Kidney cancer is one of the ten most common cancers in the developed world [1]. Several histological variants of kidney cancer are recognized by pathologists including clear cell renal carcinoma, papillary (chromophil) renal carcinoma, chromophobic renal carcinoma, and oncocytoma [2]. The identification and study of rare families that are predisposed to kidney cancer led to the identifications of genes that, when mutated in the germline, confer an increased risk of the different histological variants of kidney cancer [3, 4]. For example, germline VHL mutations are linked to an increased risk of clear cell renal carcinoma, which is the most common form of kidney cancer. This chapter describes the molecular biology of clear cell renal carcinoma with an emphasis on the role of VHL in disease pathogenesis.

2.1 VHL Tumor Suppressor Gene: Role in VHL Disease and Kidney Cancer

Von Hippel-Lindau disease was first described over 100 years ago and is characterized by an increased risk of clear cell renal carcinoma, hemangioblastomas of the retina, cerebellum, and spinal cord, and intra-adrenal paragangliomas (also called pheochromocytomas) [5, 6]. Individuals with VHL disease have typically inherited a defective allele for the VHL tumor suppressor gene, located at chromosome 3p25, from one of their parents. Less commonly VHL disease is the result of a de novo VHL mutation leading to germline VHL mosaicism [7–9]. The development of overt pathology in VHL disease is linked to somatic inactivation or loss of the remaining wild-type VHL allele in a susceptible cell type.
In keeping with this knowledge, biallelic \textit{VHL} inactivation due to mutations or, less commonly, gene methylation, is also very common in sporadic (nonhereditary) clear cell renal carcinoma \cite{10}. In most studies, at least 50\% of such tumors have sustained \textit{VHL} mutations, usually accompanied by macrodeletions affecting the remaining chromosome 3p arm. More recent studies that have employed more sensitive sequencing technologies suggest that 50\% might significantly underestimate the frequency of \textit{VHL} mutations in this population \cite{11, 12}.

Notably, the \textit{VHL} gene is ubiquitously expressed, and yet \textit{VHL} mutations are tightly linked to the development of kidney cancer but are rare in other common epithelial neoplasms. Why some cell types are susceptible to transformation after \textit{VHL} inactivation and others are not is largely a mystery. The same, however, can be said of most cancer predisposition genes.

### 2.2 Cooperating Genetic Events in Kidney Cancer

Although \textit{VHL} inactivation plays a critical role in hereditary (\textit{VHL} disease-associated) and sporadic clear cell renal carcinoma, it is not sufficient to cause this disease. This has been most convincingly demonstrated by careful natural history studies of \textit{VHL} patients. The kidneys of \textit{VHL} patients frequently harbor hundreds of preneoplastic renal cysts, only a few of which will become malignant renal carcinomas \cite{13, 14}. The development of preneoplastic renal cysts in this setting reflects the stochastic loss of the remaining wild-type \textit{VHL} allele, suggesting that biallelic \textit{VHL} inactivation is sufficient to causes cysts but not clear cell renal carcinoma. The latter presumably requires the accumulation of additional mutations affecting genes other than the \textit{VHL} gene itself. In this regard, a number of recurrent chromosomal changes have been documented in hereditary and sporadic clear cell renal carcinoma including loss of chromosome 14q and gain of chromosome 5q \cite{15–19}. In addition, cancer genome sequencing projects have revealed potentially pathogenic mutations of the known tumor suppressor genes \textit{CDKN2A}, \textit{TP53}, \textit{NF2}, and \textit{PTEN} in a subset of clear cell renal carcinomas \cite{20}. These efforts have also revealed that a subset clear cell renal carcinoma have mutations affecting chromatin modifying genes such as \textit{PRBM1}, \textit{KDM6A}, and \textit{SETD2} \cite{20, 21}. The requirement for multiple, cooperating, genetic events to cause clear cell renal carcinoma (as appears to be true for most epithelial neoplasms) presumably explains why \textit{VHL} patients typically develop kidney cancer in adulthood rather than as children.

