Inactivation of the von Hippel–Lindau tumor-suppressor protein (pVHL) is the signature “truncal” event in clear cell renal cell carcinoma, which is the most common form of kidney cancer. pVHL is part of a ubiquitin ligase that targets the α subunit of the hypoxia-inducible factor (HIF) transcription factor for destruction when oxygen is available. Preclinical studies strongly suggest that deregulation of HIF, and particularly HIF2, drives pVHL-defective renal carcinogenesis. Although HIF2α was classically considered undruggable, structural and chemical work by Rick Bruick and Kevin Gardner at University of Texas Southwestern laid the foundation for the development of small molecule direct HIF2α antagonists (PT2385 and the related tool compound PT2399) by Peloton Therapeutics that block the dimerization of HIF2α with its partner protein ARNT1. These compounds inhibit clear cell renal cell carcinoma growth in preclinical models, and PT2385 has now entered the clinic. Nonetheless, the availability of such compounds, together with clustered regularly interspaced short palindromic repeat (CRISPR)-based gene editing approaches, has revealed a previously unappreciated heterogeneity among clear cell renal carcinomas and patient-derived xenografts with respect to HIF2 dependence, suggesting that predictive biomarkers will be needed to optimize the use of such agents in the clinic.
pear that VHL loss is sufficient to cause renal dysplasia but not cancer. The same appears to be true in genetically engineered mouse models (Gnarra et al. 1997; Frew et al. 2008; Schiette et al. 2012; Albers et al. 2013). Another line of evidence has come from genomic analyses of hereditary (VHL disease) and sporadic clear renal carcinomas to identify recurrent mutations, including intragenic mutations and copy-number changes (Beroukhim et al. 2009; Guo et al. 2011; Varela et al. 2011; Donnet et al. 2012; Duns et al. 2012; Pena-Llopis et al. 2012; Cancer Genome Atlas Research Network 2013). Clear cell renal carcinomas typically include specific copy-number changes, most commonly gain of chromosome 5q, loss of chromosomes 3p and 14q, and specific intragenic mutations affecting genes linked to chromatin regulation, phosphoinositide 3-kinase (PI3K) signaling, and response to redox stress or DNA damage. Examples of such genes include PBRM1, BAP1, SETD2, KDM5C, TSC1, PIK3CA, PTEN, MTO1, and TP53. Importantly, inactivation of VHL has preceded the acquisition of these other mutations in every tumor examined to date in which deep sequencing of spatially distinct sites was used to infer its evolutionary history (Gerlinger et al. 2012, 2014; Xu et al. 2012; Fisher et al. 2014; Sankin et al. 2014). These studies have confirmed that VHL inactivation is an early, or “truncal,” lesion in clear cell renal cell carcinomas and that such tumor often display significant heterogeneity, or “branching,” with respect to the subsequent genetic events that conspired with VHL loss to cause cancer. Interestingly, VHL, PBRM1, BAP1, and SETD2 all reside on chromosome 3p. Therefore three “hits,” including two intragenic mutations and loss of chromosome 3p, can inactivate both VHL and another “Knudson 2-hit” renal cancer suppressor. For reasons that are still not well understood, VHL mutations are exceedingly rare in other neoplasms with the exceptions of pheochromocytomas and hemangioblastomas.

**THE VHL TUMOR-SUPPRESSOR PROTEIN**

The VHL gene encodes two different proteins, with apparent molecular mass of ~28 kDa and 19 kDa after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), because of alternative in-frame translational start sites (Kaelin 2007). For simplicity let us refer to both of these as pVHL in this review, because their functions appear to be highly similar, and disease-associated VHL mutations almost invariably affect both isoforms. pVHL can be found in both the cytoplasm and the nucleus, shuttling dynamically between the two compartments (Kaelin 2007). Some pVHL can also be detected associated with membranes, the endoplasmic reticulum, and mitochondria (Kaelin 2007).

Reintroducing wild-type pVHL into pVHL-defective clear cell renal carcinoma lines suppresses their ability to form tumors in nude mice xenograft assays but does not affect their viability or proliferation under standard cell culture conditions (Iliopoulos et al. 1995). pVHL does inhibit pVHL-defective clear cell renal carcinoma cells ex vivo, however, under specific conditions such as when such cells are grown as three-dimensional spheroids or at confluence under growth factor–poor conditions (Lieubeau-Teillet et al. 1998; Puse et al. 1998; Davidowitz et al. 2001).

pVHL is the substrate adapter of a ubiquitin ligase that contains elongin B, elongin C, Rbx1, and Cullin-2 (Kaelin 2007). pVHL has two domains that are hotspots for missense mutations in VHL disease: the α domain, which recruits the ubiquitination machinery, and the β domain, which serves as a substrate docking site. Although many potential pVHL substrates have been identified, the best documented substrate, and the one believed to be most tightly linked to clear cell renal carcinogenesis, is the HIF (hypoxia-inducible factor) transcription factor.

