TIMELINE

VHL, the story of a tumour suppressor gene

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Abstract | Since the Von Hippel–Lindau (VHL) disease tumour suppressor gene *VHL* was identified in 1993 as the genetic basis for a rare disorder, it has proved to be of wide medical and scientific interest. VHL tumour suppressor protein (pVHL) plays a key part in cellular oxygen sensing by targeting hypoxia-inducible factors for ubiquitylation and proteasomal degradation. Early inactivation of *VHL* is commonly seen in clear-cell renal cell carcinoma (ccRCC), and insights gained from the functional analysis of pVHL have provided the foundation for the routine treatment of advanced-stage ccRCC with novel targeted therapies. However, recent sequencing studies have identified additional driver genes that are involved in the pathogenesis of ccRCC. As our understanding of the importance of *VHL* matures, it is timely to review progress from its initial description to current knowledge of *VHL* biology, as well as future prospects for novel medical treatments for VHL disease and ccRCC.

The story of von Hippel–Lindau (VHL) disease and the *VHL* tumour suppressor gene (TSG) provides a paradigm that illustrates the relevance of studying rare inherited cancer syndromes to gain novel insights into the underlying biology of human cancers and to aid the development of therapies. Throughout most of the twentieth century, VHL disease was considered to be a rare curiosity that was of little general interest to clinicians and that had no known relevance to wider studies of cancer. However, the identification in 1993 of inherited mutations in the *VHL* gene in families with VHL disease represented the first of a series of seminal findings that have led to the *VHL* TSG assuming a central place in our current understanding of the mechanisms of cellular oxygen sensing and the pathobiology of clear-cell renal cell carcinoma (ccRCC). Here, we review the background and timelines of this narrative, and discuss the opportunities for novel therapeutic interventions in ccRCC and beyond (FIG. 1).

VHL disease

VHL disease is a hereditary, autosomal-dominant, neoplastic disease that is associated with various tumour types, including ccRCCs, central nervous system (CNS) and retinal haemangioblastomas, phaeochromocytomas (PCCs) and pancreatic neuroendocrine tumours, in addition to pancreatic and renal cysts. Ocular manifestations of the syndrome were described by Treacher Collins in 1894 (REF. 1). The name VHL disease was coined in 1936 and originates from the initial description of retinal angiomatosis in 1904 by Eugen von Hippel[2] and cerebellar and spinal haemangioblastomas in 1927 by Arvid Lindau[3]; the term has been in common use since the 1970s[4]. Clinical diagnostic criteria introduced in 1964 (REF. 5) enabled a diagnosis of VHL disease in patients who had two tumours (such as two haemangioblastomas or a haemangioblastoma and a visceral tumour) and no family history of VHL disease, and in patients who had only one tumour but family history of VHL disease. Phenotypic heterogeneity in VHL disease was first shown in 1991 (REFS 6,7). Clinically, VHL disease has been classified into type 1 or type 2 depending on the absence or presence of PCCs, respectively[8]. Type 2 VHL disease is further subdivided into type 2A (with PCCs but without RCCs), type 2B (with PCCs and RCCs) and type 2C (with PCCs only)[9] (TABLE 1). Although this classification facilitates genotype–phenotype studies (discussed below), it has limited clinical utility because families can move between subtypes: for example, from type 1 to type 2 or from type 2C to type 2B.

Identification of *VHL*

As early as 1990, a comparison of age–incidence curves for the sporadic, non-heritable forms of cerebellar haemangioblastoma and ccRCC with those for the familial forms of these tumours occurring in VHL disease indicated that the curves for tumours in VHL disease are compatible with a single, rate-limiting mutation ('one-hit') model, whereas the curves for sporadic tumours are compatible with a 'two-hit' model. This finding implicated a recessive TSG (similar to the retinoblastoma TSG, *RB1*) in the pathogenesis of these disorders[10], and the *VHL* gene, located on the short arm of chromosome 3 at cytoband 3p25–26, was identified by positional cloning in 1993 (REF. 11). Patients with VHL disease harbour a single mutant allele, and tumour development depends on the spontaneous inactivation or loss of the second, wild-type *VHL* allele. Early evidence that the *VHL* gene is a TSG came from studies of loss of heterozygosity (LOH) that showed that inactivation of both *VHL* alleles is a crucial event in the development of neoplasms in VHL disease[12,13] and sporadic non-hereditary ccRCC[14]. Almost two decades ago, studies of *VHL*-negative ccRCC cells provided biological evidence for a tumour suppressor role for the *VHL* gene product (pVHL). Reintroduction of wild-type, but not mutant, pVHL into a *VHL*-null ccRCC cell line had no demonstrable effect on cell growth *in vitro* but inhibited the ability to form tumours in nude mice[15] and restored the ability to exit the cell cycle and enter quiescence in low serum[16].

