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# Von Hippel-Lindau Tumor Suppressor Pathways & Corresponding Therapeutics in Kidney Cancer

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## Abstract

The identification and application of the *Von Hippel-Lindau* (*VHL*) gene is a seminal breakthrough in kidney cancer research. *VHL* and its protein pVHL are the root cause of most kidney cancers, and the cascading pathway below them is crucial for understanding hypoxia, in addition to the aforementioned tumorigenesis routes and treatments. We reviewed the history and functions of *VHL*/pVHL and Hypoxia-inducible factor (HIF), their well-known activities under low-oxygen environments as an E3 ubiquitin ligase and as a transcription factor, respectively, as well as their non-canonical functions revealed recently. Additionally, we discussed are how their dysregulation promotes tumorigenesis: beginning with chromosome 3 p-arm (3p) loss/epigenetic methylation, followed by two-allele knockout, before the loss of complimentary tumor suppressor genes leads cells down predictable oncological paths. This pathway can ultimately determine the grade, outcome, and severity of the deadliest genitourinary cancer. We finish by investigating current and proposed schemes to therapeutically treat clear cell renal cell carcinoma (ccRCC) by manipulating the hypoxic pathway utilizing Vascular Endothelial Growth Factor (VEGF) inhibitors, mammalian target of rapamycin complex 1 (mTORC1) inhibitors, small molecule HIF inhibitors, immune checkpoint blockade therapy, and synthetic lethality.

### Keywords

VHL; HIF; ccRCC; kidney cancer; hypoxia

Kidney cancer is among the ten most common in both men and women, with ~75,000 new cases and ~15,000 deaths every year in the United States, ~67,000 cases and ~23,000 deaths in China, and ~430,000 cases and ~180,000 deaths globally (Chen, Wanqing et al., 2016; 2020; Sung et al., 2021). Clear cell renal cell carcinoma (ccRCC) is by far the predominant type, making up to 80% of all renal cell carcinoma cases. *Von Hippel-Lindau* (*VHL*), a crucial hypoxia regulator, has a towering influence in current research: the inactivation of *VHL* occurs in ~90% of ccRCC tumors. This first silencing acts as a truncal, initial mutation from which others follow determining the subtype and form (Nickerson et al., 2008; Elias et al., 2020). The initial inactivation of *VHL* is caused mostly by the loss (typically

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via chromothripsis) of the short arm of chromosome 3, found in over 90% of patients ((Nickerson et al., 2008; Mitchell et al., 2018). This short arm (3p) carries three other two-hit tumor suppressor genes (TSG)s as well: *polybromo 1* (*PBRM1*), *SET domain containing 2* (*SETD2*), and *BRCA1-associated protein 1* (*BAP1*). *VHL* and (optionally, one or more of) the other three are fully silenced when the other allele succumbs to point mutations in 60–70% of cases, or epigenetic silencing (Dalgliesh et al., 2010). The application of VHL to ccRCC is an instructive story of biology's unifying aspect: the gene is named after a separate disease named von Hippel-Lindau syndrome, characterized by the formation of tumors in a number of different organs: e.g. retinal/craniospinal haemangioblastomas, pancreatic tumors, and epididymal cystadenomas (Lonser et al., 2003). The gene responsible for the autosomal dominant disorder was narrowed down to the 3p region in 1988 before being definitively found in 1993 (Latif et al., 1993). Given that the leading cause of death in VHL disease is metastatic ccRCC, curiosity turned to the gene's cancer connections (Maher et al., 1990). At the time, an unqualified mechanism of action was absent, but the correlative power between *VHL* and ccRCC was strong: inactivation on both alleles was ubiquitous in ccRCC patients, and the reintroduction of a functional *VHL* into tumor cells *in vivo* was sufficient to suppress the ability to form tumors in mice (Iliopoulos et al., 1995). Thus, at the turn of the millennium, it became increasingly obvious that *VHL* and ccRCC were linked, and the only remaining question was how.

## The *VHL*-HIF Canonical Signaling Axis

A fundamental part of this question was so importantly and elegantly resolved that the Nobel Committee at the Karolinkska Instituet determined to award the Nobel Prize in Physiology or Medicine to Sir Peter J. Ratcliffe, William G. Kaelin Jr., and Gregg L. Semenza 'for their discoveries of how cells sense and adapt to oxygen availability' on the Erythropoietin-VHL-HIF axis in 2019 (Zhang et al., 2019).

