

# Hypoxia Pathway Proteins are Master Regulators of Erythropoiesis

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Keywords: hypoxia, erythropoiesis, EPO, HIF, CKD

## Abstract

Erythropoiesis is a complex process driving the production of red blood cells. During homeostasis, adult erythropoiesis takes place in the bone marrow and is tightly controlled by erythropoietin (EPO), a central hormone mainly produced in renal EPO-producing cells. The expression of EPO is strictly regulated by local changes in oxygen partial pressure ( $pO_2$ ) as under deprived oxygen (hypoxia) the transcription factor Hypoxia Inducible Factor-2 induces EPO. However, erythropoiesis regulation extends beyond the well-established HIF-EPO axis, and involves processes modulated by other hypoxia pathway proteins (HPPs), including proteins involved in iron metabolism. The importance of a number of these factors is evident as their altered expression has been associated with various anemia-related disorders, including chronic kidney disease. Eventually, our emerging understanding of HPPs and their regulatory feedback will be instrumental in developing specific therapies for anemic patients and beyond.

## Introduction

Red blood cell (RBC) production mainly takes place in the bone marrow during homeostasis. This process encompasses a number of proliferation and differentiation steps starting at the hematopoietic stem cell (HSC) and down to specialized erythroid progenitors finally turning into mature RBCs. The complex process of erythropoiesis is vastly intertwined with iron metabolism [1], and regulated by a number of cytokines including the central glycoprotein hormone, erythropoietin (EPO). The primary producers of EPO are specialized fibroblast-like peritubular cells located in the adult kidney or fetal hepatocytes. Nevertheless, more recent studies reported EPO production also in astrocytes, osteoblasts, and pericytes upon transcriptional response dependent on the hypoxia pathway [2-6]. Our initial understanding of this pathway came into light almost 30 years ago when the group of Dr. Semenza identified hypoxia inducible factor (HIF) as a transcription factor responsible for the expression of EPO [7,8]. Hence, increased EPO during diminished oxygen supply, for instance at high altitude, causes our body to make more RBCs to augment delivery of oxygen. Since then, numerous hypoxia pathway proteins (HPPs) have been identified in different cells of our body, often playing critical roles in survival, growth and differentiation upon changes in  $pO_2$  [9]. Cellular oxygen sensing is therefore a fundamental biological process, necessary for adaptation of living organisms to changing physiological and pathological situations. To date a limited amount of enzymes, three HIF Prolyl Hydroxylase Domains proteins (PHD1-3) and Factor inhibiting HIF (FIH) have been identified as oxygen sensors. These oxygen-dependent enzymes act as hydroxylases and dioxygenases regulating the availability of HIF $\alpha$  subunits and in turn define when and how cells express genes in response to changing  $pO_2$  [10-12]. Under hypoxia, when the oxygen sensors are inactive, HIF $\alpha$  subunits are stabilized and translocate to the nucleus and bind to HIF $\beta$  and a number of other co-factors. Subsequently the HIF-complex binds hypoxia response elements (HRE) in the promoter/enhancer of a selection of genes, generally increasing their transcription [13-15]. This review will give a more in-depth view on the role of HPPs (i.e. HIF $\alpha$ , PHDs, HIF-controlled genes) regulating the multistage process of

erythropoiesis, and discusses the current therapeutic approaches interfering the hypoxia pathway in the treatment of anemia and/or Chronic Kidney Disease (CKD) [16].

### **HIF transcription factors and their modulators**

The heterodimeric HIF-complex is a key transcription factor constituted of an  $\alpha$  and a  $\beta$  subunit. The latter, also referred as aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed whereas HIF $\alpha$  (i.e. HIF1 $\alpha$ , HIF2 $\alpha$  (also known as endothelial PAS domain containing protein 1 - EPAS1), and HIF3 $\alpha$ ) is synthesized and constantly degraded during normoxia. Degradation of the HIF $\alpha$  subunits is tightly regulated by three PHDs (also known as EGL-9 homolog EGLN2, EGLN1 and EGLN3, respectively) that hydroxylate two specific proline residues (Pro402/Pro564 in HIF1 $\alpha$  and Pro405/Pro531 in HIF2 $\alpha$ ) in the oxygen-dependent degradation domain [11,17-19]. Consequently, this hydroxylation leads to von Hippel–Lindau tumor suppressor protein (VHL) mediated ubiquitination, and proteasomal degradation of the HIF $\alpha$  subunits [12,19,20].

