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Review

The multifaceted von Hippel–Lindau tumour suppressor protein

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ABSTRACT

Loss of von Hippel–Lindau protein (pVHL) is known to contribute to the initiation and progression of tumours associated with VHL disease as well as certain sporadic tumours including clear cell renal cell carcinoma (ccRCC). The *VHL* gene was first identified and cloned over 20 years ago and our understanding of its functions and effects has significantly increased since then. The best-known function of pVHL is its role in promoting the degradation of hypoxia-inducible factor α subunit (HIFα) as part of an E3 ubiquitin ligase complex. HIF stabilisation and transcriptional activation are also associated with various epigenetic alterations, indicating a potential role for VHL loss with changes in the epigenome. This review will highlight current knowledge regarding pVHL as well as discuss potentially novel roles of pVHL and how these may impact on cancer progression.

1. von Hippel–Lindau (VHL) disease and gene

The first indication of a tumour suppressor role for von Hippel–Lindau (*VHL*) gene was the segregation of the mutant or loss of *VHL* allele in kindreds with VHL disease. The disease was first described in the early 1900s and is named after Eugen von Hippel and Arvid Lindau. von Hippel described a family with highly vascularised tumours of the retina [1], whilst Lindau reported that these retinal tumours commonly occurred alongside lesions of the central nervous system [2]. The disease is now known to be a hereditary cancer syndrome that affects approximately 1 in 35,000 individuals. Patients with VHL disease are at a high risk of developing benign tumours most commonly found in the central nervous system (haemangioblastoma), retina (angioma) and adrenal glands (phaeochromocytoma), as well as malignant tumours of the kidney (clear-cell renal cell carcinoma; ccRCC). Although less frequent, a variety of other benign tumours are also associated with the disease including tumours of the pancreas, inner ear and bilateral papillary cystadenoma of the epidydimus in men or broad ligament in females. Despite a wide range of pathological outcomes, ccRCC is the most frequent cause of morbidity and mortality amongst these patients.

The disease is a result of germline mutations of *VHL*. Patients are heterozygotes for *VHL* with one wild-type and one defective allele. Somatic inactivation of the second functional allele in susceptible cells, and therefore loss of VHL function, leads to pathological features of disease. VHL can be described as a classic tumour suppressor gene as this outcome is in line with Knudson's two hit model of tumourgenesis, whereby tumour suppressors are recessive at the genetic level and require somatic inactivation of the remaining wild-type allele to achieve tumourgenesis [3].

2. VHL and sporadic cancer

While VHL is widely expressed in human tissue, its loss is not exclusive to VHL disease. Certain sporadic cancers are strongly associated with VHL mutation [4]. Biallelic inactivation of *VHL*, due to mutation, loss or hypermethylation, is the most frequent genetic mutation in sporadic ccRCC. ccRCC is the most common form of kidney cancer, accounting for more than 70% of all RCC cases and is often characterised by loss of chromosome 3p events [5]. The prevalence of mutated VHL is study dependent, but it is estimated that between 60–80% of sporadic ccRCC display VHL mutations [6–8]. Thus, the majority of sporadic and hereditary (VHL disease-associated) ccRCC lacks functional VHL due to loss or mutation of the *VHL* gene. VHL loss is also associated with sporadic cerebellar haemangioblastomas with prevalence between 25–50% [9,10].

$VHL^{-/-}$ mice die *in utero* due to defective placental dysgenesis [11], but targeted cell specific suppression of *VHL* is possible.

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In liver cells of mice, *VHL* suppression forms benign tumours that are highly vascular in nature [12], while intriguingly targeted *VHL* inactivation in renal proximal tubule epithelial cells and pancreatic endocrine cells results in a polycystic, pre-cancerous, pathology in murine models [13,14]. In further support of its role as a tumour suppressor gene, reintroduction of wild-type VHL into $VHL^{-/-}$ ccRCC cell lines prevents RCC cells from forming tumours in nude mice [15].

3. VHL protein (pVHL)

Located on the short arm of chromosome 3 (3p25-p26), the VHL tumour suppressor gene was first identified and cloned in 1993 [16]. The gene, which contains 3 exons, encodes a protein of 213 amino acid residues, specifically known as $pVHL_{30}$. A second wild-type isoform of 160 amino acid residues is also expressed in human cells. $pVHL_{19}$ arises from alternate translation initiation at a second AUG codon (codon 54) within the VHL open reading frame [17]. Both forms are known to display tumour suppressor abilities. Reintroduction of RCC cells with either $pVHL_{19}$ or $pVHL_{30}$ inhibits tumour development in mouse xenograft models of the disease [18]. Although both are expressed in human cells, $pVHL_{19}$ is often the more prominent form in human tissue. Interestingly VHL isoforms are also located in different compartments of the cell. While VHL_{19} is equally distributed in the nucleus and cytoplasm, VHL_{30} is found predominantly in the cytoplasm [17], suggesting that under certain circumstances they display distinct roles. However, for the remainder of this review, unless otherwise stated, both protein forms of VHL will be referred to as pVHL.