*VHL* codes for pVHL, which, after binding to elongin B and C, is linked to RING-box protein 1 and cullin-2 (Kaelin, 2002) (Fig. 1). This complex is an (E3) ubiquitin ligase that binds to hypoxia-inducible factor (HIF) α and ubiquitinates it, following which it is degraded in the proteasome (Kaelin, 2002). Thus, a decrease in pVHL leads to an increase in HIF (due to a decrease in degradation), which possesses three alpha isoforms that dimerize with a shared beta subunit. The isoforms vary in locality, function, and inhibitory pathways; HIF1α is universally expressed among cell types while HIF2α is cell-type specific, the isoforms have some overlap but also regulate some different genes each, and some pockets in the structure are conserved but not all of them are (Hara et al., 2001; Raval et al., 2005; Wu et al., 2015). The sensitivity of this pathway to hypoxia is that prior to recognition by the E3 ubiquitin ligase complex, prolyl hydroxylases of the EglN (Egl nine homolog 1; aka proyl hydroxylase domain-containing protein (PHD)) family hydroxylate proline sites on the HIFα subunits (Kaelin, 2002). This prolyl hydroxylation is crucial to recognition by the E3 ubiquitin ligase. However, these hydroxylases require oxygen as a co-substrate; under hypoxia, their activity is decreased, preventing hydroxylation and recognition by the pVHL degradation complex, and resultingly increasing HIF activity. HIF1 and 2 induce the transcription of hundreds of target genes, all with the goal of steering the cell successfully through the oxygen-poor environment (Semenza, 2007). HIF-3 is much less understood than

its two counterparts, with prior evidence suggesting it can act as a negative regulator of HIF-1/2 and recent studies indicating it functions as a complement leading to increased activation of HIF genes (Wu et al., 2015). Leading evidence suggests that while HIF-1α overexpression inhibits ccRCC, HIF-2α overexpression drives ccRCC. Whilst expression of HIF-1α in *VHL–/–* renal adenocarcinoma cell lines delayed tumor growth, overexpressing HIF-2α is both necessary and sufficient for such growth (Raval et al., 2005; Shen et al., 2011).

*VHL*-HIF signaling and the HIF-mediated transcriptional response are far more important than their connections to cancer when defective alone, as these series of regulation are part of the normal, well-functioning response to a hypoxic stimulus. The *VHL*-HIF pathway is now being investigated as a therapeutic target and finding treatments for a disease which, despite being the deadliest genitourinary cancer, until 2005 only had one United States Food & Drug Administration-approved (FDA) drug with which to combat it (interleukin-2).

## *VHL* Tumorigenesis as a Truncal Mutation

*VHL* loss is required but not solely sufficient for ccRCC (Wang et al., 2014). Parallel targeted sequencing enabled scientists to find three more tumor-suppressing genes (i.e. *PBRM1*, *SETD2*, and *BAP1)* on chromosome 3p, often deleted in tandem with *VHL*. Each of these genes encode proteins that happen to be epigenetic-related-PBRM1 is a nucleosome remodeling complex component, SETD2 is a histone methyltransferase, and BAP1 is a histone deubiquitinase. All three genes, in addition to *VHL* itself, reside within a very select region of 3p (Moore et al., 2012). With heterozygous *VHL* mutations leading to a proclivity towards tumorigenesis in humans, *VHL* possesses all the hallmarks of a Knudson two-hit suppressor gene for oncogenesis (Zbar et al., 1987; Crossey et al., 1994). In mice, ccRCC seems to require *VHL* and *Pbrm1*/*Bap1* deficiencies, as inactivation of any single gene was not sufficient (Wang et al., 2014; Turajlic et al., 2018b). Of these genes, *PBRM1* is mutated in 45–55% of human ccRCC cases, *SETD2* 20– 40%, and *BAP1* 14–19%. Interestingly, *PBRM1* and *BAP1* appear to be mutated together less than random chance would indicate, leaving them with a degree of mutual exclusivity (Gu et al., 2017). The different epigenetic genes mutated lead to different outcomes: *BAP1*-absent tumors possess worse patient survival, higher grade, and develop faster than *PBRM1*-absent tumors (Kapur et al., 2013; Turajlic et al., 2018a). *PBRM1*-loss tumors can be converted into tumors of a higher grade by inactivating *tuberous sclerosis complex 1* (*TSC1*), often mutated in ccRCC and a regulator of mammalian target of rapamycin complex 1 (mTORC1) (Kucejova et al., 2011; Gu et al., 2017). The mutually exclusive *PBRM1* and *BAP1* mutations also develop separately; *SETD2* mutations are typically found only in tumors that lack already lack *PBRM1*, and not *BAP1* (Peña-Llopis et al., 2013). Other branches from the truncal *PBRM1* mutation include modifications to the mTOR pathway (*TSC1*, *PTEN*, *PIK3CA*) and chromatin-modification genes by somatic copy number alterations/driver mutations, deletion of chromosome 9 and 14, and loss of TP53 (Elias et al., 2020). In addition, other mutations have been characterized to increase the belligerence and metastasis of PBRM1-loss tumors along mTOR, driver SCNA, and *SETD2* pathways (Turajlic et al., 2018a; Turajlic et al., 2018b).

