Genetic and Molecular Basis of Von Hippel-Lindau Disease

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Von Hippel-Lindau disease (VHL disease or VHL syndrome) is a familial multisystem neoplastic disorder, stemming from germline disease-associated variants of the *VHL* tumor suppressor gene. VHL protein is involved in oxygen sensing and adaptive response to hypoxia through the EPO-VHL-HIF signaling axis. In recent years, numerous HIF-independent pathways of VHL have been identified, expanding the role of VHL throughout several cellular processes. In addition to VHL syndrome-associated tumors, *VHL* variations have also been associated with the development of eythrocytosis. Research indicated that there is a distinction between erythrocytosis-causing *VHL* variations and *VHL* variations causing VHL disease with tumor development. Therefore, elucidating the molecular background of the pathogenic effects of *VHL* variants could help determine the best approach to VHL disease management.

VHL | VHL disease | Chuvash polycythemia | genetic variation | erythrocytosis | pheochromocytoma | renal cell carcinoma | retinal hemangioblastoma | hemangioblastoma

1. Introduction

Von Hippel-Lindau disease (VHL disease or VHL syndrome) is a familial multisystem syndrome stemming from germline disease-associated variants of the *VHL* tumor suppressor gene on chromosome 3 [1]. It is an autosomal dominant neoplastic disorder in which multiple benign and malignant tumors, as well as cysts, develop in the central nervous system (the brain, spinal cord, retina, and inner ear) and visceral organs (kidney, adrenal gland, pancreas, epididymis, and broad ligament) [2][3]. Despite its classification as a dominant disorder, the most common pattern in hereditary VHL disease is the inheritance of a germline genetic variant (herein, mutation) in one allele, followed by a second somatic change, leading to loss of the second allele [2][4][5]. In approximately 20% of cases, VHL syndrome is sporadic, caused by a de novo genetic change that arises during the formation of reproductive cells, or very early during the embryogenesis [4][6][7]. After the identification of the VHL gene in 1993, the phenotype of VHL pathogenic genetic variations was expanded to include VHL disease, dominantly inherited familial pheochromocytoma, and autosomal recessive familial polycythemia [8][9][10]. Chuvash polycythemia, a secondary congenital erythrocytosis, was the first congenital erythrocytosis linked to a VHL mutation [8]. Since then, several other VHL mutations associated with erythrocytosis have been identified [11].

2. VHL Canonical and Non-Canonical Functions

The von Hippel-Lindau tumor suppressor gene (*VHL*) is located on chromosome 3. Initially it was thought to be composed of three exons that encode the VHL protein [12][13]. Subsequent research revealed more complex transcription patterns, and an additional cryptic exon, resulting in several different functional *VHL* isoforms (**Figure 1**) [14][15]. Soon after its discovery, it was confirmed that the two most commonly isolated isoforms, VHL30 or VHL213 and VHL19 or VHL160, are both biologically active and are expressed in all tissues [16][17][18].