### 2.3 Functions of the VHL Protein

The \textit{VHL} gene encodes for two proteins by virtue of two alternative, in-frame, translation initiation sites \cite{22–24}. The long form contains 213 amino acid residues. The short form lacks the first 53 amino acid residues of the long form. In most biochemical
and biological assays, the two isoforms behave similarly, and the mutations that have been detected in kidney cancer would affect both isoforms. Therefore, “pVHL” will be used to refer to both isoforms in this chapter for simplicity.

pVHL is primarily a cytosolic protein but dynamically shuttles back and forth between the cytosolic compartment and the nucleus [25–29]. Some pVHL can also be detected in association with the endoplasmic reticulum [30] and with mitochondria [31]. pVHL has a number of functions including roles in regulating protein turnover, primary cilium maintenance, microtubule stability, and extracellular matrix formation (reviewed in [32]). For example, loss of pVHL leads to loss of the primary cilium, a specialized structure on the cell surface that plays a role in signal transduction, including mechanical signals arising from fluid pressure and flow [33–36]. Interestingly, a number of genes that, like VHL, play roles in primary cilium maintenance have been linked, when altered, to the development of visceral cysts [37]. Therefore, loss of the primary cilium might contribute to the development of the visceral cysts that are a hallmark of VHL disease. Nonetheless, some VHL mutations that have been linked to human kidney cancer do not compromise pVHL’s role in primary cilium maintenance [35]. Instead, the pVHL function that appears to be most tightly linked to suppression of kidney cancer relates to its role in the regulation of the HIF transcription factor, as described below.

2.4 pVHL and HIF

pVHL is part of a multiprotein complex that contains elongin B, elongin C, Cul2, and Rbx1 (reviewed in [32]) (Fig. 2.1). This complex serves as a ubiquitin ligase complex, meaning that it can direct the polyubiquitylation of specific substrates, which then undergo proteasomal degradation. The best documented target of the pVHL ubiquitin complex is the heterodimeric transcription factor HIF (hypoxia-inducible factor), which is a master regulator of genes that promote the adaptation to hypoxia (low oxygen) such as genes that promote glycolysis as an alternative ATP source, genes that promote erythropoiesis (such as erythropoietin), and genes that promote angiogenesis (such as VEGF).

HIF consists of an unstable alpha subunit and a stable beta subunit. In the presence of oxygen, the alpha subunit becomes hydroxylated on one (or both) of two specific prolyl residues [38–42]. Hydroxylation of either site generates a pVHL docking site and sets in motion the polyubiquitylation and destruction of HIFα. When oxygen levels are low, or pVHL is defective, HIFα is not polyubiquitylated and instead accumulates, dimerizes with HIFβ, and transcriptionally activates HIF target genes such as VEGF. Accordingly, deregulation of HIF target genes is a signature of pVHL-defective kidney cancers [43–46]. Overproduction of VEGF and erythropoietin can explain the clinical observations that kidney cancers are highly angiogenic and capable of inducing paraneoplastic erythrocytosis, respectively.

There are three HIFα genes in the human genome (HIF1α, HIF2α, and HIF3α). The products of all three of these genes have the ability to heterodimerize with HIFβ.
family members. The resulting DNA-binding complexes formed by HIF1α and HIF2α can activate transcription. HIF1α is widely expressed while the expression of HIF2α is restricted to certain cells and tissues. In addition, it is clear that some HIF-responsive genes, such as many glycolytic genes [47], are primarily regulated by HIF1α, while others, such as erythropoietin and the stem cell factor Oct4, are primarily under the control of HIF2α [48, 49]. HIF3α has been less intensively studied than HIF1α and HIF2α. It appears to undergo extensive mRNA splicing, with some mRNA isoforms encoding proteins that can block the action of HIF1α and HIF2α [50–54].