4. pVHL functions

pVHL displays no enzymatic activity, but it is known to have multiple binding partners. The protein comprises of an α and β subunit. The α-domain serves as a binding site, whereas the β-domain plays important roles in substrate recognition. Investigations into the binding partners of the protein reveal its vast array of functions, many of which are relevant to its role as a tumour suppressor protein. In addition, categorising VHL patients based on disease outcome supports the notion that the tumour suppressor roles of pVHL are diverse. Specific mutations place patients at a higher risk of developing specific tumours. Patients with type 1 VHL disease display haemangioblastoma with a low risk of phaeochromocytoma and ccRCC while type 2 patients, who also display haemangioblastoma, have a high risk of developing phaeochromocytoma. Type 2 is further subdivided into 2A (low risk of ccRCC), 2B (high risk of ccRCC) and 2C who develop phaeochromocytoma only. Taking these observations into account, research to date has led to multiple discoveries about the precise functions of pVHL. It can therefore be described as an adapter protein with both post-translational as well as transcriptional effects.

Ubiquitylation represents an efficient mechanism of tagging proteins for degradation by the 26S proteasome. Ubiquitylation of proteins is accomplished by the actions of a common ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin-ligating enzyme (E3 ligase). VHL forms a multi-protein complex with elongins B and C, cullin 2 and Rbx-1 [19–21]. This complex is structurally similar to the yeast multicomplex, SCF (Skp1/Cdc53/F-box protein). Like the SCF complex, the ECV (elongin/culin/VHL) complex displays E3 ubiquitin ligase activity, which acts to polyubiquitylate protein substrates. Within this complex, pVHL acts as a substrate recognition subunit [22]. The most extensively studied and arguably most important protein target of pVHL-mediated ubiquitylation is the hypoxia-inducible factor (HIF) family of transcription factors, which will be discussed in more detail in the following sections. However, other protein targets of the ECV have also been identified. These include certain isoforms of protein kinase C (PKC) [23], proposed to be of particular importance in the regulation of c-Jun dependent apoptosis of neurons that are potential precursors of phaechromocytoma [24]. The ECV also targets the hyperphosphorylated form of Rpb1, a subunit of RNA polymerase II, which is activated during UV radiation and associated with stress-induced transcription [25].

Not all proteins that pVHL binds to results in polyubiquitylation, indeed certain *VHL* mutations associated with cancer pathogenesis are known to display normal ubiquitylation function, including ubiquitylation of HIF. For example type 2C pVHL mutants appear to retain the ability to polyubiquitylate HIF, but still have a heightened likelihood of developing phaeochromocytoma [26]. Therefore, mutations such as these are likely to be promoting cancer progression independent of the VHL/HIF axis and the ECV complex. In support of this investigations into alternate functions of pVHL provide a diverse array of roles for this protein. pVHL assists in regulation of the extracellular matrix (ECM), where its loss in this context is proposed to promote angiogenesis by allowing vessels to easier infiltrate tumours. pVHL and fibronectin, a glycoprotein that interacts with integrin proteins to regulate the ECM, are known to bind [27]. $VHL^{-/-}$ cells secrete higher levels of fibronectin but the assembly of this fibronectin as part of the ECM is disorganised. Loss of the pVHL-fibronectin interaction is therefore associated with defective ECM formation. This is reversed upon reintroduction of wild type pVHL [27]. More recently this has been shown to be related to decreased RhoA GTPase signalling in $VHL^{-/-}$ renal cancer cells [28]. pVHL also binds to collagen IV alpha 2 (COL4α2) [29]. In this context *VHL* loss is associated with a loss of COL4α2 from the ECM [30], further deregulating the ECM architecture in the $VHL^{-/-}$ tumour environment.

Microtubules are crucial for the maintenance of cell shape and polarity, and in addition, form the mitotic spindle during cell division. pVHL associates and binds to microtubules and inhibits their depolymerisation [31]. In mammalian cells, Thoma et. al. demonstrated that pVHL localises to the mitotic spindle and that loss of this protein resulted in, amongst other things, chromosomal instability, a classic feature of cancer cells [32]. Microtubules are also essential for cilia maintenance and therefore linked to this function of pVHL is the role of the protein in cilia maintenance. Cilia are of great importance in renal epithelial cells where primary cilia play an important role in the development as well as maintaining the integrity of nephrons. pVHL loss disrupts cilia formation in mouse inner medullary collecting duct kidney cells where pVHL is needed to direct the growth of microtubules toward the cell periphery, a function that have been proven to be vital for cilia formation [33]. Likewise, human $VHL^{-/-}$ cells do not have cilia and reintroduction of pVHL into these cells results in cilia formation. Loss of cilia in adult kidney cells due to pVHL dysfunction promotes the development of renal cysts, indicative of a pre-cancerous pathology.

