

The von Hippel-Lindau Gene, Kidney Cancer, and Oxygen Sensing

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Abstract. Recent studies of a relatively rare hereditary cancer syndrome, von Hippel-Lindau (VHL) disease, have shed new light on the molecular pathogenesis of kidney cancer and, perhaps more important, on how mammalian cells sense and respond to changes in oxygen availability. This knowledge is already translating into new therapeutic targets for kidney

cancer as well as for multiple conditions, such as myocardial infarction and stroke, in which ischemia plays a pathogenic role. This review summarizes the current knowledge of the molecular pathogenesis of von Hippel-Lindau disease and the role of the VHL gene product (pVHL) in kidney cancer and the mammalian oxygen sensing pathway.

von Hippel-Lindau Disease

Approximately 100 yr ago, the British surgeon Treacher Collins described two siblings with retinal blood vessel tumors (1). Shortly thereafter, the German ophthalmologist Eugen von Hippel also described the familial occurrence of such lesions (2). It was the Swedish neuropathologist Arvind Lindau who appreciated that these familial retinal lesions, which are frequently referred to in the literature as angiomas or hemangiomas, were a marker for a systemic disorder (now called von Hippel-Lindau [VHL] disease) that also involved blood vessel tumors, called hemangioblastomas, of the central nervous system (especially the cerebellum and spinal cord) (3). It has been suggested that both the retinal lesions and central nervous system lesions be called hemangioblastomas because they are histopathologically (and probably pathophysiologically) very similar (4). Some patients with VHL disease also develop multiple visceral cysts, especially in the kidneys and pancreas. The renal cysts that develop in VHL disease are precursor lesions that can give rise, over time, to clear cell renal carcinomas (Figure 1). Indeed, hemangioblastomas and clear cell renal cell carcinomas are the two leading causes of death in this patient population (5). Other tumors linked to VHL disease include pheochromocytomas, pancreatic islet cell tumors, endolymphatic sac tumors, and papillary cystadenomas of the epididymis (males) or broad ligament (females). Although highly variable, tumors typically develop in patients with VHL disease in the second, third, and fourth decades of life.

As is true for most hereditary cancer syndromes, VHL

disease is linked to inactivation of a tumor suppressor gene (in this case, the *VHL* gene, which resides on chromosome 3p25) (6). Typically, patients with VHL disease have inherited an inactive *VHL* allele from an affected parent. In other words, patients with VHL disease are *VHL*^{+/-} heterozygotes. Some *VHL* patients without a positive family history have, upon further investigation, been found to have a parent who is mosaic for a *VHL* mutation (presumably as the result of a *de novo* mutation during early development) (7,8). Tumor development in VHL disease is linked to inactivation or loss of the remaining wild-type *VHL* allele in a susceptible cell, which leads to loss of the *VHL* gene product pVHL. This event can be documented in very early, premalignant, renal lesions (including cysts) in patients with VHL disease (9–11) (Figure 1). It is presumed that mutations that affect one or more other genes is required for conversion of these premalignant renal lesions to frank renal cell carcinomas. More than 30 yr ago, Knudson (12) and Comings (13) both predicted that the genes responsible for hereditary forms of cancer might also play critical roles in their nonhereditary counterparts. Indeed, the *VHL* gene is frequently inactivated, whether as a result of mutation or hypermethylation, in nonhereditary clear cell renal carcinomas and hemangioblastomas (14). In these settings, *VHL* gene alterations are not inherited but occur somatically.

The *VHL* gene contains three exons and encodes an approximately 4.5-kB mRNA that is ubiquitously expressed (15–17). In particular, *VHL* mRNA expression is not restricted to those tissues that give rise to tumors after *VHL* inactivation. Alternative splicing gives rise to a second transcript that is missing exon 2. Some tumors exclusively produce this second mRNA isoform, suggesting that its protein product is at least partially defective with respect to tumor suppressor activity (18). The *VHL* gene is conserved in worms, flies, and rodents (19–23). Homozygous *VHL* inactivation in the mouse is embryonic lethal (24,25). *VHL*^{+/-} mice develop blood vessel tumors of the liver that seem, as in the case of human VHL disease, to be linked to loss of the remaining wild-type *VHL* allele (25). Why

