFP-pVHL30 localization in N1E-115 cells, we performed live cell imaging and Fluorescence Recovery After Photobleaching (FRAP) as described previously [21]. The degree of mobility in these experiments can be determined by calculating the mobile fraction (Mf), which represents a ratio of absolute fluorescence recovery after photobleaching. After bleaching a small region at the cell periphery, GFP-pVHL30 was quickly recovered in this bleached region, indicating that GFP-pVHL30 is a highly mobile protein much like GFP (Figures 1B-C).

pVHL mobility is ATP-dependent

Because kinesin-2 movement along microtubules is dependent on the presence of ATP, we performed FRAP experiments of GFP-pVHL30 or GFP alone in transfected N1E-115 cells in the presence or absence of ATP. When compared

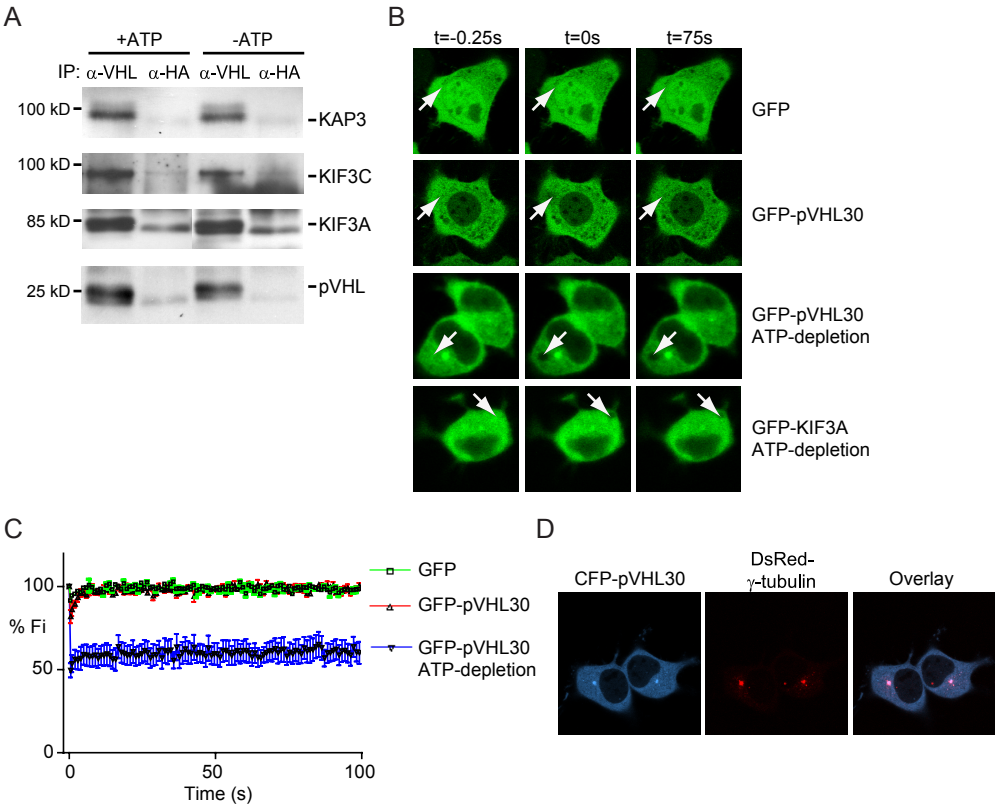


Figure 1: (A): Lysates from normally cultured and ATP-depleted N1E-115 cells immunoprecipitated with anti-hemagglutinin (anti-HA) or anti-VHL as indicated above the lanes. The KIF3A antibody recognizes an aspecific protein band running just below KIF3A. The antibody light chain runs just below pVHL. (B): Time-lapse photographs of N1E-115 cells transfected with GFP (upper row), GFP-pVHL30 (second and third row) or GFP-KIF3A (bottom row) and used for Fluorescence Recovery After Photobleaching (FRAP) experiments. Images were acquired 0.25 seconds before bleaching (left column), almost directly after bleaching (t=0, middle column), and 75 seconds after bleaching (right column). Arrows indicate bleached region. Cells in bottom two rows were subjected to ATP-depletion for 30 minutes using sodium azide and deoxyglucose. (C): FRAP curves of N1E-115 cells either transfected with GFP (green) or GFP-pVHL30 in the presence (red) or absence (blue) of ATP. This graph depicts the $\%F_i$, which represents the percentage of initial fluorescence as calculated from three independent experiments pooled in a single line. The error bars represent the standard error of the mean of each time point. (D): Live cell images of N1E-115 cells co-transfected with CFP-pVHL30 (left panel) and DsRed-γ-tubulin (middle panel) after ATP-depletion. Right panel, image overlay.

to GFP we found that GFP-pVHL30 showed a dramatically decreased mobility after ATP-depletion (Figure 1C). Furthermore, the observed response to ATP-depletion is not due to excessive expression of GFP-pVHL30; the same result was obtained using cells expressing low levels of GFP-pVHL30 (Supplemental Figure 1B).

In the absence of ATP we visually observed that a large pool of GFP-pVHL30 redistributed to a perinuclear point resembling the microtubule-organizing center (MTOC) or centrosome. A similar pattern of cellular localization was observed using GFP-KIF3A after ATP-depletion (Figure 1B). Co-transfecting CFP-pVHL30 with DsRed-labeled γ-tubulin, a major component of the centrosome [33], we observed co-localization of pVHL and γ-tubulin in ATP-depleted cells (Figure 1D), while co-localization was lost after ATP-reconstitution by replacing the medium (Supplemental Figure 1C). From these experiments we conclude that pVHL mobility and localization

are at least partly dependent on the presence of ATP, since ATP-depletion results in pVHL immobilization and centrosomal localization.

***VHL* variant alleles display differential mobility response to ATP-depletion**

The mobile fractions calculated from curves such as shown in Figure 1C can be plotted to compare protein dynamics across multiple experiments. We performed FRAP on cells transfected with GFP, GFP-pVHL30, or GFP-KIF3A, and observed high mobile fractions under normal conditions (Figure 2). Upon ATP-depletion however, the mobile fraction of both GFP-pVHL30 (Mf: 0.53 +/- 0.09) and GFP-KIF3A (Mf: 0.39 +/- 0.12) dropped significantly (both $p<0.01$), suggesting that a pool of each of these proteins exists that requires ATP for motility.

VHL disease is characterized by *VHL* mutations affecting one or more pVHL function. pVHL has been extensively described to function as an E3 ubiquitin ligase, targeting Hypoxia Inducible Factor (HIF) α-subunits for ubiquitination and subsequent degradation [1]. To test the involvement of pVHL's E3 ligase function in pVHL30's mobility response to ATP depletion, we performed *HIF1α* Response Element (HRE) reporter assays. To this end we compared GFP-pVHL30 to GFP-pVHL19, a naturally occurring pVHL variant originating from an alternative translational start site [32], or GFP-pVHL-Y112H, a VHL disease associated allele shown to have a diminished HIFα regulation [34]. GFP-pVHL19 exhibits identical E3 ligase activity like GFP-pVHL30 (Supplemental Figure 2). Interestingly, the mobility of GFP-pVHL19 remained virtually unaltered in ATP-depleted cells (Mf: 0.82 +/- 0.06). To exclude whether the E3 ligase activity of GFP-pVHL30 plays a role in pVHL mobility we tested GFP-pVHL-R167Q, described to abolish ubiquitin ligase activity by disrupting formation of the pVHL-ElonginB-ElonginC (VBC)-Cul2 complex [35]. The mobility of GFP-pVHL-R167Q was high in the presence of ATP (Mf: 0.90 +/- 0.05), however the protein did respond to ATP-depletion (Mf: 0.47 +/- 0.18) like GFP-pVHL30, excluding a role for E3 ligase activity in the mobility response of pVHL to ATP-depletion. Next we studied if increased HIFα stability does affect the response of pVHL to ATP-depletion by testing GFP-pVHL-S111N, previously shown to be defective in binding HIFα resulting in HIFα accumulation [36]. The mobility of GFP-pVHL-S111N is high in the presence of ATP and significantly decreased upon ATP-depletion (Mf: 0.53 +/- 0.18).

We have recently shown that pVHL19 is unable to bind KAP3 and while it can precipitate KIF3A, it does so very inefficiently [18]. We next asked whether pVHL's function in microtubule association and stabilization could account for pVHL30's ATP-dependent behaviour in FRAP assays. We began by measuring the *VHL* variant pVHL-Δ95-123, lacking pVHL's microtubule binding domain. FRAP-experiments of GFP-pVHL-Δ95-123 transfected cells revealed no reduction of the Mf after ATP-depletion (Mf: 0.90 +/- 0.13) (Figure 2). We subsequently tested the pVHL-Y112H patient mutation, also reported to interfere with normal microtubule stabilization. While GFP-pVHL-Y112H is a highly mobile protein in the presence of ATP, this missense variant did demonstrate a response of borderline significance to ATP-depletion (Mf: 0.74 +/- 0.20). However, when compared to GFP-pVHL30, GFP-pVHL-Y112H demonstrates a significantly higher Mf ($p<0.05$) in ATP-depleted cells (Figure 2).