VHL loss in ccRCC is also associated with genomic instability, a prominent feature in multiple cancer cells. $pVHL_{19}$ is found in the nucleus [17], suggesting a nuclear relevant role for this isoform. Recently functions for pVHL in the DNA damage response have been reported. Upon DNA damage, $VHL^{-/-}$ cells display attenuated apoptosis or abnormal cell-cycle arrest, but when pVHL is restored this response is normal [34]. Roe et. al. reported that pVHL destabilises Skp2 protein, an integral component of the Skp, Cullin, F-box-containing complex that promotes DNA synthesis in the S phase [35]. The transcription factor E2F1 is also up-regulated as part of the DNA damage response. Wei et. al. reported a feedback loop wherein pVHL regulates E2F1 activity which in turn regulates pVHL expression [36]. Recently, a possible physiologic role for pVHL in the DNA damage response was revealed whereby

suppressor of cytokine signalling 1 (SOCS1) mediates the nuclear redistribution and ubiquitylation of pVHL upon induction of double-stranded DNA breaks. *VHL* loss or mutation in this system significantly attenuated the DNA damage response resulting in increased accumulation of unrepaired DNA breaks [37].

pVHL is also proposed to be involved in modulating apoptotic regulators including the tumour suppressors p53 and nuclear factor NF-κB and the transcription factor E2F1. Under normal circumstances, p53 functions to promote cell cycle arrest and apoptosis in response to DNA damage as well as other stimuli. Roberts et. al. demonstrated that in ccRCC, HIF2α expression subsequent to VHL loss promotes Hdm2, an important mediator of p53 suppression [38], providing a potential mechanism of mutated VHL mediated survival. Similarly pVHL modulates NF-κB. pVHL loss results in increased NF-κB activity, which acts to promote oncogenesis via activation of anti-apoptotic pathways as well as proliferative pathways. Yang et. al. demonstrated that under normal circumstances pVHL that is bound to casein kinase 2 promotes the phosphorylation of Card9. Phosphorylated Card9 is an NF-κB agonist, whereby lack of phosphorylated Card9 (as is the case with pVHL loss) leads to increased NF-κB activity [39]. In further support of this mechanism, suppressing Card9 in ccRCC cells lacking function pVHL resulted in normal NF-κB activity [39]. Other roles for pVHL with relevance to cell proliferation have been suggested by Wang et. al. whom recently demonstrated that pVHL interacts with the androgen receptor, inhibiting transcriptional activity of the receptor that was linked to suppression of androgen-induced cell proliferation. Intriguingly pVHL did not affect receptor turnover [40].

Interestingly, not all VHL mutations lead to cancer indicating the multifaceted nature of this protein. A third group of patients with VHL mutations are classified as having type 3 disease. Patients with type 3 disease do not have increased propensity for cancer, but develop Chuvash polycythemia, a non-cancerous condition of erythrocytosis. Chuvash polycythemia was first identified in the Chuvash population in Russia [41] and although the mutations and disease are rare, they have since been found in patients elsewhere. The main VHL mutation identified in Chuvash polycythemia is R200W and is distinguishable by overproduction of HIF independent of tumour formation [42]. In addition, VHL-associated Chuvash polycythemia cells exhibit elevated levels of phosphorylated JAK2, leading to increased JAK2-STAT5 signalling, which likely accounts for the hypersensitivity of erythroid progenitor cells to erythropoietin. JAK2 stabilisation may be due to the attenuation of SOCS1 mediated targeting of phosphorylated JAK2 in the presence of mutated VHL [43].

5. The VHL/HIF axis

Despite a growing knowledge of the various functions of *VHL*, the most extensively characterised E3 ubiquitin ligase function of pVHL to date, and arguably its most important, is its regulation of the α-subunit of the HIF family of transcription factors [44–46]. Current evidence overwhelmingly suggests that HIF stabilisation as a consequence of *VHL* loss or mutation is an important oncogenic mechanism in VHL disease as well as sporadic ccRCC and haemangioblastomas [4].