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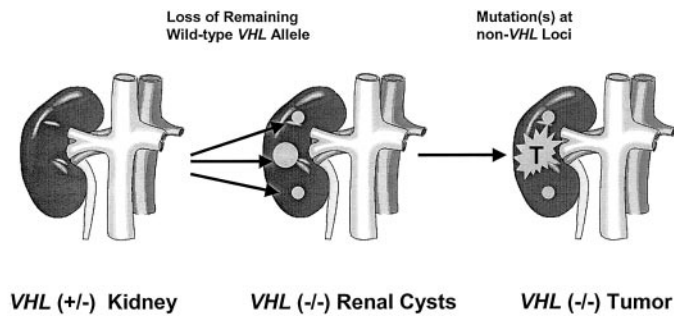


Figure 1. Renal pathology in von Hippel-Lindau (VHL) disease. Patients with VHL disease are *VHL*^{+/-} heterozygotes. Inactivation of the remaining wild-type *VHL* allele in renal tubular epithelial cells gives rise to renal cysts. Conversion of the preneoplastic renal cysts to clear cell renal carcinomas is presumed to involve additional genetic changes at other loci.

humans and mice develop tumors at different sites after *VHL* inactivation is not yet clear. A similar conundrum exists, however, for many other hereditary cancer genes.

VHL Protein

The *VHL* mRNA encodes a protein that contains 213 amino acid residues and migrates with an apparent molecular weight of 24 to 30 kD in SDS-polyacrylamide gels (26). A second protein isoform is produced as a result of internal translation from an in-frame ATG at codon 54 (27–29). This second pVHL isoform seems to be the major pVHL isoform in many cells and tissues. Both the long and short form of pVHL behave similarly in many of the assays described to date and, for this reason, are often referred to generically as “pVHL.” pVHL resides primarily in the cytosol (21,26,30–33) but can also be found in the nucleus (28,32–34), in association with the endoplasmic reticulum/membranes (35,36), and in mitochondria (37). Moreover, pVHL shuttles between the nucleus and cytoplasm in a Ras-related nuclear protein (RAN–dependent, transcription-dependent, manner (38–41). The short form of pVHL seems to have a higher affinity for the nucleus than does the long form, although the significance of this finding is not yet clear (28,32).

Tumor formation by pVHL-defective renal carcinoma cells is suppressed after restoration of wild-type pVHL function (26,29,42,43). Thus, pVHL is a tumor suppressor protein based on both genetic and functional criteria. pVHL can also suppress pVHL-defective tumor cell proliferation *in vitro* under specific experimental conditions such as growth in low serum or as three-dimensional spheroids (44–47). In some of these settings, inhibition of cellular proliferation is accompanied by decreased invasiveness/motility (48) and enhanced differentiation (44,45).

The primary sequence of pVHL does not closely resemble other known proteins and does not contain any obvious structural motifs that might provide clues to its biochemical functions. It is now clear, however, that pVHL forms stable complexes in mammalian cells with other proteins, including elongin B, elongin C, Cul2, and Rbx (also called Roc1 and Hrt1; Figure 2) (21,42,49–54). Importantly, Elledge *et al.* (55)

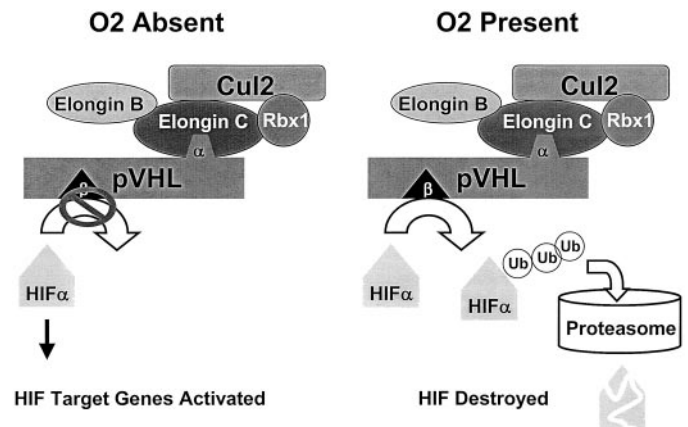


Figure 2. The pVHL ubiquitin ligase complex. pVHL forms a complex with Elongin B, Elongin C, Cul2, and Rbx1 that polyubiquitinates and hence targets for proteasomal degradation, hypoxia-inducible factor α (HIF α) subunits when oxygen is present. In the absence of oxygen, HIF α subunits accumulate and activate hypoxia-inducible genes.