To study if the response of pVHL to ATP-depletion is dependent on glycogen synthase kinase 3β (GSK3β)/casein kinase 1 (CK1)-dependent phosphorylation, we performed FRAP-experiments of cells transfected with GFP-pVHL-S68/72A, unable to be phosphorylated by GSK3β and CK1 [37]. GFP-pVHL-S68/72A responded to ATP-depletion very much like wild-type pVHL30 (Mf: 0.51 +/- 0.08; shown in Figure 2).

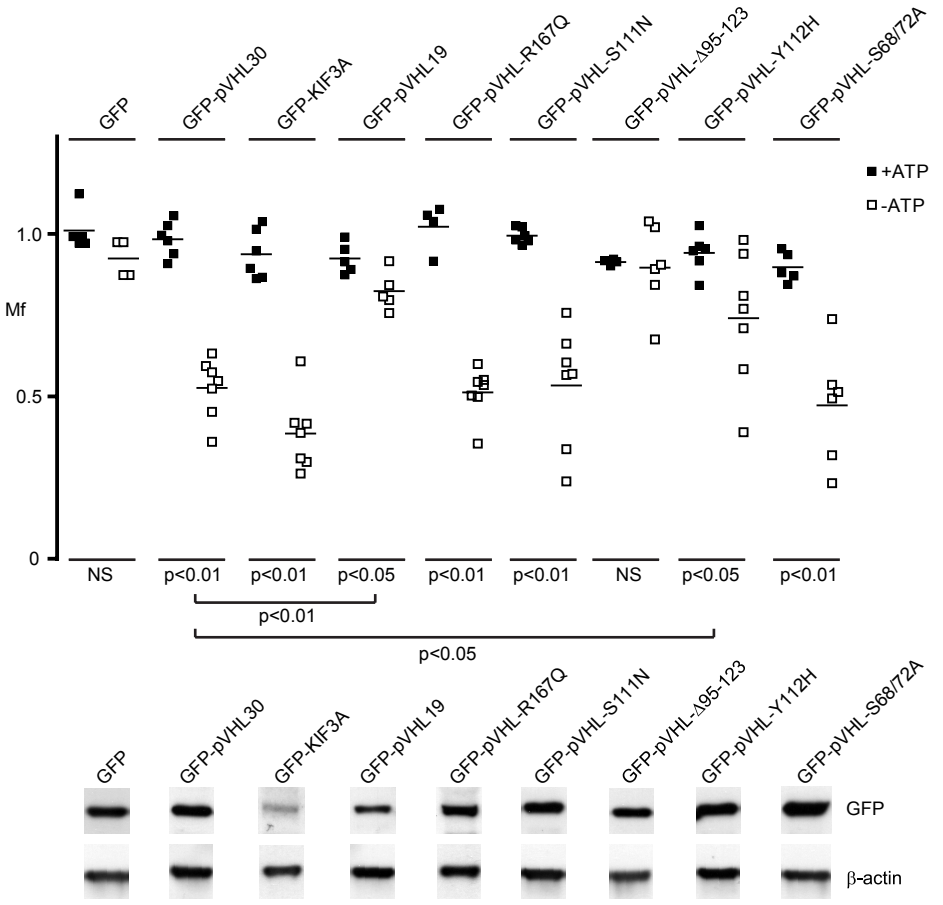


Figure 2: FRAP-derived mobility (Mf) values of GFP-tagged variants of pVHL, GFP-KIF3A or GFP transfected in N1E-115 cells. Each square represents an individual experiment before (filled squares) or after ATP-depletion (open squares). Horizontal line in each experiment represents the mean value. Significance of mobility decrease after ATP-depletion was calculated using two-tailed Student's *t*-test, and is depicted below the graph. Mf, mobile fraction; NS, not significant. Below, western blot analysis of expression levels of GFP tagged pVHL (variants), GFP-KIF3A and GFP alone; β-actin used as loading control.

We conclude from these experiments that the microtubule-associated domain of pVHL mediates the observed response to ATP-depletion. Both of the alleles not responding to ATP-depletion have been recently described to disturb primary cilia [14]. Accordingly, recent studies from our lab demonstrate that these two variant alleles are unable to bind KIF3A [18]. We hypothesized that the pVHL mobility patterns observed in Figure 2 could be mediated by kinesin-2 in a HIFα and GSK3β-independent manner.

Disrupting pVHL-kinesin-2 binding affects pVHL mobility

If the response to ATP-depletion by pVHL is regulated by kinesin-2, we hypothesized that disrupting the interaction of pVHL-kinesin-2 by means of overexpression of a dominant negative form of KAP3 would interfere with this response. This dominant negative construct, ΔN-KAP3, has been previously reported to uncouple the binding of kinesin-2 cargo to the motor proteins KIF3A and KIF3B [38].

Likewise we have observed that expression of ΔN-KAP3 inhibits binding of pVHL to KIF3A [16]. Transfecting Myc-tagged ΔN-KAP3 or empty pcDNA3-Myc vector together with GFP-pVHL30 in a 4:1 ratio, did not significantly alter pVHL mobility or subcellular distribution in normal conditions, although a greater range of Mf values were obtained in cells expressing Myc-ΔN-KAP3 (Figure 3A). Cells were screened for Myc-expression by immunofluorescence after each experiment (Supplemental Figure 3). Interestingly, GFP-pVHL30 in these cells expressing Myc-ΔN-KAP3 reacted significantly different when ATP was depleted, and accumulation of GFP-pVHL30 at the centrosome was blocked (Figure 3A). These data support a role for kinesin-2 in pVHL mobility.

We cannot entirely exclude E3 ligase activity when pVHL is bound to kinesin-2. However, our data suggest that the microtubule-associated functions of pVHL aremutually exclusive to its E3 ligase function. Firstly, immunoprecipitations of KIF3A in our lab were never able to recover HIF1α, whereas immunopreciptations of pVHL can recover both HIF1α and KIF3A (unpublished). Secondly, transduction of ΔN-KAP3 interrupts endogenous binding between full-length pVHL30 and KIF3A but does not affect E3 ligase activity or HIF1α stability [18]. While suggestive, future experiments will determine the relationship between these pVHL functions.

As an alternate approach, we constructed N1E-115 cell lines carrying doxycycline-inducible expression plasmids encoding RNAi against murine KIF3A. We tested the efficiency of the inducible RNAi construct in western blots and found a significant (approximately 75%) decrease in endogenous KIF3A levels after 48 hours of doxycycline treatment (clone M7, Figure 3B), while this did not affect cell cycle progression (Figure 3B). We chose a sister clone from the same stable transfection, M34, that failed to reduce KIF3A levels in response to doxycycline (Figure 3B) as a negative control. We then used clones M7 and M34 to determine the effect of KIF3A on GFP-pVHL30 mobility after ATP-depletion, before and after induction with doxycycline for 48 hours. Uninduced M7 cells manifested the characteristic GFP-pVHL30 immobilization after ATP-depletion. In contrast, GFP-pVHL30 in M7 cells treated with doxycycline appeared largely resistant to the effects of ATP-depletion, with significant recovery of Mf values (Figure 3B). M34 cells did not demonstrate altered Mf values in response to doxycycline, suggesting this lack of response in mobility of pVHL to ATP-depletion is a specific KIF3A-dependent effect (Figure 3B). These experiments implicate KIF3A, one of the motor subunits of kinesin-2 in pVHL cellular movement.

In the presence of ATP GFP-pVHL30 is a highly mobile protein, corresponding with published pVHL30 mobility data [17]. However in ATP-depleted cells, pVHL immobilizes where it co-localizes with γ-tubulin at the centrosome. The response to ATP suggests that pVHL mobility and localization is dependent on the presence of functional microtubule motors and or kinases, both requiring ATP for their function. This notion is supported by the endogenous binding of pVHL to kinesin-2 in these cells.