Characterization of *VHL* and pVHL

The *VHL* gene encodes two isoforms of pVHL; a 213-amino-acid, 30 kDa form ($pVHL_{30}$) and a 160-amino-acid, 19 kDa form ($pVHL_{19}$)[17–19]. $pVHL_{19}$ lacks a

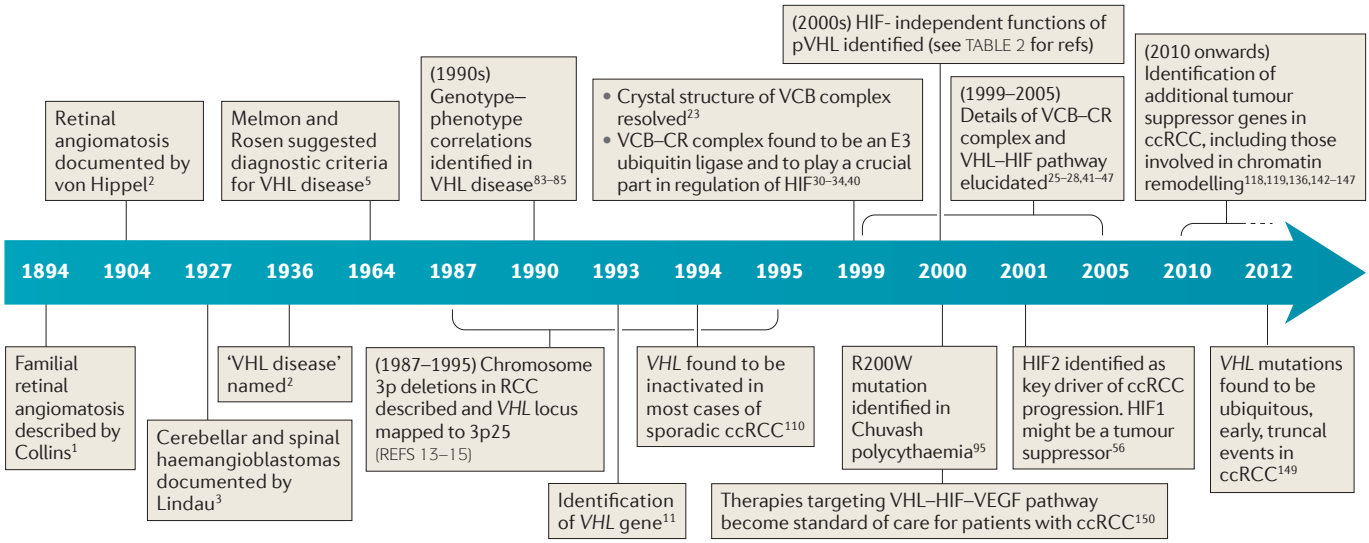

Figure 1 | **History of research on the von Hippel–Lindau (*VHL*) gene.** ccRCC, clear-cell renal cell carcinoma; HIF, hypoxia-inducible factor; VCB, pVHL–elongin C–elongin B; VCB–CR, pVHL–elongin C–elongin B–cullin 2–RBX1; VEGF, vascular endothelial growth factor.

53-amino-acid amino-terminal pentameric acid repeat domain and predominates in many tissues. Early functional studies suggested that the two isoforms have equivalent effects in assays[18], and that both isoforms have tumour suppressor activity *in vivo*[19].

pVHL structure and the VCB complex. Biochemical studies in the mid-1990s revealed that pVHL forms a ternary complex with the transcription elongation factors C and B (also known as elongin C and elongin B) termed the VCB complex[20–22]. This complex is crucial for pVHL function, and its structure was resolved in 1999 (REF. 23). pVHL consists of two tightly coupled domains, α and β (FIG. 2); the β-domain consists of two β-sheets arranged as a sandwich with an α-helix on top, whereas the α-domain, which directly contacts elongin C, consists of three α-helices. The VCB complex nucleates a complex containing cullin 2 (CUL2) and the RING finger protein RBX1 (forming the VCB–CR complex)[24–26].

Together, elongin B and elongin C act as adaptors that link the substrate-recognition subunit of the VCB–CR complex (pVHL, which binds to substrates through its β-domain) to heterodimers of CUL2 and RBX1. pVHL is stabilized by associating with elongins B and C and, in turn, elongins B and C are stabilized through their interactions with each other and with pVHL[27]. The entire VCB complex is thus resistant to proteasomal degradation. By contrast, pVHLs harbouring mutations that disrupt elongin binding are unstable and rapidly degraded by the proteasome[20–22,24]. Recently, mutations in *TCEB1* (which encodes elongin C) affecting the domains in elongin C that bind to pVHL have been described in ccRCC[28], supporting the hypothesis that the tumorigenic effects of *VHL* mutations relate to dysfunction of the VCB complex as a whole rather than dysfunction of pVHL alone.

