Forum Review Article

# NRF2 and hypoxia inducible factors: key players in the redox control of systemic iron homeostasis

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Abstract

Significance: Oxygen metabolism and iron homeostasis are closely linked. Iron facilitates the oxygen-carrying capacity of blood, and its deficiency causes anaemia. Conversely, excess free iron is detrimental for stimulating the formation of reactive oxygen species, causing tissue damage. The amount and distribution of iron thus need to be tightly regulated by the liver-expressed hormone hepcidin. This review analyses the roles of key oxygen-sensing pathways in cellular and systemic regulation of iron homeostasis; specifically, the prolyl hydroxylase domain (PHD)/ hypoxia-inducible factor (HIF) and the KEAP1/NRF2 pathways, which mediate tissue adaptation to low and high oxygen, respectively.

Recent Advances: In macrophages, NRF2 regulates genes involved in haemoglobin catabolism, iron storage, and iron export. NRF2 was recently identified as the molecular sensor of iron-induced oxidative stress and is responsible for BMP6 expression by liver sinusoidal endothelial cells, which in turn activates hepcidin synthesis by hepatocytes to restore systemic iron levels. Moreover, NRF2 orchestrates the activation of antioxidant defences that are crucial to protect against iron toxicity. On the other hand, low iron/hypoxia stabilize renal HIF2a via inactivation of iron-dependent PHD dioxygenases, causing an erythropoietic stimulus that represses hepcidin via an inhibitory effect of erythroferrone on bone morphogenetic proteins. Intestinal HIF2a is also stabilized, increasing the expression of genes involved in dietary iron absorption.

Critical Issues: An intimate crosstalk between oxygen-sensing pathways and iron regulatory mechanisms ensures that fluctuations in systemic iron levels are promptly detected and restored.

Future Directions: The realization that redox-sensitive transcription factors regulate systemic iron levels suggests novel therapeutic approaches.

## 1. Introduction:

Iron-oxygen interactions have had a major impact in the history of life on Earth since photosynthetic cyanobacteria began to use sunlight to split water and produce oxygen, about 3.4 billion years ago. By then, oceans were rich in dissolved ferrous iron, which oxidized rapidly in the presence of oxygen and precipitated in the form of ferric oxide, a process commonly referred to as the 'Rusting of the Earth'. Only ~1 Ga later, when oxidation of the ferrous iron sinks began to saturate, did atmospheric oxygen build up. The 'Great Oxygenation Event' (GOE) made molecular oxygen biologically available and marked the evolutionary transition to aerobiosis (60, 89, 132).

In our time, most living organisms require oxygen for efficient energy production. During oxidative phosphorylation in the mitochondria of eukaryotic cells, the synthesis of adenosine 5′-triphosphate (ATP) molecules is coupled with the flow of electrons through the electron transport chain. In the process, molecular oxygen functions as the final receptor for electrons. An oxygen deficit thus results in impaired aerobic energy production and a metabolic shift to glycolysis. Hypoxia is implicated in the pathophysiology of heart disease, cancer, stroke, and chronic lung disease (138). On the other hand, byproducts of oxygen redox metabolism are toxic and mutagenic. Oxygen oxidizes essential biomolecules and in the process oxygen is reduced to oxygen free radicals and other toxic reactive oxygen species (ROS). The overwhelming of the cell/organism antioxidant defences by increased ROS production results in a deleterious condition termed oxidative stress (56). To ensure that oxygen levels are tightly maintained, higher eukaryotes have evolved complex circulatory, respiratory, and neuroendocrine systems (138). Altogether, these systems promote the safe delivery of oxygen to tissues. Moreover, cells of complex