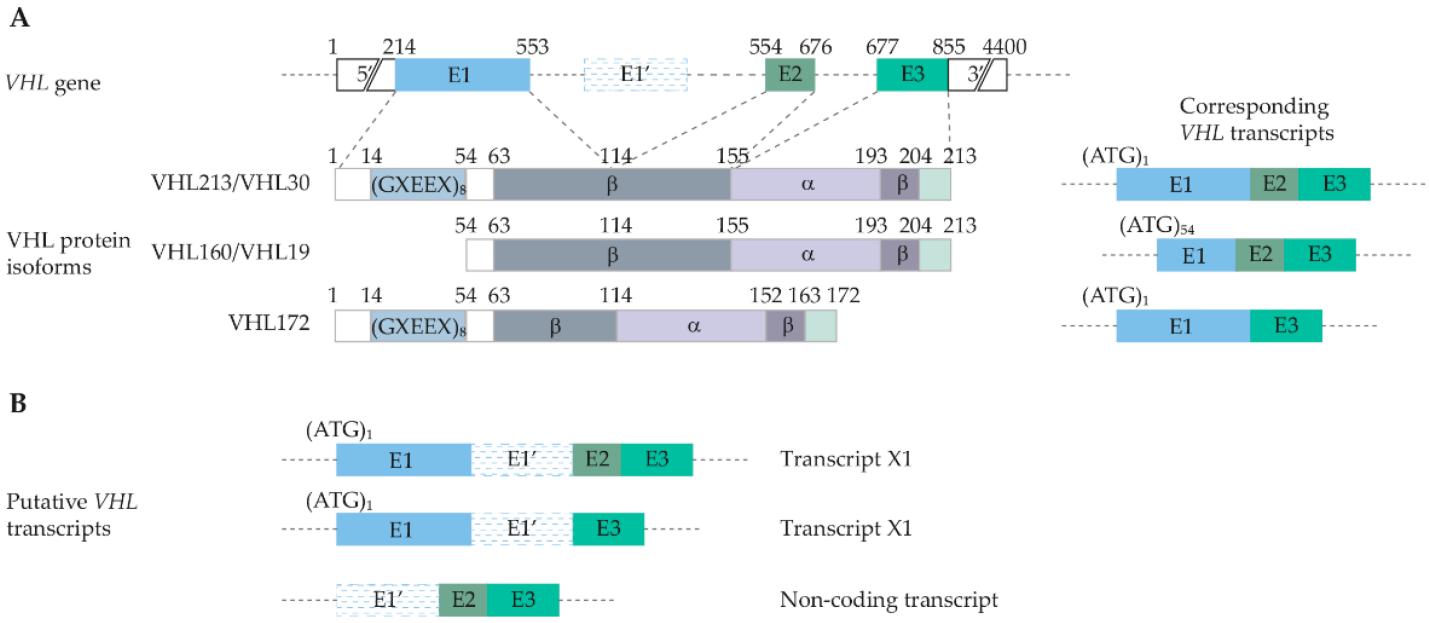

Figure 1. Schematic representation of *VHL* gene structure. (**A**). *VHL* gene structure and protein isoforms with corresponding *VHL* transcripts. (**B**). Putative *VHL* transcripts, containing exon E′, identified by cloning and sequencing [15].

VHL30 is the longest isoform, with a molecular mass of 30 kDa. It has 213 amino acids and consists of three exons, whereas VHL19 has 160 amino acids and a molecular mass of 19 kDa [16]. Iliopoulos et al. demonstrated that VHL19 is generated from an internal translational start at methionine 54 [16]. Another protein product of the *VHL* gene, VHL172, which arises from alternative splicing that joins exons one and three while excluding exon two, was also detected [17].

Recently, Lenglet et al. identified transcripts containing a cryptic exon, E1′, located in intron one of the *VHL* gene, spliced either with exon one or exons two and three [15]. They speculated that a theoretical protein consisting of 193 amino acids could exist, containing 114 amino acids encoded by exon one and 79 amino acids encoded by the cryptic exon, E1′. Their findings were linked to the aberrant retention of this cryptic exon in patients with VHL disease and erythrocytosis.

The reference sequence collection currently displays three mRNA and protein reference variants, which differ in length and exon structure. The three representative isoforms consist of 213, 172, and 193 amino acids residues, respectively [19]. UniProt database describes three isoforms, which are produced by alternative splicing and alternative initiation. Isoform one corresponds to RefSeq isoform one (VHL30 or VHL213) and isoform two

corresponds to RefSeq isoform two (VHL172), whereas isoform three corresponds to VHL19 (160 amino acids) and is produced from alternative initiation at methionine 54 [20].

The longest protein isoform has two main protein binding domains, β and α domain, which are preceded by N-terminal acidic disordered domain [21][22]. The β domain is composed of approximately 100 amino acids. This domain is rich in β strands, which form a β-sheet structure, and interacts with hydroxylated HIFs, RNA polymerase II, protein kinase C isoforms, and other proteins [23][24]. The shorter α domain contains binding sites for Elongin-B/C (BC box), Cullin-2 (Cul-2 box), p53, and other proteins [23]. The β domain contains another binding interface, interface C, which is important for VHL localization and binds TBP1 and EEF1A [23].