Although a recent *in vitro* study demonstrated that HIFs are the only targets of the PHDs [21], each PHD has a different affinity for a particular HIF subunit: HIF2 $\alpha$  is mainly regulated by PHD1, PHD2 is the major regulator of HIF1 $\alpha$  [22,23]. Noteworthy, PHD2 is the most widely expressed PHD isoform, being of crucial importance during development. Indeed, early studies in mice reported that PHD2 deficiency results in embryonic lethality between E12.5 and 14.5 due to defects in the developing placenta and heart [24,25]. Conversely, PHD1 and PHD3 deletions are not lethal, and have only limited tissue specific impact, as exhibited by their effect on cellular metabolism in skeletal muscle and altered blood pressure in the central nervous system, respectively [26,27]. Moreover, PHD2 differs from PHD1 and PHD3 in its N-terminal zinc finger, which favors recruitment of the HSP90 machinery, facilitating HIF $\alpha$  hydroxylation [28,29]. In turn, PHD1 and PHD3 can also be regulated by hypoxia inducible E3 ubiquitin ligases: the SIAH proteins [30]. HIF $\alpha$ 's availability can further be modulated at the transcriptional level through crosstalk with other signaling pathways and epigenetic regulators. For

instance, nuclear factor- $\kappa$ B (NF- $\kappa$ B) and signal transducer and activator of transcription 3 (STAT3) have been shown to bind the HIF1 $\alpha$  promoter and induce its transcription [31,32]

Whereas HIF1 $\alpha$  is expressed ubiquitously, HIF2 $\alpha$  is more restricted to specific cell types like endothelial cells, cardiomyocytes, hepatocytes, adipocytes, neurons, and interstitial cells and glomeruli of the kidney [21,33-37]. Even though both HIFs regulate many common genes, their transcriptional targets often differ, as well as their kinetics of activation and oxygen dependence. Overall, gene expression and chromatin immunoprecipitation assays showed that both HIF1 $\alpha$  and HIF2 $\alpha$  are responsible for regulating transcription of more than a thousand genes during adaptive response to hypoxia [38-40].

Conversely, HIFs have also been reported to indirectly suppress the expression of a number of genes by activation of transcriptional repressors or epigenetic regulators. For instance, Cavadas and colleagues recently showed that hypoxia mediated nuclear translocation of Repressor Element 1-Silencing Transcription factor (REST) is responsible for regulating 20% of hypoxia-repressed genes in hypoxia[41]. The other transcriptional repressors induced by hypoxia include DEC1, DEC2, Bach1, Zeb1 and Zeb2 reviewed by Cavadas *et al* [42]. Hypoxia also regulates epigenetic factors, such as DNA methylation and histone acetylation, those in turn regulate the hypoxia responsive gene expression [43]. In addition, several recent studies, including ours, have pointed towards HIF-mediated suppression by microRNAs [44-48]. Interestingly, there is a two-way communication between HIFs and microRNAs, as the latter have also been suggested to control the expression of HIFs in different cell types [49,50] (recently reviewed by Serocki and colleagues [46]).

Soon after their discovery, PHDs have been considered to play a distinctive role in regulating erythropoiesis. Somatic inactivation of PHD2 after birth causes a significant rise of serum EPO levels; an effect that can only be mimicked by simultaneous deletion of PHD1 and PHD3. Importantly, we and others discovered that this phenotype is exclusively dependent on HIF2 $\alpha$  since its targeted deficiency but not that of HIF1 $\alpha$  reverses this phenotype [34,51]. Briefly, severe HIF2-driven EPO production resulted in non-lethal hematocrit levels up to 85% upon PHD2 deletion in CD68-expressing cells, including renal

EPO-producing cells (REPCs) [34]. In humans, various heterozygous point mutations have been described in the PHD2 gene resulting in an increase of red blood cell mass, hemorrhages, strokes and enhanced risk of thrombosis [52-54]. The mutations P317R and P371H affect the substrate binding of PHD2, partially inhibiting the hydroxylation of HIF [55]. Interestingly, a patient with a heterozygous H374R mutation developed a recurrent paraganglioma that exhibited loss of PHD2 in both alleles in addition to the erythrocytotic phenotype [52].

Next to EPO and its receptor, several other genes involved in the process of erythropoiesis are directly or indirectly regulated by the PHD/HIF axis. These include genes implicated in iron metabolism, such as transferrin (TF), transferrin receptor-1 (TFR-1), ferroportin (FPN), hepcidin, divalent metal transporter 1 (DMT1), duodenal cytochrome b (DCYTB), and GATA-1 [56-61], granting this axis a central role during anemic processes associated to a wide range of diseases (Figure 1).

### **Erythropoiesis: a multistep process**

On average  $200 \times 10^9$  RBCs are generated and released into circulation every day [62]. This production is adjusted on a need basis and is tightly controlled by complex networks involving HPPs, regulators of iron metabolism and erythropoietic stress [1,63-65]. Erythropoiesis is a multistep process that includes numerous proliferation and differentiation stages starting from the HSC, down to committed erythroid progenitors and different erythroblast populations in the bone marrow (BM). This eventually gives rise to reticulocytes and enucleated mature RBCs in the blood [7,62,66]. The course of this development includes strict control by a number of transcription factors and epigenetic regulators. GATA-1 is the major transcription factor regulating differentiation and survival of erythroid progenitors [67-70], including genes involved in heme and/or globin synthesis, anti-apoptosis, cell cycle regulators, and the erythropoietin receptor (EPOR) [67-69,71-74]. In turn, GATA-1 is also regulated by HIF [73,75,76]. Additionally, GATA-2 is essential for the regulation of lineage-restricted gene expression during erythroid differentiation [70,77]. Other erythropoietic regulators that have been studied in the

context of hypoxia and HPPs are interleukin-3, certain vitamins as well as transferrin (TF)-transferrin receptors (TFR) modulating iron metabolism [59-61].