## The pVHL-HIF Regulatory Pathway

The pathways that descend from HIF are too complex and lengthy to fully mention here; the HIF system regulates hundreds of genes (Semenza, 2007). Out of all the complexity, one observation that has posed exceptionally relevant to clinical treatment is that, as a result of HIF signaling, ccRCC seems to have the largest expression of Vascular endothelial growth factor A (VEGFA) mRNA of all epithelial tumors (Jubb et al., 2004). Current therapeutic strategies aim to target every step of this pathway, from pVHL directly, to its substrate HIF, to HIF transcription targets such as VEGF. Other transcription/ translation factors also work in HIF-dependent and -independent avenues during hypoxia to maintain oxygen homeostasis. For example, Nuclear Factor-κB and HIF are known to induce each other, Activator Protein 1 cooperates with HIF, and p53 and MYC Proto-Oncogene act in a mixed role as antagonist and cooperator of HIF (Kenneth and Rocha, 2008). HIF has also been discovered to interact with chromatin-modifying proteins/complexes and exhibits control over translation (Kenneth and Rocha, 2008). On the other hand, Notch signaling appears to act independently of HIF in pVHL-loss human ccRCC cells grown *in vivo* (Sjölund et al., 2008).

As in all things, the pathway is more complicated than just HIF; pVHL has more substrates than hypoxia-inducible factor alone. For instance, Zinc fingers and homeoboxes 2 (ZHX2), Scm-like with four malignant brain tumor domains (SFMBT1), have been found to be substrates of pVHL while TANK binding kinase 1 (TBK1) was found to be a pVHL adaptor protein (Zhang et al., 2018; Hu et al., 2020; Liu, X. et al., 2020). pVHL interacts with ZHX2 and SFMBT1, like for HIF, in a process leading to ubiquitination and degradation (for instance, after SFMBT1 proline hydroxylation by PHD2). TBK1 is dephosphorylated and rendered inactive in the presence of pVHL, which is aptly similar to the previously reported finding of Akt1 phosphorylation regulation by pVHL following proline hydroxylation (Guo et al., 2016). When pVHL is suppressed, these three proteins become overactive and oncogenic. ZHX2 depletion decreased the expression of NF-κB genes (*IL6*, *IL8*, *CCL2*) and several genes related to invasion, metastasis, metabolism, and anti-apoptosis (Zhang et al., 2018). SFMBT1 had previously been found to be a transcriptional repressor in the LSD1 demethylase complex but was discovered to activate sphingosine kinase 1 (*SPHK1*), leading to tumorigenesis *in vivo* and *in vitro*. TBK1 was formerly known to interact with stimulator of interferon genes (STING) in the presence of virus, triggering TBK1 phosphorylation and subsequently, the type I IFN immune response (Liu et al., 2015). This immune signaling protein was later investigated for its role in cancer, and it was established that the molecule phosphorylates AKT and p62 with oncogenic results (Hu et al., 2020). TBK1 was additionally later found to be a target for conducting the strategy of synthetic lethality in *VHL*-loss kidney cancer (Hu et al., 2020). All of these aforementioned substrates act in a fully HIF-independent manner to promote ccRCC tumorigenesis.

Other substrates or binding partners which can be ubiquitinated and degraded or display altered activity upon pVHL binding include euchromatic histone-lysine methyltransferase 2, actin cross-linker filamin A, erythropoietin receptor, transcription factor B-Myb, ceramide kinase like protein, N-Myc downstream regulated gene 3, Card9, Akt1, and BIM-EL (Na et al., 2003; Chen et al., 2015; Lee et al., 2015; Guo et al., 2016; Heir et al., 2016; Okumura

et al., 2016; Segura et al., 2016; Casciello et al., 2017; Yin et al., 2017). The plethora of functions these substrates reminds us that the cell's response to hypoxia and cancer-caused dysregulation is broad and complex. While pVHL is more famously a contributor to the ubiquitin ligase complex, the evidence above suggests it may have other functions and can act as an adaptor protein. The search for more potential pVHL substrates, HIF target genes, and a more complete knowledge of these complex interconnected relationships will be crucial to understanding hypoxia and defeating ccRCC.

## The Modern and Multi-pronged Mechanisms to Therapeutically Address ccRCC

ccRCC has posed quite the foe, given that the disease is shockingly common yet deadly, resistant to chemotherapy, and lacked therapeutic options before 2005 (Choueiri and Motzer, 2017). However, on account of the knowledge recently gained and summarized above, researchers have at last begun to exploit these interconnected genes and proteins with the cancer's destruction in mind.

The scope of these drugs' effects should be firmly laid out; while the following treatments do extend progression-free survival (PFS) and often have an admirable partial response (PR) rate, they are rarely cures – complete responses are few and far between. The prolonging of life and improvements to quality of life nonetheless remain invaluable for patients and their loved ones. Given that, at first, the only tool at medicine's arsenal was interleukin-2, we certainly have come a long way. Subsequently, a second flurry of activity followed, with the FDA accepting six new drugs in four years. Now, given that cabozantinib, nivolumab, and lenvatinib with everolimus have been shown to be better than everolimus alone, and with the sunitinib comparison trials below also held in mind, it feels as if we have entered a third generation of safe and effective ccRCC treatments (Molina et al., 2014; Motzer et al., 2015a; Motzer et al., 2015b; Choueiri et al., 2016; Choueiri et al., 2017).

