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## Regulation of erythropoiesis by hypoxia-inducible factors

**Volker H. Haase**

Departments of Medicine, Cancer Biology, Molecular Physiology and Biophysics, Vanderbilt School of Medicine, Nashville, TN, USA

### Abstract

A classic physiologic response to systemic hypoxia is the increase in red blood cell production. Hypoxia-inducible factors (HIFs) orchestrate this response by inducing cell-type specific gene expression changes that result in increased erythropoietin (EPO) production in kidney and liver, in enhanced iron uptake and utilization and in adjustments of the bone marrow microenvironment that facilitate erythroid progenitor maturation and proliferation. In particular HIF-2 has emerged as the transcription factor that regulates EPO synthesis in the kidney and liver and plays a critical role in the regulation of intestinal iron uptake. Its key function in the hypoxic regulation of erythropoiesis is underscored by genetic studies in human populations that live at high-altitude and by mutational analysis of patients with familial erythrocytosis. This review provides a perspective on recent insights into HIF-controlled erythropoiesis and iron metabolism, and examines cell types that have EPO-producing capability. Furthermore, the review summarizes clinical syndromes associated with mutations in the O<sub>2</sub>-sensing pathway and the genetic changes that occur in high altitude natives. The therapeutic potential of pharmacologic HIF activation for the treatment of anemia is discussed.

### Keywords

Anemia; chronic mountain sickness; erythropoiesis; erythrocytosis; EPO; hepcidin; hypoxia-inducible factors; iron metabolism

### Introduction

Over 100 years ago Paul Bert and Denis Jourdanet described the association between reduced atmospheric O<sub>2</sub> pressure and elevated rbc numbers in humans and in animals, <sup>1–3</sup> which in 1890, during an high altitude expedition to the Peruvian Andes led by Francois-Gilbert Viault, was shown to result from an acute physiologic response rather than being an inherited condition. <sup>4</sup> It was the interest in understanding the molecular basis of this erythropoietic response that first led to the discovery of erythropoietin (EPO) and later on to the identification of the molecular machinery that senses pO<sub>2</sub>. The hypoxic induction of

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Address: Volker H. Haase, Department of Medicine, Division of Nephrology and Hypertension, Vanderbilt University Medical Center, C-3119A, MCN, 1161 21st Avenue South, Nashville TN 37232, USA, Tel: +1-615 343-7254, Fax: +1-615 322-6854, volker.haase@vanderbilt.edu.

#### Conflict of Interest

The author serves on the Scientific Advisory Board of Akebia Therapeutics, a company that develops prolyl-4-hydroxylase inhibitors for the treatment of anemia.

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*EPO* serves as a paradigm of O<sub>2</sub>-dependent gene regulation and the search for the transcription factor that regulates *EPO* resulted in the identification of hypoxia-inducible factor (HIF), which controls a wide spectrum of tissue-specific and systemic hypoxia responses.

Recent experimental data indicate that HIF promotes erythropoiesis at multiple levels and coordinates cell type-specific hypoxia responses. These include renal and hepatic EPO synthesis, enhanced iron uptake and utilization, as well as changes in the bone marrow microenvironment that facilitate erythroid progenitor maturation and proliferation. Because of its central role in the hypoxic regulation of erythropoiesis, pharmacological targeting of the HIF O<sub>2</sub>-sensing pathway has therapeutic potential for the treatment of anemia, in particular anemia associated with inadequate EPO production, e.g. in patients with chronic kidney disease (CKD). This review discusses recent insights into the cellular and molecular mechanisms that underlie O<sub>2</sub>-dependent regulation of EPO synthesis, iron metabolism and erythroid progenitor maturation, and examines their relevance to clinical disorders and anemia therapy.

## EPO-producing cell types

Surgical organ removal in animals identified the kidney as the major site of EPO synthesis in adults.<sup>5</sup> Although initially debated, EPO is produced by peritubular interstitial fibroblasts and not by renal tubular epithelial cells or peritubular endothelial cells.<sup>6–12</sup> Renal EPO-producing cells (REPC) can be typically found in the renal cortex (predominantly juxtamedullary region) and outer medulla (Figure 1). REPC express ecto-5'-nucleotidase (CD73) and platelet-derived growth factor receptor  $\beta$ -polypeptide (PDGFRB),<sup>9, 13</sup> both also markers of pericytes and EPO-negative interstitial fibroblasts.<sup>14</sup> *Epo* expression in tubular epithelial cells appears to be suppressed by GATA transcription factors, in particular GATA-2 and GATA-3, and can be reactivated under normoxic or hypoxic conditions when the GATA core consensus binding sequence upstream of the *Epo* transcription start site is mutated.<sup>11</sup> The kidney responds to hypoxia by increasing the number of REPC in an O<sub>2</sub>-dependent manner and therefore regulates EPO output through adjustments in REPC number.<sup>8, 11</sup> O<sub>2</sub>-dependent *Epo* transcription is controlled by distinct regulatory DNA sequences. These flank the *Epo* coding sequence on both sides, the kidney-inducibility element in the 5'-region and the liver-inducibility element in the 3'-region.<sup>15–17</sup> The 3'-hypoxia enhancer region is absolutely required for the hypoxic induction of *Epo* in the liver, as shown by genetic studies in mice.<sup>18</sup>

REPC have been visualized in BAC transgenic mice through the use green fluorescent protein (GFP). In this transgenic model the *Epo* coding sequence was replaced by GFP cDNA, which brings GFP under the control of *Epo* regulatory elements.<sup>11</sup> GFP expression was found in renal peritubular interstitial cells and in a subpopulation of hepatocytes that were localized around the central vein, supporting the notion that these two cell types represent the major sites of physiologic EPO production under conditions of systemic hypoxia. In the kidney, GFP-positive interstitial cells were unique in their morphologic appearance, as they displayed dendrite-like processes and expressed neuronal-specific markers, such as microtubule-associated protein 2 (MAP2) and neurofilament protein light polypeptide (NFL), indicating that REPC may be derived from progenitor cells of neuronal origin. This notion is furthermore supported by lineage tracing studies that utilized myelin protein zero (P0)-Cre transgenic mice, which express Cre-recombinase in neural crest-derived cells.<sup>13</sup> In keeping with this observation, Frede and colleagues established an EPO-producing renal tumor cell line with similar morphologic and molecular characteristics.<sup>19</sup> Although the hypoxic induction of *Epo* was reported in 4E cells, a mesenchymal cell clone with characteristics of embryonic kidney stromal cells,<sup>20</sup> primary REPC that retain their

EPO-producing ability are difficult to culture. The molecular mechanisms underlying this phenomenon are unclear. Transdifferentiation of REPC into myofibroblasts, which are a main source of collagen in fibrotic kidneys, has been proposed as a potential mechanism by which REPC lose their ability to synthesize EPO in CKD (Figure 1).<sup>13</sup> Myofibroblasts in kidney fibrosis appear to be derived from pericytes,<sup>21, 22</sup> which express cellular markers that are also found on REPC (e.g. CD73 and PDGFRB). To what degree these two cell populations overlap remains to be determined.