In 1998–1999 it was noted that, structurally, the VCB–CR complex resembles yeast Skp1–Cdc53–F-box protein (SCF) ubiquitin ligases[23,24]. Subsequent work showed that, functionally, both the VCB–CR complex and the SCF complex have ubiquitin ligase activity and are capable of targeting proteins for proteasomal degradation[29–33].

pVHL and HIFs. In the mid-1990s it was noted that the highly vascular tumours associated with VHL disease overproduce angiogenic polypeptides such as vascular endothelial growth factor (VEGF)[34–36]. The first biochemical evidence that pVHL might have a critical role in the transduction of signals generated by changes in ambient oxygen tension came in 1996; ccRCC cells lacking wild-type pVHL were noted to produce mRNAs encoding VEGF, glucose transporter 1 (GLUT1; also known as SLC2A1) and platelet-derived growth factor subunit B (PDGFB) under both normoxic and hypoxic conditions[37,38]. Reintroduction of wild-type but not mutant pVHL into these cells specifically inhibited production of these mRNAs under normoxic conditions, thus restoring their previously described hypoxia-inducible profile. In 1999, Maxwell *et al.* were the first to demonstrate a crucial role for pVHL in the regulation of hypoxia-inducible factor 1α (HIF1α)[39], and over the next 5 years the details of the pVHL–HIF pathway and the role of the VCB–CR complex in targeting HIFs for polyubiquitylation and proteasomal degradation were elucidated (FIG. 3).

The crystal structure of the VCB complex bound to the HIF1α carboxy-terminal oxygen-dependent degradation domain supported previous data[32] showing that the HIFα (HIF1α or HIF2α (also known as EPAS1)) peptide binds exclusively to the β-domain of pVHL[40,41]. This binding is dependent on hydroxylation of two conserved proline residues within HIFα by prolyl hydroxylase 1 PHD1 (also known as EGLN2), PHD2 and PHD3, which require oxygen as a co-substrate and are thus only active under normoxic conditions[41–45] (FIG. 3). Prolyl-hydroxylation of HIFα enables its recognition and ubiquitylation by the VCB–CR complex, and polyubiquitylated HIFs are recognized and degraded by the cellular proteasome. Under hypoxic physiological conditions (or in the absence of functional pVHL), HIFα accumulates and forms heterodimers with HIF1β. These heterodimers translocate to the nucleus, where they bind to hypoxia-response elements (HREs)[46]. According to genome-wide chromatin immunoprecipitation combined with DNA sequencing or mRNA microarray experiments, the number of direct HIF target genes is currently >800 (REFS 47,48), and many of these genes

promote adaptation to acute or chronic hypoxia[49]. HIFs also indirectly regulate gene expression by transactivating genes encoding microRNAs[50] and chromatin-modifying enzymes[47,51–53]. HIFs thus have a crucial role in cellular adaptation to reduced oxygen tension: functional pVHL is necessary to switch off this adaptation under normoxic conditions, and loss of pVHL function as a result of, for example, biallelic inactivation of the *VHL* gene impairs HIFα destabilization. This promotes inappropriate activation of downstream target genes that would normally be activated only under hypoxic conditions and thereby contributes directly to tumorigenesis. Consistent with the notion that regulation of HIFα is the key tumour suppressor function of pVHL, a large proportion of disease-associated *VHL* mutations are predicted to and have been demonstrated to significantly impair the interaction between pVHL and HIF[40,41,54–56].

Other than HIF1α and HIF2α, additional potential pVHL ubiquitylation substrates have been described, including atypical protein kinase C[57] and the large subunit of RNA polymerase II[58], although their significance in ccRCC tumorigenesis is uncertain.

The HIF transcription factors. HIF transcription factors exist as heterodimers with an α-subunit (HIF1α, HIF2α or HIF3α) and a stable β-subunit (HIF1β; also known as aryl hydrocarbon receptor nuclear translocator (ARNT))[46]. Whereas HIF1α is ubiquitously expressed, expression of HIF2α is mainly restricted to endothelial, lung, renal and hepatic cells. Both HIF1α and HIF2α are stabilized and activated by hypoxia and dimerize with HIF1β. Likewise, both isoforms activate transcription of target genes by binding to the same HRE. However, HIF1 and HIF2 are not functionally redundant. Array studies indicate that HIF1 induces apoptotic pathways that are not targeted by HIF2 and preferentially drives the expression of genes that are involved in the glycolytic pathway, whereas HIF2 preferentially promotes growth and angiogenesis[59–61]. Furthermore, the relative contributions of the two paralogues to the control of specific HIF target genes can differ in different cellular contexts[61].

Accumulating evidence from the past 10 years suggests that HIF2, rather than HIF1, is the key driver of renal cancer progression (reviewed in REF. 62). *In vitro* and cell-line xenograft studies suggest that HIF2 is both necessary and sufficient for the growth of transformed *VHL*$^{-/-}$ RCC cell lines and for much of the pathology that has been described in genetically engineered mouse models in which *VHL* has been inactivated in specific tissues[63–70]. By contrast, HIF1 is not merely dispensable in the context of ccRCC but might actually function as a tumour suppressor[9,55,60,68–73]. However, it should be noted that only HIF1, but not HIF2, has so far been shown to promote renal dysplasia and carcinogenesis in mice[74,75]. Interestingly, HIF2, but not HIF1, activates mTOR complex 1 (mTORC1)[76].

HIF-independent functions of pVHL. Although less thoroughly characterized, pVHL also has HIF-independent functions, including assembly and regulation of the extracellular matrix; microtubule stabilization and maintenance of the primary cilium; regulation of apoptosis; control of cell senescence; and transcriptional regulation (TABLE 2). Many of these roles have been discovered through biochemical interactions, but there is also evidence from *VHL* analysis in *Caenorhabditis elegans*[77] and microarray analyses in mammalian cell lines[78–81] that support the notion of HIF-independent gene expression changes induced by *VHL* loss. The extent to which HIF-independent functions of pVHL cooperate with HIF dysregulation in ccRCC tumorigenesis is currently unknown.