On the subcellular level, the strongest VHL protein expression was observed in the cytosol, nucleus, mitochondrion and endoplasmic reticulum [25]. Interestingly, Illiopoulus et al. noted that VHL, which is composed of 213 amino acids and corresponds to VHL30, localizes predominantly to cytosol, or is membrane-associated [16]. It was found only in low levels in the nucleus, whereas the 160-amino acid VHL (VHL19) was found in both the nucleus and cytosol. In contrast to the cytosolic membrane-bound VHL30, VHL19 was not associated with cell membranes [16].

RNA sequencing of 27 different normal tissues, from 95 individuals, revealed that VHL protein is ubiquitously expressed in most tissues [26]. However, protein expression studies showed that, despite the relatively high abundance of mRNA in some tissues, the levels of VHL were low or absent, indicating that complex mechanisms govern the mRNA expression, protein synthesis, and/or protein stability of VHL (https://www.proteinatlas.org/ENSG00000134086-VHL/tissue, available from v21.0 proteinatlas.org, accessed on 10 January 2022) [26][27]. This is consistent with general observations in mammalian tissues, where a number of genes exhibited very low correlations between mRNA expression levels and protein staining (Pearson's ⩽ 0.40) [26][28].

The most important canonical role of VHL, through the EPO-VHL-HIF signaling axis, is its involvement in oxygen sensing and adaptive response to hypoxia (**Figure 2**) [29][30][31][32][33]. VHL associates with Elongin-B (*ELOB*) and Elongin-C (*ELOC*), forming a VBC complex, and, together with Cullin-2 (*CUL2*) and E3 ubiquitin-protein ligase RBX1 (*RBX*, Ring-box1), constitutes a functional E3 ubiquitin ligase that specifically recognizes hydroxylated Hypoxia inducible factor (HIF) subunit α, targeting it for proteasome degradation by ubiquitination [34][35][36][37][38][39]. The VHL protein serves as a substrate (e.g., HIF1α) recruitment protein [40][41]. When oxygen levels are normal (normoxic conditions), the two proline residues on the N-terminal (NOOD) and C-terminal (COOD) regions of HIF1α, P402, and P564, respectively, are hydroxylated by the non-heme Fe^{2+} -oxygen and α-ketogluratate-dependent dioxygenase superfamily of prolyl hydroxylases (PHD1, PHD2, and PHD3) [42][43]. In vitro PHD2 catalyzes the hydroxylation of proline in COOD peptides more efficiently than the NOOD peptide proline, whereas PHD3 specifically hydroxylates only COOD proline [44].

