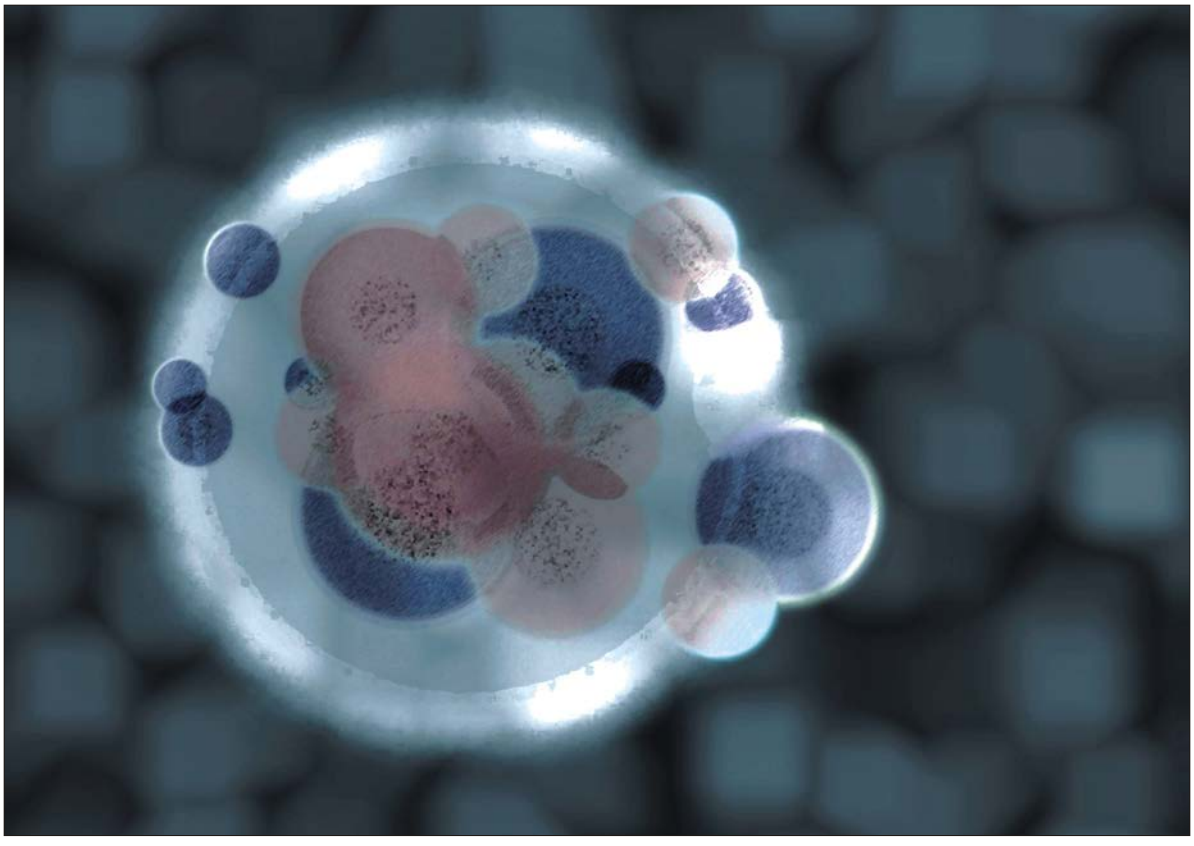

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Role of VHL in Mammalian Oxygen Sensing

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Abstract: The Von Hippel Lindau (VHL) tumour suppressor protein is a component of a multimeric protein complex VEC, which is an E3 ubiquitin ligase that specifically ubiquitylates the α subunit of Hypoxia Inducible Factor (HIF) for subsequent destruction by the 26S proteasome. This establishes VHL as a critical regulator of oxygen-mediated gene expression and a central player in mammalian oxygen sensing. This review will highlight the recent advancements in our understanding of the molecular mechanisms governing the activity of the VEC complex.