Table 1 | **Genotype–phenotype correlations in VHL disease**

VHL disease subtype	Clinical phenotype	Type of *VHL* mutation	HIF expression relative to wild type*
1	ccRCC; haemangioblastoma	Deletion, nonsense, frameshift and missense	↑↑↑
2A	Haemangioblastoma; phaeochromocytoma	Missense	↑
2B	ccRCC; haemangioblastoma; phaeochromocytoma	Missense	↑↑
2C	Phaeochromocytoma	Missense	Normal

ccRCC, clear-cell renal cell carcinoma; HIF, hypoxia-inducible factor; VHL, von Hippel–Lindau.
*'↑' indicates increased relative to wild type.

VHL genotype–phenotype correlations

Interfamilial variations in phenotype, particularly in the frequency of PCCs[82], became well recognized in the 1980s. Since 1993, germline *VHL* mutations have been reported in >900 families with *VHL* disease[83,84], and it has been possible to define genotype–phenotype correlations. The first 53 amino acids of pVHL$_{30}$ show poor evolutionary conservation, and no unequivocal mutations have been reported in this domain[83,84]. The majority of patients with truncating mutations or exon deletions have type 1 VHL disease[84] (that is, no PCCs) (TABLE 1). Interestingly, among those with type 1 VHL disease, a subgroup of patients with a contiguous deletion of all or part of *VHL* and the nearby gene *BRK1* (BRICK1, SCAR/WAVE actin-nucleating complex subunit; also known as *HSPC300* and *C3orf10*) develop retinal and CNS haemangioblastomas but have a low-risk of RCC (sometimes called the type 1B phenotype)[85–88]. Kindreds with type 2 VHL disease usually have a germline missense mutation (84%)[84]; most of these families are further characterized as having type 2B VHL disease. Subsequent analysis has suggested that amino acid substitutions on the protein surface confer a higher risk of PCC than substitution of amino acids buried deep within the protein core[89].

In vitro modelling of pVHL mutations associated with different subtypes of VHL disease suggests that the risk of developing haemangioblastoma or ccRCC is correlated with the ability of mutant pVHL to impair HIF activity[9,55,56]. Whereas type 1 and type 2B VHL disease mutations are grossly defective with respect to HIF regulation, type 2A mutations seem to be far less compromised with respect to HIF1α regulation. By contrast, certain type 2C VHL disease mutations retain their ability to downregulate HIF1α[9,55], implicating HIF-independent mechanisms in the pathogenesis of *VHL*-associated PCCs. Several studies have suggested that patients with nonsense and frameshift mutations have a higher risk of ccRCC and haemangioblastomas than patients with missense mutations[89–91]. It can be speculated that complete loss of pVHL function is lethal or disadvantageous for PCC precursor cells. In addition to the phenotypic variability associated with allelic heterogeneity, genetic modifiers might influence the phenotypic expression of VHL disease[79,92,93].

VHL and congenital polycythaemias

Almost a decade after the identification of heterozygous germline *VHL* mutations in VHL disease, it was found that some forms

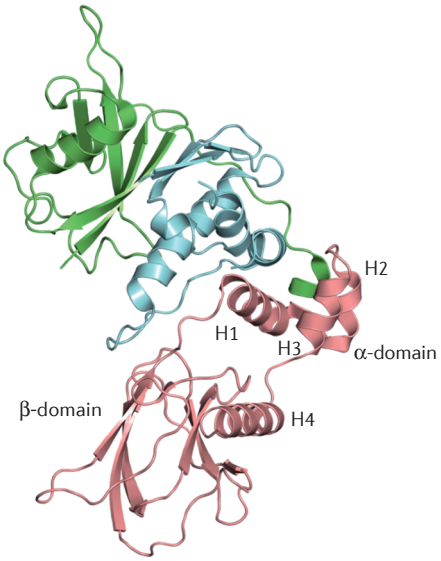

Figure 2 | **Ribbon diagram illustrating the secondary structure of the pVHL–elongin C–elongin B complex.** Von Hippel–Lindau protein (pVHL; pink) consists of two tightly coupled domains, α and β[23]. The β-domain consists of seven strands arranged in two β-sheets in a sandwich arrangement with an α-helix, and it has the properties of a substrate docking site. The α-domain consists of three α-helices and binds to elongin C (blue). The H4 helix of elongin C fits into an extended groove formed by the H1, H2 and H3 helices of the pVHL α-domain. The pVHL–elongin C complex nucleates a complex containing elongin B (green), cullin 2 (not shown) and the RING finger protein RBX1 (not shown).