noted that elongin C and Cul2 resemble two yeast proteins called Skp1 and Cdc53, which were known to regulate protein turnover. In particular, many proteins that undergo regulated destruction are first covalently modified by the attachment of a polyubiquitin tail, which serves as a signal for degradation by a multiprotein complex called the proteasome. Substrate-specific polyubiquitylation involves the sequential action of the E1 ubiquitin activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. One subfamily of E3 ubiquitin ligases in yeast is made up of Skp1 and Cdc53 bound to one of many F-box proteins (so-named because of a short, collinear, motif first identified in cyclin F) (56,57). In such SCF (Skp1/Cdc53/F-box) complexes, the F-box protein represents the substrate specificity determinant. Analogous complexes exist in mammalian cells, with the function of Cdc53 performed by a member of the Cullin family. The pVHL complex architecturally resembles an SCF complex, with pVHL assuming the role of the F-box protein, and displays ubiquitin ligase activity *in vitro* (58,59).

The search of substrates of the pVHL ubiquitin ligase complex was aided tremendously by the recognition that pVHL-defective tumors such as hemangioblastomas and renal cell carcinomas are highly vascular and frequently overproduce angiogenic peptides such as vascular endothelial growth factor (VEGF) (60–66). Moreover, hemangioblastomas, renal cell carcinomas, and pheochromocytomas occasionally cause paraneoplastic erythrocytosis as a result of ectopic erythropoietin production (67). Both VEGF and erythropoietin mRNA are induced by inadequate oxygenation and hence referred to as hypoxia-inducible genes (68,69). Earlier studies showed that the production of hypoxia-inducible mRNA is uncoupled from changes in ambient oxygen in pVHL-defective tumor cell lines and that this defect can be corrected by restoration of wild-type pVHL function (43,70–72).

pVHL-Hypoxia-Inducible Factor Connection

Many hypoxia-inducible mRNA are under the control of a heterodimeric transcription factor called hypoxia-inducible factor (HIF), which consists of an α subunit (HIF1 α , HIF2 α , or HIF3 α) and a stable β subunit (HIF1 β or ARNT) (68,69). Earlier studies showed that HIF α subunits are normally polyubiquitylated and degraded under well-oxygenated conditions. In a landmark paper, Maxwell *et al.* (73) showed that pVHL-defective cells fail to degrade HIF α subunits under well-oxygenated (normoxic) conditions and that pVHL and HIF can physically associate. Subsequently, several groups showed that HIF α subunits are polyubiquitylated by the pVHL complex under normoxic conditions (33,74–77) (Figure 2). Importantly, pVHL contains two subdomains, called α and β , that are frequently mutated in VHL Disease (78). The former represents the elongin C binding domain, whereas the latter domain is the HIF binding region.

The accumulation of HIF α subunits under hypoxic conditions reflects that the interaction of pVHL with HIF α is oxygen dependent. In particular, HIF α is hydroxylated on one of two conserved prolyl residues by members of the Egg Laying Defective Nine (EGLN) family (also called the PHD family or HPH family) in the presence of oxygen (22,79–83) (Figure 3).