While the kidney is the primary physiologic source of EPO synthesis in adults, the liver is the main site of EPO production during embryonic development. However, in adults, the liver retains its ability to produce EPO in response to moderate/severe hypoxia or to pharmacologic HIF activation.<sup>23–25</sup> Similar to the kidney, the liver responds to severe hypoxia by increasing the number of EPO-producing hepatocytes that localize around the central vein.<sup>11</sup> *Epo* has also been detected in hepatic stellate cells, which have been previously referred to as ITO cells.<sup>26, 27</sup> The timing of transition from liver to the kidney as the primary site of EPO production is species-dependent and usually occurs during late gestation or at around birth.<sup>28–31</sup> The molecular mechanisms that underlie this switch are poorly understood, but may involve transcriptional repression and/or reduced expression of certain transcriptional activators, such as GATA-4.<sup>32</sup>

In the adult liver, *Epo* mRNA levels, which are very difficult to detect at baseline, rise substantially under conditions of moderate to severe hypoxia, and account for most, if not all, physiologically relevant systemic EPO of extra-renal origin.<sup>23, 33</sup> While hypoxia-induced EPO production in the liver does not normalize Hgb values in CKD, hepatic HIF can be sufficiently stimulated by pharmacologic means to correct anemia that results from inadequate EPO production or from inflammatory conditions.<sup>24, 34</sup>

Aside from kidney and liver as the two major sources of synthesis, *EPO* mRNA expression has also been detected in the brain (neurons and glial cells), lung, heart, bone marrow, spleen, hair follicles, the reproductive tract and in osteoblasts.<sup>31, 35–46</sup> While the role of these cell types in erythropoiesis under baseline conditions has not been demonstrated, they may, to a certain degree, contribute to stress-induced erythropoiesis (Figure 1).<sup>45, 47</sup> EPO synthesized by these cells is more likely to act locally, modulating, for example, regional angiogenesis and cellular viability (for an overview of the non-hematopoietic actions of EPO see Jelkmann<sup>48</sup>).

While pO<sub>2</sub> is critical for the regulation of renal EPO synthesis, some studies have investigated the role of extrinsic signals in the regulation of EPO production. Wussow and colleagues postulated the existence of an O<sub>2</sub> sensor in the brain stem, which triggers renal EPO production through release of yet to be identified humoral factors.<sup>49</sup> More recently, HIF activation in the skin has been shown to modulate renal and hepatic EPO production indirectly through HIF-1- and nitric oxide (NO)-mediated effects on dermal blood flow, which in turn changed blood flow to kidney and liver.<sup>50</sup> Whether alterations in renal blood flow are responsible for changes in EPO production under these conditions is debatable as renal tissue pO<sub>2</sub> is kept at relatively constant levels and is not very sensitive to changes in blood flow as long as they occur within physiologic range.<sup>51</sup>

## EPO synthesis: a paradigm of hypoxic gene regulation

Human EPO is heavily glycosylated, consists of 165 amino acids and has a molecular mass of about 30 kDa, 40% of which is derived from its carbohydrate component. Its major action is to promote survival of EPO-dependent colony-forming unit-erythroid (CFU-E) cells and erythroblasts that have not yet begun to synthesize hemoglobin. Upon ligand binding, the EPO receptor (EPOR), which lacks intrinsic catalytic function and is hypoxia-

inducible,<sup>52–54</sup> associates with tyrosine kinase Janus kinase 2 (JAK2). JAK2 phosphorylates EPOR and provides multiple docking sites for signal-transducing proteins that contain src homology 2 (SH2) domains. Signaling at the EPOR occurs through multiple pathways, which include the signal transduction and activator of transcription (STAT) 5 pathway, the phosphatidylinositol-3-kinase/protein kinase B (PI-3K/AKT) and mitogen-associated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathways, as well as protein kinase C.<sup>55</sup>

EPO production is primarily stimulated by hypoxia, which, depending on severity, increases serum EPO levels up to several hundred-fold.<sup>56</sup> HIF is a heterodimeric basic helix-loop-helix (bHLH) transcription factor that belongs to the PAS (PER/aryl hydrocarbon receptor nuclear translocator (ARNT)/single minded (SIM)) family of transcription factors. It consists of an O<sub>2</sub>-sensitive  $\alpha$ -subunit and a constitutively expressed  $\beta$ -subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT).<sup>57–59</sup> Three HIF  $\alpha$ -subunits are known, HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$ . HIF-1 was first isolated from human Hep3B hepatoma cells using DNA sequences that were derived from the 3' prime hypoxia enhancer of the *EPO* gene.<sup>60,61</sup> Together with HIF-2 $\alpha$  (also known as endothelial PAS domain protein 1 (EPAS1) or HIF like factor, HLF), HIF-1 $\alpha$  facilitates O<sub>2</sub> delivery and cellular adaptation to hypoxia by stimulating a wide spectrum of biological processes that include angiogenesis, anaerobic glucose metabolism, mitochondrial biogenesis and others.<sup>62</sup> HIF-regulated genes are induced following the binding of HIF heterodimers to specific DNA consensus sequences and recruitment of transcriptional co-factors. HIF-specific DNA elements are found in the regulatory regions of many O<sub>2</sub>-sensitive genes and are referred to as hypoxia-response elements (HREs) (Figure 2). While hypoxic suppression of certain genes has been found to be associated with HIF-1 and/or HIF-2 activation, it is unlikely that HIF acts as a direct transcriptional repressor.<sup>63</sup> Under normoxia, all three HIF  $\alpha$ -subunits are targeted for rapid proteasomal degradation by the von Hippel-Lindau tumor suppressor (VHL), which acts as the substrate recognition component of an E3 ubiquitin ligase.<sup>64,65</sup> Whereas HIF-1 and HIF-2 heterodimers function as transcriptional activators, splice variants of HIF-3 $\alpha$  have been shown to be inhibitory.<sup>66,67</sup> Although HIF-1 and HIF-2 share many transcriptional targets, certain genes and processes do not appear to be co-regulated. For example, anaerobic glycolysis appears to be predominantly controlled by HIF-1,<sup>68</sup> whereas EPO synthesis and iron metabolism have emerged as HIF-2-regulated processes.<sup>24,69–73</sup> In addition to canonical HRE-mediated transcription, which requires hetero-dimerization with ARNT, HIF- $\alpha$  modulates cellular signaling pathways through interaction with proteins that do not contain PAS domains. These include, among others, tumor suppressor protein p53, the c-MYC proto-oncogene and the Notch intracellular domain.<sup>74–77</sup>

Under normal O<sub>2</sub> conditions HIF- $\alpha$ -subunits are rapidly degraded following ubiquitylation by the VHL-E3 ubiquitin ligase complex, precluding the formation of transcriptionally active heterodimers. VHL-mediated poly-ubiquitylation requires hydroxylation of specific proline residues (Pro402 and Pro564 in human HIF-1 $\alpha$ ; Pro405 and Pro531 in human HIF-2 $\alpha$ ), which are localized within its O<sub>2</sub>-dependent degradation domain (ODD).<sup>78–84</sup> Hydroxylation of HIF- $\alpha$  is carried out by three major 2-oxoglutarate (2OG)-dependent oxygenases (prolyl-4-hydroxylase domain (PHD) proteins), PHD1, PHD2 and PHD3, also known as egl nine homolog (EGLN) 2, EGLN1, and EGLN3, respectively. These enzymes belong to a larger family of proteins, in humans there are over 60 members, which couple the oxidative decarboxylation of 2OG to various chemical processes, which aside from O<sub>2</sub>-sensing, include collagen synthesis and fatty acid metabolism. In mammals, these reactions produce succinate and CO<sub>2</sub> and appear to be limited to hydroxylation and demethylation initiated by hydroxylation.<sup>85</sup> HIF 2OG oxygenases function as O<sub>2</sub> sensors as they require molecular O<sub>2</sub> for catalysis. Under hypoxia, hydroxylation is inhibited and HIF signaling is activated.<sup>86</sup> To add complexity to the regulation of this pathway, HIF increases

transcription of *PHD2* and *PHD3*. Furthermore, protein turnover of PHD1 and PHD3 is hypoxically regulated by Siah proteins, which themselves are hypoxia-inducible.<sup>87, 88</sup>

