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Erythrocytosis - the HIF Pathway in Control

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Abstract

Organisms living under aerobic conditions need oxygen for the metabolic conversion of nutrition into energy. With the appearance of increasingly complex animals, a specialized transport system (erythrocytes) arose during evolution in order to provide oxygen to virtually every single cell in the body. Moreover, in case of low environmental pO_2 the number of erythrocytes automatically increases to preserve sustained oxygen delivery. This process relies predominantly on the cytokine erythropoietin (EPO) and its transcription factor hypoxia inducible factor (HIF), whereas the von Hippel Lindau (VHL) ubiquitin ligase as well as the oxygen-sensitive prolyl hydroxylases (PHDs) represent essential regulators of this oxygen-sensing system. Deregulation of particular members of this pathway (e.g. PHD2, HIF2α and VHL) lead to disorders in blood homeostasis due to insufficient (anemia) or excessive (erythrocytosis) red blood cell production.

Introduction

High altitude is accompanied by low atmospheric oxygen pressure, which sequentially leads to insufficient oxygen uptake and reduced tissue oxygenation. In general, inadequate oxygen supply is detrimental and might lead to death of cells, tissues or ultimately even the organism. In order to avoid this complex cardiovascular, respiratory and hematological mechanisms have evolved, and one such long-term adaptation process is the elevation of erythrocyte numbers to boost the blood's oxygen transport capacity.

As early as in the 19th century, scientists recognized the correlation between low atmospheric oxygen pressure and elevated red blood cell numbers in humans and animals.[1] Some decades later, it became evident that low oxygen does not directly act on hematopoietic cells but induces the production of a soluble factor called erythropoietin (EPO). In 1977, EPO was purified from the urine of anemic patients[2] and in 1985 the corresponding *EPO* gene was isolated and cloned.[3] About two decades ago, the transcription factor HIF (hypoxia inducible factor) was first identified in hepatoma cells as the regulator of EPO through its binding to a hypoxia responsive element (HRE) present in the 3'enhancer region of the *EPO* gene.[4,5] In subsequent work, HIF was found to be expressed widespread in mammalian cells and even in lower animals that do not produce Epo or red blood cells.[6-8] In the past decennium a lot of knowledge has been acquired on the role of HIFs during red blood cell production (erythropoiesis). Accordingly, this review aims to discuss recent findings on these essential proteins in humans and mice, and the detrimental impact of their deregulation.

Erythropoietin: the driving force of erythropoiesis

EPO, a glycoprotein hormone, is the principal stimulator of erythropoiesis and is induced under hypoxic conditions. In 1957, the kidney was first identified as the primary EPO producing organ in adult mammals,[9] whereas the liver is the major source of EPO during embryogenesis (e.g. hepatocytes, Ito cells). In the kidney, a specialized EPO-producing cell (REPC) was identified in the cortex and outer medulla that was initially described as an interstitial fibroblast-like cell with neuronal characteristics.[10-14] Interestingly and in contrast to other organs, the kidney is able to increase the total amount of REPCs in an oxygen-dependent manner rather than increasing EPO expression per cell.[11,14] Neurons and glial cells in the central nervous system (CNS) represent an additional source of EPO[15,16], it has been suggested that the glycohormone functions in a paracrine fashion as a protective,[17] ventilations[18] or cognition-enhancing factor.[19]