2.5 HIF and Kidney Cancer

Deregulation of HIF2α appears to be a driving force in pVHL-defective clear cell renal carcinomas. In preclinical models, restoring the function of pVHL in pVHL-defective renal carcinoma lines suppresses their ability to form tumors in immunocompromised mice [25, 45]. Importantly, this activity of pVHL can be overridden by restoring HIF2α activity, suggesting that downregulation of HIF2α is necessary for tumor suppression by pVHL [55, 56]. Moreover, eliminating HIF2α in pVHL-defective renal carcinoma lines inhibits tumor growth, indicating that downregulation of HIF2α is also sufficient for tumor suppression by pVHL [57, 58]. Deregulation of HIF2α also appears to be necessary and sufficient for much of the pathology observed in mice that have been engineered to lack pVHL in specific tissues [59, 60]. The risk of kidney cancer associated with different VHL alleles correlates with the degree to which those alleles deregulate HIF2α (as well as HIF1α) [61] and germline HIF2α polymorphisms have been linked to the risk of sporadic kidney cancer [62].

In stark contrast, HIF1α exhibits properties of a tumor suppressor in clear cell renal carcinoma (Fig. 2.2). Although increased HIF2α levels are a hallmark of clear cell renal carcinoma cell lines and tumors, many clear cell renal carcinoma lines and
tumors produce low or undetectable levels of HIF1α [44, 63, 64]. HIF1α is located on chromosome 14q, which is deleted in ~40% of clear cell renal carcinomas, and 14q-deleted tumors exhibit a gene expression signature indicative of HIF1α loss [64]. In the majority of such tumors the remaining wild-type HIF1α allele appears to be wild type, suggesting that loss of one copy (haploinsufficiency) of HIF1α can have a biological effect. In contrast to tumors, a significant proportion of pVHL-defective renal carcinoma lines have sustained focal, homozygous, deletions, rendering them null for wild-type HIF1α [64]. This suggests that reduction to nullizygosity is a late event in tumor progression (most cell lines are established from late stage disease) and/or is selected for during the establishment and propagation of such cell lines.

Eliminating HIF1α in pVHL-defective renal carcinoma lines that express both HIF1α and HIF2α enhances their ability to proliferate in vitro and in vivo, while restoring HIF1α levels in pVHL-defective renal carcinoma lines lacking HIF1α has the opposite effect [64]. Although rare, intragenic HIF1α mutations have been described in human clear cell tumors, and when tested, these mutations impair HIF1α’s ability to suppress proliferation in such assays [20, 64, 65]. Collectively, these results indicate that HIF1α has the credentials of a tumor suppressor.

The mechanistic basis for the opposing effects of HIF1α and HIF2α is not completely understood. As discussed above, the sets of genes regulated by HIF1α and HIF2α overlap but are not entirely congruent. Perhaps one or more genes that are preferentially regulated by HIF1α can suppress renal carcinoma growth. In addition, HIF1α, and to a much lesser extent HIF2α, is subject to a second layer of oxygen-dependent regulation. Specifically, in the presence of oxygen, HIF1α becomes hydroxylated on an asparaginyl residue by the enzyme FIH-1, which diminishes the ability of HIF1α to activate transcription [66–68]. HIF2α is relatively insensitive to FIH-1 [69, 70]. Accordingly, displacement of HIF2α from HIF target genes by HIF1α in an oxygenated, pVHL-defective tumor might lead to a decrease in transcription because the former is more active than the latter as a transcriptional activator. Finally, the different effects of HIF1α and HIF2α with respect to renal suppression might relate to differential interactions with other transcription factors such as c-Myc [63, 71] and Notch [72].
In addition to HIF, pVHL regulates, at least indirectly, the oncogenic transcription factors NFκB and β-catenin. pVHL serves as an adaptor protein to promote the inhibitory phosphorylation of the NFκB agonist Card9, leading to decreased NFκB activity [73]. In addition, there is evidence for cross talk between HIF and NFκB, HIF inducing NFκB activity in some systems and NFκB, in turn, promoting the transcription of HIF1α [74–76]. Deregulation of NFκB might contribute to the resistance of clear cell renal carcinomas to cytotoxic agents. In addition, some pathogenically relevant transcriptional targets of HIF, such as VEGF and cyclin D1, are also NFκB targets, suggesting that HIF and NFκB conspire to promote tumorigenesis.