All three PHDs are expressed in the kidney, where they control HIF activity. Based on immunohistochemistry and RNA analysis their expression levels vary between different renal cell types.<sup>89</sup> mRNA transcripts of all three PHDs have been detected in FACS-sorted REPC.<sup>90</sup> A fourth potential HIF prolyl-hydroxylase, P4H-TM, localizes to the endoplasmic reticulum membrane and has been shown to hydroxylate HIF-1 $\alpha$ -derived peptides, but not type 1 collagen. P4H-TM seems to be important for normal kidney function in zebra fish and appears to be involved in the renal EPO response in mice.<sup>91, 92</sup>

The transcriptional activity of HIF is modulated by a second hypoxic switch, which operates within the carboxy-terminal transactivation domain of HIF- $\alpha$ . Factor Inhibiting HIF (FIH) is a 2OG oxygenase that catalyses the hydroxylation of an asparagine residue within the C-terminal transactivation domain of HIF- $\alpha$ , thereby inhibiting the binding of co-activators CREB-binding protein (CBP) and p300 to the HIF transcriptional complex. Conversely, FIH inactivation facilitates CBP/p300 recruitment and results in increased HIF target gene expression under hypoxia.<sup>86</sup> In the kidney FIH has been detected in REPC, podocytes and in the distal tubule.<sup>90, 93</sup> While the role of PHDs and FIH in the regulation of HIF activity is well established, alternative hydroxylation targets have been identified and are likely to impact hypoxia and EPO responses in the kidney.<sup>85, 94, 95</sup> Furthermore, it is likely that renal EPO synthesis is modulated by epigenetic changes that are carried out by non-HIF 2OG oxygenases. Although nothing is known about their role in renal physiology, 2OG oxygenases, which contain a jumonji domain, catalyze the demethylation of methylated histones,<sup>85</sup> and are likely to provide additional functional links between alterations in renal pO<sub>2</sub> levels and gene expression.<sup>96</sup>

## HIF-2 in control of EPO synthesis

Although *in vitro* approaches identified HIF-1 as the transcription factor responsible for the hypoxic induction of *EPO*,<sup>97</sup> HIF-2 has now emerged as the main regulator of EPO production *in vivo* (Figure 2). Several lines of evidence exist that support this notion: a) the location of HIF-2 $\alpha$ -expressing renal interstitial cells coincides with the location of REPC;<sup>12, 98</sup> b) genetic studies in mice have demonstrated that renal and liver EPO synthesis is HIF-2- and not HIF-1-dependent, as did siRNA and chromatin immunoprecipitation (ChIP)-based studies in certain EPO-producing cell lines;<sup>72, 99, 100</sup> c) genetic analysis of patients with inherited forms of erythrocytosis have revealed mutations in *HIF2* but not in *HIF1* (see section on HIF pathway mutations in patients with secondary erythrocytosis); and d) genetic variants of *HIF2A* have been associated with high altitude dwellers who are protected from chronic mountain sickness (see section on molecular adaptation to life at high altitude).

While HIF-1 $\alpha$  is ubiquitously expressed, HIF-2 $\alpha$  expression is more restricted. HIF-2 $\alpha$  was initially identified in endothelial cells, subsequent studies however demonstrated expression in hepatocytes, cardiomyocytes, glial cells, type-II pneumocytes, and in renal peritubular interstitial cells.<sup>98, 101</sup> The analysis of HIF-1 $\alpha$  and HIF-2 $\alpha$  knockout mice provided the first major insights into the functional differences between these two HIF homologs. Mice that lacked both copies of HIF-1 $\alpha$  died *in utero* between embryonic day (E)8 and E11 from defective neurodevelopment and cardiovascular malformations,<sup>102, 103</sup> whereas mice with homozygous HIF-2 $\alpha$  deletion died *in utero* or around birth from either severe vascular defects, defective catecholamine synthesis or abnormal lung maturation.<sup>104–106</sup> In a mixed genetic background, HIF-2 knockout mice survived into adulthood, but developed hepatic steatosis, skeletal myopathy and cardiac hypertrophy, which was associated with

mitochondrial dysfunction and defects in reactive oxygen species (ROS) scavenging.<sup>107</sup> Furthermore, HIF-2 knockout mice were pancytopenic and displayed a hypocellular bone marrow.<sup>108</sup> Further analysis revealed that anemia in these mice did not result from a cell-autonomous defect in erythroid precursor maturation, but was due to inadequate renal EPO production, indicating that HIF-2 was indispensable for systemic EPO homeostasis in adults.<sup>70</sup> In a different model, Morita and colleagues showed that local EPO production in the retina was also HIF-2-dependent,<sup>69</sup> suggesting a more general role for HIF-2 in the control of EPO regulation. While these mouse models demonstrated that EPO production in adults was HIF-2-dependent, developmental studies highlighted the importance of HIF-1 in the regulation of erythropoiesis during embryonic development. HIF-1-deficient embryos were characterized by a reduction in myeloid multi-lineage cells and committed erythroid progenitors at E9.5. This was associated with decreased *Epo* mRNA levels in the embryo proper but not in the yolk sac, while *EpoR* mRNA was decreased in both tissues.<sup>54</sup>

The most compelling support for the notion that HIF-2 is the main regulator of adult EPO synthesis comes from conditional knockout studies in mice. Utilization of a tamoxifen-inducible, ubiquitously expressed Cre-recombinase transgene permitted a direct comparison of the effects of HIF-1 and HIF-2 inactivation on erythropoiesis. Acute postnatal global ablation of HIF-2 $\alpha$  but not of HIF-1 $\alpha$ , resulted in anemia, which, similar to HIF-2 $\alpha$  germ line inactivation, was responsive to treatment with recombinant EPO.<sup>71</sup> While stimulation of renal EPO production in response to hemolysis (phenylhydrazine treatment) was blunted in HIF-2 $\alpha$ -ablated mice, postnatal deletion of HIF-1 $\alpha$  did not have any notable effect on erythropoiesis, which suggested that HIF-1 does not play a significant role in the regulation of systemic EPO homeostasis at baseline or in response to acute anemia.<sup>71</sup> Our laboratory has generated cell type-specific knockout mice to investigate the differences between HIF-1 and HIF-2 in the regulation of renal and hepatic EPO synthesis. Inactivation of HIF-2 $\alpha$  in the kidney completely ablated the renal EPO response in mice subjected to normobaric hypoxia (10% O<sub>2</sub> for 10 days), phlebotomy-induced anemic hypoxia, or treatment with a HIF activating compound.<sup>109</sup> Cell type-specific inactivation of the VHL-E3 ubiquitin ligase in hepatocytes resulted in HIF-2-, but not in HIF-1-dependent erythrocytosis, while pharmacological PHD inhibition caused a HIF-2-dependent increase in liver *Epo* mRNA levels.<sup>72</sup> Consistent with these findings are transgenic studies with non-degradable mutant isoforms of HIF-1 $\alpha$  or HIF-2 $\alpha$ . Over-expression of non-degradable HIF-2 $\alpha$  in hepatocytes produced erythrocytosis, whereas over-expression of HIF-1 $\alpha$  did not.<sup>110</sup> Taken together, multiple genetic studies in mice provide overwhelming evidence that, in the adult, renal and hepatic EPO synthesis is predominantly HIF-2- and not HIF-1-regulated. These studies have clearly identified HIF-2 as a key pharmacological target for the treatment of anemia.