**THE HIF TRANSCRIPTION FACTOR**

Active HIF is a heterodimer of two basic helix–loop–helix PAS domain–containing DNA-binding proteins, an unstable α subunit and a stable β subunit, and binds to specific DNA sequences called hypoxia-response elements (HREs) (Kaelin and Ratcliffe 2008; Semenza 2011). The human genome contains three HIFα genes (HIF1α, HIF2α, HIF3α) and two HIFβ genes (HIF1β, also called ARNT1, and HIF2β, also called ARNT2). HIF1α is widely expressed and, together with HIF1β, forms the well-studied, canonical, HIF transcription factor. The expression of HIF2α is more restricted. HIF1α and HIF2α each contain two transactivation domains, an amino-terminal transactivation domain (NTAD) and carboxy-terminal transactivation domain (CTAD), that enable them, upon DNA-binding, to recruit coactivators such as p300 and CREB-binding protein (CBP) and activate transcription. HIF3α has been far less studied, but encodes multiple splice variants that do not activate transcription but instead act as dominant-negative inhibitors of HIF-dependent transcription.

HIF regulates hundreds of genes, many of which play roles in acute or chronic adaptation to hypoxia. HIF regulates genes linked to processes such as anaerobic glycolysis, mitochondrial biosynthesis and function, autophagy, angiogenesis, cell proliferation, invasion, and migration (Fig. 1). The ability of HIF to activate some HRE-containing HIF target genes is highly cell type— and context-dependent, presumably because of the influence of other cis-acting transcription factors as well as changes in chromatin accessibility. For example, vascular endothelial growth factor (VEGF) responds to HIF in many tissues, whereas erythropoietin (EPO) in adults responds to HIF largely in dedicated cells in the kidney. Likewise, the sets of genes regulated by HIF1 and HIF2, although overlapping, are not identical. For example, some glycolytic and autophagy genes are primarily regulated by HIF1, whereas EPO is primarily regulated by HIF2 (Hu et al. 2003; Rankin et al. 2007; Kapitsinou et al. 2010).
REGULATION OF HIF BY pVHL AND OXYGEN

Under well-oxygenated conditions HIFα is prolyl-hydroxylated on one (or both) of two potential prolyl residues by members of the EglN (also called PHD) prolyl hydroxylase family, which serve as oxygen sensors (for review, see Kaelin and Ratcliffe 2008). Once prolyl-hydroxylated, HIFα binds directly to the pVHL ubiquitin ligase complex and is targeted for proteasomal degradation. Under low-oxygen conditions EglN activity is impaired, which allows HIFα to accumulate, bind to HIFβ, and activate transcription. Another layer of oxygen-dependent HIF regulation is provided by the FIH1 asparaginyl hydroxylase. In the presence of oxygen FIH1 hydroxylates a conserved asparaginyl residue located within the HIFα CTAD, preventing it from binding to coactivators. Importantly, FIH1 has a higher oxygen affinity than the EglNs, and thus can remain active at intermediate levels of hypoxia that are sufficient to stabilize HIFα. Some HIF-responsive genes depend primarily on the NTAD and others on the CTAD. Moreover, the HIF2α is relatively resistant to FIH1. Therefore, FIH1 can potentially tune the HIF response at intermediate levels of hypoxia by altering the balance of NTAD and CTAD function and possibly the balance of HIF1 and HIF2 activity.

ROLE OF HIF2 IN CLEAR CELL RENAL CARCINOGENESIS

Multiple lines of evidence suggest that HIF, and particularly HIF2, is a critical target in clear cell renal cell carcinoma. Every VHL mutation linked to clear cell renal carcinoma to date results in a protein that is defective with respect to HIF regulation, and genotype–phenotype correlations suggest that the risk of developing clear cell renal carcinoma in VHL disease families is linked to the degree to which their VHL alleles compromise pVHL’s ability to suppress HIF (Li et al. 2007; Kaelin 2008). In particular, VHL families with a low risk of developing clear cell renal carcinoma, even if at high risk of hemangioblastomas and pheochromocytomas, have VHL alleles that encode proteins that retain significant HIF ubiquitin ligase activity, whereas families at high risk of clear cell renal carcinoma are grossly defective in this regard (Kaelin 2008).