organisms are set to live within an optimum narrow range of oxygen, which is cell-type specific. Small changes in oxygen levels, either positive or negative, are promptly sensed by the cells and elicit a biochemical response that includes the induction of specific gene sets. Transcription factors NF-E2 p45-related factor 2 (NRF2; encoded by *NFE2L2*) and hypoxia inducible factors (HIFs) are the major coordinators of the cellular responses to increased oxidation or to low oxygen (hypoxia), respectively (48). NRF2 activation provides a mechanism enabling organisms to deal with metabolically induced oxidative stress and electrophilic toxicity, and achieve longevity (151). HIFs promote adaptation to low oxygen by increasing glucose uptake, angiogenesis and erythropoiesis (67, 138). Although NRF2 and HIF are constitutively expressed by cells, both are rapidly degraded under homeostatic/normoxic conditions (Figure 1). Degradation of NRF2 through the ubiquitin proteasome pathway is mediated by a repressor protein, Kelch-like ECH-associated protein 1 (KEAP1), whereas HIFa is set for 26S proteasome degradation by the von Hippel-Lindau (VHL) protein following its enzymatic hydroxylation by prolyl hydroxylases (PHDs). Notably, KEAP1 dissociates from NRF2 upon oxidation of critical cysteine residues; and PHD activity is inhibited during hypoxia. As a result, NRF2 and HIF are stabilized by oxidative stress and hypoxia, respectively. This allows cells to rapidly mount the most appropriate transcriptional response to alterations in cellular redox state by relying on pre-existing proteins, i.e. with no need for *de novo* synthesis of the master regulators (160).

Iron is the fourth most abundant element on Earth. However, since under oxygenated conditions most ferrous iron is precipitated in the form of non-reactive ferric complexes, only trace amounts of soluble iron are presently available in aqueous media. With rare exceptions, virtually all organisms from Archaea to

humans depend on iron for survival. Due to the ability to transfer one electron between its ferric ($Fe^{3+}$) and ferrous ($Fe^{2+}$) states, iron is involved in many reduction-oxidation (redox) reactions that are required for enzymatic catalysis and electron transfers. Iron serves as a cofactor in a variety of enzymes involved in critical intracellular processes, including cellular respiration (e.g. cytochromes), DNA synthesis (e.g. ribonucleotide reductase) and anti-oxidation (e.g. catalase) (56). Moreover, iron is essential for oxygen transport in most organisms across all kingdoms of life since it is a component of $O_2$-carrying molecules like haemoglobin and myoglobin. Iron deficiency eventually leads to anaemia and tissue hypoxia. On the other hand, excess free iron is toxic. Ferrous iron can react with hydrogen peroxide via Fenton chemistry, generating highly reactive hydroxyl radicals ($HO^{\bullet}$), which can damage lipids, proteins, and nucleic acids (44; 56). Iron overload contributes to the pathogenesis of cancer, diabetes, cardiovascular disease, neurodegenerative diseases, metabolic syndrome, as well as haemochromatosis and thalassaemia. In addition, high iron availability can influence host-pathogen interactions in favour of microorganisms, as iron acquisition determines the virulence of many infections (40).

In summary, iron-oxygen interactions are fundamental to life but potentially toxic if not tightly regulated. The participation of KEAP1/NRF2 and PHD/HIF in the response of cells and organisms to oxygen excess or deficiency has been extensively studied in recent years. In this review, we describe the important contribution of these two redox-sensitive signalling pathways towards the maintenance of cellular and systemic iron metabolism.

## 2. Iron homeostasis:

Individual cells and whole organisms need to maintain a sufficient iron supply to support key iron-dependent biochemical and physiological processes, and avoid the pathogenic consequences of excess iron. The mechanisms that underlie cellular and systemic iron homeostasis have been well studied, and the reader is referred to these reviews for in depth coverage (51, 57, 106, 164). Here, the basics alone will be covered to set the scene for later sections on how HIF and NRF2 interact with these regulatory circuits.