HIF-2 transactivation at the *EPO* HRE involves multiple nuclear factors that associate with the *EPO* gene.<sup>97, 99</sup> One of these factors is hepatocyte nuclear factor-4 (HNF4), which binds to the 3' *EPO* hypoxia enhancer region and is likely to interact with HIF-2 (Figure 2).<sup>99</sup> Similar to HIF-2, the cellular location of HNF4 expression coincides with the sites of EPO production in liver and kidney. Furthermore, HNF4 is required for the hypoxic induction of *EPO* in Hep3B cells.<sup>99, 111, 112</sup> The notion that HIF-2 transactivation depends on the cooperation with other transcription factors has been previously suggested and may determine whether HIF target genes are HIF-1 or HIF-2-regulated, however, specific factors that are required for HIF-2-dependent *EPO* induction have not yet been identified.<sup>113</sup>

## Molecular fine-tuning of HIF-2 regulated erythropoiesis

Post-transcriptional and post-translational modifications of *HIF2* mRNA and HIF-2 $\alpha$  protein that do not involve PHD enzymes have been shown to modulate EPO production. The molecular mechanisms that underlie these modifications, link cellular metabolism and

redox-state to hypoxia-induced erythropoiesis. HIF-2 $\alpha$  is acetylated during hypoxia and deacetylated by Sirtuin 1, a nicotinamide adenine dinucleotide (NAD)<sup>+</sup>-dependent protein deacetylase, which increases HIF-2-dependent EPO synthesis in vitro and in vivo, thus linking cellular redox and energy state to systemic hypoxia responses.<sup>114</sup> Sirtuin 1-deficient mice produced significantly lower amounts of fetal liver *Epo* mRNA, and as adults less EPO in response to severe hypoxia. Interestingly, caloric restriction, which induces Sirtuin 1 activity, suppressed EPO production in the liver.<sup>115, 116</sup> Although further studies are needed to clearly define the role of sirtuins in HIF-2-dependent erythropoiesis, these findings highlight the existence of a functional link between EPO production and cellular energy state.

Additional post-translational modifications, which impact EPO production and hypoxia-induced erythropoiesis, involve SUMOylation. SUMO (Small Ubiquitin-like Modifier) proteins are structurally related to ubiquitin and reversibly modify cellular function and localization of targeted proteins. An enzyme, which removes SUMO, is SENP (Sentrin/SUMO-specific protease). SENP 1 knockout mice are anemic and die during mid-gestation.<sup>117</sup> In this model de-SUMOylation did not occur, prevented HIF activation under hypoxic conditions and resulted in reduced hepatic EPO production.<sup>117</sup> Although SUMOylation was specifically investigated with regard to HIF-1 signaling, the presence of anemia suggested that SENP is likely to be involved in the de-SUMOylation of HIF-2 $\alpha$ .

To add more complexity to the regulation of HIF-2 activity, low intracellular iron levels have been shown to diminish HIF-2 $\alpha$  translation and thus are predicted to limit HIF-2-induced EPO production and erythropoiesis when cellular iron stores are depleted. This feedback loop makes sense physiologically, as erythropoiesis cannot occur in the absence of iron. The 5'-untranslated region (UTR) of *HIF2* mRNA contains an iron-regulatory element (IRE), a stem loop structure that binds iron-regulatory protein (IRP) when intracellular iron levels are low.<sup>118</sup> IRPs (IRP1 and IRP2) function as intracellular iron sensors that control the expression of several iron-sensitive genes, such as transferrin receptor 1 (*TFR1*), ferritin and divalent metal transporter 1 (*DMT1*).<sup>119, 120</sup> Iron is incorporated into an iron-sulfur cluster at the center of the protein and converts IRP1 to an enzyme with aconitase activity. In its aconitase form IRP1 does not bind to the IRE. In contrast, IRP2 does not convert to an aconitase and is regulated via iron-dependent proteasomal degradation.<sup>118, 121, 122</sup> Depending on the location of the IRE stem loop, the IRP/IRE complex either inhibits translation (5'-IRE), or stabilizes mRNAs when the IRE is located in the 3'-UTR (e.g. *TFR1* mRNA levels increase when intracellular iron is low). Since the IRE in *HIF2* is located in its 5'-untranslated region, HIF-2 $\alpha$  translation is inhibited when iron levels are low. This in turn limits EPO synthesis and thereby adjusts hypoxia-inducibility of erythropoiesis to iron availability.

## HIF pathway mutations in patients with secondary erythrocytosis

Mild to moderate perturbations in the HIF O<sub>2</sub>-sensing pathway lead the development of benign erythrocytoses that are associated with increased or inappropriately normal serum EPO levels. This is in contrast to primary erythrocytoses, which are characterized by suppressed serum EPO levels and are caused by molecular defects in erythroid progenitor cells or hematopoietic stem cells.<sup>123, 124</sup> Other forms of secondary erythrocytosis that associate with increased EPO production result from chronic hypoxic conditions, such as COPD, right-to-left cardiac shunts or high altitude, or can be due to EPO-producing tumors.

Abnormalities in the HIF O<sub>2</sub>-sensing pathway were first observed in patients with Chuvash polycythemia. Chuvash polycythemia is a rare autosomal recessive form of secondary erythrocytosis that is endemic but not limited to Chuvashia, a republic in central European

Russia. It is caused by a homozygous mutation in the VHL tumor suppressor at codon 200, R200W and patients with the Chuvash mutation, who are ethnically distinct from Chuvashians, have been identified in other parts of Europe, the United States and Asia.<sup>125–134</sup> Some patients are compound heterozygotes for the R200W and other VHL mutations<sup>129, 130, 135</sup>. Codon 200 is located in the C-terminus of the VHL protein and lies outside of the central groove that binds hydroxylated HIF- $\alpha$  ( $\beta$ -domain core) and outside of the helical  $\alpha$ -domain (amino acid residues 157–189), which interacts with elongin C.<sup>136</sup> Genetic alterations in these two core regions are strongly associated with the development of VHL disease, an inherited autosomal dominant tumor syndrome, where individuals who carry a *VHL* germ line mutation are predisposed to the development of highly vascularized tumors, which include renal cell carcinoma, hemangioblastomas of the CNS and retina, and pheochromocytomas.<sup>137</sup> Chuvash patients, who are homozygous for the R200W allele, are not predisposed to the development of these tumors. The ability of the R200W VHL species to capture hydroxylated HIF- $\alpha$  for ubiquitylation and subsequent proteasomal degradation is impaired, which is most likely due to changes in protein stability or conformation that impinge on the VHL-HIF- $\alpha$  interaction.<sup>138</sup> Although individuals with Chuvash polycythemia are not prone to tumor development, they suffer from premature morbidity and mortality due to pulmonary hypertension, cerebrovascular accidents and vertebral hemangiomas.<sup>139, 140</sup> Evaluation of cardiopulmonary function in a small group of Chuvash patients revealed significant abnormalities in respiratory and pulmonary vascular regulation at baseline and in response to hypoxia. Basal ventilation and pulmonary vascular tone were elevated and increases in heart rate and ventilation, as well as pulmonary vasoconstrictive responses to mild or moderate hypoxia were considerably enhanced, indicating that tight regulation of the VHL/HIF axis is required for normal cardiopulmonary physiology.<sup>141, 142</sup> Chuvash patients furthermore display abnormalities in metabolic stress responses and cytokine profiles.<sup>143–146</sup>