of congenital secondary polycythaemias (CSPs) are caused by inherited specific *VHL* mutations in an autosomal-recessive manner (that is, affected individuals are homozygous or compound heterozygous)[94–98]. The most common *VHL* polycythaemia mutation is the homozygous 598C→T mutation, resulting in the amino acid substitution R200W[94]. This mutation is endemic on the Italian island of Ischia and in the Chuvash Autonomous Republic of the Russian Federation (which has led to coining of the term 'Chuvash polycythaemia'), and sporadic cases are reported elsewhere in the world. Additional *VHL* variants associated with CSP have also been described[99]. Although *VHL*-associated CSP is considered to be a recessive disease, several independent cases of patients with CSP who are heterozygous for *VHL* mutations have been reported in the literature[99]. There have been no reports on tumour development in patients with *VHL*-associated CSP, except for two cases of isolated haemangioblastoma and a recent description of a patient who harboured compound heterozygous mutations of *VHL* (V130I and R200W) and presented with polycythaemia at age 7, then developed PCC in his 30s[100]. Heterozygous carriers of the R200W mutation have no increased risk of cancer, and parents of patients with Chuvash polycythaemia are normally healthy. A knock-in R200W transgenic mouse and a zebrafish *vhl*-null mutant also exhibit polycythaemia without tumour formation[101,102]. Polycythaemia is not a common manifestation of VHL disease, although it can occur as a paraneoplastic syndrome in individuals with familial or sporadic ccRCC, PCC or haemangioblastoma[103,104].

The molecular mechanism underlying *VHL*-associated CSPs is debated. The lack of tumorigenesis in *VHL*-associated CSPs is notable, and two main theories have been proposed to explain the pathogenesis of the two diseases. The first theory proposes that whereas the polycythaemia-associated *VHL* mutants seem to result in a relatively mild defect in oxygen sensing that might only affect a subset of HIF target genes, more-severe dysregulation of HIFα is necessary to promote tumour formation. An alternative theory proposes an involvement of different molecular pathways for the two clinical entities. Until recently, no congenital polycythaemia associated with *VHL* homozygous or compound heterozygous mutations outside *VHL* exon 3 had been described. Thus, it had been suggested that the genomic configuration of the 3′ region of *VHL* exon 3 has a specific erythropoiesis-promoting effect. This effect is thought to be independent of erythropoietin and is instead mediated by hyperactivation of tyrosine-protein kinase JAK2 (REF. 105). Most published studies suggest that, on balance, the R200W mutation results in a relatively mild but detectable defect in oxygen sensing[94,97,101,105] and that HIF2 is more important than HIF1 in the pathogenesis of *VHL*-associated CSPs[63,64,101,106–108]. Consistent with this, gain-of-function mutations in *EPAS1* can be associated with congenital polycythaemia[99].

Loss of *VHL* in sporadic ccRCC

The Knudson one-hit and two-hit models of tumorigenesis predict that sporadic cancers might be associated with somatic mutations in the same locus that is affected in the corresponding hereditary cancer[109]. Aberrant patterns in the *VHL* gene were first identified in ccRCC cell lines in 1993 (REF. 11), and it is now clear that somatic biallelic inactivation of *VHL* occurs in most sporadic ccRCCs[110–113]. The reported incidence of somatic *VHL* mutations in sporadic ccRCC varies up to 91%[28,114–118]. Mutations in non-coding regions of *VHL* have been described[119]. In addition, methylation of *VHL* that results in gene silencing occurs in 5–30% of sporadic ccRCC cases, and LOH occurs in up to 98%[111,120].

Of the somatic *VHL* mutations identified in sporadic ccRCC, 55% are frameshift or nonsense mutations. However, nearly 250 different missense mutations (accounting for 32% of all mutations) have been described in sporadic ccRCC[118]. Whereas frameshift and nonsense mutations are highly likely to result in loss of pVHL function, missense *VHL* mutations have more diverse effects, and experimental data from multiple studies support the hypothesis that individual mutations have varied effects on the integrity of pVHL and the stabilization of HIFα. Indeed, some mutant pVHL isoforms seem to behave similarly to wild-type pVHL in terms of HIFα stabilization[121], suggesting that they might represent passenger or bystander mutations rather than driver mutations that generate a growth advantage.

Somatic inactivation of the *VHL* gene is also observed in around 40% of sporadic retinal and CNS haemangioblastomas[10,122,123]. Sporadic PCCs infrequently harbour somatic *VHL* mutations[124], although up to 10% of people with apparently sporadic PCC may have germline *VHL* mutations[125].

pVHL as a biomarker in sporadic ccRCC

From 1993 to 2010, *VHL* was the only gene that was known to be frequently mutated in ccRCC, and this prompted many groups to address the question as to whether *VHL* mutational status — namely, the presence or absence of mutation, the type of mutation or alteration, or the effect of the mutation or alteration on the function of pVHL — might provide a useful biomarker in ccRCC[114]. However, so far there is no clear evidence that the presence or absence of *VHL* mutations, or the type or nature of the mutation, influences outcome in sporadic ccRCC. As yet, few studies have examined a role for *VHL* as a potential predictive marker in ccRCC[126–133], mainly because effective treatment options have come into widespread use only recently. One of the major hurdles relates to the collection of tissues of adequate quality for DNA extraction and sequencing: most clinical trials collect formalin-fixed paraffin-embedded (FFPE) tissue rather than fresh-frozen tissue, and the quality of DNA extracted from FFPE tissue is generally inferior. Therefore, the frequency of *VHL* mutations reported in many of these studies is lower than might be expected from other studies, implying a possible skewing of the results.