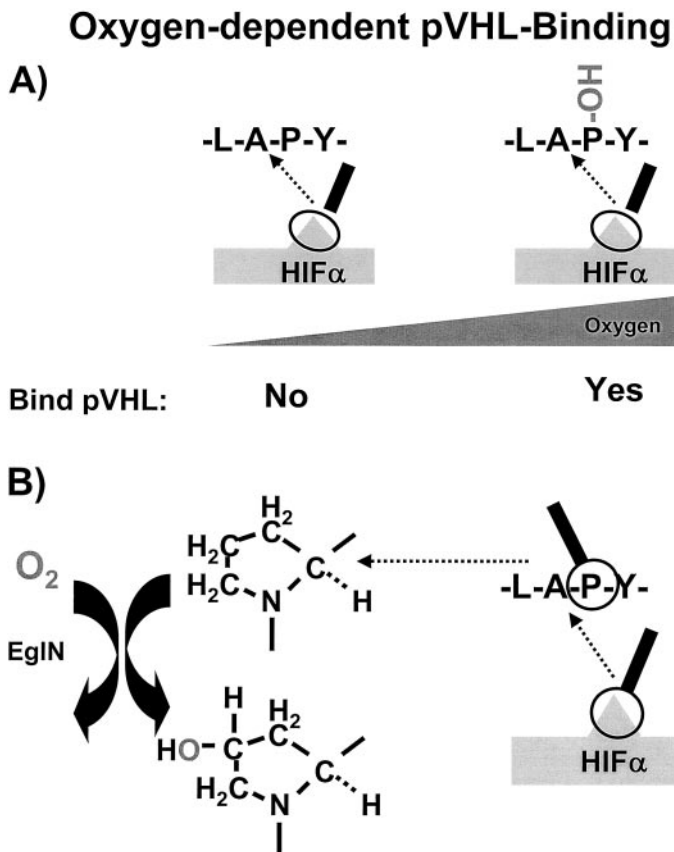


Figure 3. Oxygen-dependent pVHL binding. (A) In the presence of oxygen, HIF α subunits are hydroxylated on one of two conserved prolyl residues. pVHL binds exclusively to hydroxylated HIF α . (B) Hydroxylation of HIF α is carried out by members of the Egg Laying Defective Nine (EGLN) family.

Hydroxylation serves as a signal for recruitment of pVHL. This reaction is inherently oxygen dependent because the oxygen atom of the hydroxyl group is derived from molecular oxygen (Figure 3). Moreover, the EGLN prolyl hydroxylases require iron and 2-oxoglutarate as co-factors. The former explains the hypoxia mimetic effects of iron chelators and antagonists. The crystal structure of pVHL bound to HIF reveals that selective binding to hydroxylated HIF is the result of critical hydrogen bonds between the prolyl hydroxyl group and two hydrophilic pVHL residues within the otherwise hydrophobic pVHL β domain (84,85).

Several lines of evidence suggest that HIF is a/the critical downstream target of pVHL with respect to tumor formation. First, pVHL mutants associated with hemangioblastoma and renal cell carcinoma have, when tested, been defective with respect to HIF polyubiquitylation (74–76,86,87). Second, pVHL tumor suppressor activity can be neutralized by peptides that bind to the pVHL β domain (88). Both of these observations suggest that HIF, or perhaps some other substrate recognized by the β domain, is an important pVHL target. The importance of HIF *per se* with respect to renal carcinogenesis is underscored by the finding that a HIF1 α variant that escapes recognition by pVHL can override pVHL’s tumor suppressor activity *in vitro* (but not *in vivo*) (88), whereas a similar HIF2 α variant can override pVHL’s tumor suppressor activity *in vivo* (89). Thus, in the context of renal cell carcinoma, inhibition of HIF is necessary for tumor suppression by pVHL.

Emerging genotype-phenotype correlations in VHL disease strongly suggest that pVHL has functions unrelated to HIF. VHL disease can be subdivided into type 1 (low risk of pheochromocytoma) and type 2 (high risk of pheochromocytoma) (90). Type 2 is subdivided into type 2A (low risk of renal cell carcinoma), type 2B (high risk of renal cell carcinoma), and type 2C (pheochromocytoma only). Type 2 disease is almost invariably associated with VHL missense mutations, suggesting that pheochromocytoma reflects a VHL “gain of function” or that complete loss of pVHL function is incompatible with pheochromocytoma development. When tested, type 2C mutants retain the ability to regulate HIF, in contrast to mutants associated with types 1, 2A, and 2B disease (86,87). Thus, no biochemical gain of function or loss of function has been revealed so far for type 2C mutants. Types 2A and 2B mutants are comparably defective with respect to HIF regulation and yet confer different risks for renal cell carcinoma. One recent report suggested that type 2A mutants, in contrast to 2B mutants, are defective with respect to associating with and stabilizing microtubules (32). Finally, one form of familial polycythemia, called Chuvash polycythemia, was recently linked to a homozygous, hypomorphic, VHL mutation (R200W), which encodes a pVHL mutant that is quantitatively defective with respect to HIF regulation (91). It is interesting that these families do not seem to be at markedly increased risk for tumor formation. Perhaps in keeping with this observation, forced activation of HIF target genes in animal models has not given rise to tumors in the tissues examined to date (92–94). Collectively, these considerations point to pVHL’s having multiple functions, with site-specific tumor risk determined by

the degrees to which these various functions are quantitatively or qualitatively altered.

pVHL Targets Other than HIF

Indeed, a number of functions have been ascribed to pVHL, although in some cases, these functions may ultimately be linked to HIF dysregulation. pVHL has been reported to interact with fibronectin and clearly plays a role in regulation of extracellular matrix assembly and turnover (36,44,45,48). How and to what degree these two findings are linked are still unclear. In addition to affecting fibronectin matrix assembly, pVHL regulates a variety of genes, such as TGF- β , tissue inhibitors of metalloproteinase, and matrix metalloproteinases, that affect matrix turnover and may also affect certain integrins (48,95,96).