Further mutational analysis of the HIF O<sub>2</sub>-sensing pathway in patients with idiopathic erythrocytosis led to the identification of families with heterozygous mutations in *HIF2*, *PHD2* or *VHL* (non-R200W); for a summary of non-R200W *VHL* mutations the reader is referred to Lee and Percy.<sup>135</sup> Interestingly, mutations in HIF-1 $\alpha$  have not been described to date, underscoring the importance of HIF-2 in the regulation of EPO synthesis in humans. Most gain-of-function mutations in HIF-2 $\alpha$  are in direct proximity to proline residue 531, which is one of the two main hydroxylation sites (the other major hydroxylation site is proline 405).<sup>147–154</sup> Biochemical analysis demonstrated that the originally identified G537W mutation impaired recognition and hydroxylation by PHD2 and thus interaction with VHL.<sup>155</sup> Two recently identified HIF-2 $\alpha$  gain-of-function mutations, A530T and A530V, were associated with polycythemia, paraganglioma and/or somatostatinoma.<sup>156</sup> Conversely, several PHD2 missense mutations have been identified that resulted in diminished hydroxylase activity.<sup>157, 158</sup> Patients, who were heterozygous for these mutations were characterized by increased rbc values in the presence of inappropriately normal serum EPO levels.<sup>157–162</sup> Some of these mutations (P317R, H374R) are likely affect iron-chelation at the catalytic center, which is critical for PHD enzymatic activity. Furthermore, H374R was associated with paraganglioma development, indicating that PHD2 may function as a tumor suppressor.<sup>158, 161</sup>

## Molecular adaptation to life at high altitude

Chronic mountain sickness (CMS), also known as Monge's disease, affects long-term high-altitude (> 2500 m) residents or natives, and is associated with excessive erythrocytosis (females, Hgb 19 g/dL; males, Hgb 21 g/dL), hypoxemia, pulmonary hypertension, right-sided heart failure and neurologic symptoms, such as headache, fatigue, tinnitus, insomnia, paresthesia and loss of memory.<sup>163–165</sup> The disease was first described in high altitude



163, 165 CMS is usually alleviated by descent to low altitude or by phlebotomy. 163, 164 While the disease is prevalent in the Andean population, it is less common in native Tibetans, who live at comparable altitude. In contrast, Tibetan residents of Han Chinese descent are much more frequently affected by CMS, which represents a major public health burden. 165–168 Prevalence of CMS is higher in men than in women, increases with altitude and age, and is more likely to develop in the presence of lung diseases, smoking and environmental pollution. 165 The pathogenesis of CMS is thought to result, at least partly, from an abnormal, i.e. blunted, ventilatory response. 165 Aside from differences in susceptibility to CMS, native Tibetans and Andeans differ in their baseline physiologic responses to high altitude. Native Tibetans have higher resting ventilation and hypoxic ventilatory response at comparable altitudes, lower oxygen saturation of arterial hemoglobin and lower hemoglobin concentrations (15.6 g/dL versus 19.2 g/dL in males) 169, 170 There is also less intrauterine growth retardation and better neonatal oxygenation among native Tibetans compared to native Andeans or Han Chinese. 167, 171 Furthermore, differences in energy metabolism have been described, which need further characterization. 172 These differences in physiologic phenotypes reflect divergence in genetic adaptation and selection, which result from differences in length of high-altitude habitation (~ between 25,000 and 50,000 years for native residents on the Tibetan plateau, compared to ~10,000 years for the Andean Altiplano and ~ 60 years for Tibetan residents of Han Chinese descent), the degree of geographical isolation (Tibetan plateau > South American Altiplano) and gene pool stability. 167 Genome-wide searches for single-nucleotide polymorphisms (SNP), candidate gene approaches and exome sequencing have identified genetic variations in the *HIF2A*, *PHD2* and *PPARA* genes that associate with lower hemoglobins in native Tibetans compared to Han Chinese and with high altitude adaptation in other populations. 173–180 Although the precise biological function of these alleles is not known, they are predicted to confer adaptation to the hypoxic environment and to modulate susceptibility to CMS and other high altitude-associated diseases.

## HIF coordinates EPO production with iron metabolism

Iron demand in the bone marrow increases when erythropoiesis is stimulated by HIF-2-mediated EPO production in kidney and liver. The need for additional iron necessitates an increase in intestinal iron uptake and serum iron binding capacity, as well as enhanced mobilization of iron from internal stores. HIF-2 has not only emerged as the key regulator of renal and hepatic EPO production, but it furthermore plays a critical role in iron uptake and utilization as it directly regulates *DMT1* and duodenal cytochrome b (*DCYTB*) (Figure 3). This has been demonstrated in animal models of iron-deficiency and hemochromatosis. 73, 181, 182 DMT1 transports iron into the cytoplasm of cells and DCYTB reduces ferric iron to its ferrous form ( $\text{Fe}^{2+}$ ) before it is taken up from the gut lumen into intestinal cells via DMT1. Other bona fide HIF-regulated genes involved in iron metabolism include transferrin, which transports serum iron in its ferric form ( $\text{Fe}^{3+}$ ), its high affinity receptor *TFR1*, 183–185 ceruloplasmin, which oxidizes  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  and is important for iron transport, 186 and heme-oxygenase-1, which is critical for the recycling of iron from phagocytosed erythrocytes. 187

A critical  $\text{O}_2$ -sensitive regulator of systemic iron homeostasis that has received much attention is hepcidin, a small 25 amino acid containing peptide, which is mainly produced by hepatocytes, where its transcription is iron- and  $\text{O}_2$ -sensitive. Hepcidin suppresses intestinal iron uptake and release of iron from internal stores by facilitating the degradation and internalization of the only known cellular iron exporter, ferroportin, which is expressed on the surface of enterocytes, hepatocytes and macrophages. 188 Thus, in iron-deficient states (e.g. iron-deficiency anemia) and/or under hypoxic conditions (e.g. ascent to high altitude)

the liver makes less hepcidin and intestinal iron uptake is enhanced (Figure 3). Chronically elevated serum hepcidin levels are frequently associated with inflammatory states (interleukin-6 induces hepcidin transcription via JAK/STAT signaling) and lead to reduced ferroportin surface expression and hypoferremia, lending support to the notion that hepcidin has a key role in the pathogenesis of anemia of chronic disease.<sup>188, 189</sup> In contrast, constitutively low hepcidin production in the liver, e.g. due to genetic defects in iron-signaling pathways, results in persistent hyperferremia and the development of hemochromatosis.<sup>190</sup>