As understanding of the molecular pathways downstream of *VHL* expands, various groups are investigating whether a combination of *VHL* mutational status and other molecular markers (for example, expression of HIF-target genes) might prove more useful as prognostic markers than *VHL* alone, but no unequivocal prognostic biomarkers have so far been identified. An alternative way of classifying *VHL*-deficient tumours was described by Gordan *et al.*[71], who analysed *VHL* genotype, as well as HIF1α, HIF2α and MYC expression, in 160 primary tumours and subdivided the tumours into 3 groups with distinct molecular characteristics: tumours with wild-type *VHL* alleles and undetectable HIFα protein expression (designated *VHL* WT); *VHL*-deficient tumours expressing detectable HIF1α and HIF2α proteins (designated H1H2); and *VHL*-deficient tumours expressing only HIF2α (designated H2). H2 tumours displayed enhanced MYC activity and higher rates of proliferation relative to H1H2 tumours regardless of stage, and also displayed different gene expression profiles, implying the existence of two biologically distinct types of *VHL*-deficient ccRCCs: those that produce HIF1α and those that do not.

Other mutations in ccRCC

In contrast to most other epithelial tumour types, mutations in genes such as *BRAF*, *TP53*, *PTEN*, *RB1*, epidermal growth factor receptor (*EGFR*) and *ERBB2* are uncommon in ccRCC[134,135]. Until 2010, basic research into ccRCC was dominated by studies focused on *VHL*, the HIF transcription factors and putative target genes with HREs, although it had been documented that *VHL* inactivation alone was insufficient for ccRCC development[136,137]. Segments of chromosome 3p that show recurrent loss in ccRCC include 3p12, 3p13–14.2, 3p21 and 3p25–26 (in which *VHL* is located) and, 15 years ago, allelic loss at 3p21 was hypothesized to be important in ccRCC development[138,139].

Recent studies using massively parallel sequencing technologies have implicated several novel driver genes in ccRCC. Additional TSGs have been identified on chromosome 3p: BRCA1-associated protein 1 (*BAP1*; mutated in 8–11% of ccRCCs)[116,140,141], SET domain-containing 2 (*SETD2*; mutated in 3–12% of ccRCCs)[117,134,140–143] and polybromo 1 (*PBRM1*; mutated in 41% of ccRCCs)[117,140,141,144,145]. Mutations in *BAP1* and *PBRM1* are usually mutually exclusive and are associated with different tumour biology and patient outcomes[146]. The three genes all have important functions in chromatin biology, and ccRCC-relevant mutations have also been described in other genes encoding chromatin-modifying enzymes, including lysine-specific demethylase 5C (*KDM5C*; mutated in 4–9% of ccRCCs)[134,140] and *KDM6A* (mutated in 1–7% of ccRCCs)[117,134,142,147]. Furthermore, mutations relevant to ccRCC have been described in genes involved in the ubiquitin-mediated proteolysis pathway[140]. In addition, in approximately 20% of ccRCCs, mutations have been found in several genes encoding key regulators in the mTORC1 pathway, including *MTOR*, tuberous sclerosis 1 (*TSC1*), *PIK3CA* (which encodes the PI3K catalytic subunit-α) and *PTEN*[146]. Additional pathways and components that are recurrently dysregulated in ccRCC include DNA methylation, p53-related pathways and mRNA processing[28,148]. Remodelling of cellular metabolism has been highlighted as a recurrent pattern in ccRCC that correlates with tumour stage and severity[148].

Within ccRCC tumours there is significant mutation heterogeneity, which raises the possibility that tumour subclones harbouring different mutations might have different responses to treatments[149]. Somatic mutations are classified into ubiquitous, shared and private mutations according to their prevalence[149]. Ubiquitous mutations, such as those in *VHL*, indicate early, truncal events and are present in every tumour cell. Shared and private mutations are found in progressively smaller subclones. In ccRCCs that harbour *VHL* mutations, chromosome 3p loss and *VHL* mutations occur ubiquitously throughout the tumour[149].

Therapeutic implications of pVHL

The discovery of the *VHL* gene and the identification of its crucial role in regulating the HIF-mediated response to hypoxia have facilitated considerable changes in ccRCC treatment over the past 15 years. Drugs that modulate the pVHL–HIF–VEGF pathway have proven benefit in treating ccRCC and are now the standard of care for patients with metastatic disease, with established superiority over cytokine therapies[150]. Such drugs include multiple tyrosine kinase inhibitors that target the VEGF receptors (such as sunitinib, sorafenib, pazopanib and axitinib, among others), inhibitors of the mTOR pathway (such as temsirolimus and everolimus) and the monoclonal anti-VEGF antibody bevacizumab.