In the laboratory, activating HIF2-responsive genes, such as by expressing a HIF2α variant that cannot be prolyl-hydroxylated, can override pVHL’s tumor-suppressor activity in nude mice xenograft assays (Kondo et al. 2002; Raval et al. 2005; Biswas et al. 2010). Conversely, down-regulating HIF2α with short-hairpin RNA (shRNA) technology suppresses tumor formation by pVHL-defective clear cell renal carcinomas in mice (Kondo et al. 2003; Zimmer et al. 2004; Gordan et al. 2008).

Currently there are no faithful, highly penetrant, genetically engineered mouse models for VHL2/2 clear cell renal carcinomas. Nonetheless, the pathological changes induced after inactivating VHL in various mouse tissues has, when tested, been prevented by concurrent inactivation of HIF2α (Rankin et al. 2007, 2008, 2009) and can, when tested, be mimicked by expressing a version of HIF2α that cannot be prolyl-hydroxylated in wild-type mice (Kim et al. 2006). Notably, a human genetic polymorphism linked to HIF2α is associated with the risk of developing clear cell renal cell carcinoma (Purdue et al. 2011).

In stark contrast HIF1α appears capable of suppressing clear cell renal carcinomas. Forced expression of HIF1α, unlike HIF2α, does not antagonize pVHL’s tumor-suppressor activity in nude mouse experiments (Maranchie et al. 2002; Raval et al. 2005; Biswas et al. 2010; Shen et al. 2011). Interestingly, HIF1α resides on chromosome 14q, which is often deleted in clear cell renal cell carcinoma, and many clear cell renal carcinoma lines have
sustained homozygous deletions that specifically inactivate HIF1α such that they solely produce HIF2α (Shen et al. 2011). Restoring HIF1α expression in such cell lines suppresses tumor growth, whereas eliminating HIF1α in lines that retain a wild-type HIF1α allele promotes tumor growth (Shen et al. 2011). Intragenic HIF1α mutations have rarely been identified in clear cell renal cancers and, when tested, have uniformly been loss of function (Morris et al. 2009; Dalgliesh et al. 2010; Shen et al. 2011). Moreover, the appearance of HIF2α, and apparent loss of HIF1α, in preneoplastic renal lesions in VHL patients correlates with worsening cellular atypia and signs of impending transformation (Mandriota et al. 2002; Schiette et al. 2012). Interestingly, genetic ablation of HIF1α worsens the renal pathology associated with papillary renal cancers driven by fumarate hydratase loss in the mouse, providing a precedent for HIF1α as a renal cancer suppressor (Adam et al. 2011).

Most 14q deleted clear cell renal cancers, in contrast to clear cell renal carcinoma cell lines, retain one HIF1α allele and the remaining HIF1α allele is usually wild-type. It is not yet clear whether the homozygous HIF1α deletions detected in cell lines occurred in vivo, possibly associated with disease progression, or were selected for ex vivo. Nor is it clear whether HIF1α can act as a haploinsufficient tumor suppressor in vivo.

Nonetheless, these findings suggest that HIF1α and HIF2α have nonidentical, and probably opposing, roles in clear cell renal cancer (Keith et al. 2012). In further support of this conclusion, HIF2α has been shown in the renal carcinoma setting to cooperate with c-Myc and to suppress p53, whereas HIF1α has the opposite effects (Keith et al. 2012). These different roles presumably reflect differences in their ability to activate different HIF target genes, for the reasons cited above, as well as differences with respect to other noncanonical functions.

**TARGETING HIF2-RESPONSIVE GENE PRODUCTS WITH SMALL MOLECULES**

Drugs exist for a number of HIF2-responsive gene products suspected of playing a role in clear cell renal carcinogenesis including VEGF, PDGF B, c-Met, TGFA (ligand for EGFR), cyclin D1, and both SDF1 and its receptor, CXCR4. Among epithelial cancers, clear cell renal carcinomas have the highest VEGF levels, presumably driven by pVHL loss and HIF2 deregulation. Moreover, the induction of VEGF early during the evolution of clear cell renal carcinomas probably minimizes the selection pressure to activate alternative angiogenic factors. Perhaps for this reason, clear cell carcinomas have proven to be particularly sensitive to drugs that inhibit VEGF or its receptor, kinase insert domain receptor (KDR). Six such agents have now been approved by the U.S. Federal Drug Administration (FDA) for this indication (Table 1).