A number of studies have indicated that pVHL can interact with certain atypical PKC members (97–102). It has been suggested that pVHL directly inhibits PKC activity or acts as a PKC ubiquitin ligase. pVHL also inhibits cyclin D1, although there is no evidence that this involves a direct interaction between pVHL and cyclin D1 (103,104). Moreover, the effects of pVHL on the cell cycle and cell-cycle regulators might be confounded because some HIF targets, such as TGF- α , are potent renal epithelial mitogens (105–108). Other potential direct targets of pVHL include transcription factor SP1 (109,110), the large subunit of RNA polymerase II (111), two deubiquitinating enzymes called VDU1 and VDU2 (112,113), and the RNA-binding protein hnRNP A2 (114). Most of these other pVHL interactors await independent verification.

Implications for Therapy

As described above, inactivation of pVHL is an early, causal event in a significant percentage of clear cell renal carcinomas, and preclinical studies indicate that restoring pVHL function in pVHL-defective renal carcinoma cells is sufficient to suppress tumorigenesis. Inhibition of HIF target genes is necessary for tumor suppression by pVHL. Whether it is likewise sufficient is not yet known, although inhibition of HIF in most but not all cancer models has been sufficient to suppress tumor growth. Thus, there is a strong rationale for developing therapies directed against HIF or its downstream targets in renal carcinoma. Unfortunately, sequence-specific DNA-binding transcription factors such as HIF have not yet proved to be highly tractable drug targets. Fortunately, however, a number of HIF-responsive genes implicated in tumorigenesis encode growth factors that might (along with their receptors) be amenable to pharmacologic agents (Figure 4). These include paracrine-acting angiogenic factors such as the VEGF and PDGF, which are thought to stimulate endothelial cells and supporting pericytes, respectively, as well as autocrine growth factors such as TGF- α . These three growth factors bind to membrane-bound receptors that become active as tyrosine kinases upon ligand binding. Treatment with a VEGF neutralizing antibody led to delayed disease progression in a cohort of patients with metastatic renal carcinoma in a randomized phase II trial and is now being tested in phase III (115). A number of drugs that inhibit the tyrosine kinase activity of the VEGF receptor KDR

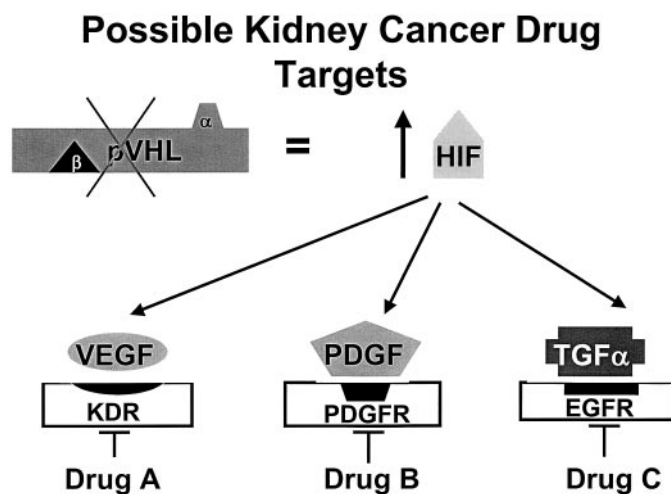


Figure 4. Possible kidney cancer drug targets. Inactivation of pVHL leads to overproduction of HIF target genes. Some of these genes encode growth factors, such as vascular endothelial growth factor, PDGF B, and TGF- α , that bind to specific receptors. Signal transduction by these receptors is linked to their ability to serve as tyrosine kinases and can be blocked with drugs currently being tested in humans.

and the PDGF receptor, which are similar proteins because they are phylogenetically related, are currently being tested in human kidney cancer patients. Examples include PTK787 and SU11248 (116–119). In addition, recent studies indicate that the mTOR inhibitor rapamycin and histone deacetylase inhibitors such as trichostatin A also lead to downregulation of VEGF in tumor cell lines and might be tested in this setting as well (120–122). In theory, treatments aimed at VEGF and/or PDGF might be combined with agents such as Iressa that inhibit EGFR (96,123), which is the receptor for TGF- α . Of note, TGF- α was sufficient to cause renal cysts in one mouse model and thus might provide a link between pVHL inactivation and premalignant cyst formation (124). EGFR inhibitors have been effective in some renal cyst models (125). Also, there is evidence for molecular cross-talk between EGFR signaling and HIF (126). Therefore, drugs that inhibit EGFR might be additive or synergistic with drugs that inhibit other HIF targets.