pVHL also stabilizes the putative tumor suppressor Jade1, which has been implicated in polyubiquitylation of β-catenin [77, 78]. In addition, loss of pVHL leads to increased signaling downstream of receptors such as c-Met, leading to enhanced β-catenin activity [79]. Finally, there is evidence that HIF can, directly or indirectly, increase the transcription of β-catenin target genes [80]. β-Catenin likely contributes to abnormal proliferation and enhanced invasiveness.

2.6 Therapeutic Targets

2.6.1 HIF

In general, DNA-binding transcription factors, with the exception of steroid hormone receptors, have historically been difficult to inhibit with drug-like small organic molecules. A number of drugs have been identified, however, that indirectly downregulate HIF activity [81–89]. A caveat, however, is that HIFα has a very high metabolic turnover rate and, accordingly, is one of the first proteins to disappear from cells when transcription or translation are impaired. It is not clear whether some of the purported HIF inhibitors are specific for HIF or would have similar effects on other short-lived proteins. A second caveat is that most of the reported HIF inhibitors have been studied primarily with respect to HIF1α rather than HIF2α. For the reasons outlined above, it will be important to inhibit HIF2α in clear cell renal carcinoma.

2.6.2 mTOR

Rapamycin-like mTOR inhibitors can downregulate HIF [90–95] and have been approved for the treatment of kidney cancer in patients who have failed treatment with VEGF inhibitors (see below). The antitumor activity of rapalogs is likely to reflect direct effects on tumor cells [96, 97], including downregulation of HIF, as well as effects on signaling downstream of VEGF in endothelial cells. pVHL-defective renal carcinoma cells appear to be more sensitive to rapalogs than identical cells that
contain wild-type pVHL [97]. Two factors, however, might limit the effectiveness of rapalogs in clear cell renal carcinoma. First, mTOR exists in a rapamycin-sensitive complex called TORC1 and a relatively insensitive complex called TORC2 [98]. Loss of TORC1 activity primarily affects HIF1α, while loss of TORC2 primarily affects HIF2α [99]. For this reason, ATP-competitive mTOR inhibitors capable of inhibiting both TORC1 and TORC2 might be more active than rapalogs for the treatment of clear cell carcinoma. A recent preclinical study supports this view [100]. Secondly, inhibition of TORC1 disrupts a negative feedback loop that normally serves to suppress signaling by specific receptor tyrosine kinases [101–103]. As a result, use of rapalogs has been associated with a paradoxical increase in receptor tyrosine kinase signaling. This increase, at least in theory, could be blunted by dual inhibition of PI3K and/or TORC2.

2.6.3 VEGF

Increased angiogenesis is a hallmark of kidney cancer and is associated with massive overproduction of the canonical HIF target VEGF. Multiple agents that inhibit VEGF itself, such as bevacizumab [104], or its receptor KDR, including sunitinib [105], sorafenib [106], and pazopanib [107, 108], have demonstrated activity in the treatment of this disease. Approximately, 70% of kidney cancer patients treated with VEGF inhibitors will experience disease stabilization and some degree of tumor shrinkage. The percentage of patients achieving a partial response by RECIST criteria varies, however, among the different agents. This might reflect differences in potency or fortuitous off-target effects that contribute to tumor regression.