Targeting HIF2α. As HIF2 seems to be crucial in the development of ccRCC, targeting HIF2α would seem to be a sensible therapeutic strategy to treat this type of cancer. However, with the exception of the steroid hormone receptors, targeting DNA-binding transcription factors with drug-like small organic molecules has historically been relatively unsuccessful. Nevertheless, several potential strategies to inhibit HIF2α have been identified. Small molecules that allosterically inhibit HIF2α and that antagonize HIF2 heterodimerization and DNA binding

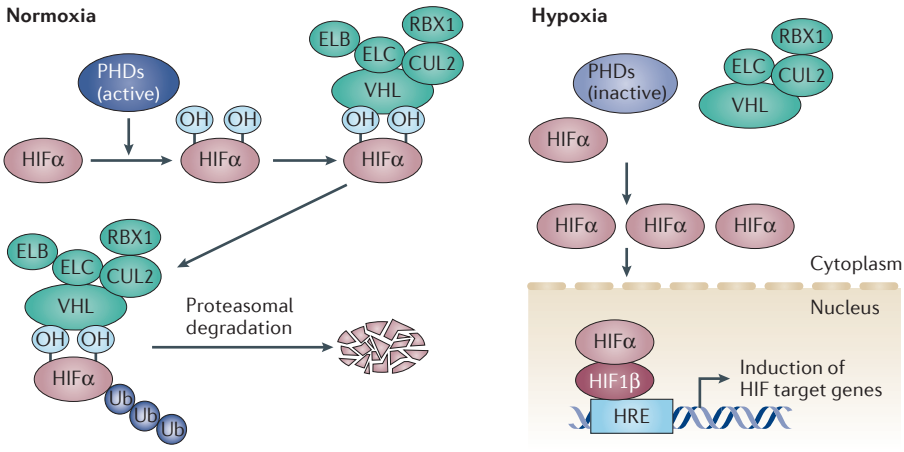

Figure 3 | **Oxygen-dependent hypoxia-inducible factor regulation.** In normoxic conditions, hypoxia-inducible factor-1α (HIF1α) and HIF2α are hydroxylated on one or both of two conserved proline residues by prolyl hydroxylase 1 (PHD1), PHD2 and PHD3. Prolyl-hydroxylated HIFα is recognized by the pVHL–elongin C (ELC)–elongin B–cullin 2 (CUL2)–RBX1 (VCB–CR) E3 ubiquitin ligase complex and targeted for ubiquitylation (Ub) and proteasomal degradation. In hypoxic conditions, PHD1, PHD2 and PHD3 are inactive (oxygen is an essential cofactor). HIFα therefore accumulates and forms heterodimers with HIF1β. These heterodimers translocate to the nucleus, bind to hypoxia-response elements (HREs) and induce the transcription of genes involved in adaptations to hypoxia.

have been described[151,152]. Proof-of-concept experiments suggest that it might be possible to target HIF2α with DNA-binding polyamides that disrupt the HIF2α–DNA interface, although at present the bioavailability of such agents is inadequate[153–155]. Acriflavine is a small molecule that inhibits the ability of HIF1α and HIF2α to dimerize with HIF1β and has been shown to inhibit tumour growth and vascularization[156]. Alternatively, if reliable methods for systemic delivery of small interfering RNA (siRNA) become available, siRNA targeting of HIF2α might become a future therapeutic option. Two groups have been screening for drugs that indirectly inhibit HIF2α in *VHL*-null ccRCC cells, although the specificity of these compounds remains to be established[157–160]. Many other compounds indirectly inhibit HIFα, including mTOR inhibitors, heat shock 90 kDa protein (HSP90) inhibitors and histone deacetylase (HDAC) inhibitors[161].

pVHL and synthetic lethality. Two genes are synthetically lethal if mutation of either alone is compatible with cell viability but mutation of both leads to cell death[162]. Synthetic lethality thus provides a framework to discover drugs that might preferentially kill cancer cells harbouring a cancer-relevant gene yet leave normal cells unharmed. Results from two synthetic-lethality screens targeting *VHL*-deficient cells have been reported. A cell-based, small-molecule synthetic-lethality screen identified a compound, STF-62247, that selectively induces autophagic cell death in *VHL*-deficient cells but not in those expressing wild-type *VHL*[163]. From the same screen, a second compound, STF-31, was identified that inhibits glucose uptake by GLUT1 and exhibits enhanced cytotoxicity against *VHL*-deficient ccRCC[164]. The second synthetic-lethality screen, which used short hairpin RNAs targeting 88 kinases, reported that silencing of cyclin-dependent kinase 6 (CDK6), MET (also known as HGFR) and MEK1(also known as MAPKK1) preferentially inhibited the growth of *VHL*-null cells compared with their counterparts in which wild-type pVHL had been reconstituted[80]. Interestingly, in both screens, the selective killing of cells lacking *VHL* was HIF-independent, suggesting that therapies targeting these pathways might cooperate with those targeting HIF. Another study showed that lack of a functional *VHL* gene product sensitizes RCC cells to the apoptotic effects of the protein synthesis inhibitor verrucarin A[165].