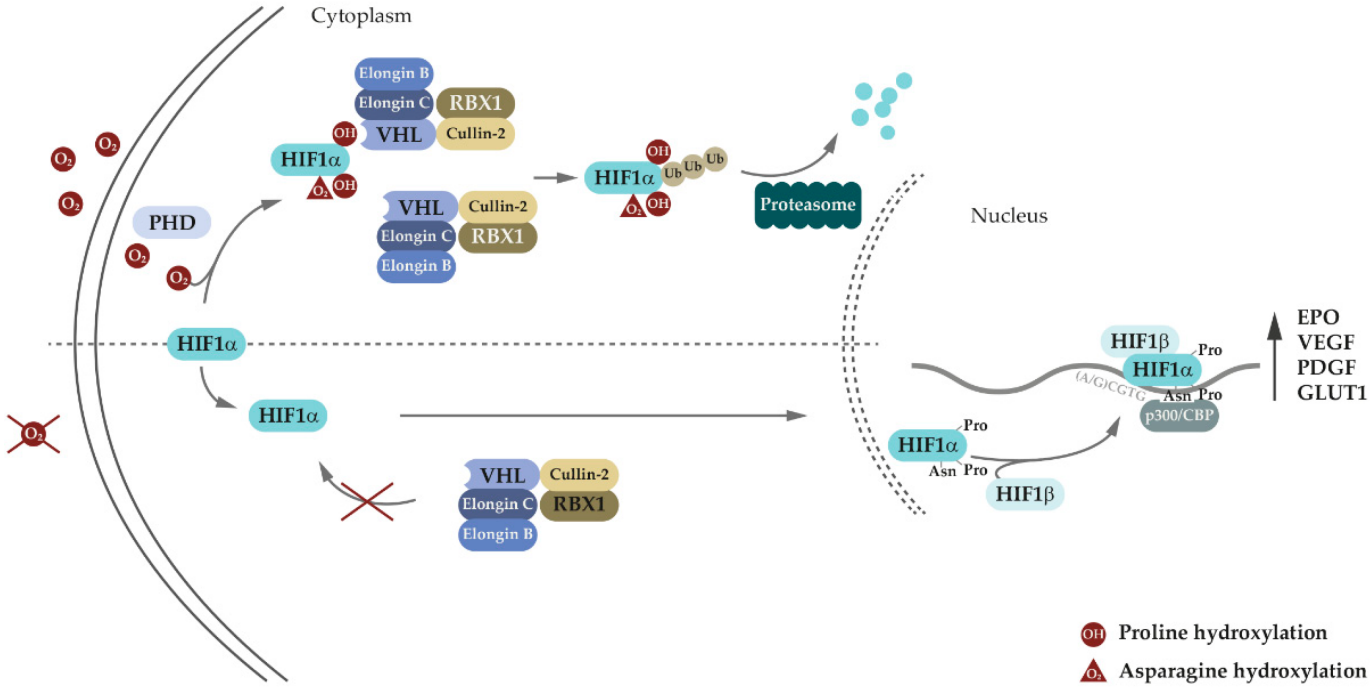

Figure 2. Role of VHL in adaptive response to oxygen levels.

The efficiencies of P402 and P564 hydroxylation differ, and when the level of oxygen falls, the hydroxylation of HIF1α prolines is gradually diminished, with P402 more affected than P564 [45][46]. VHL recognizes both hydroxylated prolines with similar binding affinities, resulting in interaction between HIF1α and VBC complex in a 1:2 stoichiometric ratio [47]. HIF1α can therefore bind two VBC complexes or one VBC complex, according to the status of P402 and P564 hydroxylation, at different oxygen levels [45][46][47]. The decrease in VHL targets could lead to gradual increase in HIF1α.

Additionally, in normoxia (normal levels of oxygen), the asparaginyl residue N803 is hydroxylated by factor inhibiting HIF (FIH) or asparaginyl hydroxylase, preventing HIF1α interaction with transcriptional coactivator p300 (*EP300*) [48][49]. Under low levels of oxygen (hypoxia), HIF1α-specific prolyl and asparaginyl residues are not hydroxylated by PHDs and FIH, respectively. Non-hydroxylated HIF1α subunits can enter the nucleus through specific importins, where they form heterodimeric complexes with Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT, also known as Hypoxia-inducible factor 1, β subunit, HIF1β) [43]. The HIF1α-ARNT complexes act as transcriptional regulators, and bind to consensus 5′-(A/G)CGTG-3′ sequence or HIF ancillary sequences 5′-CAGGT-3′ in hypoxia response elements (HRE), within promoters of several genes involved in adaptive response to hypoxia [42][43].

VHL is also involved in numerous HIF-independent pathways, including protein stabilization [50][51], regulation microtubule stability, and ciliogenesis, as well as stability of the mitotic spindle and chromosomal instability [52][53][54], endocytosis [55], apoptosis [56], protein phosphorylation [57], regulation of gene transcription [58][59][60], regulation of extracellular matrix assembly and tight junctions [61][62][63][64], RNA stability [65][66][67], regulation of senescence [68][69], and, in cytokine signaling pathways, mediating DNA damage response and initiating DNA repair [70] or JAK/STAT signaling [71]. These diverse roles of VHL confirm its implication in several crucial cellular processes,

including hypoxia-related cellular adaptations, such as hematopoiesis, metabolic adjustments in oxygen deprived environments, and hypoxia-unrelated mechanisms, such as metabolic rewiring, response to oxidative stress, DNA damage, particularly double-stranded breaks, inflammation, and so on, in the absence or presence of reduced oxygen levels.