HIFs are basic helix–loop–helix–PAS domain proteins of which there are three HIFα members (HIF1-3α) expressed in mammalian cells along with the constitutively expressed HIF1β (also called aryl hydrocarbon receptor nuclear translocator; ARNT). The HIFα and β subunits contain the same basic subunits, a bHLH domain for DNA binding, a Per-ARNT-Sim (PAS) involved in dimerization and a c-terminus that facilitates binding with other regulatory proteins (C-terminal transactivation domain; CAD). Under adequate oxygen tension, HIFα subunits are expressed but the protein is rapidly hydroxylated at two conserved proline residues by a family of oxygen dependent enzymes called the prolyl hydroxylases (PHDs or EGLNs) [47]. The PHDs, of which there are 4 expressed in mammalian cells (PHD1-4), add an –OH group to proline 402 and proline 564 of the HIFα subunit [46,48]. Only hydroxylated HIFα is recognised and bound via the β-domain of pVHL as part of the ECV complex. pVHL E3 ligase ubiquitylates HIFα via physical interaction with the core of the HIFα oxygen-dependent degradation domain (ODD). Polyubiquitylation of HIFα results in rapid degradation by the 26S proteasome such that HIFα is negligibly detectable in normoxic cells (Fig. 1). In the majority of circumstances where *VHL* is mutated or lost, HIFα is no longer recognised by the ECV and escapes degradation. Thus in $VHL^{-/-}$ tumours, HIF isoforms are constitutively stabilised.

However, it should be noted that various other factors also affect the stabilisation and expression of HIF. The most important of which is the availability of oxygen. Frequently tumours are highly hypoxic due to hyperactive tumour growth with a lagging, or inappropriately directed, blood vessel growth resulting in inadequate oxygen supply. Such environments mean that oxygen levels in a given tumour are highly heterogeneous. Measurements as low as 5 mmHg pO_2, which corresponds to approximately 0.7% O_2, have been recorded in regions of tumours [49]. During such circumstances of compromised oxygen availability, or hypoxia, the oxygen-dependent PHDs are inhibited. HIFα is no longer hydroxylated and therefore escapes ubiquitylation and degradation and is stabilised in the cell. Thus, HIF stabilisation, as a consequence of VHL inactivation or hypoxia, is an important event in a wide variety of cancers.

6. HIF-mediated transcription

Once expressed in a cell HIFα dimerises with the constitutively expressed HIFβ and this complex translocates to the nucleus. In the nucleus HIF recruits coactivators p300/CBP [50] and subsequently binds to a consensus hypoxia-responsive element (HREs; 5′-[A/G]CGTG-3′). HIF induced gene transcription is further controlled by factor inhibiting HIF (FIH) during adequate oxygen availability. Similar to the PHDs, the 2-oxoglutarate (2OG) and Fe(II)-dependent dioxygenase, FIH acts to hydroxylate an asparaginyl (Asn) residue of HIFα in the C terminal transactivation domain [51]. Such hydroxylation acts to restrict transcriptional activation of HIF by blocking the recruitment of p300/CBP. Therefore during periods of oxygen limitation FIH no longer catalyses this reaction, resulting in heightened HIF transcriptional binding and activation at hypoxia responsive gene promoters or HIF binding sites.

HIF acts to up-regulate a variety of hypoxia responsive genes that are involved in promoting an adaptive response to low oxygen tension. Genomic analysis of direct HIF binding sites suggests that HIF can directly target as many as 500 sites although exact numbers vary depending on the cell type interrogated [52,53]. Genes directly affected by HIF binding include those that promote energy metabolism (e.g. glucose transporter 1; *GLUT1*, phosphoglycerate kinase; *PKG*, lactose dehydrogenase A; *LDHA*), genes that up-regulate new blood vessel growth/angiogenesis (e.g. vascular endothelial growth factor; *VEGFA*), erythropoiesis (e.g. erythropoietin; *EPO*) and cell survival (e.g. transforming growth factor-α; *TGFα*). To date over 70 genes have been validated as direct HIF target genes [54], although given more recent genome wide studies it is apparent that the actual number may be significantly higher.

7. HIF stabilisation and cancer progression

Tumours associated with VHL disease as well as sporadic ccRCC and haemangioblastoma display an erratic vascular network