Erythropoiesis is a complex multistep process during which erythroid progenitors enucleate and develop into mature red blood cells. Upon Epo binding to its receptor, the EpoR signaling through the Janus kinase 2 (JAK2) activates multiple pathways including Stat5, phosphoinositide-3 kinase (PI-3K)/Akt, and p42/44 mitogen-activated protein kinase (MAPK). This reduces apoptosis and promotes expansion and differentiation of the progenitors.[20] In adult mammals, erythropoiesis is mainly carried out by the bone marrow. However, in response to stress (e.g. anemia / bone marrow transfer / certain diseases), erythropoiesis may extend to extramedullary sites, such as spleen and liver, thereby increasing erythrocyte output. As shown in mice, stress erythropoiesis is characterized by massive self-renewal of BFU-E (burst-forming unit-erythroid) cells and is regulated by additional extrinsic factors like the stress hormone cortisol, stem cell factor (SCF) and the bone morphogenetic protein 4 (BMP4).[21-24] In humans, analogous pathways have not yet been identified, and the molecular basis is also not well described. However, recently, erythroblastic island macrophages have been reported to facilitate human stress and pathological erythropoiesis.[25]

The HIF pathway

The HIF pathway is present in virtually every cell of the body, orchestrating a whole cascade of downstream genes that allow acclimatization to reduced levels of oxygen. The alpha subunit of the HIFs (mainly HIF1α and HIF2α) becomes stabilized nearly instantaneously during low oxygen conditions[26] and translocates to the nucleus where it dimerises with the constitutively expressed HIFβ subunit and promotes transcription of genes containing a HRE.[27] In human cells, pan-genomic analyses of HIF binding to DNA have now revealed the existence of more than 500 direct transcriptional targets of HIF in a given cell line.[28,29] More than a decade ago, the groups of Dr. Ratcliffe and Dr. Kaelin discovered that both HIF1α and HIF2α are regulated at the post-transcriptional level by the HIF prolyl-hydroxylase domain enzymes (PHDs). These oxygen sensors hydroxylate the alpha-subunits and prime them for poly-ubiquitination by the von Hippel-Lindau (VHL) tumor suppressor complex which ultimately leads to proteolytic degradation (Figure 1).[30,31] To date, four PHDs have been identified in mammals, of which PHD2 (gene name: *Egl nine homolog 1 (Egln1)*) has been described as the key limiting enzyme targeting HIFα for degradation under normoxic and mild hypoxic conditions.[32-34]

HIF1α exhibits a ubiquitous expression pattern whereas HIF2α is found in a limited number of cell types including endothelial cells, cardiomyocytes, hepatocytes, glial cells and interstitial cells of the kidney.[35] Both isoforms have overlapping sets of target genes, but can also play non-redundant roles, depending on the cell type and oxygen concentrations.[36] Accordingly, HIF1α has been suggested to represent the response to acute hypoxia, while HIF2α is the predominant subunit to chronic exposure to low oxygen as occurring at high altitude.[37] In addition, several studies have demonstrated that both HIF isoforms can even display opposing roles in vivo, for instance in renal cell carcinoma growth and metastasis

formation.[38,39] Glycolysis enzymes like phosphoglycerate kinase 1 (PGK1) and lactate dehydrogenase A (LDHA) are predominantly HIF1α-dependent.[36] In contrast, HIF2α has been described to induce matrix metallopeptidase 9 (MMP9) and the transcription factor oct-4, that is involved in stem cell function and in the elevation of hemoglobin gene expression in humans (Figure 1).[40-42] Until recently, it was unclear which of the HIF and PHD isoforms regulated erythropoiesis and the expression of EPO in particular. Only with knowledge gained from patients with erythrocytosis and transgenic mice it became evident that the HIF2α isoform and not HIF1α is the key player in EPO gene expression and erythropoiesis-enhancing processes (e.g. iron absorption and transport).

Mutations in HIF pathway proteins can lead to erythrocytosis in humans

Erythrocytosis is an aberrant increase in red blood cell number and comprises a heterogeneous group of disorders. A general distinction is made between the hypersensitivity of the erythroid progenitors to EPO (primary erythrocytosis) and the excessive activation of EPO gene transcription (secondary erythrocytosis). The most common example of primary erythrocytosis is Polycythemia Vera (PV). Here, erythroid progenitors carry a gain-of-function mutation in the *JAK2* gene, which leads to constitutive activation of the EPO signaling pathway at the EPO-R level. On the other hand, patients bearing point mutations in specific members of the HIF pathway can develop secondary erythrocytosis (Table 1).