Our FRAP-data show that approximately 50% of the GFP-pVHL30 pool is mobilized via ATP-dependent mechanisms, while the other half remains diffuse. The short isoform of pVHL (pVHL19), lacks the N-terminal domain and is not phosphorylated by GSK3β [37]. Since pVHL-S68/72A responds to ATP-depletion similar to pVHL30, and does not affect binding to microtubules [37], we conclude that the N-terminal domain of pVHL is necessary for the response to ATP-depletion. CK2-dependent phosphorylation of serine residues 33, 38 and 43 of pVHL in the N-terminal domain of pVHL, has been implicated in the tumor suppressor function in mouse xenograft assays [19]. Therefore, it will be interesting to examine the effect of

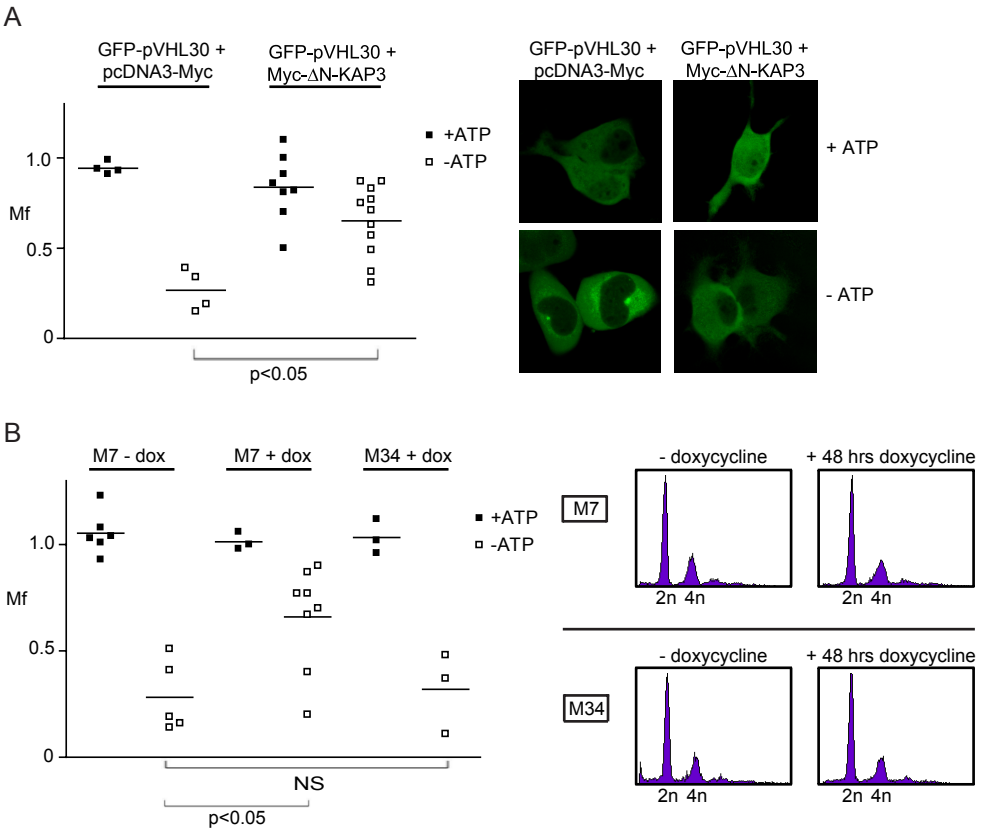


Figure 3: (A): FRAP-derived Mf values for GFP-pVHL30 transfected into N1E-115 cells in the presence or absence of Myc-ΔN-KAP3 as indicated above the graph. Each square represents an individual experiment before (filled squares) or after ATP-depletion (open squares). Horizontal line in each experiment represents the mean Mf value. Significance of mobility decrease after ATP-depletion was calculated using a two-tailed Student's *t*-test, and is depicted below the graph. Photographs to the right illustrate that ΔN-KAP3 blocks centrosomal localization of pVHL in response to ATP-depletion. (B): Mf values of GFP-pVHL30 transfected into N1E-115 cells stably transfected with a doxycycline-inducible KIF3A RNAi (M7) or a sister clone that failed to reduce KIF3A levels in response to doxycycline (M34), treated with (+dox) or without (-dox) doxycycline for 48 hours, and before (filled squares) or after ATP-depletion (open squares). Each square represents an individual measurement and the mean is illustrated by a horizontal line. Significance of mobility decrease after ATP-depletion was calculated using a two-tailed Student's *t*-test, and is depicted below the graph. Right panel: Western blot of endogenous KIF3A (upper panel) of N1E-115 cells in M7 and M34 cells before and after stimulation with doxycycline (dox) for 0, 24 and 48 hours (below). Lower panel, western blot for mitogen activated protein (MAP) kinase serves as loading control. Right, cell cycle profiles of fixed M7 and M34 cells with or without doxycycline stimulation for 48 hours.

CK2-dependent phosphorylation of pVHL, regarding proper localization and mobility of pVHL, which might be necessary for microtubule-dependent functioning of pVHL.

Acknowledgements

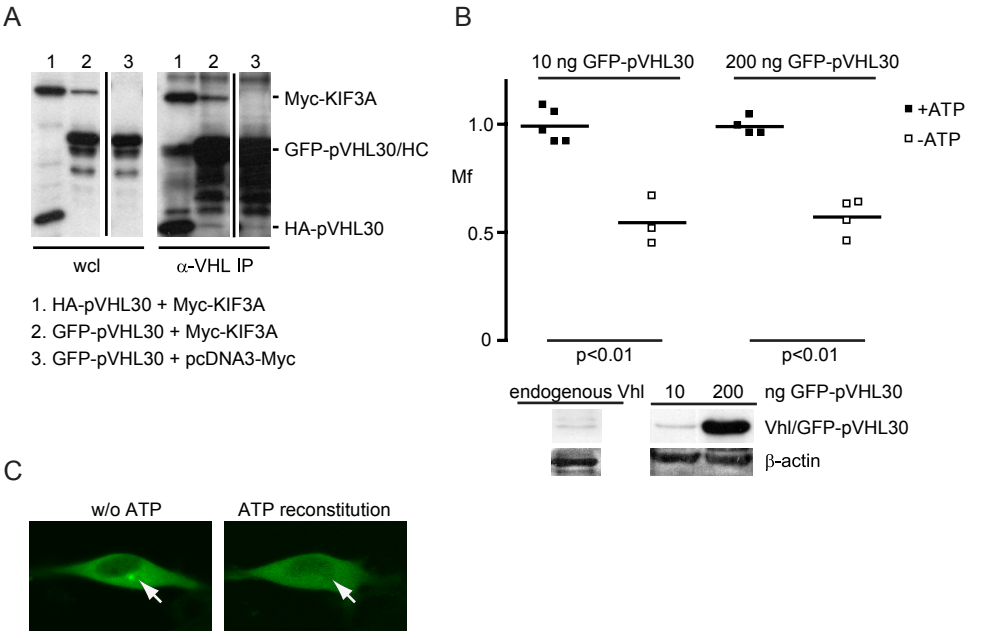
We thank the Dept. of Cell Biology of the University of Utrecht, the Netherlands for the use of the live cell imaging station. This work is supported by the Dutch Cancer Society (Grants UU 1999-1879/2114 to E.V.) and the Netherlands Organization for Scientific Research (NWO grants: AGIKO grant 920-03-179 to M.L. and VIDI award 016.066.354 to R.G.).

References

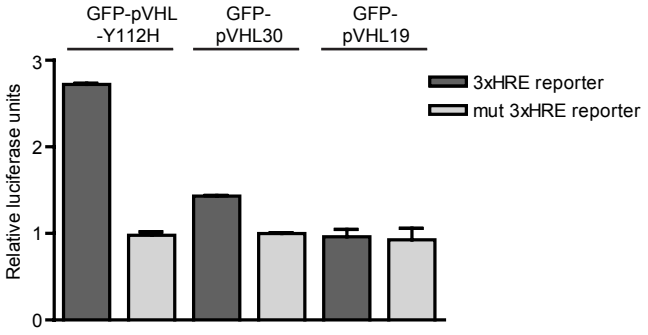
1. **Kaelin, W.G., Jr.** The von Hippel-Lindau protein, HIF hydroxylation, and oxygen sensing. *Biochem Biophys Res Commun* 338, 627-638 (2005).
2. **Kanno, H. *et al.*** Role of the von Hippel-Lindau tumor suppressor protein during neuronal differentiation. *Cancer Res* 60, 2820-2824 (2000).
3. **Murata, H. *et al.*** Von Hippel-Lindau tumor suppressor protein transforms human neuroblastoma cells into functional neuron-like cells. *Cancer Res* 62, 7004-7011 (2002).
4. **Iliopoulos, O., Kibel, A., Gray, S. & Kaelin, W.G., Jr.** Tumour suppression by the human von Hippel-Lindau gene product. *Nat Med* 1, 822-826 (1995).
5. **Lolkema, M.P. *et al.*** The von Hippel-Lindau tumor suppressor protein influences microtubule dynamics at the cell periphery. *Exp Cell Res* 301, 139-146 (2004).
6. **Ohh, M. *et al.*** The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol Cell* 1, 959-968 (1998).
7. **Lee, S. *et al.*** Nuclear/cytoplasmic localization of the von Hippel-Lindau tumor suppressor gene product is determined by cell density. *Proc Natl Acad Sci U S A* 93, 1770-1775 (1996).
8. **Lee, S. *et al.*** Transcription-dependent nuclear-cytoplasmic trafficking is required for the function of the von Hippel-Lindau tumor suppressor protein. *Mol Cell Biol* 19, 1486-1497 (1999).
9. **Groulx, I., Bonicalzi, M.E. & Lee, S.** Ran-mediated nuclear export of the von Hippel-Lindau tumor suppressor protein occurs independently of its assembly with cullin-2. *J Biol Chem* 275, 8991-9000 (2000).
10. **Duan, D.R. *et al.*** Characterization of the VHL tumor suppressor gene product: localization, complex formation, and the effect of natural inactivating mutations. *Proc Natl Acad Sci U S A* 92, 6459-6463 (1995).
11. **Pause, A. *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 U S A* 94, 2156-2161 (1997).
12. **Shiao, Y.H., Resau, J.H., Nagashima, K., Anderson, L.M. & Ramakrishna, G.** The von Hippel-Lindau tumor suppressor targets to mitochondria. *Cancer Res* 60, 2816-2819 (2000).
13. **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* 5, 64-70 (2003).
14. **Thoma, C.R. *et al.*** pVHL and GSK3beta are components of a primary cilium-maintenance signalling network. *Nat Cell Biol* 9, 588-595 (2007).
15. **Schermer, B. *et al.*** The von Hippel-Lindau tumor suppressor protein controls ciliogenesis by orienting microtubule growth. *J Cell Biol* 175, 547-554 (2006).
16. **Lolkema, M.P. *et al.*** Allele-specific regulation of primary cilia function by the von Hippel-Lindau tumor suppressor. *Eur J Hum Genet* (2007).
17. **Mekhail, K. *et al.*** Regulation of ubiquitin ligase dynamics by the nucleolus. *J Cell Biol* 170, 733-744 (2005).
18. **Lolkema, M.P. *et al.*** The von Hippel-Lindau tumour suppressor interacts with microtubules through kinesin-2. *FEBS Lett* 581, 4571-4576 (2007).