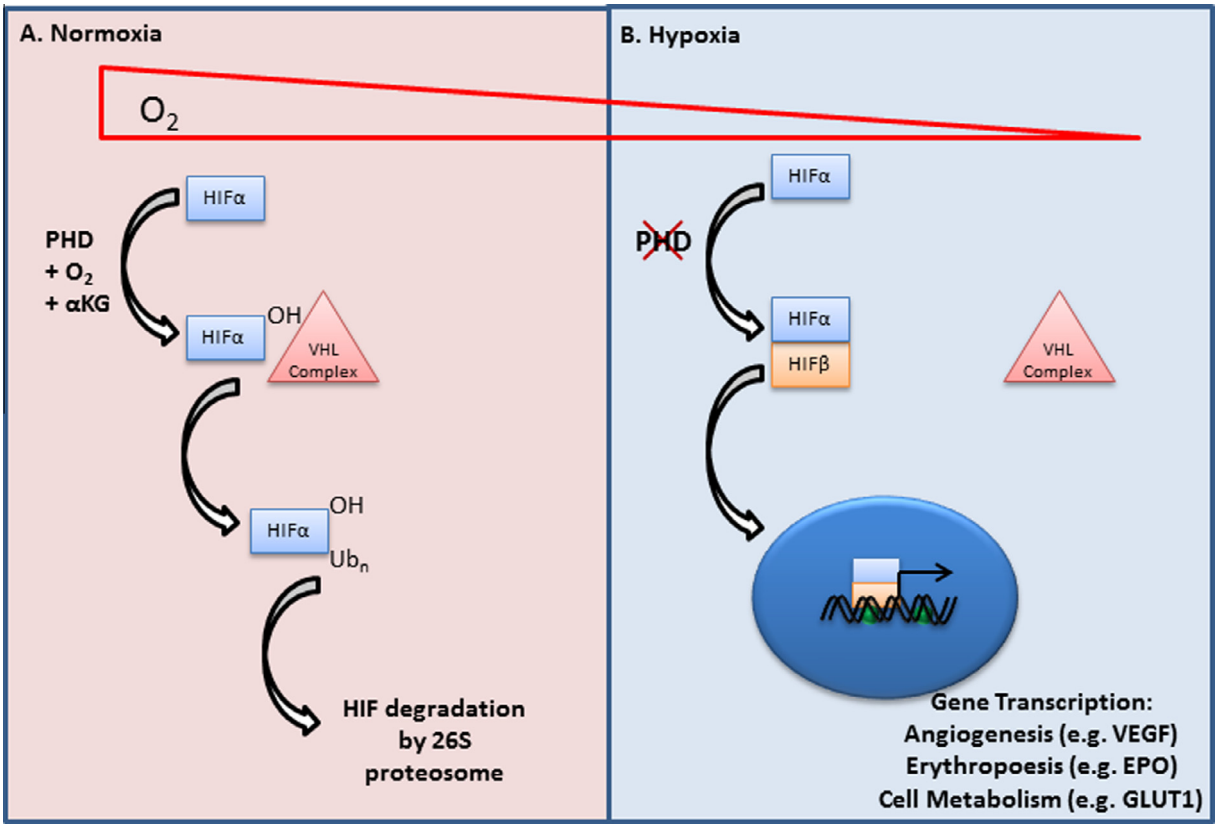

Fig. 1. Regulation of HIF protein. (A) During situations of normal oxygen tension, normoxia, HIF isoforms are expressed. However HIFα becomes rapidly hydroxylated at two proline residues by a family of oxygen and α-ketoglutarate dependent enzymes called the prolyl hydroxylases (PHDs). Hydroxylated HIFα is subsequently recognised by von Hippel–Lindau (VHL) tumor suppressor protein as part of the ECV complex. This results in rapid polyubiquitylation and degradation by the 26S proteosome. (B) When oxygen levels in a cell are decreased, in hypoxic situations, the PHDs are inhibited. This allows HIFα isoforms to dimerise with the constitutively expressed ARNT (also called HIFβ). This transcription factor translocates to the nucleus where it binds to hypoxia responsive elements (HREs) to initiate transcription of HIF responsive genes. These include genes involved in promoting angiogenesis, cell metabolism and erythropoiesis. Similarly, when VHL is mutated/lost the ability of the ECV complex to recognise hydroxylated HIF is lost, resulting in an increase in HIF expression as well as HIF mediated gene transcription.

associated with increased *VEGF* expression [55]. In addition, haemangioblastoma along with ccRCC and phaeochromocytomas are characterised by increased production of EPO [56]. Such characteristics can be largely attributed to the HIF dependent functions of pVHL, which under normal circumstances would act to suppress expression of such genes. Thus loss of *VHL*, or indeed hypoxia, activates a pathway that, amongst other functions, promotes angiogenesis and cell survival, advantages utilised by cancer cells.