It is hoped that second generation VEGF inhibitors that display increased potency and/or specificity will ultimately proof even more efficacious and less toxic than the currently approved agents. A caveat, however, is that early clinical data suggest that on-target toxicities, such as endothelial and cardiac dysfunction, will ultimately limit the degree to which VEGF signaling can be safely disrupted in man [109–113].

VEGF inhibitors favorably change the natural history of kidney cancer but are not curative. Kidney cancers treated with VEGF inhibitors will eventually develop resistance, as would be expected when treating a genetically complex neoplasm with a single agent. The mechanisms underlying the development of resistance to VEGF inhibitors in this setting are poorly understood. One recent preclinical study suggested a role for increased expression of interleukin-8, which is an angiogenic factor that can cooperate with VEGF [114, 115]. Interestingly, a recent clinical study linked interleukin-8 polymorphisms to the therapeutic efficacy of VEGF inhibitors [116].

There are conflicting studies with respect to importance of VHL status as a predictive biomarker with respect to the use of VEGF inhibitors for kidney cancer [117–120]. This might reflect, at least partly, variability among these studies with respect to histological inclusion criteria, response criteria, and the methods used to
determine VHL status. Suffice it to say that VHL status should not currently be used to guide the use of VEGF inhibitors in kidney cancer, especially given the paucity of alternative treatments.

2.6.4 PDGF

In preclinical models newly sprouting blood vessels become less sensitive to VEGF blockade once they are properly invested with surrounding pericytes [121–123], which respond to the HIF-responsive growth factor PDGF B [124–126]. Many of the available KDR inhibitors also inhibit the PDGF receptor, and hence, they might be ideally suited for blocking HIF-induced angiogenesis. On the other hand, the PDGFR inhibitor imatinib has not demonstrated activity in the treatment of kidney cancer, either alone or combined with bevacizumab [127–129].

2.6.5 TIE2

The TIE2 tyrosine kinase influences the response of endothelial cells to VEGF. TIE2 is under the control of two ligands, called angiopoietin 1 and angiopoietin 2 [130]. The former, which acts as a TIE2 agonist, stabilizes blood vessels, while angiopoietin 2, which acts as an antagonist, destabilizes blood vessels and primes them for sprouting in response to signals such as VEGF. At the same time, loss of TIE2 activity renders cells hypersensitive to VEGF withdrawal. Although there are conflicting data with respect to the regulation of angiopoietins by pVHL [131, 132], these considerations suggest that TIE2 inhibition might augment the activity of VEGF antagonists.

2.6.6 HGF and c-MET

pVHL-defective clear cell renal carcinoma cells are hypersensitive to the c-Met ligand HGF, leading to enhanced proliferation and invasiveness [133]. pVHL might regulate c-Met itself, which has been suggested to be a HIF target gene, as well as signaling downstream of c-Met [134–136]. Interestingly, both HGF and c-Met are located on chromosome 7, which is often amplified in clear cell renal carcinoma, and pVHL-defective cells are more dependent on c-Met for survival than are isogenic cells in which pVHL function has been restored [137]. c-Met has also been implicated in tumor angiogenesis. All of these considerations suggest that c-Met blockade, alone or in conjunction with a VEGF antagonist, would have activity in clear cell kidney cancer. c-Met inhibitors are already being tested in papillary renal cancer because some hereditary papillary renal cancers are linked to activating
germline c-Met mutations [3]. On the other hand, c-Met mutations appear to be rare in sporadic papillary renal cancers.

### 2.6.7 TGFα and EGFR

Kidney cancers frequently overproduce the HIF-responsive growth factor TGFα and its receptor EGFR [138–147]. Moreover, inhibiting this growth factor or its receptor in preclinical models suppresses pVHL-defective tumor growth [147, 148]. Nonetheless, the activity of small molecule EGFR inhibitors in the treatment of human kidney cancer has so far been disappointing [129, 149–151], although one study suggested a possible benefit for those with the highest expression of EGFR [150]. It should be borne in mind, however, that ATP-competitive EGFR inhibitors, such as erlotinib, have so far only proven efficacious for human cancers that have EGFR point mutations (in contrast to tumors driven by receptor overexpression). It is possible that anti-EGFR antibodies will be more efficacious in this setting, such as appears to be the case in colorectal cancer (where EGFR mutations are rare). On the other hand, the clinical results with two such agents as monotherapy, ABX-EGF and C225 (cetuximab), have thus far also been disappointing [152, 153].