Several studies in the field of erythropoiesis have also explored the role of micro-RNAs, including during the process of enucleation [78,79]. A recent study by Rivkin and colleagues reported that the hematopoietic specific mir-142 plays a critical role in maintaining the typical biconcave shape of RBCs through the control of cytoskeletal regulators and directly influencing the overall lifespan of the erythrocyte [80]. In another study, mir-142 was shown to directly target HIF1 $\alpha$  [81]. Although more research is required to unambiguously link mir-142, HIF1 $\alpha$ , and the shape of the RBCs, other studies are available reporting HIF's participation in the regulation of micro-RNAs involved in different stages of erythropoiesis [82-85] (Table1).

### **EPO/EPOR axis: hypoxia pathway in action**

EPO is the central hormone during erythropoiesis and is mainly responsible for survival, proliferation, and differentiation of committed progenitors, ultimately enhancing the oxygen-carrying capacity of blood [86]. EPO promotes survival of colony-forming unit-erythroid cells (CFU-E) and early erythroblasts. Moreover, erythroid precursors are differentially sensitive to EPO and respond differently to increasing levels. At higher levels, cells survive and differentiate, whereas at lower EPO levels less sensitive cells die due to caspase-mediated apoptotic death [71].

After birth, EPO is mainly produced by REPCs, whereas hepatocytes are the main source during embryo development [87-90]. In the BM, EPO binds to its homodimeric receptor EPOR on erythroid progenitor cells, triggering its association with Janus kinase (JAK2). In turn, JAK2 phosphorylates the cytoplasmic tail of EPOR, providing multiple docking sites for signal-transducing proteins, which subsequently lead to STAT-5 phosphorylation [91]. Additionally, it was shown that the phosphatidylinositol-3-kinase/protein kinase B (PI-3k/AKT) and mitogen-associated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathways can also be activated in this way [92].

Severe hypoxia or loss of PHD2 in hepatocytes can also result in dramatic liver EPO production in adults [93-96]. However, in contrast to HIF2 $\alpha$ -mediated EPO production, HIF stabilization in epithelial cells of the kidney results in suppression of EPO, leading to anemia. The lab of Dr. Haase showed that proximal nephron specific deletion of VHL (Pax8-rtTA-cre) results in a reduction of REPCs and renal anemia, suggesting a cellular crosstalk in the tubule-interstitial compartment in the kidney, changing the production of EPO [97].

Since HIF2 $\alpha$  is a major player in erythropoiesis, most of the studies have focused on this subunit as a facilitator of EPO production. Interestingly, also HIF3 $\alpha$  was recently shown to be involved in EPO regulation [98,99]. Until now, only limited studies provided insight into the complex alternative splicing of *HIF3 $\alpha$*  mRNA resulting in short and long variants either inhibiting hypoxia response or possessing transactivation capacities [100,101]. *In vitro* knockdown or overexpression of the long HIF-3 $\alpha$ -2 splice variant in EPO-producing cell lines resulted in downregulation or upregulation of EPO respectively, involving the canonical HRE [99,100]. However, further research is required to define whether HIF3-mediated EPO regulation also occurs *in vivo*.

Whereas HIF2 $\alpha$ -dependent EPO regulation is well-established, the precise molecular mechanism responsible for tight expression control and the regions within the EPO locus responsive to HIF-mediated regulation remain the subject of continuous research efforts. It is known that oxygen-dependent regulation of the EPO gene is controlled by distinct regulatory sequences in liver and kidney and involves a region of 0.4 kb of the 5'-sequence (promoter) and a 0.7 kb region of the 3'-sequence in the liver [102,103] whereas a highly conserved regulatory element responsible for renal EPO expression has been suggested to be located between 14 and 6 kb in the 5'-region [104,105]. However, the strict tissue-specific and conditional EPO transcriptional regulation strongly suggests that additional mechanisms might be involved. Moreover, studies on single gene scale [106] and at pan-genomic level [107] showed that long-range enhancer-promoter chromatin looping occurs independently of HIF and involves the recruitment of transcriptional factors and coactivators, further suggesting that EPO regulation might be more complex.



Accordingly, Smythies et al [40] illustrated that the tissue-specific role of HIF2 $\alpha$  is related to association with tissue-specific transcription factors. More recently, Orlando et al reported that multiple, distal and proximal HREs cooperate in oxygen-regulated *EPO* gene expression and that EPO regulation in renal cells may have more in common with neuronal cells than with hepatic cells, further illustrating the context-dependent complexity of EPO regulation [108].