Platelet-derived growth factor subunit B (PDGFB) acts to support vascular pericytes, and immature vessels without pericyte coverage are more sensitive to VEGF withdrawal than are mature vessels (Benjamin and Keshet 1997; Benjamin et al. 1998, 1999). Many of the currently available KDR inhibitors also inhibit the PDGF receptor, which could theoretically enhance their activity. On the other hand, PDGF receptor inhibitors have not proven to be active in clear cell renal carcinoma, either alone or added to VEGF inhibitors (Polite et al. 2006; Vuky et al. 2006; Hainsworth et al. 2007; Ryan et al. 2011).

HIF2, in ways that are still not completely understood, enhances signaling by c-Met and its ligand hepatocyte growth factor (HGF) (Koochekpour et al. 1999; Pennacchietti et al. 2003; Nakaigawa et al. 2006), and pVHL-defective renal carcinoma cells are hypersensitive to c-Met depletion with small interfering RNAs (siRNAs) (Bommi-Reddy et al. 2008). Cabozantinib, which inhibits KDR and c-Met, is highly active against clear cell renal carcinoma, including in patients who have failed multiple VEGF inhibitors (Choueiri et al. 2015, 2016). It is unclear, however, whether this is truly because of its ability to inhibit c-Met. It is formally possible, for example, that cabozantinib is simply a better KDR inhibitor in vivo than other drugs in this class.

Clear cell renal carcinomas express high level of the HIF2-responsive growth factor transforming growth factor-α (TGF-α) as well as its receptor, epidermal growth factor receptor (EGFR) (Uhlman et al. 1995; Ramp et al. 1997; Knebelmann et al. 1998; de Paulsen et al. 2001; Merseburger et al. 2005; Smith et al. 2005). HIF2 promotes the translation of EGFR (Franovic et al. 2007; Uniacke et al. 2012), whereas pVHL has been reported to promote the endocytosis and degradation of EGFR (Zhou and Yang 2011; Uniacke et al. 2012). Although clear cell renal carcinoma lines are sensitive to EGFR inactivation in mouse models, EGFR inhibitors have thus far been essentially inactive against clear cell renal carcinoma patients. The reason for this conundrum is unclear, but it could be due, at least partly, to selection for EGFR dependence ex vivo during cell line selection and propagation.

The cells giving rise to clear cell renal carcinoma are unusual in that HIF2α can induce cyclin D1 in this

**Table 1.** List of genes associated with methylation imprint, which are confirmed by knockout mouse studies

| Agent          | U.S. Brand Name | Company             | Description | Approval Year for Kidney Cancer |
|----------------|-----------------|---------------------|-------------|---------------------------------|
| Sorafenib      | Nexavar         | Onyx/Bayer          | TKI         | 2005                            |
| Sunitinib      | Sutent          | Pfizer              | TKI         | 2006                            |
| Bevacizumab    | Avastin         | Genentech           | Anti-VEGF Ab| 2009                            |
| Pazopanib      | Votrient        | GlaxoSmithKline     | TKI         | 2009                            |
| Axitinib       | Inlyta          | Pfizer              | TKI         | 2012                            |
| Cabozantinib   | Cabometrix, Cometriq | Exelixis          | TKI         | 2016                            |
context, whereas HIF and hypoxia usually down-regulate cyclin D1 (Bindra et al. 2002; Zatyka et al. 2002; Baba et al. 2003). Moreover, pVHL-defective clear cell renal carcinoma cells appear to be more dependent on cdk6 than isogenic cells in which pVHL has been restored and are sensitive to shRNA-mediated depletion of cyclin D1 (Bommi-Reddy et al. 2008; Zhang et al. 2013). Finally, a human polymorphism linked to the risk of clear cell renal cell carcinoma alters the binding of HIF2α to a cyclin D transcriptional enhancer element (Schodel et al. 2012). It will be of interest to see if newer, more specific, cdk4/6 inhibitors, such as palbociclib and abemaciclib, are active in this disease.

CXCR4 has been implicated in the maintenance of clear cell renal carcinoma cancer–initiating cells, in tumor cell invasion and metastasis, and in mobilization of myeloid-derived suppressor cells that have been linked to resistance to VEGF blockade (Staller et al. 2003; Zagzag et al. 2005; Pan et al. 2006; Struckmann et al. 2008; Gassenmaier et al. 2013; Panka et al. 2013; Vanharanta et al. 2013; Micucci et al. 2015). A number of CXCR4 inhibitors have been proven safe in man, and clear cell renal carcinoma trials are contemplated.

**DEVELOPMENT OF DIRECT HIF2 ANTAGONISTS**

In theory targeting HIF2 itself would be more efficacious than targeting any one HIF2-responsive gene product. Unfortunately, however, DNA-binding transcription factors have classically been viewed as undruggable by the pharmaceutical industry. The one exception to this rule has been the steroid hormone receptors, which have ligand-binding hydrophobic pockets that can serve as an entry point for drug design.