Experimental studies in cultured cells, in animal models and in human subjects have established that hepcidin production is O<sub>2</sub>-sensitive and involves the HIF pathway.<sup>126, 191–193</sup> Several models have been proposed to explain the hypoxic suppression of hepcidin, including direct HRE-mediated regulation by HIF-1, regulation by dioxygenases, signaling via EPOR or through humoral factors that are released from the bone marrow when erythropoiesis is stimulated.<sup>193–197</sup> Other studies have linked hypoxia to iron signaling pathways and have proposed that hypoxia diminishes signals that normally increase hepcidin production in hepatocytes. Activation of signaling through the hemochromatosis protein HFE, TFR1, TFR2, or hemojuvelin (HJV), which acts as a co-receptor for bone morphogenetic protein 6 (BMP6), increases hepcidin transcription in a SMAD-dependent fashion.<sup>190, 198–202</sup> Recent *in vivo* studies have shown that HIF induces furin, a proprotein convertase that cleaves HJV and generates a soluble form of HJV, which suppresses hepcidin by antagonizing BMP6 signaling.<sup>203, 204</sup> Similarly, transmembrane protease serine 6 (TMPRSS6), also known as matriptase-2, was reported to be HIF-regulated and is predicted to blunt BMP6/HJV-mediated signals under hypoxic conditions.<sup>205–207</sup> Our laboratory has used a genetic approach to dissect the role of HIF in the regulation of hepcidin. We have created conditional knockout strains, in which we disengaged HIF activation from EPO synthesis and found that hypoxia/HIF-mediated suppression of hepcidin required EPO.<sup>208</sup> However, we determined that the induction of EPO synthesis alone was not sufficient to suppress hepcidin in this model. Hepcidin suppression under conditions of hypoxia and hepatic HIF activation was dependent on erythropoietic activity in the bone marrow. Our data established that HIF activation in hepatocytes suppresses hepcidin indirectly through EPO-mediated stimulation of erythropoiesis and is consistent with previous studies from Pak and colleagues in phlebotomized animals.<sup>196</sup> In the context of anemic hypoxia, both HIF-1 and HIF-2 are activated.<sup>24</sup> HIF-2 induces EPO production in kidney and in liver (depending on the severity of hypoxia), resulting in increased serum EPO levels and stimulation of erythropoiesis, which subsequently leads to the suppression of hepcidin in the liver.<sup>209</sup> HIF-2 is a direct regulator of both renal and hepatic EPO synthesis, but regulates hepcidin only indirectly via stimulation of bone marrow activity (Figure 3).<sup>197, 208, 210</sup>

It is plausible that serum iron levels modulate the suppression of hepcidin under hypoxic conditions, although this has not been sufficiently addressed experimentally. Serum iron and ferritin levels are decreased in Chuvash patients and in individuals sojourning at high altitude for 10–12 days.<sup>191, 211</sup> Time course analysis, however, indicated that the acute decrease of serum hepcidin at high altitude preceded changes in serum ferritin and transferrin saturation, suggesting that under hypoxic conditions hepcidin regulation is, at least initially, iron-independent.<sup>212</sup> Support for this notion furthermore comes from patients with  $\beta$ -thalassemia, who have low serum hepcidin levels despite iron overload.<sup>213</sup>

Growth differentiation factor 15 (GDF-15) and twisted gastrulation homolog 1 (TWSG1) have been identified as candidate erythrokinins, although not erythroblast-specific, that have the potential to suppress hepcidin under conditions of increased erythropoietic activity.<sup>214–216</sup> GDF15 is an iron- and O<sub>2</sub>-regulated (HIF-independent) member of the

TGF- $\beta$  superfamily, which is secreted from maturing erythroblasts and has been shown to suppress hepcidin transcription in primary human hepatocytes and hepatoma cells (Figure 3).<sup>214, 217</sup> While increased GDF15 serum levels associate with syndromes of ineffective erythropoiesis, for example  $\alpha$ - and  $\beta$ -thalassemia, its role in hepcidin regulation under physiologic conditions and in other forms of anemia remains unclear.<sup>214, 216, 218–220</sup> Therefore, it was proposed that GDF15 may be a marker of bone marrow stress or erythroblast apoptosis.<sup>216</sup> Elevated serum GDF15 level have also been found in patients with heart failure,<sup>221</sup> which adds complexity to this model. We found that recombinant murine GDF15 suppressed hepcidin in Hep3B cells at a concentration of 750 pg/ml.<sup>208</sup> This is in contrast to previous reports where higher doses of GDF15 were needed to achieve hepcidin suppression in human HuH-7 hepatoma cells and in primary hepatocytes, while low dose GDF15 treatment increased hepcidin.<sup>214</sup> While demonstrated in mice, studies in humans receiving recombinant EPO have not yet shown a significant inverse relationship between serum hepcidin and GDF15 levels, which may relate to the EPO doses administered, study size, complexity of regulation and species-dependent differences.<sup>208, 222</sup> In the context of iron-deficiency anemia, Tanno and colleagues found that GDF15 serum levels were not elevated,<sup>223</sup> while Lakhal and colleagues reported that patients with low serum iron had elevated GDF15 levels compared to iron-replete controls (mean of 1048 pg/ml vs. 542 pg/ml).<sup>217</sup> Similarly, increased serum GDF15 levels were found following DFO treatment, suggesting iron-dependent regulation.<sup>217</sup> Furthermore, temporary increases in serum GDF15 levels associated with increased serum EPO following ascent to high altitude.<sup>212</sup>

In addition to regulating iron metabolism, hypoxia has direct effects on the bone marrow. It promotes erythropoiesis by modulating erythroid progenitor maturation and proliferation.<sup>224, 225</sup> Hypoxia stimulates EPOR expression and regulates components of the hemoglobin synthesis pathway.<sup>52–54, 226, 227</sup> Hypoxia also modulates the interaction of erythroid progenitors with other cell types and thereby regulates stem cell maintenance, lineage differentiation and maturation. Recent studies have highlighted a role for endothelial HIF-2 in this regulation,<sup>225</sup> as mice with globally reduced HIF-2 expression displayed a defect in erythroid maturation, which was dependent on the expression levels of vascular adhesion molecule (VCAM)-1, an integrin receptor that binds very late antigen-4 (VLA4) on erythroblasts and supports erythroid maturation. This finding lends more support to the concept that HIF-2 acts a central regulator of hypoxia-induced erythropoiesis, which coordinates EPO synthesis with iron metabolism and erythroid progenitor maturation.

## The HIF axis as a novel therapeutic target for the treatment of anemia

Over the last 20 years EPO therapy has transformed the lives of millions of patients who suffer from anemia. Therapy with recombinant EPO eliminates the need for rbc transfusions, improves cardiovascular function and cognitive ability. The US Food and Drug Administration (FDA) approved recombinant EPO in 1989, initially for use in patients with renal anemia then for use in cancer patients (FDA approval 1993). Since then its administration has become standard of care. However, despite its clinical effectiveness and success, recent randomized controlled clinical trials have raised significant safety concerns, resulting in several black box warnings issued by the FDA. These studies showed that aiming for normal Hct values in the dialysis patient population (Hct target of 42%) increased the risk of serious cardiovascular complications or adverse composite outcomes.<sup>228</sup> In pre-dialysis CKD patients with or without diabetes higher Hgb targets, particularly in patients with poor initial Hgb responses, were also associated with increased cardiovascular risk.<sup>229–231</sup> Furthermore, in the Oncology setting high dose recombinant EPO administration was found to be associated with tumor growth and increased overall mortality, a concerning finding, which currently lacks sufficient explanation (for recent

reviews on this topic see <sup>232, 233</sup>). Cardiovascular safety concerns in the CKD/ESRD patient population have changed EPO-prescribing practices, and have resulted in a decrease in recombinant EPO use and not surprisingly in an increase in rbc transfusions (<http://www.usrds.org/2012/slides/indiv/v2index.html#/176/>). The underlying mechanisms for the increase in cardiovascular mortality are unclear, but may relate to the EPO dose administered and the clinical conditions that associate with EPO hyporesponsiveness. There is much debate about what represents a “safe” Hgb target and individual patient needs and lifestyle choices has to be taken into account when prescribing recombinant EPO. Despite these dilemmas, the clinical success of recombinant EPO therapy has been a major incentive for the development of new erythropoiesis stimulating agents and the design of novel therapeutics that boost synthesis of endogenous EPO. <sup>234</sup>