### Anti-VEGF Therapy

The first modern therapies to be approved by the Food & Drug Administration took advantage of the extremely high *VEGFA* expression in ccRCC tumors by inhibiting the VEGF receptor. VEGFA promotes angiogenesis and cell growth; inhibiting it or its receptor could therefore slow uncontrolled proliferation of ccRCC. Sorafenib, a tyrosine-kinase inhibitor (TKI) targeting the VEGF receptors, was the first of these treatments to be approved, heralding a new, second age of ccRCC therapeutics (See Table 1 for drug clinical profiles) (Escudier et al., 2007). The TKI sunitinib and bevacizumab (a monoclonal antibody targeting VEGFA) were approved thereafter (Motzer et al., 2007; Summers et al., 2010). Two more TKIs – axitinib and pazopanib – have also been approved for the treatment of ccRCC, with evidence suggesting that, in comparison to sunitinib, the latter is safer and has better quality-of-life for patients, and the former has a better PFS (Motzer et al., 2013a; Motzer et al., 2013b). Other more recently approved TKIs include cabozantinib and lenvatinib (Motzer et al., 2015a; Choueiri et al., 2016). These six TKI drugs (all excluding monoclonal antibody bevacizumab) inhibit additional receptors (Table 1); a strategy targeting several receptors can often be more comprehensive and effective

while reducing the possibility of tumor resistance. For example, cabozantinib also inhibits the hepatocyte growth factor receptor (HGFR/c-Met) while a series of converging factors in ccRCC heavily depend upon and utilize this pathway. MET protein is overexpressed and phosphorylated by *VHL*-deficient cells, induces tumor growth following oncogenesis (which it promotes), is implicated in resistance to the commonly-used drug sunitinib, and is necessary for ccRCC survival (Nakaigawa et al., 2006; Bommi-Reddy et al., 2008; Zhou et al., 2016). This approach of targeting multiple vulnerable, hyper-dependent pathways would later be the basis of synthetic lethality targeting.

## HIF-2a Inhibition

HIF-2α was long thought to be "undruggable" as it lacked a ligand-binding domain, but recent groundbreaking crystallography found the existence of several pockets in the protein that a small molecule could fit into to disrupt its dimerization with HIF-1β (Scheuermann et al., 2009; Wu et al., 2015). If a small molecule inhibitor could be found, this could hypothetically inhibit not just a target like *VEGFA*, but the entirety of HIF-2α-mediated transcriptional activity. Screens of hundreds of thousands of compounds and drug fragments in screens yielded a class of similarly structured molecules with allosteric inhibitory ability (Scheuermann et al., 2013). The general characteristics of the ligands discovered found from this study were used later in a mixture of intentional modification and structure selectivity to create compound PT2385, the first HIF-targeting small-molecule inhibitor to face a human clinical trial (Wehn et al., 2018). The safety profile was favorable, with none of the 51 patients choosing to leave the experiment as a result of severe adverse effects or a dose-limiting event (Courtney et al., 2018). The overall response rate was 14%; the disease control rate was 66%. There was a significant difference in PFS between those who had a trough concentration of PT2385 over 0.5 μg/mL 12 hours after receiving the oral dose on day 15 as opposed to those that did not, with those with the higher concentration faring better. This, another study on glioblastoma, and a combination study with nivolumab illustrated a frustratingly high drug exposure variability due to differing metabolism of the drug betwixt patients, with a higher exposure being correlated with better PFS (Rini et al., 2019a; Strowd et al., 2019). This variability left some patients underexposed and made decisions around dosage of PT2385 more difficult.

PT2977 (later known as MK-6482, Belzutifan) was developed to improve upon this first step, with higher HIF pocket affinity, less lipophilicity, less affinity for serum proteins, and lower glucoronidation (shown to be the primary divergent metabolic path causing exposure variation) (Xu et al., 2019). Despite only a small structural change, the pharmacokinetics were far improved, and a phase 3 trial for the molecule is currently in the recruiting phase (NCT04195750 & NCT04586231). A phase I/II and a phase II study of PT2977 suggest similar safety levels as PT2385, along with promising efficacy rates even in pretreated cases. ORR was 24% (I/II) and 28% (II), the disease control rate was 80% (I/II), 67% (I/II) and 87% (II) of patients had tumor shrinkage, the 12-month PFS rate was 98% (II), and 81% had responses greater than six months (I/II) (Choueiri et al., 2020b; Jonasch et al., 2020). Other clinical trials for PT2977 and PT2385 are ongoing.

One drawback to the small-molecule inhibitor approach is that the mechanism of action of this technique depends on the structure of HIF-2, which can change due to *de novo* mutation. Indeed, two mutations have been found – one on HIF-2α decreasing small-molecule affinity and the other on HIF-1β increasing affinity for dimerization with HIF-2α – that convey a level of acquired resistance to these treatments (Chen, Wenfang et al., 2016). Both of these mutations have been found in tumorgrafts following exposure to PT-2399 and the former mutation in two human patients during a PT2385 clinical trial (Chen, Wenfang et al., 2016; Courtney et al., 2020). However, several more pockets have been found in HIF-2α, which presents an opportunity for combination treatments and the development of additional inhibitors (Wu et al., 2015; Wu, D. et al., 2019). While acquired resistance to these inhibitors is unfortunate, we must remember to compare them against the existing treatments today; notably, acquired resistance to sunitinib developed within 60 days in mice tumorgrafts but took over 120 days for PT-2399, representing significant progress in ccRCC therapeutics (Chen et al., 2016).