At a cellular level, sensing of iron concentrations is achieved through the combined action of two proteins, iron-regulatory proteins 1 and 2 (IRP1 and IRP2) that control post-transcriptional expression of genes mediating iron uptake, storage, utilisation and release (57). The IRPs, which do not bind each other, and act independently, each bind stem loop structures, called iron regulatory elements (IRE), which are located at the 3' or 5' untranslated regions (UTRs) of target RNAs (Figure 2). Transferrin receptor (TFR1) mRNA contains multiple IREs in the 3' UTR, so that upon IRP binding in iron starved conditions the mRNA is stabilized and TFR1 protein levels increase. Some genes have an IRE at the 5' UTR of their mRNAs, including ferritin heavy and light chains (FTH1 and FTL), 5-aminolevulinate synthase 2 (ALAS2), ferroportin (FPN1), aconitase 2 (ACO2) and HIF2a. Therefore, relative suppression of translation of these genes occurs in iron starved conditions, which in the case of ferritin and FPN1, supports liberation of iron from intracellular stores and its retention within the cell.

IRP1 and IRP2 are homologues but are regulated by different mechanisms. When cellular iron is sufficient, IRP1 binds an Fe-S cluster and functions as an enzyme,

cytoplasmic aconitase. Loss of the Fe-S cluster in iron deficient conditions causes a structural change in IRP1, which converts it into an IRE-binding protein (128).

IRP2 is regulated by iron-dependent protein degradation. Fbox/LRR-repeat protein 5 (FBXL5) binds IRP2 and facilitates its ubiquitination and proteasomal degradation. However, in iron deficient cells, FBXL5 loses its N-terminally bound iron and is itself degraded, resulting in IRP2 accumulation (130, 162). IRP2 accumulation can also be driven by low oxygen concentrations, independently of HIF (101).

The precise regulomes of the IRPs is yet to be determined, both in scope and in specificity – for example variation within IREs exist such that interaction with IRP1 rather than IRP2 is favoured (71). Cell-type effects of IRPs (singly and in combination) may also occur but are poorly understood, and some heterogeneity is likely to be present; for example, the interaction between IRPs and HIF2a is particularly important in the kidney and the intestine (6). Nevertheless it is clear that IRP1 and IRP2, by their combined actions, together exert considerable control over cellular iron homeostasis via sensing cellular iron levels.

Systemic iron homeostasis is governed by the iron regulatory hormone hepcidin, a peptide synthesized and secreted by hepatocytes that acts by binding and inhibiting the only known cellular iron exporter, FPN1 (109) (Figure 3). FPN1 is expressed widely across different cells, but at different levels (39). High expression of FPN1 is apparent on the basolateral membrane of duodenal enterocytes and on a subset of macrophages, notably those involved in erythrophagocytosis in the spleen (and liver). Hepcidin blocks the transfer of dietary iron into plasma, and recycling of iron from degraded red blood cells (RBC). Control of hepatic hepcidin expression is complex; at least four major arms of regulation exist, each with several components. Iron stimulates hepcidin expression, but unveiling the precise mechanisms by which

iron is detected and understanding how this information is converted into regulation of hepcidin has proved problematic. The saturation of transferrin is sensed at the hepatocyte cell membrane. Several players in this process have been identified via genetic approaches, namely the homeostatic iron regulator (HFE), TFR1, TFR2, bone morphogenetic protein (BMP) receptor complexes (including hemojuvelin, HJV), and the serine protease Matriptase-2, and the result of their action is to generate an intracellular Small Mothers Against Decapentaplegic (SMAD)-mediated signal to regulate transcription of the HAMP gene that encodes hepcidin (16, 115) (Figure 3). Nevertheless the mechanistic roles of these proteins individually and *in toto* remain uncertain. Liver iron stores also regulate hepcidin expression, at least partly via synthesis of BMP6 (70, 102), in a process involving NRF2 that is described later.