Otto Warburg first observed, over 70 years ago, that cancer cells metabolize glucose in a manner that is in contrast from that of cells in normal tissues [57]. Now termed the Warburg effect, it is well recognised that cancer cells utilise anaerobic metabolic pathways to generate adenosine triphosphate (ATP) producing, as a consequence, high levels of lactate. This occurs independent of oxygen availability and is in contrast to normal cellular metabolism, which in the presence of oxygen, metabolizes glucose to carbon dioxide by oxidation of glycolytic pyruvate in the mitochondrial tricarboxylic acid (TCA) cycle. Aerobic metabolism produces NADH, which then fuels oxidative phosphorylation to maximise ATP production. In contrast to anaerobic metabolism, this reaction results in minimal synthesis of lactate. pVHL loss and subsequent HIF stabilisation play an important role in promoting such phenotypic changes that characterise tumours. HIF mediated gene transcription significantly promotes anaerobic metabolism in a cell by activating genes such as *GLUT1*, *LDHA* and *PKG*, which promote glucose uptake and oxygen independent metabolism.

In addition to the aforementioned functions, HIF stabilisation as a consequence of VHL loss encourages other pro-oncogenic pathways including those involved in epithelial mesenchymal transition [58,59] as well as invasion and metastasis [60]. Thus, HIF stabilisation is a common occurrence in tumour biology and is associated with an adverse prognosis [61]. Investigating the contribution of HIFα to tumour initiation and progression continues to be an area of active research, particularly understanding the exact targets of both HIF1α and HIF2α. Despite multiple overlapping transcriptional targets of HIF1α and 2α, research investigating ccRCC suggests that gene targets unique to one or other isoform may enable pro- or anti-oncogenic arms of the HIF pathway [62]. The strongest evidence for this has been highlighted in *VHL* associated ccRCC. Interestingly, arm level losses on chromosome 14q, associated with loss of *HIF1α*, which originally was predicted to drive more aggressive disease in fact is considered a protective mechanism. One recent study demonstrated that patients with ccRCC that lacked both VHL and HIF1α harboured significantly more aggressive tumours than patients that had VHL loss alone. Patients with 14q deletion (HIF1α loss) had worse outcomes than patients that retained *HIF1α* [63]. This is supported by various *in vitro* and *in vivo* experiments. ccRCC cells and xenografts with either HIF1α or HIF2α demonstrate different phenotypes. As expected HIF2α promotes growth, however, HIF1α results in decreased growth [64]. The transcriptional targets of HIF1α and 2α provide insight into why this is the case. HIF2α mediates expression of pro-oncogeneic genes such as *cyclin D1*, *VEGF* and *TGFα*, while HIF1α activates expression of pro-apoptotic genes such as *BNIP3* [65]. In further support of this theory, the expression and behaviour of one HIF isoform appears to influence that of the other.

For example increased HIF2α expression suppresses HIF1α and vice versa, suggesting that these two transcription factors do not necessarily work in unison [65]. Such contrasting properties have led researchers to suggest that in ccRCC, HIF1α may have tumour suppressor functions while HIF2α promotes disease progression [62].

Given the importance of HIF in VHL disease, sporadic ccRCC and phaeochromocytoma as well as its importance in multiple other cancers when stabilised due to hypoxia, such observations have highlighted the need to dissect the pVHL/HIF axis in order to truly understand what promotes and controls HIF1α and HIF2α mediated transcription. One exciting new avenue of research investigates the role of chromatin remodelling and epigenetic modification in mediating this response.

8. Chromatin remodelling and gene transcription

As is the case with any transcription factor, in order to bind, the DNA must be arranged in a manner that is conducive to allow easy access to specific sequences. This is determined by chromatin structure, which is governed by a variety of factors. Included and essential to these are epigenetic modifications that play a vital role in regulating chromatin structure and thus controlling the accessibility of proteins, including transcription factors to chromatin. Epigenetic modifications encompass a variety of alterations that include DNA methylation and histone modifications (Fig. 2). Regulating the extent of DNA methylation at cytosine residues or modifying histone tails deems transcription factor binding sites either active (available for transcription) or inactive (unavailable for transcription). Thus, the response of a cell to a given transcription factor can be significantly influenced by the extent of transcriptionally active or inactive loci available to it. In this context, one could expect that epigenetic modifications would effect HIF mediated transcription in a similar manner [66].

Investigations into the genetic mutations in ccRCC demonstrate that in addition to VHL mutations, many of these tumours harbour mutated genes that are involved in chromatin remodelling. These include a component of the SWI/SNF chromatin remodelling complex *PBRM1* (encoding Polybromo 1) [67], the histone methyltransferase *SETD2* and the histone demethylases *UTX (KDM6A)* and *JARID1C (KDM5C)* [68,69]. Although the histone methyltransferase and demethylase mutations are present in less than 15% of tumours, *PBRM1* mutations were found in 41% of the samples analysed making it the second most commonly mutated gene in ccRCC after *VHL* [67–69]. Such modifications contribute possibly to an epigenetic landscape that allows for the promotion of tumour growth and cancer progression.