Another mechanism to directly target HIF-2α utilizes siRNA. Arrowhead Pharmaceuticals develops siRNA to target ligands found only in ccRCC. This RNAi clinical mechanism has been known for some time, but one of the largest challenges was engineering a manner to deliver the siRNA effectively to the target (Bobbin and Rossi, 2016). In a strategy known as Dynamic PolyConjugates, researchers can link the targeting siRNA with a vehicle that targets the cancer cells by recognizing the intermembrane proteins αvβ3 and αvβ5 commonly overexpressed in ccRCC, enabling the effective delivery of siRNA. This method inhibited tumor growth in mouse xenografts, and, if successful, could help overcome acquired resistance via HIF-2α/ HIF-1β structural changes and decrease toxicity by seeking out ccRCC ligands directly (Wong et al., 2018). A small phase Ib trial was conducted utilizing these techniques with mixed results on advanced hepatocellular carcinoma (Wu et al., 2019). On account of failing to meet the primary endpoint of HIF-1α mRNA silencing after a single dose, the trial was ended early. On the other hand, one of the nine patients exhibited a PR enduring 72 weeks following the study start date. Another phase 1b trial is currently recruiting patients (NCT04169711). Hopefully, this creative delivery mechanism that targets ccRCC and evades one mechanism of tumor resistance will eventually provide better outcomes to patients.

## Immunotherapeutics

Yet another approach has been to use immune-checkpoint inhibitors to restore T cell antitumor activity; this line of attack has led to a recent flurry of FDA-approved drugs within the past few years. CTLA-4 is a T cell protein receptor that transmits inhibitory signals to nearby T cells, promoting immunotolerance. Ligand PD-L1, expressed in tumors, acts upon T cell surface receptor PD-1 contributing to immunosuppression and tumor survival – not surprisingly, both of these receptors are upregulated in cancers. Current immunotherapies use monoclonal antibodies to target these T cell receptors and ligands: nivolumab and pembrolizumab targeting PD-1, avelumab targeting PD-L1, and ipilimumab targeting CTLA-4. Nivolumab and pembrolizumab were both approved as singular treatments for RCC following studies comparing them to a placebo, but ipilimumab was approved in combination with nivolumab after being shown to be more effective than sunitinib (Motzer

et al., 2015b; Tykodi et al., 2019; Motzer et al., 2020). Avelumab also followed this latter path, being accepted with axitinib following a comparison trial to sunitinib (Choueiri et al., 2020a).

The connection between the effectiveness of immunotherapy and the varying forms of ccRCC caused by different mutations is currently under dispute. While it is logical that tumors with separate paths to tumorigenesis and different expression of genes and proteins would be commensurately distinctly prone or invulnerable to immunotherapy, the jury is still out as to which is true for *PBRM1*-loss ccRCC. While one team of researchers found that loss of this gene defined a resistance to immune checkpoint inhibitor (ICI) therapy, another laboratory found the opposite was true, and that clinical benefits to anti-PD-1 therapy was correlated with loss of the same gene (Miao et al., 2018; Liu, X.-D. et al., 2020). More work remains to be done to provide specific, targeted therapy to the various strains of cancer previous research has elucidated.

## mTORC Inhibition

Other trials have aimed to inhibit mTORC1, which regulates cell survival and proliferation and is one of the most frequently dysregulated pathways in human cancers (Xu et al., 2014). The drugs termsirolimus and everolimus act as analogs of rapamycin (rapalogs) and inhibit mTORC1. Both drugs have been successful over interferon-α treatment (a common previous standard of treatment), were approved by the FDA, and represent some of the first few bold steps taken after 2005 (Hudes et al., 2007; Motzer et al., 2008).

There are significant structural and functional differences between mTORC1 and its related protein complex, mTORC2, which both contain mTOR. For instance, current mTORC1 inhibitors are not effective against mTORC2, and while HIF-1α expression relies upon both complexes, HIF-2α is regulated by mTORC2 alone. Trials attempting to target both mTORC1 and 2 have generally failed due to overly high toxicity, which is all too unfortunate because inactivation of mTORC1 results in the loss of negative feedback inhibition of mTORC2 (Santoni et al., 2014).

## Combinatorial Treatments & Synthetic Lethality

This idea of combining treatments as in the mTORC1-mTORC2 or ICB-ICB (immune checkpoint blockade) studies above is hardly new but is occasionally like a siren's song – alluring yet deadly. The advantages of this strategy could potentially be vast, as attacking ccRCC in multiple ways could increase effectiveness, decrease the possibility of resistance, and yet yield no significant increase in toxicity by spreading the load across several separate systems. Another primary matter of consideration is giving thought to how the pathways stemming from both drugs might interact with each other. The challenge of implementing this stratagem so far has been managing the toxicity. For instance, the administration of drugs that inhibit both mTOR and VEGF was too toxic, and blocking VEGF with both bevacizumab and sunitinib led to peril in its patients (Feldman et al., 2009; Flaherty et al., 2015). Additionally, the original testing of termsirolimus with and without interferon-α found no significant difference in survival (Hudes et al., 2007). On the other hand, there is broad reason to think that using combination therapy to treat ccRCC has a bright future.