Fortunately, pioneering work by Rick Bruick and Kevin Gardner identified a potentially druggable hydrophobic pocket in the HIF2α PAS B domain (Scheuermann et al. 2009, 2013). Moreover, these investigators identified chemical matter that can bind to this pocket and allosterically prevent dimer formation between HIF2α and aryl hydrocarbon receptor nuclear translocator (ARNT) (Scheuermann et al. 2009, 2013; Rogers et al. 2013). These discoveries formed the basis for medicinal chemistry efforts at Peloton Therapeutics that led to the HIF2α inhibitor PT2385 and the highly related tool compound PT2399 (Chen et al. 2016; Cho et al. 2016; Wallace et al. 2016).

PT2385 and PT2399 inhibit HIF2α-ARNT1 dimerization and HIF2α-dependent transcription in cells at high-nanomolar/low-micromolar concentrations (Chen et al. 2016; Cho et al. 2016; Wallace et al. 2016) (Fig. 2). Importantly, these effects are highly specific because these two compounds do not affect HIF1 (Chen et al. 2016; Cho et al. 2016; Wallace et al. 2016). PT2399 also inhibits the growth of pVHL-defective clear cell renal carcinoma cells in soft agar assays, in subcutaneous and orthotopic xenograft assays, and in lung colonization assays aimed at modeling established pulmonary metastases (Chen et al. 2016; Cho et al. 2016; Wallace et al. 2016). These effects of PT2399 on transcription and tumor growth are on-target because they can be reversed with HIF2α variants that contain single missense mutations that prevent them from binding to PT2399 (Chen et al. 2016; Cho et al. 2016). PT2385 and PT2399 are also active in patient-derived xenograft models, including some models that are relatively resistant to the KDR inhibitor sunitinib (Chen et al. 2016; Cho et al. 2016). Based on these data PT2385 has entered human clinical trials, with promising early signs of activity (Chen et al. 2016).

Nonetheless, it is already clear that some pVHL-defective clear cell renal carcinoma lines and patient-derived xenografts are resistant to PT2385/PT2399, as are some patients (Chen et al. 2016; Cho et al. 2016). This is because of, at least in part, previously unappreciated variability with respect to HIF2α dependence among

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**Figure 2.** Control of hypoxia-inducible factor 2 (HIF2) by von Hippel–Lindau tumor-suppressor protein (pVHL) and PT2385/PT2399. In the presence of oxygen pVHL binds directly to the HIF2α subunit and polyubiquitylates it, thereby targeting HIF2α for proteasomal degradation. In pVHL-defective cells HIF2 accumulates inappropriately. PT2385 and the related compound PT2399 bind directly to HIF2α and induce an allosteric change that prevents HIF2α from binding to its obligate partner, aryl hydrocarbon receptor nuclear translocator 1 (ARNT1).
pVHL-defective clear cell renal carcinoma lines and tumors and suggests that predictive biomarkers will be needed to optimize the use of such agents in the clinic (Chen et al. 2016; Cho et al. 2016). In the preclinical models the most sensitive renal carcinoma lines and patient-derived xenografts had the highest HIF2α levels, suggesting these two properties are linked (Chen et al. 2016; Cho et al. 2016). Conversely, the presence of p53 mutations predicts for resistance (Chen et al. 2016; Cho et al. 2016). Although p53 mutations are relatively rare in primary clear cell renal carcinomas, their true prevalence in metastatic disease is not known. Moreover, it is possible that p53 mutations are selected for by prior therapy, including therapy with VEGF inhibitors.

**CONCLUSION**

Biallelic inactivation of the VHL tumor-suppressor gene is the most frequent initiating, or truncal, event in clear cell renal carcinoma. The VHL gene product, pVHL, is the substrate recognition subunit of a ubiquitin ligase that targets HIFα subunits for proteasomal degradation when oxygen is present. Loss of pVHL leads the inappropriate accumulation of active HIF1 and HIF2. HIF2α drives clear cell renal carcinogenesis, and drugs that inhibit the HIF2-responsive gene product VEGF are now cornerstones of kidney cancer therapy. HIF2α was recently found to have a druggable hydrophobic pocket, and a first-generation HIF2α inhibitor that blocks its dimerization with ARNT, and hence inactivates HIF2α, has now entered the clinic based on promising preclinical data. Nonetheless, HIF2α dependence among clear cell renal carcinomas appears to be heterogeneous. Moreover, p53 pathway mutations confer resistance to HIF2α antagonists in preclinical models. These two observations imply the need for predictive biomarkers to optimize the use of HIF2α antagonists in the clinic.

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