VHL

In 1997, the first type of erythrocytosis related to the HIF pathway was discovered by Dr. Prchal and colleagues. They described 103 individuals suffering from erythrocytosis that belong to 81 families living in the Chuvash region (Russia).[43] A number of patients were studied in detail and displayed markedly increased hematocrit levels accompanied by significantly higher EPO levels. However, molecular analysis failed to demonstrate mutations

in the *EPO-R* or previously described erythrocyte alterations (e.g. high oxygen affinity hemoglobin). Subsequent genetic studies revealed a homozygous mutation in the *VHL* gene (C598T leading to the R200W amino acid change) in all affected individuals. This resulted in reduced affinity of VHL for the hydroxylated HIFα subunit and subsequent increase of EPO and red blood cells.[44,45] Recently, the underlying molecular mechanism was discovered: the R200W VHL mutation alters the affinity of VHL for suppression of cytokine signaling 1 (SOCS1), which prevents the degradation of the EPO-R coupled kinase pJAK2.[46] This illustrates that VHL, as part of the oxygen sensing machinery, does not only influence the production of EPO but also regulates erythropoiesis at different levels. The clinical presentation of Chuvash erythrocytosis patients has been carefully studied and includes a wide range of hematological and vascular abnormalities but no tumors. Chuvash patients suffer from complications such as thrombosis, major bleeding episodes and higher systolic pulmonary artery pressure, which collectively lead to premature lethality.[47-49] However, it has been suggested that thromboembolic events in patients with VHL mutations might be associated with a subsequent gain in HIFα activity rather than the increase in red blood cell mass. For instance, VEGF and PAI-1, two HIFα targets, are upregulated in the serum of Chuvash erythrocytosis patients, and might have an impact on coagulation pathways.[50,51] Further studies have revealed higher homocysteine levels in Chuvash erythrocytosis patients, which could be an additional cause for the observed elevated blood pressure and thrombosis.[52] Later, another cohort with the same mutation was identified on an island in the Bay of Naples, Italy, which suggested for a founder mutation. Indeed, single nucleotide polymorphism (SNP)-analysis near the VHL gene on individuals from different ethnic backgrounds confirmed this, indicating that the R200W mutation arose between 14,000 and 62,000 years ago in a single ancestor.[53,54] Apart from the R200W mutation, two additional homozygous VHL mutations (Croatian H191D and P138L) and several (compound) heterozygous

mutations have been discovered in single patients, resulting in very similar phenotypes observed in classical Chuvash patients.[55-59] Conversely, the well-known autosomal dominant cancer-predisposition von Hippel-Lindau (VHL) syndrome, with over 1500 known VHL mutations, does not lead to erythrocytosis and is due to inheritance of a single mutated allele of VHL.[60]

PHD2

Since 2006, several patients and families with heterozygous loss-of-function mutations in the *PHD2* gene have been described.[55,61-64] The first mutations that were discovered are the P317R and the P371H variants, that affect the catalytic rate and substrate binding of PHD2, leading to partial inhibition of HIF hydroxylation.[64-66] A few of the reported PHD2 mutations, apart from erythrocytosis, also led to other pathologies such as superficial thrombophlebitis[64], sagittal sinus thrombosis[66] and hypertension[67]. However, the number of such patients is currently still too small to draw firm conclusions. Only in one case, PHD2 has also been described to be associated with tumor formation - in particular, a recurrent paraganglioma. This patient is a heterozygous carrier of a PHD2 germline mutation (H374R) which affects one of the three conserved amino acids that coordinate Fe^{2+} binding, therefore contributing to the functionality of the enzyme.[68] Interestingly, sequence analysis of the removed tumor mass showed that not one but both PHD2 alleles were mutated in the tumor cells (loss of heterozygosity). Functional analysis of the described PHD2 variants revealed that only the H374R variant has a detrimental effect, and all other studied PHD2 mutations show only weak deficiency in HIFα regulation.[63] Such functional differences may permit PHD2 to act as a tumor suppressor in patients.