Due to its involvement in numerous cellular processes, VHL interacts with several proteins, constituting more or less complex molecular relationships (**Figure 3**). The occurrence of several VHL isoforms further augments the complexity of VHL biological functions. For example, it has been determined that VHL30, but not VHL19, interacts with tumor suppressor ARF (*CDKN2A* gene) through the acidic disordered N-terminus. VHL19 lacks the first 53 N-terminal amino acids in this region, and does not interact with ARF [72][73]. ARF promotes interaction between VHL30 and PRMT3, an arginine methyltransferase which can monomethylate or asymmetrically dimethylate several proteins, including p53 and 40S ribosomal protein RPS2 [72]. Methylated p53 arginine residues affect p53 response by influencing the specificity of p53 binding to promoters [74]. Furthermore, VHL isoforms directly interact with p53, which competes for binding to the VHL α region with Elongin-C and ATM, an important cell cycle checkpoint kinase, as well as AURKA, another cell cycle kinase, which is involved in microtubule formation and the regulation of centrosomes, spindles and kinetochores, and centrosomal proteins, e.g., CEP86 [75][76][77]. VHL isoforms, particularly VHL19, have been shown to interact with collagen, fibronectin, and enzymes involved in collagen biosynthesis [72].

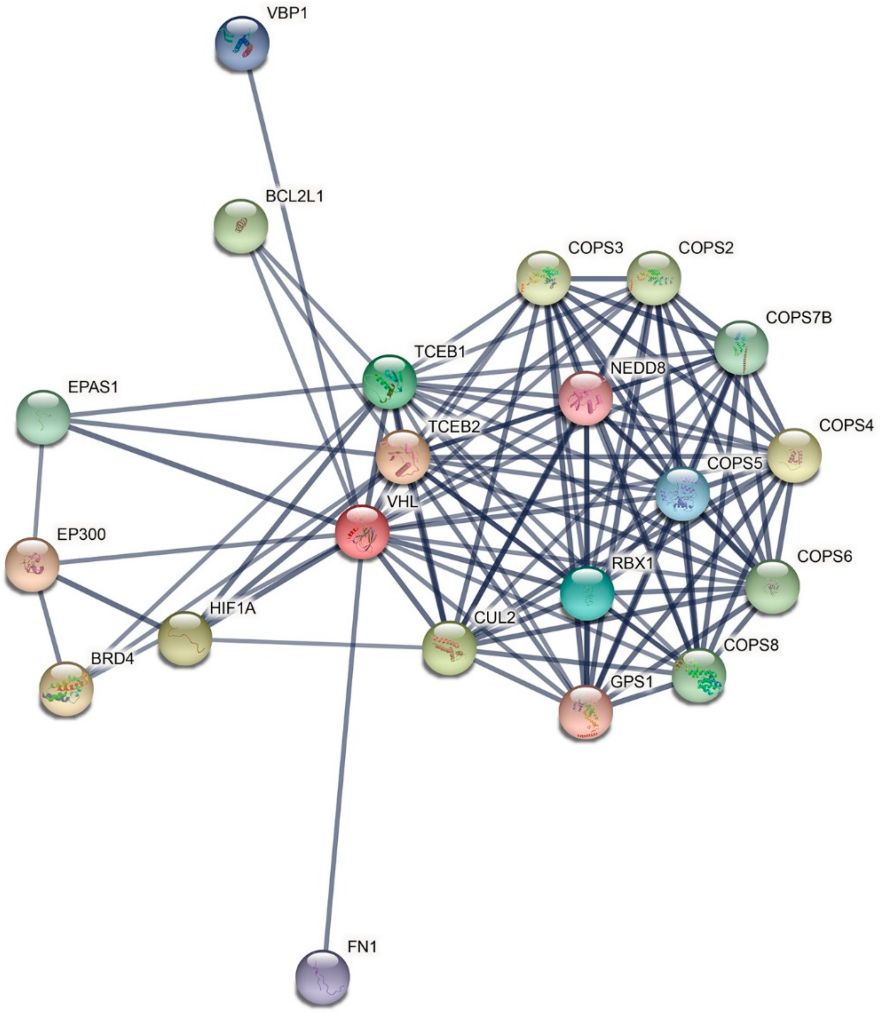

Figure 3. STRING network of VHL protein–protein interactions. Settings: Network type, full STRING network; meaning of network edges, confidence (line thickness indicates the strength of data support); active interaction sources, experiments; minimum required interaction score, high confidence (0.700); max number of interactors to show, no more than 20 interactors; organism, *Homo sapiens* [78].