19. **Lolkema, M.P. *et al.*** Tumor suppression by the von Hippel-Lindau protein requires phosphorylation of the acidic domain. *J Biol Chem* 280, 22205-22211 (2005).
20. **Giles, R.H. *et al.*** Interplay between VHL/HIF1alpha and Wnt/beta-catenin pathways during colorectal tumorigenesis. *Oncogene* 25, 3065-3070 (2006).
21. **Lippincott-Schwartz, J., Snapp, E. & Kenworthy, A.** Studying protein dynamics in living cells. *Nat Rev Mol Cell Biol* 2, 444-456 (2001).
22. **Cole, D.G. *et al.*** Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in Caenorhabditis elegans sensory neurons. *J Cell Biol* 141, 993-1008 (1998).
23. **Kozminski, K.G., Beech, P.L. & Rosenbaum, J.L.** The Chlamydomonas kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. *J Cell Biol* 131, 1517-1527 (1995).
24. **Piperno, G. & Mead, K.** Transport of a novel complex in the cytoplasmic matrix of Chlamydomonas flagella. *Proc Natl Acad Sci U S A* 94, 4457-4462 (1997).
25. **Kondo, S. *et al.*** KIF3A is a new microtubule-based anterograde motor in the nerve axon. *J Cell Biol* 125, 1095-1107 (1994).
26. **Takeda, S. *et al.*** Kinesin superfamily protein 3 (KIF3) motor transports fodrin-associating vesicles important for neurite building. *J Cell Biol* 148, 1255-1265 (2000).
27. **Yamazaki, H., Nakata, T., Okada, Y. & Hirokawa, N.** KIF3A/B: a heterodimeric kinesin superfamily protein that works as a microtubule plus end-directed motor for membrane organelle transport. *J Cell Biol* 130, 1387-1399 (1995).
28. **Fan, S. *et al.*** Polarity proteins control ciliogenesis via kinesin motor interactions. *Curr Biol* 14, 1451-1461 (2004).
29. **Nishimura, T. *et al.*** Role of the PAR-3-KIF3 complex in the establishment of neuronal polarity. *Nat Cell Biol* 6, 328-334 (2004).
30. **Muresan, V. *et al.*** KIF3C and KIF3A form a novel neuronal heteromeric kinesin that associates with membrane vesicles. *Mol Biol Cell* 9, 637-652 (1998).
31. **Yang, Z. & Goldstein, L.S.** Characterization of the KIF3C neural kinesin-like motor from mouse. *Mol Biol Cell* 9, 249-261 (1998).
32. **Iliopoulos, O., Ohh, M. & Kaelin, W.G., Jr.** pVHL19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. *Proc Natl Acad Sci U S A* 95, 11661-11666 (1998).
33. **Joshi, H.C., Palacios, M.J., McNamara, L. & Cleveland, D.W.** Gamma-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature* 356, 80-83 (1992).
34. **Cockman, M.E. *et al.*** Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem* 275, 25733-25741 (2000).
35. **Iwai, K. *et al.*** Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proc Natl Acad Sci U S A* 96, 12436-12441 (1999).
36. **Ohh, M. *et al.*** Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol* 2, 423-427 (2000).

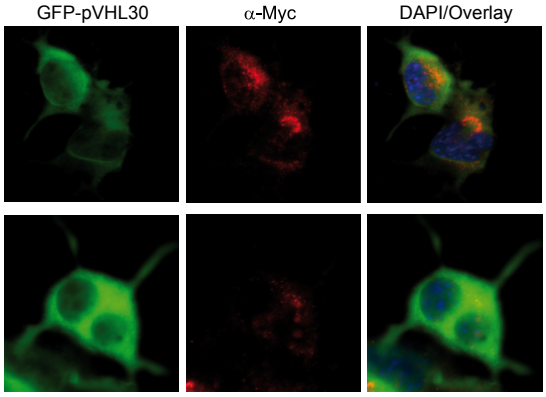
37. **Hergovich, A. *et al.*** Priming-dependent phosphorylation and regulation of the tumor suppressor pVHL by glycogen synthase kinase 3. *Mol Cell Biol* 26, 5784-5796 (2006).
38. **Jimbo, T. *et al.*** Identification of a link between the tumour suppressor APC and the kinesin superfamily. *Nat Cell Biol* 4, 323-327 (2002).



Supplemental Figure 1: (A): α-VHL IPs of HEK293T cells transfected with HA-pVHL30 and Myc-KIF3A, GFP-pVHL30 and Myc-KIF3A or GFP-pVHL30 alone. Left panel, transfection efficiency for Myc-KIF3A, GFP-pVHL30 or HA-pVHL30. Right panel, KIF3A and pVHL recovery in α-VHL IP fractions. HC, heavy chain. (B)**:** FRAP-derived mobility (Mf) values of N1E-115 cells transfected with either 10 ng GFP-pVHL30 and 990 ng pcDNA3 or 200 ng GFP-pVHL30 and 800 ng pcDNA3. Each square represents an individual experiment before (filled squares) or after ATP-depletion (open squares). Horizontal line in each experiment represents the mean value. Significance of mobility decrease after ATP-depletion was calculated using two-tailed Student's *t*-test, and is depicted below the graph. Mf, mobile fraction. Below, western blot analysis of expression levels of endogenous Vhl and GFP-pVHL30; β-actin used as loading control. (C): Photographs of an ATP-depleted cell before and after ATP-reconstitution.



Supplemental Figure 2: *HIF1α* Response Element reporter (3xHRE reporter; dark gray bars) or mutated (mut) 3xHRE reporter (light gray bars) in HEK293T cells transfected with 200 ng GFP-pVHL-Y112H, GFP-pVHL30 or GFP-pVHL19 and 50 ng Myc-HIF1α.



Supplemental Figure 3: Confocal images of N1E-115 cells transfected with GFP-pVHL30 in combination with Myc-ΔN-KAP3 or pcDNA3 used for FRAP analysis. Nuclei stained with DAPI.

Chapter 7

Summarizing Discussion

pVHL: a multifunctional tumor suppressor

The *von Hippel-Lindau* (*VHL*) tumor suppressor gene was mapped to chromosome band 3p26-25 by Latif and colleagues in 1993 [1]. Subsequent molecular and animal studies pointed to the tumor suppressor activity of the protein encoded by *VHL* (pVHL) as being primarily ascribed to its role in the degradation of HIFα subunits alone, by functioning as an adaptor of an E3 ubiquitin ligase [2]. Recent insights into the functionality of pVHL support the hypothesis that pVHL's tumor suppressing activity probably extends beyond HIFα regulation [2].

From microtubule stability to primary cilia regulation

In the past, we have developed cell biological techniques to quantify the stabilization of peripheral microtubules by pVHL. As a consequence, we were able to ascertain the extent to which plus-end microtubule dynamics are affected by pVHL in a HIF1/2α-negative RCC background. An interesting link between microtubule plus-end stability, pVHL function, and the multiple cyst/tumor formation in patients with VHL disease was found to be the primary cilium; a detailed description of the composition and function of this organelle is given in Chapter 3. The hypothesis that loss of a cilium and/or ciliary function will stimulate cells to proliferate, ultimately becoming cysts, has driven us and others to focus on primary cilia regulation by pVHL in kidney epithelial cells.