The FDA approved a dual treatment of lenvatinib (inhibitor of VEGFR and FGFR) and everolimus (mTORC1 inhibitor), which is more effective than everolimus alone, even as the two drugs must be dosed at lower concentrations together than they would have to be if administered separately (Molina et al., 2014; Motzer et al., 2015a). Five of the FDA-approved drugs for ccRCC are approved only as a part of combination treatment (Table 1). Moreover, five separate combinations of ICB/VEGF inhibitors have greater benefits in regards to PFS, overall survival (OS), and response rates, depending on the combination, compared to sunitinib alone (Motzer et al., 2018; Motzer et al., 2019; Rini et al., 2019b; Choueiri et al., 2021; Motzer et al., 2021).

Several more studies are currently underway to continue pushing this paradigm by utilizing a small-molecule HIF-2α inhibitor and a tyrosine kinase inhibitor at once, looking at the combination of PT2385 and nivolumab, PT2385 and cabozantinib, and PT2977 and cabozantinib (NCT02293980, NCT03634540). The campaign to find more effective and new combinations of already established and verified drugs lives on and will continue to be a valuable resource in oncology.

Synthetic lethality - the approach of looking for and targeting genes which have become essential for a cell after *VHL* loss - is another leading strategy in fighting kidney cancer. This plan is appealing for its cytotoxicity, specificity, and its capacity to evade acquired ccRCC resistance. At this time, several compounds have been found to be selectively deadly to *VHL*-absent ccRCC. STF-62247 was found in a compound screen, and is selectively lethal to *in vivo* and *in vitro* ccRCC cells via a HIF-independent autophagy-inducing mechanism (Turcotte et al., 2008). The authors also discovered another compound, STF-31, which possessed the same synthetic lethality and decreased tumor growth in mice *in vivo* (Chan et al., 2011). STF-31 directly binds and inhibits glucose transport protein, GLUT1 (encoded by the gene *SLC2A1s).* GLUT1 is overexpressed in ccRCCs, and *SLC2A1s* is upregulated by HIF factors, leading to a differential dependency on glycolysis (Chan et al., 2011).

Thompson et al. conducted another screen to find that inhibitors of ROCK1 had a cytotoxic effect inhibiting *in vivo* mouse tumor growth in *VHL*-deficient ccRCC but only under hypoxia, suggesting that it utilizes a HIF-dependent mechanism of action (Thompson et al., 2017). These same researchers have also separately found that some statins (HMG-CoA reductase inhibitors) are lethally synthetic due to their inhibition of mevalonate synthesis (Thompson et al., 2018).

A third team conducted lethality screens in fly and human cancer lines and discovered that the inhibition of both *CDK4* and *CDK6*, in combination with *VHL*, possessed a synthetic relationship independent of HIFs (even though HIF-2α is known to induce CDK partner cyclin D1) (Nicholson et al., 2019). When a CDK4/6 dual inhibitor (palbociclib) was combined with HIF-2α small-molecule inhibitor PT-2399, they operated synergistically and by some metrics outperformed the use of either drug individually in mice xenografts (Nicholson et al., 2019). Previously, this team had found that shRNA inhibition of *CDK6* alone, *MET*, and *MAP2K1/MEK1* each preferentially inhibited two different VHL-deficient ccRCC lines in a partially HIF-independent fashion, and a dual *CDK4/6* inhibitor limited

*in vitro* growth in these lines (Bommi-Reddy et al., 2008). This follows multiple studies showing inhibitors ademaciclib and palbociclib had deleterious effects on ccRCC, although the particular mechanism of action was not yet definitively proven (Nicholson et al., 2019). This same Dana-Farber Cancer Institute group has also discovered that HIF drives many histone lysine demethylases and is hyperdependent on some portions of this change in expression; loss of *EZH1* – a H3K27 methyltransferase – was synthetically lethal (Chakraborty et al., 2017).

Omacetaxine mepusuccinate (homoharringtonine) had already been approved by the FDA to treat chronic myeloid leukaemia before it was found to be synthetically lethal with ccRCC, making it an especially interesting compound to consider (Wolff et al., 2015).

Yet another screen found that the selenocysteine biosynthesis pathway was dysregulated in ccRCC; five of six genes in the pathway had depleted sgRNAs targeting them in *VHL*-absent ccRCC compared to cells with *VHL* (Sun et al., 2019). The researchers also found that the DNA damage response (DDR) was a key dysregulated player in cancer; loss of pVHL leads to genetic instability and the upregulation of DDR to repair and maintain the cancer cell's DNA. Knockout of several genes in both the selenocsysteine and DDR pathways were found to be synthetically lethal (Sun et al., 2019). Further studies have found that DNA repair and the SWI/SNF chromatin remodeling complex inhibitors are synthetically lethal to *PBRM1*-deficient cancers, adding another layer of complexity and discernment to the treating of various strains of kidney cancer (Sasaki and Ogiwara, 2020; Chabanon et al., 2021).