Negative regulation of hepcidin expression occurs during iron deficiency, and also due to anaemia (50, 116). Decreased oxygen transport capability either due to low haemoglobin levels or reduced oxygen concentrations, leads to stimulation of erythropoiesis, which requires iron. Erythropoietin (EPO)-activated erythroblasts secrete a hormone, erythroferrone (ERFE), which sequesters BMP6 and so impairs hepcidin production, facilitating iron uptake from the diet and iron release from macrophage stores (8, 9, 69).

Lastly, hepcidin expression is increased by inflammatory cytokines, especially by interleukin 6 (IL6), which acts via Signal transducer and activator of transcription 3 (STAT3) transcription factors (108, 174). However inflammatory induction of hepcidin by IL6 also requires an active BMP-SMAD signaling pathway (45, 82, 147), and can be countermanded by strong increased erythroid demand for iron in order to facilitate oxygen carriage (157).

The mechanisms of both cellular and systemic iron homeostasis reflect the key interaction of iron and oxygen. Notably IRPs do not directly regulate hepcidin, however IRPs do control expression of the target of hepcidin, FPN1, and a key regulator of hepcidin, ERFE, is downstream of HIF-mediated induction of EPO, and HIF2a expression is controlled by IRPs. Therefore, as might be expected, cellular and systemic iron homeostasis are not independent of each other, and their interactions are mediated by hypoxia and by oxidative stress, which in turn is controlled by NRF2.

## 3. The KEAP1/NRF2 antioxidant response:

The transcription factor NRF2 is an ancient protein that has evolved over geological timescales as changes to atmospheric oxygen occurred and life diversified (49). NRF2 is a ubiquitously expressed member of the human cap'n'collar (CNC) basic-region leucine zipper transcription factor family. In homeostasis, NRF2 is sequestered in the cytoplasm by KEAP1, an adaptor subunit of Cullin3 E3 ubiquitin ligase, and targeted for ubiquitination and rapid proteasomal degradation (32, 73, 182) (Figure 1). The short half-life of NRF2 protein (~15–40 min) explains its low abundance in unstressed conditions. Importantly, KEAP1 contains several highly reactive cysteines that, upon modification by electrophilic molecules, disrupt its capacity to bind NRF2. Oxidative stimuli thus destabilize the NRF2-KEAP1-Cullin3 complex and block NRF2 degradation, allowing *de novo* synthesized NRF2 to translocate into the nucleus and, following dimerization with small musculoaponeurotic fibrosarcoma oncogene homologue (sMAF) proteins, induce the expression of antioxidant response elements (AREs)-containing genes (63) that encode ~250 proteins with cellular protective properties (31). Both NRF2 and KEAP1

are ubiquitously expressed, and the NRF2–KEAP1 axis is recognized as the major thiol-driven switch of systemic oxidative stress responses (144). Thus, NRF2 regulates the expression of enzymes involved in the synthesis and use of reduced glutathione (GSH) (catalytic and modulator subunits of glutamate-cysteine ligase, glutathione reductase, glutathione peroxidase and glutathione S-transferases), members of the redoxin family (thioredoxin, thioredoxin reductase, peroxiredoxin and sulfiredoxin), and enzymes that provide the reducing equivalents required for antioxidant reactions (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme 1 and isocitrate dehydrogenase 1). In addition to proteins/enzymes that are directly involved in the neutralization of ROS, the NRF2-KEAP1 axis regulates genes encoding enzymes that limit the redox cycling of quinones and glutathione depletion (NAD(P)H:quinone oxidoreductase 1), that promote the elimination of GSH-conjugated electrophiles (multidrug resistance-associated proteins), or that are involved in the breakdown of haem and concomitant production of the antioxidant bilirubin (haem oxygenase 1, HMOX1; biliverdin reductase).