HIF2α

A new form of familial erythrocytosis was discovered in a family where the phenotype was associated with a heterozygous missense mutation in the HIF2α gene (EPAS1). The mutation is predicted to produce a G537W change in the amino acid sequence of HIF2α, which is very close to the primary site of hydroxylation (Pro-531).[69] The resulting impairment of the hydroxylation of HIF2α and its subsequent VHL binding leads to an aberrant stabilization of this transcription factor during normoxia. Further studies have revealed numerous other HIF2α alterations, all near the primary hydroxylation site, typically leading to elevated EPO levels and erythrocytosis in the affected patients.[55,70] In addition, numerous SNPs in the HIF2α gene are found in Tibetans and are associated with only a moderate increase in hemoglobin concentrations. This adaptation to high altitude strengthens the link between HIF2α and erythropoiesis.[71,72] Contrarily, mutations of the HIF1α isoform have not been associated with altered red blood cell production.

Interestingly, mutations of the HIF2α gene have not only been shown to lead to erythrocytosis but have also been recently described to cause neoplasia. In particular, one patient carrying an inherited gain-of-function mutation in HIF2α (F374Y) displayed erythrocytosis, with additional recurrent multiple paragangliomas.[73] In addition, two erythrocytosis patients with paragangliomas, one of them with an additional somatostatinoma, have also been described to carry somatic HIF2α mutations (A530T and A530V), which increase the half-life of the HIF2α subunit and enhance HIF downstream signaling.[74] The mutation was found in DNA from the tumor cells only and not in other cell types nor in the patients' parents, which argues for a causative postzygotic event.[74] Screening of patients with chromaffin-cell tumors (paragangliomas, pheochromocytomas) led to the discovery of numerous other somatic HIF2α mutations which are only partially accompanied by erythrocytosis.[75-78] This predicts a direct oncogenic role for HIF2α, independent of its impact on red blood cell production.

Taken together, patients bearing a polymorphism in VHL, PHD2 or HIF2α collectively highlight the importance of the HIF signaling pathway in red blood cell homeostasis. Both somatic and germline mutations in HIF pathway members have been shown to lead to erythrocytosis. In some cases, erythrocytosis was accompanied by neuroendocrine tumors whose molecular basis remains to be unraveled (Table I).

Genetically modified mice reveal important players in erythropoiesis

Only a limited amount of erythrocytosis-associated mutations in the HIF pathway proteins in humans have been described so far – and most of them only very recently. To unravel the effective role of the different HIF pathway proteins during erythropoiesis, various genetically modified mice have been developed in the past 15 years (Table 2).

HIFαs, PHDs and VHL

Although HIF1α was initially discovered as the isoform that activates EPO transcription,[5] it was only after both systemic and cell-type specific HIF1α and HIF2α knockout mice were made that the distinct role of both these transcription factors in erythropoiesis became clear. HIF1α knockout mice ($HIF1\alpha^{-/-}$) are only viable up to E11.5 and these embryos show major defects of the cardiovascular system and the neural tube.[79,80] However, the lack of HIF1α doesn't lead to complete abolishment of erythropoiesis, but rather to multiple disturbances in the adaptive responses to hypoxia. Conversely, HIF2α deficient mice revealed that the observed pancytopenia is caused by abnormally low plasma EPO levels and impaired renal EPO induction.[81] Ablation of this subunit after birth resulted in anemia accompanied by decreased circulatory EPO.[82] Interestingly, even heterozygous deficient mice ($HIF2\alpha^{+/-}$) show a mild form of anemia (K.F. and B.W., unpublished data, October 21, 2011). The group of Dr. Haase was able to demonstrate that the regulation of erythropoiesis is essentially driven by