Lastly, we have found that TBK1 is hyper-activated in ccRCC, independently of HIF, upon pVHL loss or under hypoxic conditions, and tumors from patients revealed that TBK1 phosphorylation is notably increased (Hu et al., 2020). Conversely, a direct interaction with pVHL mediated by EglN-mediated hydroxylation was established to lead to lower levels of phosphorylation observed in the wild-type. The loss of TBK1 via targeted sgRNAs or shRNAs led to decreased cell proliferation and growth defects in *VHL*-null cells while leaving *VHL*-proficient cells unaffected – the hallmark of synthetic lethality. TBK1 was previously known to be an innate immune function actor, but the mechanism of action in this case was not related to these pathways. TBK1 was discovered to phosphorylate and contribute to stabilization of p62/SQSTM1 (an oncogene overexpressed in kidney cancer), a crucial protein for cell proliferation. This and many other synthetic lethal targets will hopefully lead to better outcomes for patients, laying low tumor cells while leaving normal cells unscathed. In the mean while, the strategy of finding lethal partners for ccRCC will continue to consist of the repetitive methods described above (screening tens of thousands of compounds) until computational technology acquires the ability to meaningfully induce possible drug candidates (Murali et al., 2021).

## Conclusion

Our ability to treat kidney cancer has only truly advanced insofar as our knowledge of the underlying causes and impacts of the disease has greatly improved. The implications of this work lie far beyond a single type of renal cell carcinoma; hypoxia is both a normal response

to a common deficiency and a condition found in most solid cancers. These common, ubiquitous cellular pathways have proven connotations to other cancers and dysfunctions and will continue to be a deserved subject of research going forward. With all of the excitement to be found in the last decade in treating this former apex predator, we remain cautiously optimistic that this coming decade will lead to more interesting discoveries and better outcomes for patients using the strategies and therapeutics described above.

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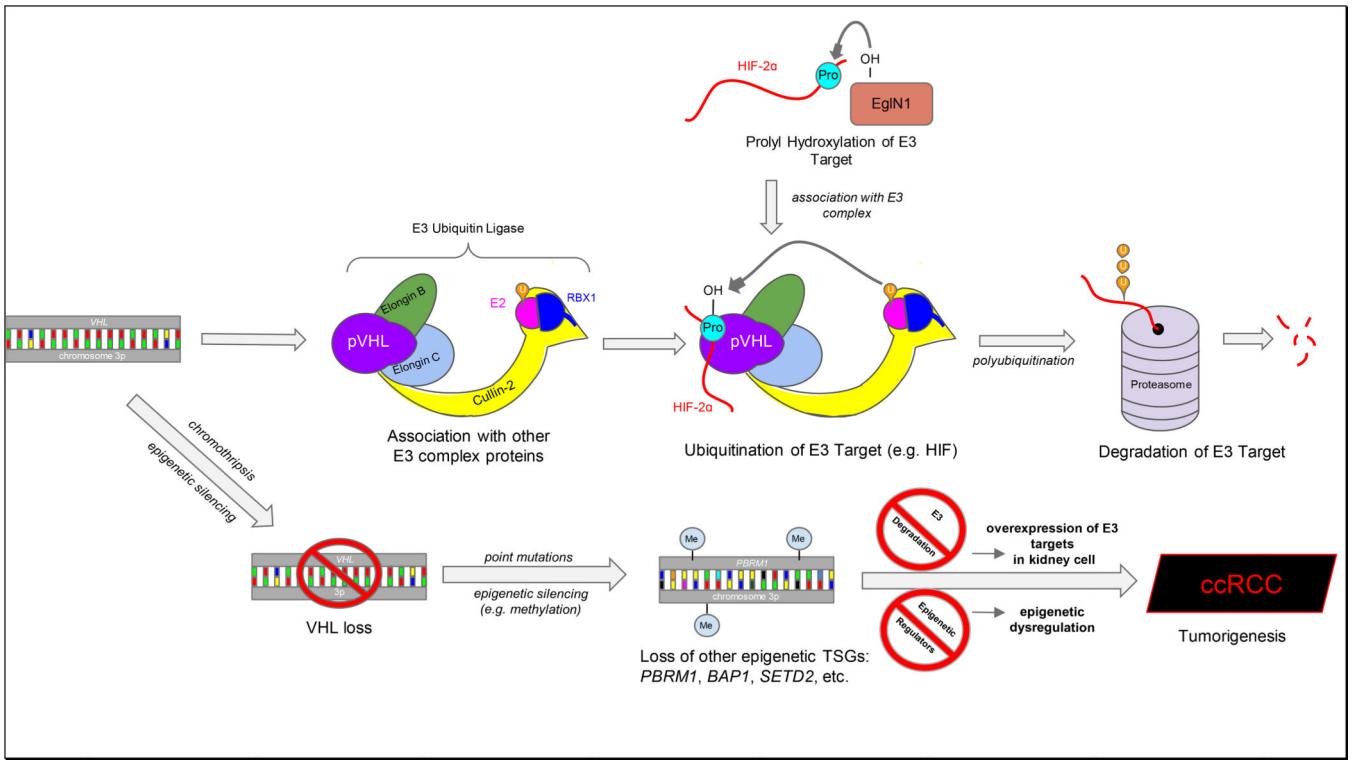