We analyzed primary cilia regulation by re-introducing pVHL into RCC cells devoid of HIF1/2α [3], which increased the number of functional cilia, as measured by calcium-flux assays. Removal of exogenous pVHL resulted in a decrease in cilium length, however not in cilia numbers, suggesting that pVHL maintains cilia function (Chapter 4). This finding is the first evidence that pVHL affects ciliary function in kidney epithelial cells. Analysis of pVHL variants unable to protect microtubules from nocodazole treatment, [4] including VHL type 2A disease mutant Y112H, showed that these proteins were unable to localize at the cilium or to restore ciliary function in RCC cells, arguing that the microtubule stabilization activity of pVHL is closely linked to pVHL's ciliary localization and function. Interestingly, the short isoform of pVHL (pVHL19), missing the first 53 amino acids of the full-length protein [5, 6], did not localize along the ciliary axoneme and could not restore ciliary function to the extent of full-length pVHL. This finding suggests that the N-terminal acidic domain is likely to be responsible for proper ciliary localization and ciliary function of pVHL, independent of HIF1/2α expression.

Notably, this N-terminal domain has shown to exert a tumor suppressing function. Phosphorylation of pVHL at three serines (S33, S38 and S43) via CK2, has shown to be essential to suppress tumor formation in mouse xenografts [7]. Regarding this finding, it will be interesting a) to study the role of CK2-dependent phosphorylation pVHL functioning in microtubule stability and ciliary function and b) to analyze sporadic clear-cell RCCs for reduced levels of CK2 function.

Unraveling the mechanism behind cilia regulation by pVHL

The group of Maxwell (London, UK) was the first to publish the pVHL-cilia connection [8]. By using retroviral transductions of full-length pVHL in *VHL*-negative RCC cells, they observed increased numbers of primary cilia, and suggested this was dependent on HIF1/2α expression, since siRNAs targeting HIF1α and HIF2α separately resulted in increased cilia numbers in *VHL*-negative RCC cells. Other research groups, including us, show that pVHL is able to regulate primary cilia

formation and maintenance in a HIFα-independent manner [9-12]. The discussion is still open concerning the HIFα-involvement in this respect. It might be possible that pVHL forms and maintains primary cilia via a dual pathway, with respect to HIFα dependency.

Trying to unravel the mechanism behind cilia regulation by pVHL, we performed yeast-2-hybrids to identify novel interactors of pVHL with known cilia regulation function. We identified KIF3A in our screen, and were able to confirm the pVHL/KIF3A interaction endogenously via immunoprecipitations (Chapter 5). KIF3A, subunit of the kinesin-2 microtubule motor complex, is involved in ciliogenesis by transporting proteins, including the IFT particles and the Par3-Par6-aPKCζ complex [13] along the ciliary axoneme to the ciliary tip [13]. Supportive of the cilia-cyst hypothesis, a mouse model with a targeted deletion for *KIF3A* in the kidney results in kidney cyst formation [14]. We found that pVHL binds all kinesin-2 subunits and found that KIF3A is necessary for pVHL to bind microtubules. This interaction does not affect KIF3A stability nor affects the E3 ubiquitin ligase function of the pVHL-ElonginC/B-Cul2 complex (Chapter 5). We conclude that KIF3A is not a classical target for pVHL, but might be essential for pVHL's functioning in regulating cilia. The domains within pVHL important for binding kinesin-2 have been identified to be a) the N-terminal domain, previously described to be required for tumor suppression [7], which binds KAP3, and b) the microtubule-binding domain [4], also shown to exert a tumor suppressor function [15], which binds KIF3A (Chapter 5) [16]. These findings correlate well with the regions and mutations of pVHL described as being deficient in stabilizing microtubules [4] and ciliary function [9].

Together, these data suggest that kinesin-2 is involved in tumor suppression by pVHL. Notably, the only protein reported to bind the N-terminal acidic domain of pVHL, kinesin-2 subunit KAP3 (Chapter 5), has been shown to repress malignant transformation of neural progenitor cells, in the manner of a tumor suppressor [17]. Whether these two tumor suppressors influence each others' function has yet to be determined. Furthermore, it is tempting to analyze the mutational status of kinesin-2 in *VHL*-associated tumors, sporadic clear-cell RCCs, kidney/pancreatic cysts or pheochromocytoma, suggesting a model in which kinesin-2 walks hand-in-hand with pVHL to prevent cyst and tumor formation.

Interestingly, Schermer *et al*. found that pVHL interacts with the Par3-Par6-aPKCζ polarity complex. Surprisingly, they were unable to detect KIF3A in this complex, known to transport this complex along microtubules via direct binding to Par-3 controlling cell polarity [18]. Furthermore, they also found that pVHL controls correct orientation of microtubules in interphase cells [11]. Incorrect microtubule orientation, with consequent kinesin-2 disturbances, might result in improper localization of organelles, polarity proteins and/or signaling molecules which may affect major signaling pathways and polarizing events, e.g. junction formation, known to be regulated by pVHL [19]. Unpublished observations (by Laura Daenen and myself), regarding improper localization of Par3-Par6-aPKCζ in *VHL*-negative RCC cells, point towards this model. Further studies, e.g. siRNA experiments, need to be conducted to analyze the (positive) effect of pVHL on kinesin-2 plus-end directed transport.

Thoma *et al*. [12] show that the primary cilium-maintenance signaling network requires either pVHL or signaling via GSK3β. Inactivation of either pVHL or GSK3β alone did not affect cilia maintenance, however combined inactivation of these components leads to loss of cilia in RCC cells. Furthermore, they showed that *VHL* mutation and GSK3β inactivation (through serine-9 inhibitory phosphorylation) is

correlated with a reduction in the frequency of cilia in epithelial cells lining kidney cysts in VHL patients. These findings suggest that secondary mutations at non-*VHL* loci resulting in GSK3β inactivation are required to lose cilia maintenance function.

The connection between GSK3β and pVHL has been recently identified. GSK3β phosphorylates pVHL at serine-68 after a priming-phosphorylation by CK1 at serine-72. Phosphorylation of pVHL by GSK3β inhibits local microtubule stabilizing function of pVHL, however not binding to microtubules [20]. These data suggest that GSK3β does not affect VHL binding to kinesin-2, however might affect the functionality of this complex.

In Chapter 6, we attempt to study the effect of kinesin-2 binding and GSK3β phosphorylation on the mobility of cytoplasmic pVHL. In these experiments we removed ATP to block microtubule motor/kinase activity, and identified that ~50% of cytoplasmic pVHL30 is mobilized via ATP-dependent mechanisms, while the other half remains diffusely cytoplasmic. *VHL* disease alleles displaying less binding to kinesin-2 (Chapter 5) respond significantly less to ATP-depletion, and are not immobilized at the centrosome. RNAi targeting KIF3A or a dominant negative for pVHL-KIF3A binding (ΔN-KAP3) also affect the response to ATP-depletion, indicating that kinesin-2 regulates a subpopulation (~50%) of cytoplasmic pVHL30. However, a phosphorylation mutant of pVHL (S68/72A) responded like wildtype pVHL, indicating that the mobility of pVHL is not dependent on phosphorylation via GSK3β, HIFα stability (as shown with S111N), or the E3 ubiquitin ligase function of pVHL (as shown with R167Q). Since many VHL patients suffer from cyst and tumor development, possibly as a result of defects in ciliary regulation/peripheral microtubule stability, it is tempting to analyze whether treatment with low-dose microtubule stabilizing drugs, like Paclitaxel, prevents cyst and/or tumors in these patients.

Cilia regulation related to genotype/phenotype correlation in VHL disease?

The recent discoveries by multiple groups that pVHL regulates primary cilia has immediately led to the analysis of *VHL* disease associated alleles in their ability to regulate primary cilia. Recent observations by our group and by the Krek lab in Zurich indicate that *VHL* type 2A disease alleles Y98H and Y112H have lost their ability to regulate cilia [9, 12] however, this is not observed by Lutz and Burk (New York) [10]. *VHL* type 2B mutations (Y98N and Y112N) [12] and type 2C mutations (V84L and L188V) [8] seem to retain their ability to regulate primary cilia, however Lutz and Burk show that type 2B mutation W117R is unable to regulate cilia in RCC cells [10]. Furthermore, the only *VHL* type 1 disease allele tested so far (N78S) has lost the ability to regulate primary cilia [8]. Unfortunately, different culture conditions and RCC cells, displaying different HIF1/2α expression, have been used for these experiments. Further studies will establish uniform conditions in which cilia regulation by VHL disease mutated alleles can be comprehensively addressed.

***VHL* loss: losing cilia and increasing cell proliferation?**

In Chapter 3 we describe that ciliary function is essential in guiding downstream signaling, such as planar cell polarity (PCP), Sonic hedgehog (Shh) or PDGFRα-signaling. Loss of a ciliary component might therefore trigger an aberrant signaling response affecting one or more transcriptional program. An example of this is KIF3A which, when deleted in mouse embryos, produces a robust Shh-signaling defect [21]. Interestingly, sporadic clear-cell RCCs show an increased expression of “classic ”Shh-targets, including PAX2 [22], SNAIL [23] and MCM2, the last of which can

be used as a prognostic marker in RCC [24]. One might speculate that intervening with a potential illegitimate Shh signaling activated by pVHL-dependent cilia loss might offer efficient novel therapies for patients with RCC.