3. Genetic and Molecular Basis of VHL Disease

The spectra of pathogenic variations in VHL disease is diverse, and the associations between genotypes and phenotypes—the development of specific tumors—are not always predictable [79]. VHL databases, such as VHLdb (http://vhldb.bio.unipd.it/, accessed on 23 December 2021) and the UMD-VHL mutations database (www.umd.be/VHL/, accessed on 23 December 2021), have collected detailed information on over 1600 different pathogenic variations [80]. The COSMIC (https://cancer.sanger.ac.uk/cosmic, accessed on 23 December 2021) database lists more than 1800 entries for genetic variation in *VHL* gene [81]. Based on the diverse roles VHL exerts in cellular homeostasis in normoxic and hypoxic conditions, the elucidation of the effect of pathogenic variations at the molecular level is a daunting task. Over the years, the efforts of several research groups enabled greater understanding of the molecular mechanisms underlying different types of pathogenic genetic variants.

The most common VHL disease-associated genetic changes include deletions of exons, in-frame insertions and deletions, truncating point mutations, missense mutations, splice-site mutations, and frameshift insertions and deletions, as well as structural variations and even gene fusions [9][80][82][83]. In general, from the perspective of VHL genetic modifications, type 1 is characterized by exonic deletions and truncating mutations, as well as missense mutations, associated with VHL instability and high HIF activity [1][12][83]. In type 2, regardless of the subtype, missense mutations, which have generally been found to retain partial functionality of VHL protein, are predominantly found [1][83]. Furthermore, patients with nonsense or frameshift mutations are at higher risk for the development of RCC and hemangioblastomas. Clinical investigations demonstrated that, in addition to the type of the *VHL* genetic variant, the disease type is also age dependent, with a penetrance of over 90% by the age of 65 [84].

Studies involving patients with different manifestations of VHL disease, as well as functional studies of genetic aberrations of *VHL,* have clearly indicated that some aberrations segregate with distinct phenotypes (**Table 1**). For example, Ong et al. demonstrated that the risk of developing PCC was associated with *VHL* mutations that change surface amino acids (termed surface mutations), compared to mutations that change amino acids buried deeper within the structure (termed deep mutations), and/or large deletions and truncating (frameshift and nonsense) mutations [9]. Furthermore, the mean age at diagnosis was lower in PCC patients harboring surface mutations than in those carrying other types of mutations in the *VHL* gene. They also discovered that RCC and retinal hemangioblastoma patients with deleterious mutations were older at the diagnosis of the disease [9]. Hacker et al. determined that p.R167Q and p.D121G type 2B mutations, located in the Elongin-C binding region, probably retained the ability to bind VBC complex proteins Cullin-2 and Elongin-B, as shown in co-immunoprecipitation experiments [85]. Despite the unstable association of proteins that constitute VBC complexes, this research showed that a p.R167Q VHL mutant could ubiquitinylate HIF1α. Buart et al. showed that an R167Q *VHL* mutation leads to

molecular changes related to more pronounced cancer cell stemness and tumor plasticity. VHL-R167Q expressing RCCs are associated with a poor survival [86].

Table 1. Selected *VHL* genetic variants in VHL disease and their association with phenotype.