**Fig. 1. The *VHL*-pVHL-E3-ccRCC axis.**

The *VHL* gene is transcribed into the pVHL protein, which associates with several other proteins to form the E3 Ubiquitin Ligase complex. When a hydroxylated substrate is recognized, it is polyubiquitinated, which is a signal leading to degradation into oligonucleotides in the proteasome. This proline hydroxylation is crucial for recognition by the E3 complex, and the use of oxygen as a co-substrate in this step makes it sensitive to oxygen concentration. Under hypoxia (where oxygen is absent) or following VHL loss, this complex is no longer available to degrade its substrates in the usual way. When VHL loss (leading to overexpression of E3 complex substrates) is combined with the loss of other tumor-suppressing genes relating to epigenetic regulation, this leads kidney cells down a sure path leading to tumorigenesis and ccRCC. (p)VHL, von Hippel-Lindau tumor suppressor (protein); E2, Ubiquitin-conjugating enzyme; RBX1, RING-box protein 1; HIF-2, Hypoxia-inducible factor 2; EglN1, Egl nine homolog 1; TSG, tumor-suppressor gene; *PBRM1*, *polybromo 1*; *BAP1*, *BRCA1-associated protein*; *SETD2*, *SET domain containing 2*; ccRCC, clear cell renal cell carcinoma; U, ubiquitin; Pro, proline; Me, methyl group.

**Table 1.**

Selected studies demonstrating clinical profiles of ccRCC treatments.

| Trial ID | Compound / FDA Approval Date | Target(s) | *n* / Phase of Trial | ORR (in %) | OS (Median in months) | PFS |
|---|---|---|---|---|---|---|
| | ***Rapalogs*** | | | | | |
| NCT00065468 | Temsirolimus (2007) ^ | mTORC1 | 626/III | 8.6 *v* 4.8 | 10.9 *v* 7.3 | 3.8 *v* 1.9 |
| NCT00510068 | Everolimus (2009) | mTORC1 | 410/III | NR *v* 8.8 | NR *v* 8.8 | 4.0 *v* 1.9 |
| | **VEGF Inhibitors** | | | | | |
| NCT00073307 | Sorafenib (2005) | VEGFR, PDFGR, Flt-3, B-Raf | 902/III | 10 *v* 2 | 17.8 *v* 15.2 | 5.5 *v* 2.8 |
| NCT00083889 | Sunitinib (2006) ^ | VEGFR, PDFGR, c-Raf | 750/III | 47 *v* 12 | 26.4 *v* 21.8 | 11 *v* 5 |
| NCT00738530 | Bevacizumab (2009) * | VEGFA | 649/III | 30 *v* 12 | 23.3 *v* 21.3 | 10.2 *v* 5.4 |
| NCT00334282 | Pazopanib (2009) | VEGFR, PDGFR, c-Kit, FGFR | 435/III | 30 *v* 3 | 22.9 *v* 20.5 | 9.2 *v* 4.2 |
| NCT00678392 | Axitinib (2012) ^ | VEGFR, PDGFR, c-Kit | 723/III | 19 *v* 9 | 20.1 *v* 19.2 | 8.3 *v* 5.7 |
| NCT01865747 | Cabozantinib (2016) ^ | VEGFR, c-Met, AXL | 658/III | 17 *v* 3 | 21.4 *v* 16.5 | 7.4 *v* 3.8 |
| NCT01136733 | Lenvatinib (2016) *^ | VEGFR, FGFR | 153/II | 43 *v* 6 | 18.5 *v* 16.5 | 14.6 *v* 5.5 |
| | ***Immune Checkpoint Inhibitors*** | | | | | |
| NCT02231749 | Nivolumab (2018) ^ | PD-1 | 821/III | 23 *v* 4 | 25.8 *v* 19.7 | 4.2 *v* 4.5 |
| NCT02231749 | Ipilimumab (2018) *^ | CTLA-4 | 1096/III | 42 *v* 27 | NR *v* 26.0 | 11.6 *v* 8.4 |
| NCT02853331 | Pembrolizumab (2019) *^ | PD-1 | 861/III | 59.3 *v* 35.7 | NR *v* NR | 15.1 *v* 11.1 |
| NCT02684006 | Avelumab (2019) *^ | PD-L1 | 886/III | 51.4 *v* 25.7 | NR *v* NR | 13.8 *v* 8.4 |
| | ***HIF-inhibitors*** | | | | | |
| NCT02293980 | PT-2385 | HIF-2α | 51/I | 14 *v* NR | NR *v* NR | NR *v* NR |
| NCT03401788 | MK-6482 | HIF-2α | 61/II | 27.9 *v* NR | NR *v* NR | NR *v* NR |
| *RO7070179* | ARO-HIF2 | HIF-2α | 8/Ib | NR *v* NR | NR *v* NR | NR *v* NR |

Asterisked drugs are not monotreatments; comparison treatment is placebo unless marked ^.

ORR, overall response rate; OS, overall survival; PFS, progression-free survival; NR, not reached/not available.