### Hypoxia-mediated EPO production at other sites

Apart from REPCs and embryonic hepatocytes also other cell types have been shown to produce EPO in response to hypoxia. As an example, hypoxia-induced EPO production in the brain and certain cell types of the central nervous system (CNS) has come into light in the past years [109,110]. Urrutia and colleagues showed that pericyte specific (NG2:cre) VHL or PHD2/PHD3 deficiency leads to polycythemia. This was reversed in mice additionally lacking HIF2 $\alpha$ , emphasizing the essential role of HIF2 $\alpha$  for EPO production in other cell types [5,109,111]. Rankin and colleagues showed that specific deletion of VHL in osteoprogenitors in mice (OSX:cre-VHLf/f) presented elevated EPO expression in bone, which was also accompanied by polycythemia. Also in this setting, HIF2 $\alpha$  was the central HIF isoform [4].

It is noteworthy that the majority of EPO-producing cells, such as pericytes, astrocytes, as well as REPCs are of neurological origin [5,6,34,112]. Recent unpublished data from our lab also described EPO production in the adrenal gland chromaffin cells upon HIF2 $\alpha$  activation; cells known to be derived from neural crest. In line with this, polycythemia is one of the initial traits recognized in pheochromocytoma (the tumor of the adrenal medulla) relate to VHL or loss-of-function mutations or HIF2 gain-of-function mutations is [113]. In addition to HIF-mediated increased EPO expression in tumors, EPOR is also significantly increased in the hypoxic region of tumors, suggesting a HIF-mediated modulation of EPOR [114,115].

## EPOR and HIF axis

EPO will bind to erythroid progenitors (i.e. erythroblasts) in the BM/Spleen via the EPOR to meet the enormous daily production of RBCs. Remarkably, an *in silico* study identified almost thirty genetic variants of the EPOR, and seven variants of the EPO gene associated with erythrocytosis or increased hematocrit in humans. Mutations in EPOR have been shown to activate the receptor constitutively, resulting in enhanced production of erythroid colonies and erythrocytosis but reduced EPO synthesis [116].

In addition to its expression on erythroblasts, EPOR has even been identified on non-erythroid cells such as neural cells, endothelial cells, skeletal muscle myoblasts, macrophages, and white adipose tissue [3,117-119]. Although under normal conditions EPOR expression is very low in the nervous system, mild episodes of hypoxia can significantly increase its expression [120]. Similarly, unpublished data from our lab suggest HIF-mediated EPOR expression in the adrenal medulla.

Furthermore, Su and colleagues recently showed that hypoxia induces EPOR expression via HIF1 $\alpha$  in lung carcinoma cells. According to this study, HIF1 $\alpha$  interacts with the transcription factor Early Growth Response 1 (EGR1) to negatively regulate EPOR in the early phase of hypoxia, and with SP1 to promote EPOR expression in the later phase [115]. Thus, the hypoxia pathway is crucial in modulating the expression of EPO and its receptor. Both are not only directly involved in erythropoiesis, EPO is also crucial in regulation of the iron metabolism via hepcidin suppression [121] (see further).

## Iron metabolism: HPP as crucial contributors

Hemoglobin is the most essential component of erythrocytes as it carries oxygen bound to heme groups throughout the body. Central in heme is iron, which makes up about 70% of all available iron. Therefore, iron transport and metabolism is of utmost importance and in turn is also regulated by a number of HRE-containing genes, directly linking it to the hypoxia pathway [122]. For instance, to maintain necessary iron levels in other cells, HIF2 $\alpha$  plays a pivotal role promoting cellular iron uptake. Particularly, HIF2 $\alpha$

upregulates divalent metal transporter 1 (DMT1) and duodenal cytochrome b (DCYTB), where DMT1 is responsible for iron transport into cells, while DCYTB reduces the ferric form of iron to ferrous iron [123,124]. HIFs also regulate the expression of transferrin (TF) and transferrin receptor-1 (TFR-1), where TF catches up to two iron atoms from circulation and transports it into the cells via TFR-1 [60,61,125,126]. FPN, a critical factor that facilitates iron export from iron-storing cells (i.e. enterocytes and macrophages), is inhibited by the peptide hormone hepcidin [127]. Therefore, it is crucial that hepcidin production is strictly controlled for normal erythropoiesis to occur. To ensure this, erythroblasts produce and secrete the hormone erythroferrone (ERFE) upon EPO signaling, suppressing hepcidin production [121,126,128-130]. A more recent study by Arezes and colleagues suggests direct interaction of ERFE with BMP5, BMP6 and BMP7, leading to inhibition of BMP/SMAD signaling, and consequent hepcidin suppression [131] (Figure 2 and Table 1). More detailed insights into the regulation and action of ERFE has been recently reviewed by Coffey and Ganz [128].