Recently, we identified that a direct upstream component of MCM2, transcription factor E2F1, is down-regulated by reconstitution of pVHL in two *VHL*-negative RCC cell lines. In multiple experiments, a robust drop was observed at the mRNA and protein levels, translating to subsequent negative regulation of E2F1 transactivation potential (Chapter 2). The transcription factor E2F1 has a dual role. On one hand, E2F1 is a cell cycle related protein, expressed in late G1 and is responsible for the transactivation of downstream components important for G1/S transition like minichromosome maintenance proteins (MCMs) [25-27], which are components of the DNA-replication initiation complex (RC) [28]. On the other hand, DNA damage or stress will trigger an E2F1 transcriptional program, resulting in the transactivation of proteins involved in the response to DNA damage (e.g. BRCA1, Rad51) or apoptosis (e.g. p73, Apaf-1) [29]. We observed that E2F1 activity is negatively regulated by pVHL in a HIFα-independent manner, arguing that E2F1 is involved in the transactivation of proteins involved in cell cycle progression. Indeed, MCM3 was downregulated after re-introduction of pVHL. However, we were unable to detect cell cycle differences in the RCC cells with or without exogenous pVHL, which might be explained by their tumorigenic highly proliferative background. Interestingly, the *in vitro* regulation of E2F1 by pVHL seems to carry over to the *in vivo* pathobiology; *VHL* disease-associated clear-cell RCCs display significantly higher nuclear E2F1 expression, than in non-*VHL* associated clear-cell RCCs. Given the functional relationship of E2F1 with other factors involved in renal tumorigenesis, including Sp1 [30, 31], c-Myc [32-34], p53 [35] and cyclin D1 [34], future studies will be left to reveal whether increased E2F1 expression and transactivity in *VHL*-associated clear-cell RCCs is resulting from cilia-loss. Furthermore, it will be interesting to determine whether E2F1 is able to trigger a program leading to the accumulation of further genetic abnormalities in kidney epithelial cells and to which extent E2F1 expression relates to cancer progression in *VHL*-associated clear-cell RCCs.

References

1. **Latif, F. *et al.*** Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 260, 1317-1320 (1993).
2. **Frew, I.J. & Krek, W.** Multitasking by pVHL in tumour suppression. *Curr Opin Cell Biol* 19, 685-690 (2007).
3. **Bluyssen, H.A. *et al.*** Fibronectin is a hypoxia-independent target of the tumor suppressor VHL. *FEBS Lett* 556, 137-142 (2004).
4. **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* 5, 64-70 (2003).
5. **Iliopoulos, O., Ohh, M. & Kaelin, W.G., Jr.** pVHL19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. *Proc Natl Acad Sci U S A* 95, 11661-11666 (1998).
6. **Schoenfeld, A., Davidowitz, E.J. & Burk, R.D.** 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 U S A* 95, 8817-8822 (1998).

7. **Lolkema, M.P. *et al.*** Tumor suppression by the von Hippel-Lindau protein requires phosphorylation of the acidic domain. *J Biol Chem* 280, 22205-22211 (2005).
8. **Esteban, M.A., Harten, S.K., Tran, M.G. & Maxwell, P.H.** Formation of primary cilia in the renal epithelium is regulated by the von Hippel-Lindau tumor suppressor protein. *J Am Soc Nephrol* 17, 1801-1806 (2006).
9. **Lolkema, M.P. *et al.*** Allele-specific regulation of primary cilia function by the von Hippel-Lindau tumor suppressor. *Eur J Hum Genet* 16, 73-78 (2008).
10. **Lutz, M.S. & Burk, R.D.** Primary cilium formation requires von hippel-lindau gene function in renal-derived cells. *Cancer Res* 66, 6903-6907 (2006).
11. **Schermer, B. *et al.*** The von Hippel-Lindau tumor suppressor protein controls ciliogenesis by orienting microtubule growth. *J Cell Biol* 175, 547-554 (2006).
12. **Thoma, C.R. *et al.*** pVHL and GSK3beta are components of a primary cilium-maintenance signalling network. *Nat Cell Biol* 9, 588-595 (2007).
13. **Hirokawa, N., Tanaka, Y., Okada, Y. & Takeda, S.** Nodal flow and the generation of left-right asymmetry. *Cell* 125, 33-45 (2006).
14. **Lin, F. *et al.*** Kidney-specific inactivation of the KIF3A subunit of kinesin-II inhibits renal ciliogenesis and produces polycystic kidney disease. *Proc Natl Acad Sci U S A* 100, 5286-5291 (2003).
15. **Datta, K., Sundberg, C., Karumanchi, S.A. & 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* 61, 1768-1775 (2001).
16. **Lolkema, M.P. *et al.*** The von Hippel-Lindau tumour suppressor interacts with microtubules through kinesin-2. *FEBS Lett* 581, 4571-4576 (2007).
17. **Teng, J. *et al.*** The KIF3 motor transports N-cadherin and organizes the developing neuroepithelium. *Nat Cell Biol* 7, 474-482 (2005).
18. **Nishimura, T. *et al.*** Role of the PAR-3-KIF3 complex in the establishment of neuronal polarity. *Nat Cell Biol* 6, 328-334 (2004).
19. **Calzada, M.J. *et al.*** von Hippel-Lindau tumor suppressor protein regulates the assembly of intercellular junctions in renal cancer cells through hypoxia-inducible factor-independent mechanisms. *Cancer Res* 66, 1553-1560 (2006).
20. **Hergovich, A. *et al.*** Priming-dependent phosphorylation and regulation of the tumor suppressor pVHL by glycogen synthase kinase 3. *Mol Cell Biol* 26, 5784-5796 (2006).
21. **Huangfu, D. *et al.*** Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature* 426, 83-87 (2003).
22. **Hueber, P.A., Waters, P., Clark, P., Eccles, M. & Goodyer, P.** PAX2 inactivation enhances cisplatin-induced apoptosis in renal carcinoma cells. *Kidney Int* 69, 1139-1145 (2006).
23. **Li, X. *et al.*** Snail induction is an early response to Gli1 that determines the efficiency of epithelial transformation. *Oncogene* 25, 609-621 (2006).
24. **Rodins, K., Cheale, M., Coleman, N. & Fox, S.B.** Minichromosome maintenance protein 2 expression in normal kidney and renal cell carcinomas: relationship to tumor dormancy and potential clinical utility. *Clin Cancer Res* 8, 1075-1081 (2002).
25. **Tsuruga, H. *et al.*** HsMCM6: a new member of the human MCM/P1 family

encodes a protein homologous to fission yeast Mis5. *Genes Cells* 2, 381-399 (1997).
26. **Leone, G. *et al.*** E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev* 12, 2120-2130 (1998).
27. **Ohtani, K. *et al.*** Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. *Oncogene* 18, 2299-2309 (1999).
28. **Maiorano, D., Lutzmann, M. & Mechali, M.** MCM proteins and DNA replication. *Curr Opin Cell Biol* 18, 130-136 (2006).
29. **Tsantoulis, P.K. & Gorgoulis, V.G.** Involvement of E2F transcription factor family in cancer. *Eur J Cancer* 41, 2403-2414 (2005).
30. **Karlseder, J., Rotheneder, H. & Wintersberger, E.** Interaction of Sp1 with the growth- and cell cycle-regulated transcription factor E2F. *Mol Cell Biol* 16, 1659-1667 (1996).
31. **Lin, S.Y. *et al.*** Cell cycle-regulated association of E2F1 and Sp1 is related to their functional interaction. *Mol Cell Biol* 16, 1668-1675 (1996).
32. **Leone, G. *et al.*** Myc requires distinct E2F activities to induce S phase and apoptosis. *Mol Cell* 8, 105-113 (2001).
33. **O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V. & Mendell, J.T.** c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 435, 839-843 (2005).
34. **Oswald, F., Lovec, H., Moroy, T. & Lipp, M.** E2F-dependent regulation of human MYC: trans-activation by cyclins D1 and A overrides tumour suppressor protein functions. *Oncogene* 9, 2029-2036 (1994).
35. **Powers, J.T. *et al.*** E2F1 uses the ATM signaling pathway to induce p53 and Chk2 phosphorylation and apoptosis. *Mol Cancer Res* 2, 203-214 (2004).