Besides the induction of antioxidant and drug-metabolizing genes, NRF2 is also known to promote homeostasis by regulating cellular bioenergetics and autophagy. Through the regulation of genes involved in carbohydrate metabolism, NRF2 increases the availability of substrates and reducing equivalents for mitochondrial respiration, thus contributing to an efficient cellular energy metabolism (61). Through the modulation of proteasome subunits (79) and autophagy genes, NRF2 contributes to the elimination of damaged proteins and organelles in response to oxidative stress or nutritional starvation (113). One of the targets is the cargo recognition protein SQSTM1/p62 (66, 75), which upon binding to KEAP1 promotes its transport to the

autophagosome for degradation, thus sparing NRF2 from KEAP1-mediated degradation (154). For a more comprehensive list of NRF2 target genes with cytoprotective functions, readers are referred to a recent review (31).

## 4. NRF2 and iron homeostasis:

NRF2 was originally cloned during the search for proteins that could bind to the nuclear factor erythroid 2 (NF-E2)/AP-1 motif in the locus control region located 5′ of the β-globin gene cluster (104). However, the generation of global knockout mouse strains did not reveal alterations in haematological markers compared to wild-type (22, 63). *Nrf2*-deficient mice grew normally and were fertile with no apparent phenotype, except for the decolourization of the incisors. Interestingly, incisors of wild-type mice are brownish yellow due to iron deposition in the enamel surface layer. In *Nrf2*-null mice, incisors are white in colour as a result of lower iron deposition on the surface of mature enamel (176). Iron-deficient incisors were thus the first hint of an interaction between NRF2 and iron homeostasis. Most research that followed has focused on the ability of NRF2 to orchestrate the transcriptional induction of phase II detoxification genes carrying an ARE, but a number of studies have started to disclose the contribution of NRF2 to iron and/or haem metabolism.

Reticuloendothelial cells, comprising splenic macrophages and Kupffer cells, promote the recycling of haem-derived iron from senescent RBC. Within reticuloendothelial cells, NRF2 is believed to play an important role in the haem degradation pathway (Figure 4). Following engulfment of senescent or injured erythrocytes, haemoglobin is degraded in the phagolysosome (13). Haem is then released out of the phagolysosome into the cytosol through the lysosomal

membrane-bound transporter haem-responsive gene 1 (SLC48A1, HRG1) (121, 171), which is regulated by NRF2 (17). Moreover, NRF2 is a well-established transcriptional regulator of *HMOX1* (3). Within macrophages, HMOX1 metabolizes cytosolic haem from RBC into ferrous iron and biliverdin. This important reaction allows the recycling of ferrous iron for fresh erythrocyte production. NRF2-mediated expression of *HMOX1* is negatively controlled by BTB domain and CNC homolog 1 (BACH1), a BTB-basic leucine zipper transcription factor that competes for the same binding sites on *HMOX1* as NRF2 (112). Importantly, BACH1 is both a transcriptional repressor and a haem sensor. Upon binding haem, BACH1 undergoes ubiquitination and proteosomal degradation (181), which allows NRF2 binding to the *HMOX1* gene promoter (125).

NRF2 and BACH1 were also shown to modulate the expression of FPN1, the mammalian iron exporter that is responsible for iron mobilization from haemoglobin-recycling macrophages (38), in RAW264.7 murine macrophages treated with haem (92). This work suggested that FPN1 expression is regulated by two overlapping mechanisms: a transcriptional control mediated by the binding of NRF2 to a putative ARE that was identified approximately −7 kb from the *Fpn1* promoter, which is iron-independent and requires the release of the haem-sensitive BACH1 repressor; and a translational control by the IRE/IRP system in response to iron levels. Altogether, the BACH1-NRF2 interaction provides an additional level of regulation that ensures that NRF2 cooperates with iron-containing haemin to induce both HMOX1 and FPN1, thus promoting haem iron recycling by macrophages.