pVHL proteostasis. Following synthesis on ribosomes, nascent pVHL is shuttled from the ribosomal machinery with the assistance of HSP70 (REF. 166). Formation of the VCB complex is then mediated by the hetero-oligomeric chaperonin TCP1 ring complex (TRiC; also known as chaperonin-containing TCP1 (CCT))[166,167]. TRiC facilitates pVHL folding, thereby enabling its association with elongin B and elongin C to form the VCB complex[166]. Failure to generate a correctly folded pVHL or a mature VCB complex results in degradation of pVHL through the ubiquitin–proteasome system. pVHL degradation specifically requires another chaperone, HSP90, which does not participate in pVHL folding[168]. As distinct chaperone pathways mediate the folding and quality control of pVHL, an enhanced understanding of the mechanisms by which destabilized

Table 2 | **pVHL functions**

Mechanism	HIFα-dependent functions	HIFα-independent functions
Angiogenesis	Regulation of VEGF[37,38], PDGF[38] and adrenomedullin-159, among others	None
Glucose uptake and metabolism	Regulation of GLUT1 and GLUT3 (also known as SLC2A3)[38], hexokinase 2, phosphoglycerate kinase 1 (REF. 59), LDHA[59], phosphofructokinase 1 and pyruvate dehydrogenase[59], among others	None
Chemotaxis	Regulation of SDF1 (REF. 172) and CXCR4 (REF. 173)	None
Cell proliferation and survival	Regulation of TGFα[174] and EGFR[175]	None
Homeostasis	Regulation of external pH through CAIX[136]	None
Assembly and regulation of the extracellular matrix	Regulation of E-cadherin[176–178] and MMPs[179,180]	Regulation of fibronectin[9,55,176,181–185], collagen IV[176,182], adherens, tight junctions and integrins[178,186,187], and MMPs[183]
Microtubule stabilization and maintenance of the primary cilium	Primary cilia modulation[180]	Association and stabilization of microtubules[188–196]
Regulation of apoptosis	HIF modulation of p53 (REFS 197,198–200) and NF-κB[201–203] activity, and suppression of BNIP3 (REF. 60)	Activation of p53 transcriptional activity[204,205], modulation of NF-κB activity[206] and downregulation of JUNB (which is known to blunt neuronal apoptosis during NGF withdrawal)[207]*
Cell senescence	None	Control of cell senescence through RB and the SWI2/SNF2 chromatin remodeller p400 (REFS 208,209)
Transcriptional regulation	None	Involvement in ubiquitylation of the large subunit of RNA polymerase II in response to oxidative stress[58,21], control of influence on HuR[211–213], binding to SP1 transcription factor[214–216]
Erythropoiesis	Regulation of erythropoietin[64]	None
Cell cycle progression	Regulation of cyclin D1 (REFS 60,79,217,218)	None
Lipid metabolism	Adipose differentiation-related protein[59]	None

BNIP3, BCL2/adenovirus E1B-interacting protein 3; CAIX, carbonic anhydrase IX; CXCR4, CXC-chemokine receptor 4; EGFR, epidermal growth factor receptor; GLUT, glucose transporter; HIF, hypoxia-inducible factor; HuR, human antigen R (also known as ELAV1); LDHA, lactate dehydrogenase A; MMPs, matrix metalloproteinases; pVHL, von Hippel–Lindau protein; NF-κB, nuclear factor-κB; NGF, nerve growth factor; PDGF, platelet-derived growth factor; SDF1, stromal-cell derived factor 1 (encoded by *CXCL12*); TGFα, transforming growth factor-α; VEGF, vascular endothelial growth factor. *Dysregulation of this pathway is speculated to be important in the pathogenesis of phaeochromocytomas.

pVHL mutants are targeted for proteasomal degradation could provide strategies for refolding and stabilization of such mutants to allow their incorporation into the VCB complex and potentially restore their tumour suppressor activity. The proteasome inhibitors bortezomib and MG132 are both capable of increasing *VHL* expression levels, and a cell-based screen of the Prestwick Chemical Library compounds has identified several compounds that upregulate VHL^{W117A} in VHL^{W117A}-infected cell lines[169]. A recent study showed that the protein levels of $pVHL^{R167Q}$ (a recurrent mutation in type 2B VHL disease) dictate its ability to downregulate HIF2α and suppress tumour growth, and that the proteasome inhibitors bortezomib and carfilzomib stabilize VHL^{R167Q} and increase its ability to downregulate HIF2α[170].

Summary and conclusions

The identification of the *VHL* TSG in 1993 led to the elucidation of the genetic basis for a rare genetic disorder, and this finding has been shown to be of broad medical and scientific interest. *VHL* is frequently inactivated at an early stage in sporadic ccRCC. Insights gained from functional analysis of the *VHL* gene product, pVHL, have afforded novel insights into the molecular mechanisms of cellular oxygen sensing and provided the basis for the introduction of novel targeted therapies into the routine clinical treatment of advanced ccRCC. In the past 5 years, several additional potential driver genes have been identified in ccRCC, one of which has already been demonstrated to interact with the pVHL–HIF axis[171]. Future challenges lie in linking the pathways implicated by these genes with the effects of dysfunctional pVHL that result from *VHL* mutations, and with the ultimate aim of establishing a roadmap of tumour ontogeny for ccRCC.

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Competing interests statement
The authors declare competing interests: see Web version for details.

DATABASES
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