Key words: VHL, VEC, HIF, hypoxia, NEDD8

INTRODUCTION

Inheritance of one mutant Von Hippel Lindau (VHL) allele gives rise to the development of the autosomal dominant VHL disease, which affects approximately 1 in 40000 individuals[1]. In this setting tumours arise from the mutational inactivation, gene silencing, or loss of the remaining wild-type VHL allele, in keeping with Knudson's 'Two-hit' model. VHL patients are predisposed to develop tumours in multiple organs including the retina, CNS (brain and spinal chord), adrenal gland and kidneys[1]. However, virtually all VHL patients develop retinal and CNS haemangioblastomas, the two cardinal traits of VHL disease. Moreover, bi-allelic loss and/or mutation of the VHL gene have been observed in the vast majority of sporadic kidney cancer, establishing VHL as a critical 'gatekeeper' gene in kidney tumourigenesis[1]. VHL-associated tumours upregulate a host of hypoxia-responsive genes, such as Vascular Endothelial Growth Factor (*VEGF*), glucose transporter 1 (*GLUT1*) and erythropoietin (*EPO*). These genes along with others aid to improve cell survival under hypoxia via neo-vascularization, anaerobic metabolism and the recruitment of oxygen-carrying red blood cells[1].

VHL classification system: Intragenic mutations that give rise to VHL disease have been found over the entire open reading frame of the VHL gene. Despite the heterogeneity of these mutations, a phenotypic pattern that corresponds to specific mutations has arisen[1]. VHL disease can be divided into two subcategories depending on the risk of developing phaeochromocytoma. Individuals with Type 1 VHL disease are not predisposed to develop phaeochromocytoma, while Type 2 patients develop phaeochromocytoma. Type 2 VHL disease is further subdivided into Type 2A, 2B and 2C: Type 2B patients also develop Renal Clear Cell carcinoma (RCC) and Type 2C patients exclusively develop phaeochromocytoma. In addition, VHL patients with Types 1, 2A and 2B develop the two cardinal features of the disease, retinal and CNS haemangioblastomas. Type 1 VHL disease is often associated with mutations resulting in gross truncations or even a complete loss of VHL. Whereas mutations that cause Type 2 VHL disease are frequently missense mutations. These differences in susceptibility to develop phaeochromocytoma suggest a gain of function mutation or that complete loss of VHL function is not permissible for the development of phaeochromocytoma. Furthermore, these phenotypic variations may also indicate multiple functions of VHL, tissue-specific function(s), or variations of one function.

The VHL gene and protein: In 1988, Seizinger and colleagues mapped the locus of the putative VHL tumour suppressor gene to a narrow region of chromosome 3p, in accord, deletions in this region have been observed in RCC[2]. Latif and colleagues in 1993 identified and cloned the gene defective in VHL patients[3]. The VHL gene contains three axons that produce a 4.5 kb mRNA. However, two translation products are observed. A full-length VHL of 213 amino acids with a molecular weight of 30 kDa (VHL_{30}) and an internally translated shorter 160 amino acid VHL of 19 kDa (VHL_{19}), which results from an alternative start site at codon 54[4,5].

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There are several notable differences between VHL_{30} and VHL_{19}. For example, VHL_{19} is equally distributed in the nucleus and cytoplasm, while VHL_{30} is found primarily in the cytoplasm with minor fractions localized in the nuclear and membrane compartments[4]. VHL_{30} has the ability to shuttle between the nucleus and the cytoplasm[6]. Recently it has been shown that under acidic conditions VHL_{30} and VHL_{19} can be sequestered to nuclear loci and upon reinstatement of neutral pH, VHL_{30} and VHL_{19} return to the cytoplasm[7]. To date, all disease-causing VHL_{30} mutants have failed to bind and promote the assembly of an extracellular matrix protein fibronectin, underscoring the importance of fibronectin matrix assembly in the development of VHL disease[8-10]. Interestingly, VHL_{19} does not bind fibronectin, indicating that VHL_{19} and VHL_{30} have non-overlapping function(s)[4]. In addition, VHL_{19}, but not VHL_{30}, associates with the microtubules *in vivo*. Unless otherwise specified, VHL will henceforth refer to both VHL_{19} and VHL_{30}.