Interestingly, hepcidin functionality has been linked to HIF2 $\alpha$  activity [132]. Initial studies pointed towards a direct role of HIF during hepcidin production, where hypoxia inversely regulates hepcidin expression [133] through the element CRE [134] or direct suppression of hepcidin by HIF binding to HRE and suppressing *HAMP* (encoding hepcidin) transcription [135]. Vice versa, deletion of hepatic hepcidin significantly increased FPN leading to cellular iron efflux, decreased PHD activity and eventually HIF2 $\alpha$  upregulation [136].

Further, a negative feedback loop of EPO by increased iron levels has been proposed. Iron excess induced by injection or feeding resulted in decreased renal EPO gene expression, which is mediated through reduced HIF2 $\alpha$  levels in renal interstitial fibroblasts [137]. In addition to regulation by PHDs, translational regulation of HIF2 $\alpha$  is inhibited by binding of iron regulatory proteins (IRPs) on iron response element (IRE) in its 5'UTR [93,138]. Taken together, these studies suggest a Fe-HIF2 $\alpha$ -EPO-ERFE-hepcidin axis, underlining the importance of HIF2 $\alpha$  in iron metabolism [136,139,140]. PHDs and HIFs can be potentially targeted for the treatment of iron-related disorders such as anemia, chronic kidney

disease (CKD), and polycythemia, where HIF2 $\alpha$  is an essential target for polycythemia-related disorders [141].

Chronic kidney disease (CKD) is one of the leading causes of death and arises mainly as a result of diabetes and hypertension [142]. Although anemia can be caused by a large variety of reasons, anemia due to kidney damage is attributed to decreased production of EPO by kidneys; leading to suppression of erythropoiesis [87]. HIF stabilizers act by restoring EPO levels and reducing hepcidin, and have been used effectively to enhance hemoglobin levels in patients suffering from anemias, including CKD patients [143].

### **Anemia and CKD – the PHD-HIF axis and therapeutic targeting**

Anemia can have different etiologies and is a common feature during the course of numerous pathologies. As such, the success of specific anemia treatments depends on the disease context. In many cases, including CKD-related anemia, treatment includes erythropoiesis-stimulating agents (ESA) [71]. However, there is not always a clear inverse correlation between the degree of anemia and EPO levels in the serum. In fact, many patients suffering from renal anemia do not respond to treatment with ESA [144]. Together, this suggests that modulating EPO levels in serum is not sufficient to treat anemia in CKD patients, and that new therapeutic approaches need to be developed. In line, a recent study showed that anemia in rats led to increased expression of HIF2 $\alpha$  and EPO in kidney and bone marrow, whereas rats suffering from CKD failed to show these increases.

An important factor contributing to the progression of CKD is incomplete or maladaptive tissue repair following acute kidney injury (AKI). In this setting, HIF stabilization through pharmacological PHD inhibitors enhances kidney recovery in several types of AKI including AKI caused by nephrotoxicity and renal reperfusion, the most common causes of AKI. Importantly, other studies suggest

that HIF1 $\alpha$  accumulation might be harmful during sepsis-associated AKI. This indicates that the success of HIF stabilization depends on the type of AKI, and possibly also other factors [145].

Since PHDs need 2-Oxoglutarate (2-OG) for their hydroxylase activity, most of the clinically advanced HIF stabilizers are 2-OG derivatives (reviewed by [146]). To date, several drugs that inhibit PHD activity have progressed into clinical trials. Importantly, these drugs do not target FIH, which preferentially inhibits HIF1 $\alpha$  transcriptional activation, allowing for increased HIF2 $\alpha$  stabilization. HIF stabilization induces a transient increase in EPO levels, achieving concentrations closer to the physiological range than those obtained with current ESA therapy. This suggests that treatment with HIF stabilizers could be more suited for certain patients (for recent reviews [147-150] ). Despite the promising future HIF stabilizers hold for treatment of anemia in CKD patients, concerns have arisen regarding their chronic use because of the significant pool of genes targeted by HIFs as well as the higher risk of hyperkalemia or the development of iron deficiency [147,151,152]. Therefore, further research is of utmost importance to better understand the impact of long-term HIF stabilization and to identify drugs targeting specifically HIF1 $\alpha$  and/or HIF2 $\alpha$  isoforms.