Variant	Protein Change	Codon	VHL Type/Phenotype	Functional Consequence	Reference
c.191G>C	R64P	64	Type 2C	Increased aPKC JUNB levels; impaired binding to fibronectin.	[87][88]
c.194C>T	S65L	56	Type 2B	Impaired HIF1α binding; impaired HIF2α regulation.	[89][90][91]
c.208G>A	E70K	70	Type 1	Impaired HIF1α binding.	[92][93]
c.233A>G	N78S	78	Type 1	Impaired HIF1α regulation.	[94][95]
c.239G>A	S80N	80	Type 2C	No known consequence.	[94]
c.245G>C	R82P	82	Type 2B	Loss of function of VHL.	[96]
c.250G>C	V84L	84	Type 2C	No known consequence.	[94]
c.262T>A c.262T>C	W88R	88	Hemangio-Blastoma [1]	No known consequence.	[90]
c.269A>T	N90I	90	Type 2B	Impaired HIF1α regulation.	[90][94][97] [98]
c.292T>C	Y98H	98	Type 2A	Impaired HIF1α regulation; defective microtubule stabilization.	[87][94]
c.292T>A	Y98N	98	Type 2B	Impaired HIF1α regulation; impaired GLUT1 suppression.	[97]
c.334T>A	Y112H	112	Type 2A	Impaired HIF1α regulation; decreased VHL stability.	[85][99]
c.334T>A	Y112N	112	Type 2B	Reduced stability of the Vhl-Elongin B/C complex; impaired HIF1α regulation; elevated HIF2α, GLUT1, and cyclin D1 expression in normoxic conditions.	[99][100] [101]
c.334T>G	Y112D	112	Type 2C	No known consequence.	[94]
c.340G>C	G114R	114	Type 2B	Reduced stability of the Vhl-Elongin B/C complex.	[102]
c.349T>C c.349T>A	W117R	117	Type 2B	Impaired HIF1α regulation; impaired binding to fibronectin; elevated HIF2α and	[62][101] [103]

Variant	Protein Change	Codon	VHL Type/Phenotype	Functional Consequence	Reference
				GLUT1 expression in normoxic conditions.	
c.355T>C c.357C>G c.357C>A	F119L	119	Type 2B	Decreased VHL stability; impaired HIF1α regulation.	[104]
c.407T>C	F136S	136	Type 2B	No known consequence.	[94]
c.407T>A	F136Y	136	Type 2B	No known consequence.	[94]
c.408T>G	F136L	136	Type 2B	Decreased VHL stability; impaired HIF1α regulation.	[104][105]
c.482G>C	R161P	161	Type 2B	Reduced stability of the Vhl-Elongin B/C complex; defective microtubule stabilization.	[106][107]
c.482G>A	R161Q	161	Type 2A; Type 2B	Reduced VHL stability.	[108]
c.486C>G	C162W	162	Hemangio-Blastoma [1]	Impaired HIF1α regulation.	[90][109]
c.499C>T	R167W	167	Type 2B	Decreased binding to Elongin B/C and Cullin-2; impaired ubiquitination and degradation of ESR1.	[62][110]
c.500G>A	R167Q	167	Hemangio-Blastoma [1]	Decreased binding to Elongin C; impaired HIF2α regulation.	[90][111][112]
c.562C>G	[115] L188V		Type 2C	Impaired binding to fibronectin; elevated RWWD3, aPKC, and JUNB levels.	[87][88][113][114]

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RCC formation. These mutations cause abnormal, often excessive, blood vessel remodeling, and data from a study performed by Arreola et al. suggested that they have different effects on the nature of vascular changes during the development of retinal vasculature [111]. They analyzed embryonic stem cell-derived blood vessels with *Vhl*$^{-/-}$, *Vhl*$^{2B/2B}$, and WT backgrounds in constructed mouse models with genotypes (i) conditional *Vhl*-null genotype, (ii) one wild-type *Vhl* allele and a second mutant *Vhl* allele with a type 2B p.G518A mutation (equivalent to the VHL p.R167Q protein variation in humans), and (iii) mutant *VHL* p.G518A allele and conditional deletion of the wild type *Vhl* allele (mimicking loss of heterozygosity). Non-mutant mouse models were used as controls. The conditional *Vhl*-null mutation resulted in accelerated arterial vessel maturation, whereas the type 2B *Vhl* p.G518A mutation caused an increase in vessel-branching complexity and disrupted Notch and Vegf signaling, also demonstrated by increased Vegfa, Hey2, and Notch3 mRNA levels in enriched endothelial cells *Vhl*$^{2B/2B}$ extracted from embryonic stem (ES) cell cultures. In comparison, the expression profiles of Vegfa and Notch pathway components in *Vhl*$^{-/-}$ endothelial cells were different, indicating that aberrant Vegfa and Notch signaling pathways in different genetic backgrounds differ, and thus influence the morphological differences in the development of vasculature. Examination of postnatal mouse retinas, obtained at different postnatal development stages, demonstrated that conditional *Vhl*-null mutation had a profound effect on the reduction of arterial and venous