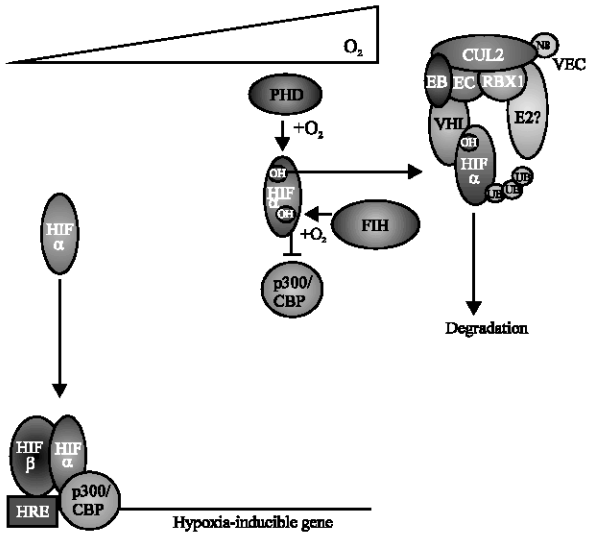

Fig. 1: VEC/HIF pathway
EB: elongin B, EC: elongin C, UB: ubiquitin, N8:NEDD8

VHL-associated proteins and the VEC complex: As the initial analysis of the protein and gene sequence did not give any clues as to possible functions of VHL, efforts to find VHL-associated proteins were made with the supposition that these interacting proteins will have known functions or contain motifs with predicted functions. It is now known that VHL forms a multiprotein complex with elongin C, elongin B, Rbx1 and Cul2[1]. The VHL complex (VEC; Fig. 1) has high structural similarity with a yeast multiprotein complex called SCF (Skp1/Cdc53/F-box protein). Cul2 and Cdc53 are members of the Cullin family. Elongin C is an orthologue of yeast Skp1 and both VEC and SCF contain a ring-finger protein Rbx1. SCF is a known E3 ubiquitin ligase complex that targets substrates recruited via the F-box protein for ubiquitylation. Polyubiquitin-tagged proteins are then degraded by a common 26S proteasome[1]. Thus, VEC is thought to function as a mammalian E3.

The crystal structure of VHL/elongin B/elongin C was determined in 1999 and showed that VHL had two functional domains[11]. The α domain (so named for the α helices that form this domain) was predicted to function as an elongin C-binding site. The β domain (so named for the β-pleated sheets that form this domain) was predicted to function as a protein-protein interaction interface. Tumour-derived mutations frequently occur on the surface residues within the α and β domains, suggesting the significance of these regions for the tumour suppressor function of VHL[11]. Thus, VHL is thought to function as an F-box protein conferring substrate specificity and binding to elongin C, which acts to nucleate the VEC complex by binding to the scaffold component, Cul2 (Fig. 1).

VEC targets and PHD: Tumours devoid of VHL show an upregulation of many Hypoxia-responsive genes[12]. Hypoxia Inducible Factor (HIF) is a major regulator mediating the adaptive response to changing oxygen tension[12]. HIF is a heterodimeric transcriptional activator, composed of constitutively stable HIF-β subunit and labile HIF-α subunit, which is stabilized under hypoxia. Thus, the activity of HIF is conferred by the oxygen-dependent stability of HIF-α. There are three members of the HIF-α family: HIF-1α, -2α and -3α[12]. Under hypoxia, HIF dimers bind to the Hypoxia Responsive Elements (HRE) contained in the promoter/enhancer regions of many hypoxia-responsive genes including the aforementioned *VEGF*, *GLUT1* and *EPO*.