Pharmacological inactivation of PHD enzymes with 2OG analogs increases serum EPO levels in animal models and in humans, and has the potential to benefit patients with anemia that results from inadequate EPO production. <sup>24, 34, 92, 235, 236</sup> In vitro and in vivo studies have suggested that pharmacological or genetic targeting of individual PHD enzymes has differential effects on renal and hepatic EPO synthesis. Inducible, global deletion of PHD2 in adult mice resulted in severe erythrocytosis from a dramatic increase in renal EPO production (Hct values > 80%), as well as other organ pathologies, in particular when PHD3 was inactivated simultaneously. <sup>237–241</sup> PHD1- and PHD3-deficient mice, which in contrast to conventional PHD2 knockout mice survive into adulthood, developed mild to moderate erythrocytosis (Hct of 67% compared to 53% in control mice) only when both enzymes were inactivated simultaneously, the liver being the source of EPO and not the kidney. <sup>25, 240</sup> Genetic or pharmacologic inactivation of all three PHDs, however, is required to produce a strong and sustained erythropoietic response. <sup>25, 34</sup> This is in contrast to the kidney where inactivation of PHD2 alone is sufficient to produce severe erythrocytosis. <sup>239, 240</sup> While these tissue-specific differences are not well understood, functional diversity between individual PHDs is expected, because of differences in cellular localization, hypoxia-inducibility and biochemical behavior (for a review see <sup>86, 242</sup>). Furthermore, PHD1 and PHD3 appear to preferentially target HIF-2 $\alpha$  in vitro and in vivo, which offers potential for therapeutic exploitation under conditions where HIF-1 activation is non-desirable. <sup>240, 243</sup>

Aside from stimulating endogenous EPO synthesis, pharmacological inhibition of HIF prolyl-hydroxylation is likely to have beneficial effects on iron uptake and utilization (see section on HIF and iron metabolism), and may therefore be superior to the administration of recombinant EPO alone, especially in renal anemia patients, who often suffer from chronic inflammation, functional iron deficiency and EPO resistance. <sup>244</sup> The beneficial effects on iron metabolism are most likely produced with systemic administration of HIF stabilizing PHD inhibitors, which would target multiple organs including kidney, liver, gut and the bone marrow. A potential downside to this approach, however, is that HIF transcription factors regulate a multitude of biological processes, and intermittent HIF activation over prolonged periods of time may lead to changes in glucose, fat and cholesterol metabolism, promote angiogenesis and have other adverse effects. <sup>245–250</sup> Liver-specific PHD inhibition using siRNA has been shown to correct Hgb values in preclinical models of renal anemia and anemia of chronic inflammation, which was associated with decreased hepcidin expression in the liver. <sup>34</sup> The latter, however, is most likely a reflection of increased erythropoietic activity. <sup>208</sup> Whether tissue-specific targeting of PHD enzymes as efficacious as systemic therapy remains to be investigated. Irrespective of tissues targeted, the short-term and long-term effects of HIF stabilizing compounds on the human body will have to be carefully evaluated in clinical trials that are currently ongoing or have been recently completed in patients with renal anemia, but also through well-controlled physiologic studies in normal individuals.

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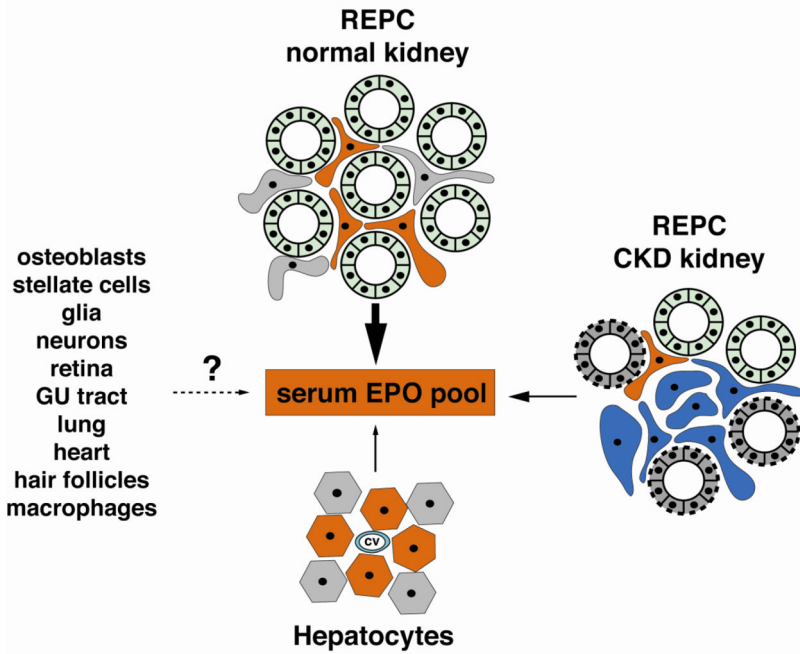


**Practice Points**

- Recognize the role of HIF-2 as a central regulator of hypoxia-induced erythropoiesis.
- Recognize the role of genetic factors in the pathophysiology of high altitude sickness.
- Recognize the role of hepcidin in the regulation of iron uptake and utilization.
- Patients with secondary erythrocytosis of unclear etiology should be evaluated for mutations in the HIF O<sub>2</sub>-sensing pathway.
- High Hgb targets in renal anemia therapy are associated with increased cardiovascular risk; the optimal Hgb target is not known.