Interestingly the SWI/SNF complex, of which *PBRM1* is a part, appears to be necessary for mediating transcriptional responses to hypoxia as well as directly promoting HIF1α expression [70]. Thus it is tempting to predict that a modified epigenetic profile, as is found in cancer cells, may act to promote more aggressive disease by in part influencing HIF mediated transcription, although little is known about the precise effects of PBRM1 mutations or indeed any other chromatin remodelling mutations on HIF transcription. Interestingly, what is known is that while epigenetic modifications may help determine the extent of HIF mediated transactivation in a given cell, HIF itself also directly targets genes that are involved in chromatin remodelling [71].

9. Epigenetic involvement in the VHL/HIF axis

Comprehensive molecular profiling of over 400 ccRCC tumour samples identified a globally hypomethylated profile as a signature of the disease [72]. McRonald et. al. investigated promoter specific methylation in renal carcinoma patients and found significant changes in the DNA methylation levels compared to normal renal tissue [73]. DNA methylation involves the addition of a methyl group (CH_3) to the 5th carbon of the cytosine ring, becoming 5 methylcytosine (5MeC). Classically associated with gene silencing, the DNA methylation profile is significantly altered when a variety of cell types including colorectal cancer cells [74], benign prostate epithelial cells [75] and fibroblasts [76] are grown in hypoxia. The precise mechanism promoting such changes are less well

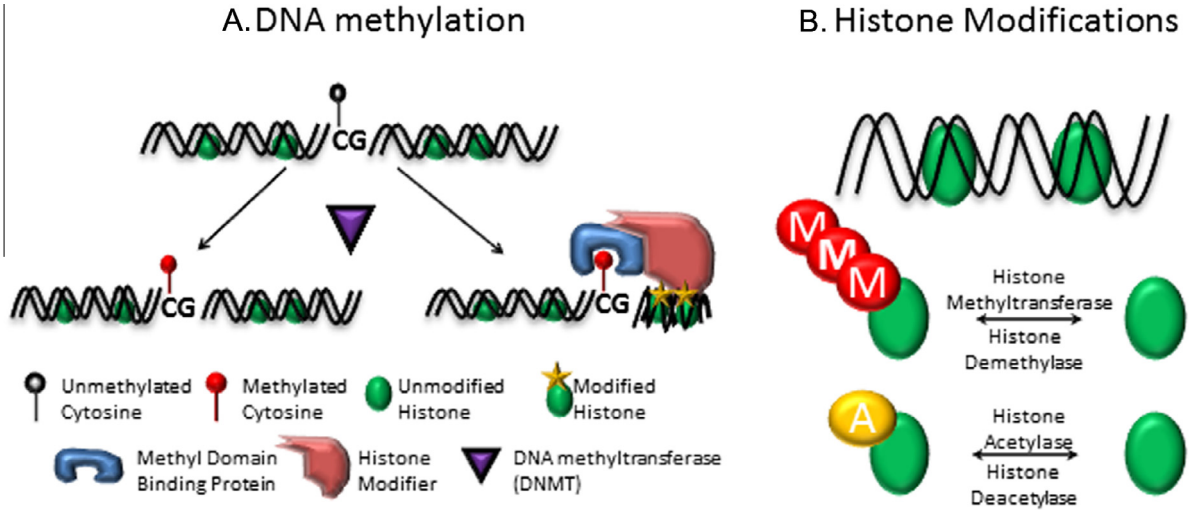

Fig. 2. DNA methylation and histone modifications represent two important mechanisms of epigenetic regulation. (A) DNA methylation occurs at cytosine (C) residues, where a CH3 group is added to the 5th carbon of the pyramidine ring, resulting in 5methylcytosine (5MeC). This reaction is catalysed by a family of enzymes called the DNA methyltransferases (DNMTs; purple) of which there are three active forms found in mammalian cells – DNMT1, DNMT3a and DNMT3b. DNA methylation is associated with repressive chromatin, where methylated cytosines act as a binding platform for methyl-domain binding proteins (blue) and subsequent recruitment of other histone modifying proteins (pink) resulting in a condensed chromatin structure. (B) The N-terminals of histone proteins (green) can undergo an array of modifications. These include mono-di- and tri-methylation as well as acetylation which are catalysed by histone methyltransferases and histone acetyltransferases respectively. Histone demethylases and deacetylases remove these marks. Unlike histone acetylation that is associated with active transcription and a relaxed chromatin structure, histone methylation is mark dependent. For example H3K9me3 is associated with gene repression and a condensed chromatin structure, whereas H3K4me3 is associated with gene transcription and relaxed chromatin.