As predicted from the VHL crystal structure, the β domain of VHL was necessary and sufficient to bind the α subunit of HIF. However, this interaction was strictly dependent on oxygen tension[13]. That is, under normal oxygen pressure VHL recognized HIF-α, while under reduced oxygen pressure VHL failed to recognize HIF-α, explaining why HIF-α is no longer degraded under hypoxia. The selectivity of VHL binding of HIF-α was determined to be dependent on the hydroxylation of conserved Proline residues (402 and 564 based on HIF-1α sequence) within the LAPYIXMD motif found within and near the oxygen-dependent degradation (ODD) domain of HIF-α[14-16]. Prolyl-hydroxylation was carried out by a newly identified class of enzymes called prolyl hydroxylases (PHD) 1, 2 and 3 in the presence of oxygen[17]. Interestingly, it was recently shown that PHDs are upregulated during hypoxia[18]. The current explanation for the reason of the upregulation of PHDs is that upon recovering oxygen homeostasis, there will be an

abundance of PHDs ready to rapidly hydroxylate HIF-α for subsequent ubiquitin-mediated destruction, thus curtailing the hypoxic response.

Moderation of VEC activity by NEDD8: Cdc53 is covalently modified by an ubiquitin-like molecule called Related-to-ubiquitin-1 (Rub1) and this modification was demonstrated to increase the E3 activity of the yeast SCF complex. Similarly, Cul2 of the VEC complex is neddylated on Lysine at position 689 by a NEDD8 conjugating enzyme Ubc12[19]. NEDD8 modification of Cul2 was shown to dramatically enhance the ability of VEC to ubiquitylate HIF-α[19]. For example, the addition of a dominant-negative Ubc12 prevented the neddylation of Cul2 and attenuated the ability of VEC to ubiquitylate HIF-α. The modification of Cul2 by Nedd8 did not affect the assembly or stability of the VEC complex. The current working hypothesis is that the neddylation of Cul2 increases the recruitment of an E2 ubiquitin-conjugating enzyme, thereby promoting the overall E3 activity of VEC (Fig. 1).

VEC-independent regulation of HIF: C-TAD domain of HIF-α and FIH: The C-terminal transactivation domain (C-TAD), which is present in HIF-1α and HIF-2α, but not HIF-3α[20], interacts with co-activators p300/CBP to effectively induce the transcription of hypoxia-inducible genes via HRE[12]. Recently, it was shown that C-TAD was subjected to hydroxylation at an Asparagine residue at position 803 (based on HIF-1α sequence) under normoxia by Factor Inhibiting HIF-1 (FIH)[21,22]. Importantly, asparaginyl-hydroxylation of C-TAD prevented the recruitment of p300/CBP via steric hindrance. This represents an added preventive mechanism to suppress the transcriptional activity of HIF under normoxia, thus ensuring inadvertent triggering of hypoxia response during normal oxygen tension.

The importance of HIF is underscored by the finding that HIF is frequently over-expressed in many human cancers. This is, in part, due to the inevitable hypoxic conditions of growing tumours as the physical distance of the tumour tissue to the nearest blood vessel surpasses the diffusional capacity of oxygen. Hypoxia and the resulting rise in HIF expression are likely to explain the angiogenic features of tumours and have been correlated with metastasis and poor prognosis. VHL is a substrate-conferring component of an E3 VEC complex that targets HIF-α for ubiquitin-mediated destruction under normoxia. Thus, tumour cells devoid of functional VHL, as in VHL patients or in the majority of kidney cancer cells, show elevated levels of HIF-α and HIF-target genes and profound angiogenesis and likely explains the hypervascular phenotype of VHL-associated tumours. Thus, studies designed to uncover the molecular mechanisms of HIF regulation will not only assist in the development of more specific and targeted therapeutic techniques, but also advance our understanding of how mammalian cells are able to sense oxygen.

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