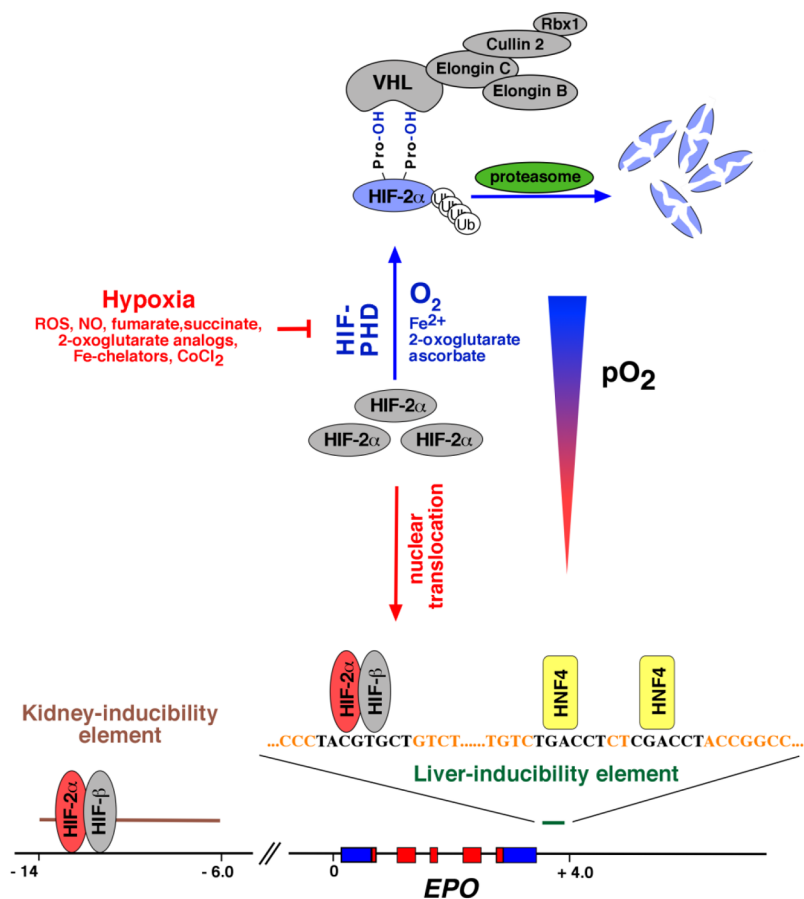
### Research Agenda

- Molecular and cellular mechanism underlying the pathogenesis of renal anemia.
- EPO-associated cardiovascular and oncologic risk.
- Regulators of iron metabolism as a therapeutic target in anemia therapy.
- Physiologic, metabolic and genetic studies of human populations that live at high altitude.
- Physiologic studies in patients with mutations in the O<sub>2</sub>-sensing machinery.
- HIF and non-HIF 2OG oxygenases in the regulation hypoxia responses.
- HIF prolyl-4-hydroxylases as therapeutic targets.



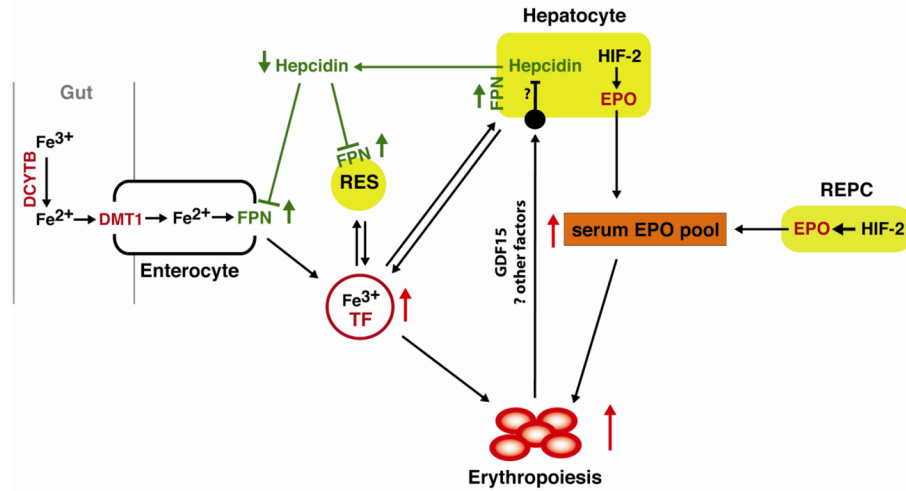
**Figure 1. Cellular sources of EPO**

Shown is a schematic overview of cell types and tissues with EPO-producing capacity. In adults, the kidney and liver are the two major contributors to the serum EPO pool with the kidney being the main physiologic site of EPO synthesis. While *EPO* transcripts are not detectable at baseline, the liver produces EPO when stimulated with either moderate to severe hypoxia or pharmacologically. The contribution of other cell types to erythropoiesis under stress conditions is not clear. A list of cell types and tissues in which *EPO* transcripts have been detected under various experimental conditions is shown on the left. Renal EPO-producing cells (REPC) are peritubular interstitial fibroblasts (shown in orange). Tubular epithelial cells do not produce EPO and are shown in green. Under chronic injury conditions, REPC undergo trans-differentiation into collagen-producing myofibroblasts (blue) and lose their ability to produce EPO; injured renal tubuli are shown in grey color. In CKD kidneys, the number of REPC is reduced, which results in inadequate EPO production in response to hypoxic stimuli and leads to the development of anemia. **Abb.:** cv, central vein.



### Figure 2. EPO is HIF-2-regulated

Shown is an overview of *EPO* gene regulation by HIF-2. The VHL-E3-ubiquitin ligase complex targets hydroxylated HIF-2 $\alpha$  for proteasomal degradation (shown are key components of this complex). Hydroxylated HIF- $\alpha$  binds to the  $\beta$ -domain of VHL, which is contained within amino acid residues 64–154. The C-terminal  $\alpha$ -domain links VHL to the E3-ligase via elongin C. HIF-2 $\alpha$  hydroxylation is carried out by O<sub>2</sub>- and iron-dependent HIF prolyl-4-hydroxylases (HIF-PHD). In the absence of molecular O<sub>2</sub>, HIF-2 $\alpha$ , which is constitutively synthesized, is no longer degraded and translocates to the nucleus where it forms a heterodimer with HIF- $\beta$  also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-2 $\alpha$ / $\beta$  heterodimers bind to the HIF consensus binding site 5'-RCGTG-3' and increase *EPO* transcription in the presence of transcriptional coactivators, such as CREB-binding protein (CBP) and p300. Hypoxic induction of *EPO* in the liver is mediated by the liver-inducibility element located in the 3'-end of the *EPO* gene. The hypoxic induction of *EPO* in REPC requires the kidney inducibility element, which is located 6–14 kb upstream of its transcription start site. Nitric oxide, reactive O<sub>2</sub> species, succinate and fumarate, cobalt chloride and iron chelators such as desferrioxamine inhibit HIF-PHDs, which results in increased *EPO* transcription. Boxes depict *EPO* exons. *EPO* coding sequences are shown in red, non-translated regions are shown in blue. Numbers indicate distance from transcription start site in kilobases (kb; not drawn to scale). Also shown are 3'-HNF4 binding sites. **Abb.:** CoCl<sub>2</sub>, cobalt chloride; Fe<sup>2+</sup>, ferrous iron; HNF4, hepatocyte nuclear factor 4; NO, nitric oxide; ROS, reactive oxygen species; ub, ubiquitin.



### Figure 3. HIF coordinates EPO production with iron metabolism

Shown is a schematic overview of changes in iron metabolism as a consequence of HIF activation. HIF-regulated genes are indicated in red. HIF-2 activation (either by hypoxia, pharmacologic means or as a result of mutations) induces renal and hepatic EPO synthesis, which leads to an increase in serum EPO levels and stimulation of erythropoiesis. Iron metabolism is adjusted to match iron demand from increased erythropoiesis. In the duodenum, duodenal cytochrome b (DCYTB) reduces ferric iron (Fe<sup>3+</sup>) to its ferrous form (Fe<sup>2+</sup>), which is then transported into the cytosol of enterocytes by divalent metal transporter-1 (DMT1). *DCYTB* and *DMT1* are both HIF-2-regulated. Absorbed iron is released into the circulation by ferroportin and is then transported in complex with transferrin (TF) to the liver, reticulo-endothelial cells (RES), bone marrow and other organs. TF is HIF-regulated and hypoxia increases its serum levels. EPO-induced erythropoiesis inhibits hepcidin synthesis in the liver and reduces serum hepcidin levels. As a result of low serum hepcidin ferroportin (FPN) cell surface expression is increased (hepcidin promotes ferroportin degradation). As a result, more iron is released from enterocytes, hepatocytes and RES cells. Growth differentiation factor 15 (GDF15) is made by erythroid precursor cells and has been shown to suppress hepcidin in hepatocytes.