It is interesting that another HIF target, CAIX, encodes a renal carcinoma antigen previously called G250 (10,127,128). Antibodies against G250 might be used to localize tumors and, in time, as therapeutics. Gene expression profiling has uncovered a wealth of additional genes that are differentially expressed by cells that do or do not contain wild-type pVHL, including genes encoding secreted or membrane-bound proteins (104,129–131). Thus, loss of pVHL and consequent overproduction of HIF target genes might provide a foundation for new immunotherapeutic as well as chemotherapeutic approaches to kidney cancer.

HIF is conserved among metazoans and presumably evolved to enhance the survival of cells exposed to acute or chronic hypoxia (68,69). HIF induces changes in glucose uptake and

metabolism to allow for continued ATP generation in a hypoxic environment, changes in carbonic anhydrase secretion to compensate for increased lactic acid production resulting from anaerobic glycolysis, and changes in red blood cell production and angiogenesis to enhance oxygen delivery. In theory, a HIF agonist might therefore be beneficial in diseases, such as myocardial infarction and stroke, that are characterized by acute or chronic ischemia (Figure 5). EGLN belongs to a superfamily of iron and 2-oxoglutarate-dependent dioxygenases (132–134). Small molecule EGLN inhibitors that act as iron or 2-oxoglutarate antagonists lead to HIF stabilization and activation of HIF target genes (22,80,81). Of note, a single administration of one such compound (FG-0041) was efficacious in preserving myocardial function in a rat model of myocardial infarction (80,135). Open questions are whether the salutary effects of FG-0041 in this model were truly related to HIF and whether chronic administration of a HIF agonists would be sufficient to produce pathologic changes in the tissues, such as the eye, brain, and kidney, that are affected in VHL disease.

Conclusion

Inactivation of the *VHL* tumor suppressor gene is an early, causal event in the development of clear cell renal cell carcinomas and hemangioblastomas. Its protein product, pVHL, is part of an E3 ubiquitin ligase complex that targets HIF α subunits for destruction in the presence of oxygen. Accordingly, pVHL-defective tumor cells overproduce a variety of HIF target genes, which have been implicated in metabolism, mitogenesis, and angiogenesis. The products of some of these genes might be suitable targets for pharmacologic or immunological approaches to kidney cancer. Notably, inhibition of HIF is necessary for renal carcinoma suppression by pVHL in xenograft models. The interaction of pVHL with HIF is governed by prolyl hydroxylation of one of two conserved prolyl residues within the HIF α subunits. This reaction is carried out by members of the EGLN family and is inherently oxygen dependent. Acute inhibition of EGLN might theoretically be useful in the treatment of diseases characterized by inadequate oxygen delivery. It is likely that HIF-independent functions, in a tissue-specific manner, also contribute to tumor development

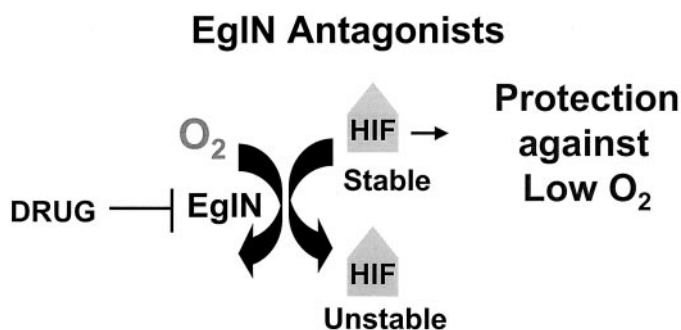


Figure 5. EGLN Antagonists. In theory, drugs that blocked EGLN activity would lead to activation of HIF target genes, which might be beneficial in diseases characterized by inadequate tissue oxygenation.

after pVHL inactivation. Further elucidation of these functions should lead to enhanced understanding of renal carcinogenesis and should also speak to the advisability of pharmacologically manipulating HIF activity in humans.

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