renal HIF2α.[83] Indeed, specific deletion of HIF2α in the kidney resulted in EPO-dependent anemia, which was only partially compensated by hepatic HIF2α.[83] Moreover, although both HIF isoforms are expressed in the kidney, only HIF2α is found in the peritubular interstitial cells,[32,74] and co-localized with EPO mRNA in these cells.[84] At the molecular level, it was shown that HIF2α is actually the major isoform binding the 3' enhancer of the *EPO* gene in its native form, whereas HIF1α primarily binds to the isolated HRE, as initially described.[4,5,85] Moreover, the existence of additional transcription factors that bind to sites outside the actual HRE which promote the preferential binding of HIF2α has been proposed, too.[85] Recently, the group of Dr. Lee presented a new mouse line bearing a G536W missense mutation in HIF2α that corresponds to the first such human mutation identified (G537W). Remarkably, these mice not only showed elevated hematocrit and pulmonary hypertension, these findings attest that missense mutations in HIF2α can indeed cause erythrocytosis.[86]

The HIFα subunits are regulated by different PHDs- the oxygen sensors. However, it is only after the mutant mouse lines were made that the functional differences between the family members became clear. Indeed, systemic deletion of PHD2, leads to embryonic lethality due to placental and heart defects, whereas PHD1 and PHD3-specific knockout mice do not show any apparent abnormalities.[87] Inducible PHD2-deficient mice on the other hand, develop severe erythrocytosis and show decreased life expectancy.[88,89] Mice that are systemically deficient for either PHD1 or PHD3 do not display increased hematocrit values, and only mice lacking both these isoforms simultaneously develop a moderate form of erythrocytosis. In the latter mice, plasma EPO and renal EPO expression is decreased while hepatic EPO mRNA is induced.[89] Thus, PHD1 and PHD3 appear to have only minor roles in the regulation of EPO expression, although their additional loss in the background of PHD2-deficiency can ameliorate the erythrocytosis phenotype.[90,91] Our research group recently developed a

conditional PHD2-deficient mouse line displaying severe but non-lethal erythrocytosis.[92] Using different genetic approaches (PHD2/HIFα double deficient mice) we could show that the EPO-dependent red blood cell increase is driven by HIF2α, which is in line with other observations made in familial erythrocytosis.[69,93] Conversely, we found that HIF1α actually serves as a protective factor in these PHD2-deficient mice via the local induction of PHD3.[92]

Mice carrying a homozygous deletion of the VHL gene die *in utero* due to a defect in placental vasculogenesis.[94] A liver specific VHL deletion led to hepatic vascular tumors and erythrocytosis, which was accompanied by increased EPO levels.[95] The increase in erythrocytes was not reversible by additional hepatic HIF1α deletion,[96] but only by deletion of HIF2α.[85] Mice with an astrocyte specific deletion of VHL not only exhibit a significant increase in cerebral EPO mRNA but also a significant induction of plasma EPO and erythrocytosis.[16] The additional deletion of HIF1α did not correct this increase in red blood cell count but rather made the phenotype more severe and shortened the survival time of these double deficient mice. On the other hand, elimination of HIF2α along with VHL normalized the red blood cell count and most of the cerebral EPO transcript.[16] Recently, ablation of VHL in osteoblasts led to HIF2α-dependent EPO induction in these cells, accompanied by erythrocytosis and enhanced bone formation.[97] In 2007, a mouse line carrying the homozygous R200W mutation (leading to Chuvash erythrocytosis in humans) was created. Interestingly, this point mutation resulted in moderate erythrocytosis accompanied by splenic erythropoiesis.[98] Embryonic stem cells carrying this mutation exhibited normoxic stabilization of HIF2α, which was accompanied by up-regulation of HIF2α-targets like VEGF.