### **HIF-EPO-FGF23 axis: PHD2/HIF inhibition**

As discussed above, HIF-dependent EPO regulation is crucial for appropriate erythropoiesis in homeostatic and pathological conditions. Considering that adult erythropoiesis occurs in BM, it is noteworthy to mention another important player in this axis: Fibroblast Growth Factor-23 (FGF-23). FGF-23 is a bone-derived hormone essential for regulating vitamin D and phosphate concentrations [153]. Two forms of FGF23 have been described: a full-length biologically active protein (iFGF23) that upon cleavage results in a C-terminal inactive fragment (cFGF23) [154]. While iFGF23 interacts with FGF receptor 1 (FGFR1) to decrease blood phosphate levels in mice, cFGF23 can directly increase BM erythroid cell numbers in the same manner as treatment with recombinant human (rhEPO) [155]. Accordingly, FGF-23 constitutes the link between erythropoiesis and bone homeostasis in both

physiological, as well as in a variety of pathological conditions [156,157]. Indeed, we recently showed increased FGF-23 levels associated with ineffective erythropoiesis and impaired bone mineralization in myelodysplastic syndromes (MDS) [158]. Similarly, in CKD mouse models the expression of *Fgf23* mRNA is significantly increased in bone cells, resulting in reduced bone mineralization [159]. Additionally, it has been reported that FGF-23 expression can be regulated by inflammation and iron deficiency. Induction of inflammation in mice using IL1 $\beta$ , LPS and/or TNF correlates with increased expression levels of FGF-23 in osteocytes, both in humans and in mice [160] but also in CKD and inflammation in other organs [161]. David et al showed that IL1 $\beta$ -induced inflammation resulted in an FGF-23 increase accompanied by a significant ferritin increase and decrease of iron in the serum. Interestingly, the same study demonstrated that increased skeletal FGF-23 expression during inflammation remained even after administration of the PHD inhibitor FG-4592 [162] (roxadustat – one of the PHD inhibitors currently in clinical trials for CKD treatment [163]). Conversely, another study in bone showed increased *Fgf23* mRNA expression upon HIF1 $\alpha$  stabilization [164]. Noonan et al. studied the role of the HIF/EPO/FGF-23 axis in iron metabolism in an early-stage CKD mouse model. They reported that, upon treatment with HIF-PHD inhibitor FG-4592, iFGF-23 serum levels dropped remarkably. As a result, deficiency of iron metabolism stabilized through decreased liver *ferritin*, *Bmp6*, and *hepcidin* mRNAs [165]. Taken together, FGF-23 constitutes an important regulator of erythropoiesis and bone homeostasis. Although there is supporting evidence linking the regulatory role of HIFs and FGF-23 expression, additional studies are required to unravel the molecular mechanisms linked to these HPPs (Figure 2).

## Conclusions

Taken together, hypoxia pathway proteins play direct and indirect roles during the complex process of erythropoiesis. Therefore, the multifaceted regulation of all these proteins is essential in order to preserve the enormous demand of red blood cells needed under steady state but also stress situations.

The most crucial member of this system is EPO, primarily produced in kidney and directly regulated by the PHD2/HIF2 $\alpha$  axis. Besides its impact on the development of red blood cells, EPO plays an essential role on iron metabolism by regulating hepcidin expression in the liver. In addition, EPO steers the production and processing of other growth factors (e.g. FGF23) essential during erythropoiesis and in that way regulating its own production. Due to the essential roles of HPPs in erythropoiesis, the development of HIF stabilizers and PHD inhibitors as therapeutic agents are under detailed investigation for the treatment of anemias of various etiologies. However, more research is necessary to better understand the wide impact of the central HPPs especially for chronic intervention of this pathway.

## Acknowledgments

This work was supported by grants from the DFG (German Research Foundation) within the CRC/Transregio 205/1, Project No. 314061271 - TRR205, “The Adrenal: Central Relay in Health and Disease“ (A02) to B.W.; DFG grants WI3291/5-1 and 12-1 to B.W. and RA1923/12-1 to M.R. This work was supported by grants from the German-Israeli-Foundation to M.R., and B.W.; the DFG priority program  $\mu$ BONE 2084 to M.R. and B.W.; BW was further supported by the DFG Heisenberg program (WI3291/13-1).

## Authors' contributions

D.W., D.G, D.R. and B.W. wrote the manuscript. D.H. and M.R. contributed to the discussion and edited the manuscript.

## Conflict of interest

The authors have declared that no conflict of interest exists.