branching in late stages, and very little effect in early stages. Retinal vessels in *Vhl* heterozygous mice, harboring wild type allele and a type 2B *Vhl* mutation, showed increased arterial, but not venous, branching, whereas in conditional *Vhl* homozygous mice, carrying a type 2B *Vhl* mutation and conditional deletion of second *Vhl* allele, both arterial and venous branching were observed. Collectively, these results indicated the differential effect of aberrant Vegfa and Notch signaling linked to *Vhl* missense mutations, or conditional deletion of *Vhl,* on vascular (dys)morphogenesis [111][116]. These findings could lead to the identification of novel treatment targets in VHL disease, characterized by extensive vascularization due to overproduction of VEGF.

Genotype–phenotype correlations in VHL disease suggest that oxygen-dependent HIF regulation by VHL mutant proteins, as well as HIF-independent VHL functions, modulate the risk of tumor development. It has been established that certain mutations in the VHL gene resulted in a state of pseudohypoxia with elevated levels of HIF proteins, and subsequent activation of HIF-dependent genes, which upregulate angiogenesis, increased cell proliferation and shifted metabolism toward glycolysis, the pentose phosphate pathway, and glutamine-dependent fatty acid biosynthesis, whereas other mutations preferentially affected HIF-independent pathways, without inducing pseudohypoxia [12][87][117]. The HIF signaling pathway is most frequently activated by inactivating mutations of the VHL gene [118]. Abnormally elevated transcriptional activities of the HIF1α and HIF2α genes have been shown to increase tumor survival in solid tumors [119]. Increased hemoglobin concentrations can occasionally occur because of tumor (hyper)production of erythropoietin, as observed in hemangioblastomas, RCC, and PCC [33][118][120][121]. Interestingly, however, despite the fact that elevated HIFs in the background of certain *VHL* mutations has been associated with erythrocytosis, this condition is not a common feature of VHL disease.

The complexity of phenotype–genotype associations between VHL aberrations and disease is further demonstrated by research data that *VHL* genetic aberrations follow the so-called continuum model of tumor suppression, which accounts for the zygosity status of genetic change and tissue specificity [108][122]. Indeed, the research performed by Couve et al. indicated that disease phenotype, in the background of specific *VHL* mutations, can be dependent on the gradient of VHL loss of function, and can show an additive effect in the context of double mutants [108]. Their research resolved intriguing family cases who were classified as having type 2B VHL disease, based on the presence of CNS and retinal hemangioblastomas, RCC, PCC, and pancreatic neuroendocrine tumors. Initially, only heterozygous p.R200W change was found. This change, in hetero- or homozygous form, was firmly associated with normal phenotype and/or erythrocytosis (Chuvash polycythemia), respectively [123][124][125]. Subsequent analyses revealed another change, p.R161Q, located in the same allele together with the p.R200W change, in diseased probands. Protein change p.R161Q was previously associated with type 2A VHL disease, with low risk for the development of RCC. However, the presence of both mutations in the same allele abrogated HIF2α binding, whereas a single p.R161Q mutant showed only partially impaired binding, and p.R200W binding to HIF2α was within the normal range [123][124][125]. Therefore, the simultaneous presence of these two changes in the *VHL* gene affected the HIF signaling pathway more profoundly and carriers of double mutations were susceptible to type 2B VHL disease.

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