Nederlandse Samenvatting

Curriculum Vitae

List of Publications

Dankwoord

Nederlandse Samenvatting

Dit proefschrift behandelt de vraag hoe de uitschakeling van het *von Hippel-Lindau* (*VHL*) gen kan leiden tot het ontstaan van tumoren. De erfelijke ziekte waarbij het *VHL* gen betrokken is, is in het begin van de 20ste eeuw ontdekt door Dr. Eugen von Hippel en later beschreven door Dr. Arven Lindau. Hen was opgevallen dat in een aantal patiënten tumoren voorkomen in de ogen, het centraal zenuwstelsel en de nieren. De patiënten met deze met name vaatrijke tumoren bleken allen familie van elkaar te zijn, suggererend dat het ontstaan van deze tumoren een erfelijke of familiare oorzaak heeft. De ziekte VHL is in de loop van de vorige eeuw verder beschreven en kenmerkt zich onder andere door: bloedvatgezwellen (hemangiomen) van het netvlies en centraal zenuwstelsel (met name kleine hersenen en ruggemerg), niercel tumoren, bijniertumoren (pheochromocytomen), alvleesklier tumoren, binnenoor tumoren en cysten in de nieren, alvleesklier en (bij mannen) in de bijballen. Uit het kapsel van een cyste kan een gezwel ontstaan: een cystadenoom. Deze gezwellen zijn meestal goedaardig en kunnen bij VHL patiënten ontstaan in de bijbal en soms in de alvleesklier. De gevolgen van deze aandoeningen kunnen ernstig invaliderend zijn voor de patiënt, door functie verlies van aangedane organen zoals de nieren, kleine hersenen of ogen.

Een tumor kan ontstaan doordat een bepaald gen, een stuk DNA wat codeert voor de uiteindelijke vorming van een eiwit, zijn functie meer of beter uitvoert. Dit noemt men een oncogen. Voorbeelden hiervan zijn genen die de cel aanzetten tot celdeling. Hierbij is het vaak zo dat één gemuteerde kopie van dit gen (welke altijd actief is), leidt tot ongeremde celdeling wat uiteindelijk kan resulteren in de vorming van een tumor. *VHL* is echter een gen dat bij normale functie juist de ontwikkeling van tumoren onderdrukt. *VHL* behoort hiermee tot de klasse van tumor suppressor genen, waarvan wordt aangenomen dat een goed functionerende kopie van dit gen voldoende is om tumoren te voorkomen. Echter, de inactivatie van een tumor suppressor gen leidt in het algemeen tot het actief worden van meerdere processen die tot de vorming van een tumor kunnen leiden. Wanneer mensen een ‘foute’ kopie van een tumor suppressor gen van hun ouders erven, hoeft er nog maar één kopie te worden veranderd om een normale cel tot een tumor cel te transformeren. In alle cellen die gedurende het leven celdelingen doormaken worden fouten gemaakt bij het ‘overschrijven’ van de informatie in het DNA. Deze toevallige fouten leiden normaliter gesproken niet tot problemen omdat de kans dat twee kopieën van een tumor suppressor gen tegelijkertijd worden aangedaan in een lichaamscel uitermate klein is. Echter, als iedere cel al een ‘foute’ kopie bij zich draagt, wordt de kans op inactivatie van beide kopieën van een tumor suppressor gen in een lichaamscel een stuk groter. Deze ‘tweede hit’ zou verklaren waarom mensen met één ‘foute’ kopie van een tumor suppressor gen, een verhoogde kans hebben op het krijgen van een tumor. Sinds de ontdekking dat de inactivatie van het *VHL* gen op chromosoom 3, verantwoordelijk is voor het ontstaan van VHL ziekte in 1988 is sindsdien veel onderzoek gedaan naar de functies van dit gen. *VHL* codeert voor twee von Hippel-Lindau eiwitten; een lange vorm (pVHL30) en een korte vorm (pVHL19); samen pVHL genoemd. Een belangrijke functie van pVHL heeft betrekking op het afbreken van Hypoxia Inducible Factor (HIF)α eiwitten. Deze eiwitten zijn belangrijk voor de nieuwvorming van bloedvaten (angiogenese) in een zuurstofarme omgeving. Mutaties in *VHL* zorgen er echter voor dat ook in een zuurstofrijke omgeving angiogenese kan optreden

door een verhoogde stabiliteit van HIFα eiwitten.

In **hoofdstuk 2** beschrijven we de rol van pVHL30 in het verlagen van de expressie van E2F1 in niercellen. Het E2F1 eiwit functioneert als transcriptie factor voor een scala aan eiwitten, betrokken bij enerzijds celdeling en anderzijds bij de respons op cellulaire DNA schade of stress. Een verlaging van E2F1 expressie door pVHL30 is zichtbaar op mRNA en eiwit niveau. Daarnaast wordt de activiteit van E2F1 significant verlaagd door pVHL30, welke onafhankelijk lijkt te zijn van HIFα stabiliteit. *VHL*-geassocieerde niertumoren hebben een significant verhoogde E2F1 expressie in de celkern vergeleken met niet *VHL*-geassocieerde tumoren, wat suggereert dat een verhoogde expressie van E2F1 in de kern betrokken is bij de pathobiologie van de ziekte VHL. In **hoofdstuk 3** geven we een overzicht van de huidige kennis op het gebied van cilia in relatie tot tumor formatie. Een cilium wordt vergeleken met een antenne op de cel, gezien zijn vorm en functie. Een belangrijke functie van het cilium is het registreren van stroming buiten de cel, zoals urine dat langs tubulaire cellen stroomt. Deze tubulaire cellen zijn uiteindelijk de bron voor de vorming van *VHL*-geassocieerde niertumoren. Defecte cilia resulteren in een afwijkende signalering in cellen, wat kan leiden tot de vorming van cysten en lijkt betrokken zijn bij tumor formatie. In **hoofdstuk 4** laten we zien dat pVHL30 betrokken is bij het uitgroeien van een cilium en regulatie van cilium functie, onafhankelijk van HIFα. Mutaties in *VHL*, welke geassocieerd zijn met VHL ziekte, resulteren in de vorming van gemuteerde pVHL eiwitten. Laatstgenoemde eiwitten hebben de cilium regulatie functie verloren en localiseren niet meer in het cilium, evenals pVHL19 wat erop duidt dat de eerste 54 aminozuren van pVHL30 belangrijk zijn voor deze functie. In **hoofdstuk 5** beschrijven we de binding tussen pVHL30 en kinesin-2, een eiwitcomplex welke direct aan microtubules gebonden is. Microtubules zijn buisvormige structuren die te vergelijken zijn met snelwegen van de cel. Over de microtubules vindt transport plaats van een scala aan eiwitten vanaf en naar de celuiteinden. Kinesin-2 is betrokken bij het transport van eiwitten naar de celuiteinden, waaronder (de top van) het cilium. pVHL30 bindt via kinesin-2 aan microtubules. Twee domeinen in pVHL30, waaronder de eerste 54 aminozuren, lijken belangrijk te zijn voor interactie met kinesin-2. Mutaties in deze domeinen, welke tot een verstoorde binding met kinesin-2 resulteren, localiseren niet in het cilium en hebben niet de mogelijkheid om functionele cilia te reguleren. In **hoofdstuk 6** tonen we aan dat de localisatie en mobiliteit van pVHL30 afhankelijk is van kinesin-2. Kinesin-2 gebruikt ATP om over microtubules te lopen. Het verstoren van kinesin-2 functie, via depletie van ATP, resulteert in de immobilisatie van pVHL30. Echter, mutaties in pVHL30 of pVHL19 welke een verstoorde kinesin-2 binding hebben, immobiliseren niet in de afwezigheid van ATP. Dit gedrag lijkt onafhankelijk te zijn van de mogelijkheid om HIFα te binden.

De resultaten in dit proefschrift dragen bij aan een verbeterd inzicht in de functionaliteit van het *VHL* tumor suppressor gen. Het ontstaan van cysten (en mogelijk tumoren) in VHL patiënten zou verklaard kunnen worden door een verstoorde uitgroei en functionaliteit van cilia. Een verstoorde signalering via het cilium is gedetecteerd in onder andere niertumoren, wat nieuwe perspectieven biedt voor de ontwikkeling van therapieën gericht op het aanpakken van deze verstoorde cilium signalering voor VHL patiënten en overige patiënten met (nier)kanker.

Curriculum Vitae

Dorus Mans werd geboren op 4 maart 1978 te Best. Na het behalen van het VWO diploma aan het Heerbeeck College te Best in 1996, begon hij in datzelfde jaar met de studie Microbiologie aan de Fontys Hogescholen te Eindhoven. Na het afronden van deze studie werd in 2000 aangevangen met de studie Medische Biologie aan de Vrije Universiteit te Amsterdam. Gedurende deze studie werd onderzoekservaring opgedaan via een onderzoeksstage in het UCSF Cancer Center te San Francisco (onder begeleiding van Prof. dr. Donna Albertson en Prof. dr. Dan Pinkel). Na het afronden van deze studie werd in juni 2003 gestart met het in dit proefschrift beschreven onderzoek onder begeleiding van Dr. Rachel Giles en Prof. dr. Emile Voest. Dit onderzoek is uitgevoerd op de afdeling Medische Oncologie van het Universitair Medisch Centrum Utrecht. Per 1 april 2008 zal Dorus Mans zijn onderzoek voortzetten in de groep van Dr. Ronald Roepman aan het UMC St. Radboud te Nijmegen.