Conclusion

Deregulation of EPO transcription due to mutations in HIF pathway proteins is an important underlying cause of erythrocytosis in patients. Moreover, these mutations can also result in other pathologies like tumor development. Recently, various point-mutations in the *HIF2α/EPAS1, VHL* and *PHD2* genes have been identified and additional studies have led to new insights into the HIF pathway. Complementary to these mutations, many genetically modified mice have provided a powerful tool to study the effect and location of HIF pathway members in relation to erythropoiesis and additional risk factors. Furthermore, it might be of great interest to develop new mouse models for erythrocytosis and related diseases including mice carrying specific point mutations found in humans (as mentioned above for the R200W VHL and very recently the G537W HIF2α mutation).

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Author contribution

K.F. and B.W. wrote the manuscript. M.G. provided helpful discussions and helped write the manuscript.

Conflict of interest

The authors declare that there are no competing financial interests.

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Figure 1: Oxygen-dependent regulation of HIFα and its target genes. If oxygen demand is covered, HIFα becomes constantly hydroxylated by PHDs, and subsequently undergoes proteasomal degradation after binding with VHL. Under hypoxic conditions, HIFα is stabilized, translocates to the nucleus, binds to its heterodimerization partner HIFβ as well as to other co-factors, and leads to the transcriptional activation of target genes that harbor HRE sequences in their promoter region. HIF1α and HIF2α share target genes (green) but have certain preferences (preferentially induced by HIF1α in yellow; by HIF2α in blue) (HIF: hypoxia inducible factor, PHD: prolyl hydroxylase, VHL: von Hippel-lindau, CBP: CREB-binding protein, HRE: hypoxia responsive element).

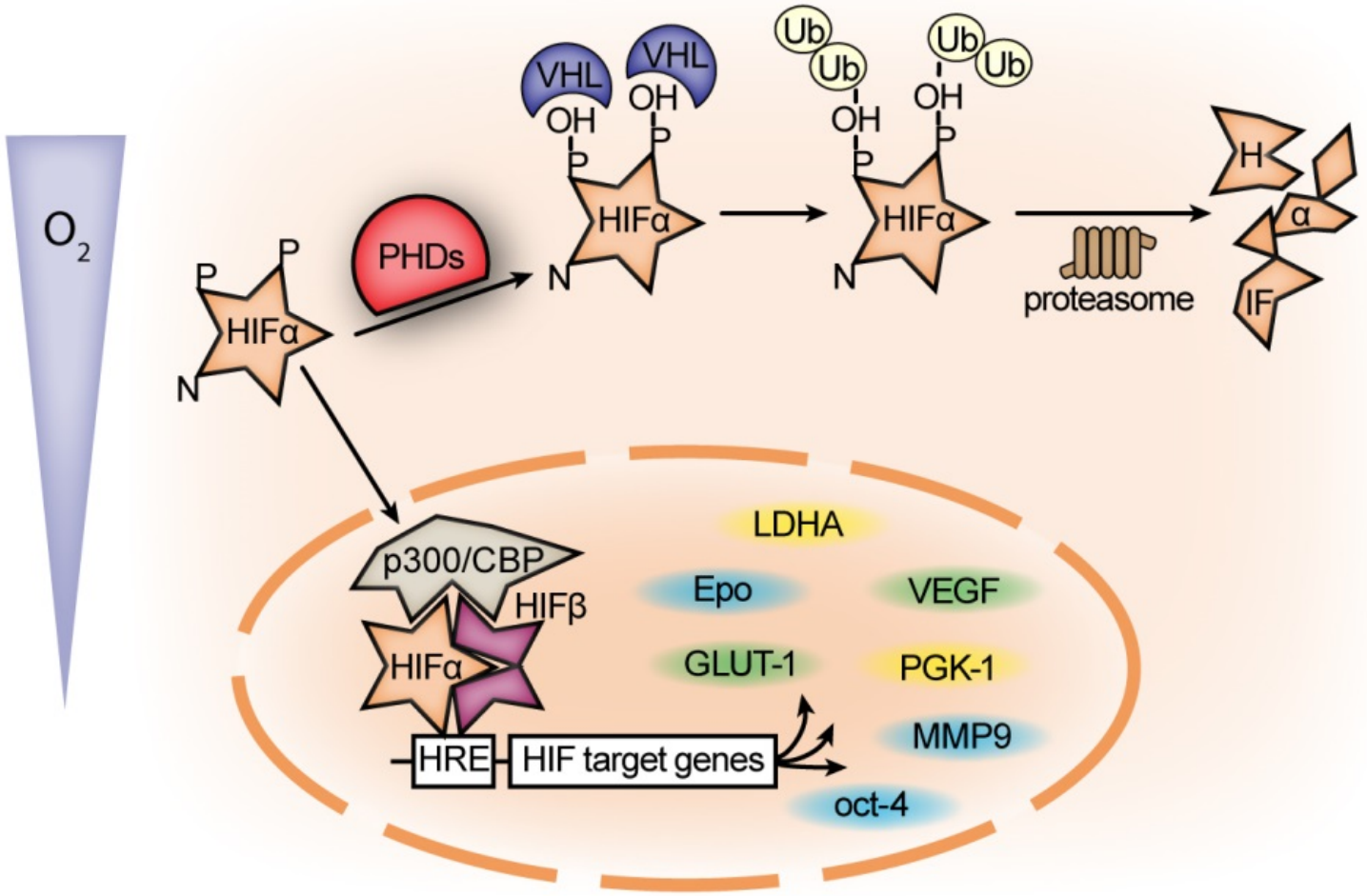
Table 1: HIF pathway related mutations that have resulted in erythrocytosis and/or tumor development in humans.