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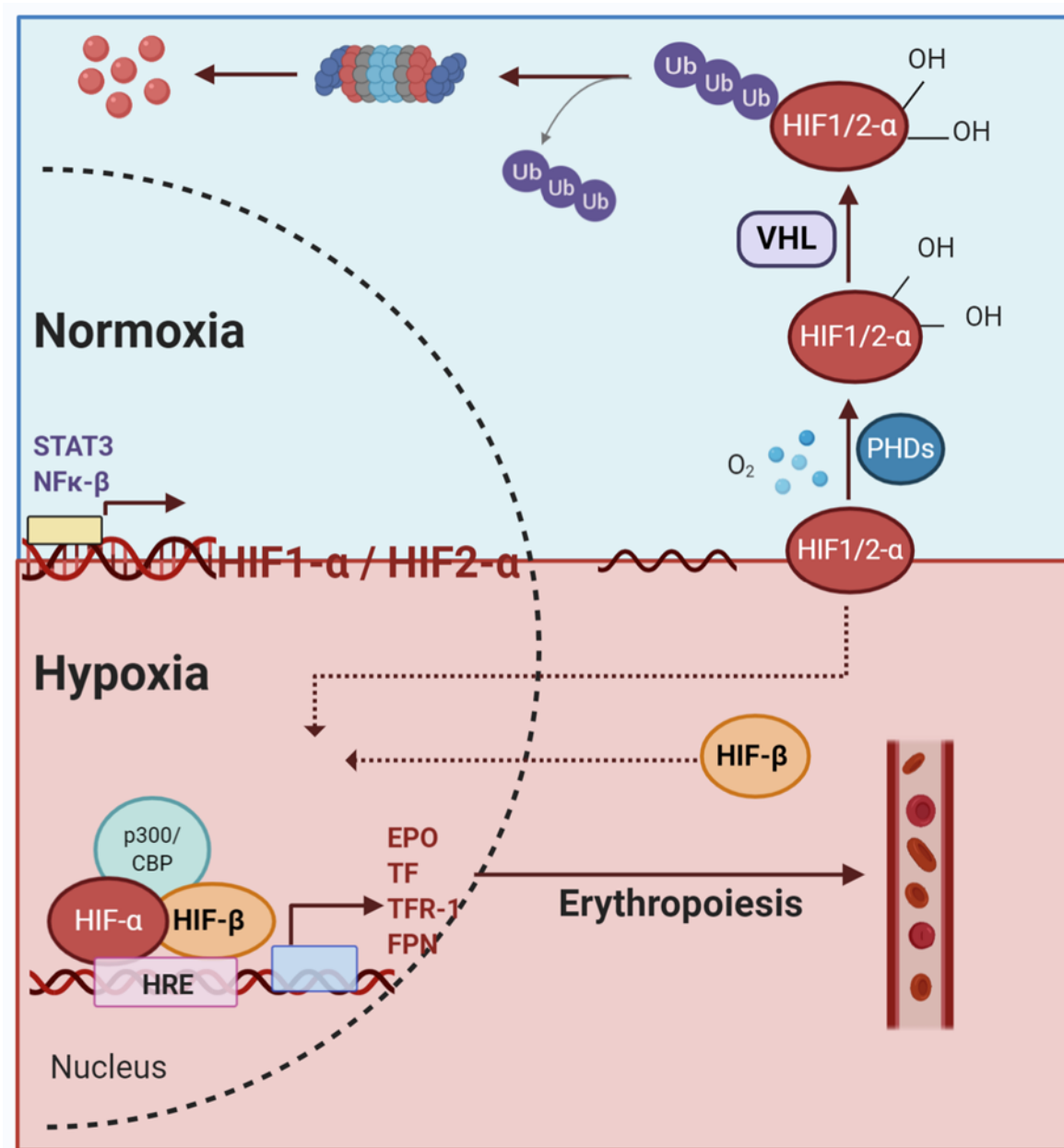
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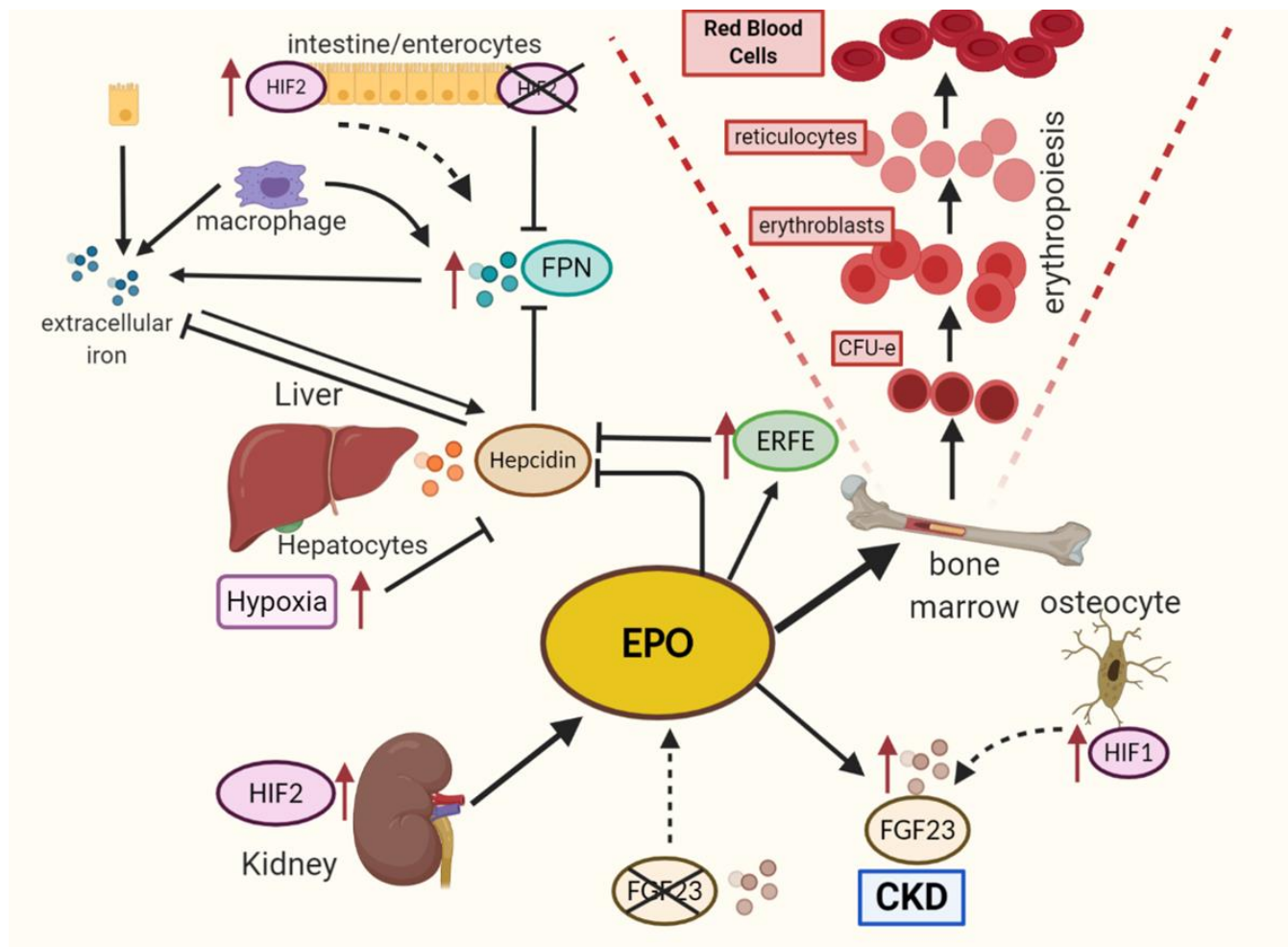
**Table1: Regulators of erythropoiesis**