understood, although it is tempting to hypothesise that these may be HIF mediated responses given the fact the enzymes involved in catalysing the DNA methylation reaction, the DNA methyltransferases (DNMTs), all contain putative HREs in their promoters, while studies have demonstrated that they display altered expression in response to hypoxia [77,78]. Adding an extra layer of complexity to the interaction between HIF and DNA methylation is the fact that DNA methylation modifications are reversible [79]. This is thought to occur via two mechanisms. The first is by passive demethylation, which could occur as a consequence of reduced DNMT expression or activity. The second is the more recent discovery that methylated cytosines residues can be actively demethylated by the activity of a group of dioxygenases called TETs (ten eleven translocation) enzymes [80]. TETs catalyse the sequential hydroxylation of 5MeC to 5-hydroxymethylcytosine (5hMeC), 5-formylcytosine and 5-carboxylcytosine, leading to eventual DNA demethylation. Interestingly, like the PHDs, TETs are a family of Fe(II) and α-KG-dependent dioxygenases utilising molecular oxygen to catalyse the DNA demethylating reaction. Given the significant changes in oxygen levels that occur in tumours, the activity and expression of TETs, along with the levels of 5hMeC, likely contribute to the dynamic DNA methylation profile evident in cancers [81]. Gene specific DNA methylation observations are in support of a model whereby HIF mediated gene transcription is impacted by levels of DNA methylation. Indeed within the consensus HRE sequence is a CpG site, which is known to be of functional importance in the regulation of both *EPO* and *CA9*. The HRE of both have been reported as methylated in specific cell types where HIF mediated transcription of such genes requires a methylation free HRE [82,83]. In addition, Vanharanta et. al. demonstrated that HIF mediated activation of cytohesin 1 interacting protein (*CYTIP*) in $VHL^{-/-}$ cells is enabled by loss of DNA methylation implicating loss of DNA methylation as a feature of an enhanced metastatic cell phenotype when associated with VHL loss and HIF stabilisation [84].

HIF relevant epigenetic modifications are not exclusive to DNA methylation events. Perhaps the most convincing evidence of epigenetic involvement in the HIF mediated response is the fact that many histone-modifying enzymes are direct HIF target genes. Residues of histone tails can undergo a variety of modifications. These include lysine residues that can become mono-, di- or tri- methylated, a reaction mediated by histone methyltransferases and reversed by histone demetylases. A significant proportion of the demethylases, specifically numerous enzymes that are JmjC domain-containing proteins, are direct HIF1α target genes [85,86]. Like the TETs and PHDs, these enzymes also require oxygen to perform their demethylase activity although their sensitivity to oxygen levels remains to be further investigated. Interestingly there have been reports that increased expression of such enzymes is necessary to induce a complete HIF mediated response. Luo et. al. reported that in breast cancer cells HIF1α interacts with the histone demethylase jumonji domain containing protein 2C (*JMJD2C*). *JMJD2C* acts to demethylate H3K9me3, a repressive histone mark. The authors reported that this demethylation was required at the promoters of some hypoxic responsive genes in order to elicit full HIF mediated expression of these genes [87]. It is, however, paradoxical that a histone demethylase that requires oxygen is seemingly operating to enhance HIF-dependent transcription under hypoxia.

10. Conclusion

The role of VHL as a tumour suppressor protein is well documented. In the last two decades, researchers have made significant contributions to our understanding of the functions of this protein and how its loss contributes to cancer pathogenesis. However, the full extent of pVHL's tumour suppressive contribution is not fully understood and this remains an area of active research. To date, anti-tumor functions suggest roles for pVHL in protein degradation, extracellular matix regulation, cilia formation, microtubule maintenance, cellular proliferation as well as roles in the DNA damage response. Indeed it is likely that there remains much to learn about pVHL as even with current knowledge, understanding is lacking as to how exactly certain mutations lead to heightened likelihood of specific cancer forms. Despite the multifaceted nature of pVHL, there is no doubt that central to its role as a tumor suppressor is its regulation of HIFα subunits. HIF stabilisation significantly promotes a pro-oncogenic environment and dissecting the precise mechanisms via which HIF performs such functions are important in order to treat and understand cancer pathologies. Given the fact that tumourigenesis classically manifests with both genetic and epigenetic changes in malignant cells, it is tempting to speculate that HIF activation contributes to the epigenetic landscape that has direct consequence to oncogenesis. This is of particular interest as it appears that in parallel the epigenome regulates the HIF mediated response. Investigations to determine the extent of which VHL loss or HIF stabilisation contributes to the epigenome provides an exciting avenue of research and may offer a new perspective on the multifaceted roles of pVHL.

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The authors declare that they have no conflict of interest.

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