A possible explanation for the ineffectiveness of EGFR blockade in kidney cancer relates to c-Met. There is a growing appreciation that c-Met activation can confer resistance to EGFR inhibitors in other settings [154–156], and as described above, c-Met is likely active in clear cell renal carcinoma. In this regard, it is worth noting that mouse models can underestimate the importance of c-Met, because mouse HGF does not effectively engage the human c-Met present on implanted human tumor cells [157]. These considerations warrant testing EGFR inhibitory antibodies in conjunction with c-Met inhibitors for the treatment of clear cell renal carcinoma.

### 2.6.8 Cyclin D1 and Cdk6

In renal epithelial cells, but not in many other cell types, the increased HIF levels observed upon pVHL loss drives the overproduction of cyclin D1 [158, 159]. Cyclin D1, bound to either cdk4 or cdk6, drives cell proliferation by phosphorylating, and thereby inactivating, the RB tumor suppressor protein. Cdk6 is located on a region of chromosome 7 that is amplified in clear cell renal carcinoma, and pVHL-defective renal carcinoma cells display increased sensitivity to cdk6 loss compared to cells in which pVHL function has been restored [137]. Some clear cell renal carcinomas harbor chromosome 9p deletions that target the cdk4/6 inhibitor p16 (also called Ink4A) [18, 160]. Importantly, however, mutations of pRB itself are rare in clear cell carcinoma, suggesting that they would retain sensitivity to inhibition of cdk4 and cdk6 (these kinases appear to be dispensable for oncogenesis in cells that lack wild-type pRB).
2.6.9 **IL-6**

Interleukin-6 is frequently overexpressed in clear cell renal carcinoma and might contribute to the unexplained fevers observed in a subset of kidney cancer patients [161–165]. IL-6 has also been reported to be regulated by pVHL [158]. IL-6 can act as an autocrine factor to stimulate renal carcinoma proliferation through activation of the JAK-STAT pathway [166]. In one clinical study, disease stabilization was noted in a subset of kidney cancer patients treated with a neutralizing anti-IL-6 antibody [167].

2.6.10 **Lactate Dehydrogenase A and Monocarboxylate Transporters**

HIF both reduces oxidation phosphorylation and increases the rate of glycolysis. The conversion of pyruvate to lactate is catalyzed by the HIF-responsive gene product LDH A, and the maintenance of intracellular pH is accomplished, at least partly, by upregulation of the HIF-responsive monocarboxylate transporter MCT4 [168–170]. Preclinical studies, conducted largely in other tumor types, suggest that blocking LDH A or MCT4 in pVHL-defective tumors would have antitumor effects [171–174].

2.7 **Summary**

Inactivation of the VHL tumor suppressor gene is a signature lesion in clear cell renal carcinoma. pVHL has multiple functions including targeting HIF family members for polyubiquitylation and proteasomal degradation. Deregulation of HIF2α is a driving force in pVHL-defective tumors. HIF1α, in stark contrast, exhibits properties of a tumor suppressor and appears to be one of the targets of the chromosome 14q deletions that are common in this disease. Drugs that inhibit the HIF target VEGF are now approved for the treatment of kidney cancer as are two rapamycin-like drugs that inhibit mTOR. The activity of mTOR inhibitors is probably due, at least in part, to tumor cell-intrinsic effects as well as effects on angiogenesis. A number of other HIF targets, in addition to VEGF, are suspected of contributing to tumor growth and can now be explored in the clinic.

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