Gene	Type of mutation	mutation	erythrocytosis	Tumor type	Ref.
VHL	Germline (Homo>>Hetero)	**R200W**	+	-	45,55
	Germline (Homo)	**H191D P138L**	+	-	57-59
	Germline ((Compound) Hetero)	**Various (including R200W)**	+	-	55,56
	Somatic/ Germline (Hetero)	**various**	**-**	e.g. spinal hemangioblastoma, renal cell carcinoma (RCC) and pheochromocytoma	60
PHD2	Germline (Hetero)	**various**	+	-	55,61-64,66,67
	Germline (Hetero)	**H374R**	+	paraganglioma	68
HIF2α	Germline (Hetero)	**Various (including G537W)**	+	-	55,69,70
	Germline (Hetero)	**F374Y**	+	pheochromocytoma/ paraganglioma	73
	Somatic (Hetero)	**A530V A530T**	+	paragangliomas/ somatostatinoma	74
	Somatic (Hetero)	**various**	**+/-**	Pheochromocytomas / paragangliomas / somatostatinoma	75-78

Table 2: Available genetically modified mice illustrating the impact of a deregulated HIF system on murine erythropoiesis.
* in anemic mice and during early postnatal development

Gene	Type of modification	Phenotype mice	Ref.
VHL	Liver specific KO	erythrocytosis	95
	Brain specific KO	erythrocytosis	16
	R200W mutation	erythrocytosis	98
PHD2	Induced complete KO	erythrocytosis	88,89
	Conditional KO (including EPO-producing cells in kidney and brain)	erythrocytosis	92
	Heterozygosity	mild erythrocytosis	99
HIF2α	Complete KO	pancytopenia	100
	Induced complete KO	anemia	82
	Liver specific KO	anemia	* 85 * 83
	Kidney specific KO	anemia	83
	Heterozygosity	mild anemia	unpublished
	G536W mutation	erythrocytosis	86

Figure 1


O2
HIFα
P
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PHDs
VHL
VHL
OH
OH
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Ub
OH
OH
P
P
HIFα
N
proteasome
H
α
IF
p300/CBP
HIFβ
HIFα
HRE
HIF target genes
LDHA
Epo
VEGF
GLUT-1
PGK-1
MMP9
oct-4