Stage of erythropoiesis/iron metabolism	Factors	Key role in erythropoiesis	Regulation by HIF/EPO	References
Early stages of development of erythroid progenitors	GATA-1	Initiates erythropoiesis Regulates the transcription of several erythroid differentiation-related genes	HRE present in GATA-1 and GATA-1 regulation by HIF1.  EPO protects GATA-1 from caspase-induced degradation	[62,70,75,82]
Pro-erythroblasts to late erythroblasts	EPO	Key cytokine essential for growth, survival and differentiation of RBC's	HIF2 directly regulates EPO	[34,95,113,166,167]
	EPOR	Essential for erythropoiesis and action of EPO	Some evidence on regulation by hypoxia and HIFs	[115,120,168]
Late stage maturation/Apoptosis	FAS, FAS- L	Apoptosis and arrest maturation	Down regulation by Erythropoietin	[169]
Iron metabolism	Hepcidin	A crucial regulator of iron metabolism; suppresses Ferroportin	Direct and indirect suppression by hypoxia, HIF and EPO	[130,134,136,170,171]
	Ferroportin (FPN)	A critical factor that facilitates iron export from the cells	Regulated by HIF2a and hepcidin	[132]
	Erythroferrone (ERFE)	Suppresses hepcidin production	Upregulation by EPO	[121,131]
	Transferrin (TF)	Required for transporting iron	Regulation by HIF1	[61]
	Transferrin receptor (TFR1)	Plays role in erythroid differentiation/ role in iron uptake	Induced by HIF1 and hypoxia	[59,60]
	FGF23	Potential role in erythropoiesis. blockage of FGF23 results in increased erythropoiesis	Increased by erythropoietin	[155,172,173]



**Figure 1: Schematic overview of the hypoxia pathway under normoxia and hypoxia.** The HIF- $\alpha$  subunits are synthesized and subsequently degraded under normoxia (Blue). In the presence of physiological oxygen levels, PHDs mediate hydroxylation of HIFs leading to subsequent their binding to VHL. VHL facilitates the ubiquitination of hydroxylated HIFs resulting in proteosomal degradation.

However, under hypoxia (red), the hydroxylation of the alpha subunits is inhibited, stabilizing the HIF- $\alpha$  subunits. The stabilized alpha subunits bind to the beta subunit and translocate to the nucleus. The HIFs together with other co-factors, bind HREs promoting the transcription of genes essentially involved in erythropoiesis.



**Figure 2: Hypoxia pathway proteins mediated regulation of erythropoiesis.** EPO, the crucial factor involved in erythropoiesis is mainly HIF2-dependently regulated in the kidney. EPO produced in the kidney acts on erythroblasts in BM via EPOR and is essential in the development of red blood cells, especially in the late phases. In addition to its crucial role in the development of RBCs, iron metabolism is indispensable. More information can be found in the text.