List of Publications

1. Mans DA, Voest, EE, and Giles RH.
All along the watchtower: is the cilium a tumor suppressor organelle?
BBA - Reviews on Cancer, In press

2. Mans DA, Lolkema MP, van Beest M, Daenen LG, Voest EE, and Giles RH.
Mobility of the von Hippel-Lindau tumour suppressor protein is regulated by kinesin-2.
Experimental Cell Research, In press

3. Mans DA, Lolkema MP, Ulfman LH, Volpi S, Voest EE, and Giles RH.
Allele-specific regulation of primary cilia function by the von Hippel-Lindau tumor suppressor.
European Journal of Human Genetics (2008) Jan;16(1):73-8

4. Mans DA, Lolkema MP, Snijckers CM, van Noort M, van Beest M, Voest EE, and Giles RH.
The von Hippel-Lindau tumour suppressor interacts with microtubules through kinesin-2.
FEBS Letters (2007) Oct 2;581(24):4571-6

5. Giles RH, Lolkema MP, Snijckers CM, Belderbos M, van der Groep P, Mans DA, van Beest M, van Noort M, Goldschmeding R, van Diest PJ, Clevers H, and Voest EE.
Interplay between VHL/HIF1alpha and Wnt/beta-catenin pathways during colorectal tumorigenesis.
Oncogene (2006) May 18;25(21):3065-70

6. Snijders AM, Fridlyand J, Mans DA, Segraves R, Jain AN, Pinkel D, and Albertson DG.
Shaping of tumor and drug-resistant genomes by instability and selection.
Oncogene (2003) Jul 10;22(28):4370-9

Dankwoord

Dit is de gelegenheid om die mensen te bedanken die op zijn/haar manier een bijdrage hebben geleverd aan het tot stand komen van dit proefschrift. Allereerst wil ik mijn directe begeleider en co-promotor Rachel Giles bedanken. Rachel, we hebben 't uiteindelijk gefixed: pVHL/kinesin-2/cilia is publiceerbaar! Het begeleiden van je eerste AIO, is voor ons allebei een learning curve geweest. Bedankt voor je enthousiasme, creativiteit, dynamiek en gezelligheid. Je hebt me overtuigd van de charme van het cilia-onderzoek. Veel succes met het *vhl1*/*lrrc50* project en met je carrière naar de top! Ten tweede wil ik mijn promotor Emile Voest bedanken. Emile, als geen ander weet je klinische expertise te verenigen met kennis van de moleculaire biologie. Bedankt voor je enthousiasme, helikopterview en vertrouwen!

Alle (oud)collega's van het lab Experimentele Oncologie hebben bijgedragen aan een gezellige tijd in het lab. Beste René, drie jaar geleden ben je met je onderzoeksgroep naar Utrecht verhuisd en dat heeft ons allen goed gedaan. Je scherpte, creativiteit en feedback zijn een motivatie voor iedere onderzoeker. Bedankt!
Sander, helaas heb ik maar even van je zangtalenten kunnen genieten...(d'oh!)... bedankt voor de gezellige momenten en overheerlijke stukken taart! Marlies, Joost en Jeanine, het onderzoek naar de perfecte biomarker voor (nier)kanker is door jullie in volle hevigheid losgebarsten. Succes met het vervolg van jullie promotie-onderzoek! Ellen en Ive, bedankt voor de mooie (*vhl1*) visverhalen op donderdagochtend. Henk en Kristy, succes in het VUMC! M'n oud-kamergenoten: Marvin, golfend of niet, je slaat je door je promotie heen. Je enthousiasme over microtubules is volkomen terecht! Wanneer gaan we nu eens samen publiceren? Rob, bedankt voor het cheffen van cd's/dvd's en je duikverhalen/foto's. Wanneer komt er een labuitje, onder water? Libor, the cloning expert. When will someone call for you? Good luck with your projects! Renske, je bent er bijna. Succes met de laatste loodjes! Susanne L., je hebt met verve je eerste AIO afgeleverd; hopelijk gaan er nog veel meer volgen! Patrick, succes met het opzetten van je eigen groep in Utrecht! Miranda en Ron, succes met jullie project! Aniek en Martijn V., bedankt voor de 'brabantse gezelligheid'! Arne en Anneloes succes met jullie onderzoek! Monica, do you really like the Simpsons? Good luck with your research! Livio, waarom hebben we een radio, we hebben jou toch? Bedankt voor de gezelligheid. Noëline en Sylvana, bedankt voor het uit handen nemen van veel administratief werk.

De mannen van de chirurgie. Onno, bedankt voor je goede kritische blik op biochemische proeven. Ga door met het scoren van mooie papers en doelpunten; wanneer sta je weer in het Parool? Frederik, bedankt voor een fijne tijd in en buiten het lab. Succes met de proeven! Winan, je gaat als een trein. Over 9 maanden het volgende project afronden? Menno, Benjamin, Maarten, Nikol en Angelique: bedankt voor een leuke tijd in het lab. Succes met jullie onderzoek! Judith, succes met het opzetten van je eigen groep! Stephanie, een fijn promotie-traject toegewenst. Geert, je hebt een vliegende start gemaakt in Utrecht. Succes met het uitbouwen van je groep! Nannette, San Francisco, Utrecht... wie weet waar we elkaar de volgende keer treffen? Saskia en Merel succes met jullie project!

Verder wil ik alle oud-collega's bedanken voor hun bijdrage aan dit proefschrift. Cristel, je lijkt het wel heel gezellig te vinden op de derde verdieping van het UMC. Bedankt voor een gezellige tijd in het lab! Elianne, Niven, Niels eindelijk ben ik aan de beurt! Bedankt voor de gezelligheid in en buiten het lab! Stefano, eeeh, it was fun to have you around. When shall we drink a beer together? Moniek, je hebt me

in de wereld van de biochemie geïntroduceerd. Eindelijk hebben we de IPs gepubliceerd! Mascha, bedankt voor de vele microtubule co-seds die je gedraaid hebt. Het Krek lab had het dus toch bij het verkeerde eind! Gerben, ik heb zo'n donkerbruin vermoeden...dat je je draai vindt in Boston, samen met Barbara. Succes met jullie carrière! Laura, de stabiele RCC10 clones zijn een goudmijn. Bedankt voor al je hulp en succes met de laatste loodjes in Toronto! Susanne van S., bedankt voor het kloneren van al die *VHL* mutanten en de gezelligheid die je bracht in ons lab! Maak er een mooie tijd van in Dundee! Jamila en Veronica, good luck with your research in Amsterdam and New York, respectively! Arna, IP expert, bedankt dat ik je mocht begeleiden gedurende je stage! Bettina, Barend, Colinda, Laurens, Maartje, Arie, Haiko, Anita, Yvonne, Carin, Marcel, Marie, Alexandra, Jarmila, Liesbeth, Marjolein en Mirjam bedankt voor een leuke tijd! De labs van Bos, Burgering, Timmers, Holstege, van Diest en de Bruin: bedankt voor jullie wetenschappelijke input!

Verder wil ik vrienden en familie bedanken voor hun steun. Ruud, de omslag en boekenlegger zien er super uit; bedankt! Nico en Ann, wat was het een mooie tijd in Californië en later in de Caribbean! Relaxen en over onderzoek babbelen: een ideale combi. Bedankt! Antoine and Sindy, I had a great time with you guys in San Francisco. Thanks a lot! Mark en Koen: bedankt voor de mooie momenten in de Lange Nieuwstraat 74. Dennis, we zijn uiteindelijk allebei goed terecht gekomen. Bedankt voor je vriendschap! Bertil, Els, Annemieke en Johan, enorm bedankt voor jullie steun tijdens m'n promotie! De XPS-M1210 is een uitkomst! Gert, Marja, Maikel en Imara, bedankt voor jullie interesse! Ans en Jan, de bevalling zit er op! Bedankt voor jullie steun! Verder wil ik mijn paranimfen Erik en Martijn bedanken. Erik, je bent diegene die me met succes door het VWO heeft gesleurd! Van tafelvoetbal spelen tot laat in de kroeg hangen. 'Meneer van der Wal en buurman' zal ik niet snel vergeten... Martijn, jouw creativiteit heeft ons beide niet onberoerd gelaten. Uiteindelijk heeft pVHL/kinesin-2 ons voor veel jaren (en nog steeds) bezig gehouden. Bedankt voor de vele discussies en mooie tijd!

Oma, bedankt voor zoveel vertrouwen, gezelligheid en vele 'bekskes koffie' in de afgelopen jaren! Fijn dat u dit mee mag maken! Een promotietraject is zoals GTST, met ups en downs, maar gelukkig eindigt het dit keer positief! Pap en mam, jullie vertrouwen, steun, liefde en positiviteit heeft me geholpen deze periode met succes af te ronden! Bedankt voor alles! Liggend op de verwarming, terwijl ik aan het typen ben, onze harige huisgenoot Jip. Met jou is het typen zelfs een leuke aangelegenheid geworden! Tot slot wil ik diegene bedanken die onvoorwaardelijk achter me staat en er altijd voor me is! Marjolijn, we hebben het samen voor elkaar gebokst! Het zit er echt op! Ik kijk uit naar een toekomst samen met jou!

Dorus