The degradation of haemoglobin is expected to increase the cellular pool of labile iron, which cells may use for biosynthetic processes such as haem or iron–sulfur cluster generation. Besides inducing genes that are involved in the safe degradation

of haem, NRF2 may also promote the reincorporation of iron into haem via the upregulation of genes involved in haem biosynthesis (Figure 4). The ATP binding cassette subfamily B member 6 (ABCB6), which imports porphyrins from the cytosol into the mitochondria (77, 136), was shown to be regulated by NRF2 in lung-derived (A549, BEAS-2B) and hepatoma cell lines (HepG2) treated with the dietary isothiocyanate sulforaphane, a well-known inducer of phase II enzymes via the NRF2 pathway (17). Ferrochelatase (FECH), a mitochondrial enzyme that inserts ferrous iron into protoporphyrin IX in the last step of haem biosynthesis (72), is induced by NRF2 activation in mouse liver (175).

Importantly, iron in the labile pool remains redox-active. Therefore, if not used for biosynthetic processes, it must be safely stored, to avoid the formation of oxygen-derived free radicals through Fenton reaction. Ferritin, the major iron storage protein, is able to sequester up to 4500 iron atoms into its core in a non-toxic form (156). It is a 24 subunit spherical shell protein comprising a variable number of light (FTL) and heavy chain (FTH1) subunits. FTH1 contains a ferroxidase active site, which promotes the oxidation of $Fe^{2+}$ to $Fe^{3+}$ for storage in the central core, whereas FTL may assist in the electron exchanges that are required for iron mineralization/demineralization (20, 156). When iron is in demand, ferritin can be broken down to release it, in a process known as ferritinophagy (91). Notably, AREs were identified within the promoter regions of murine Ftl and Fth1 genes (161, 170), and basal mRNA levels of both genes were lower in *Nrf2*-deficient mice than in wild-type mice (78, 158). Moreover, the induction of both Ftl and Fth1 in primary mouse embryo fibroblasts following administration of chemopreventive dithiolethiones was NRF2-dependent (118). Hence, NRF2 may alter iron homeostasis by increasing iron storage in ferritin, thus decreasing the cellular labile iron pool.

In keeping with its potential important role in haem catabolism, NRF2 may also favour the conversion of biliverdin, the remaining haem metabolite, to an antioxidant free radical scavenger, bilirubin (148). Bilverdin reductase (BLVRB), the enzyme responsible for this conversion, is an NRF2 target gene. Transcription of BLVRB was shown to increase in mice with liver-specific Keap1 knockout (175) and in human breast cancer MCF10A cells upon KEAP1 knockdown or treatment with sulforaphane (1).

Overall, there is wide evidence that NRF2 participates in the transcriptional regulation of several genes involved in haem metabolism, but whether this correlates with any physiopathological outcomes is something that remains to be demonstrated. Future research should provide definite proof that the KEAP1/NRF2 pathway regulates the mobilization of iron from haem during haemoglobin catabolism and/or its reutilization.

Recently, NRF2 was demonstrated to play a critical role in toxic iron sensing by the liver (Figure 5). The liver is essential for the metabolism of many endogenous chemicals and exogenous toxicants, and loss of NRF2 increases the sensitivity to multiple hepatotoxicants (153). It also plays a central role in iron homeostasis: it stores excess body iron and regulates hepcidin levels in response to changes in iron levels (126). Elevated hepatic iron content was firstly shown to activate NRF2 and increase the expression of cytoprotective genes in mice fed high-iron diets (105, 145). Recently, Lim et al. (84) have demonstrated that NRF2 is activated by iron-induced, mitochondria-derived pro-oxidants. Moreover, it drives iron-induced BMP6 expression in liver sinusoid endothelial cells (LSECs). BMP6 secreted from LSECs in turn increases hepcidin synthesis by neighbouring hepatocytes (18), which eventually reduces circulating iron levels due to iron sequestration within

reticuloendothelial macrophages and abrogation of dietary iron absorption via FPN1 degradation (57). Whilst *Nrf2* knockout mice present no major alterations in systemic iron homeostasis in basal conditions, the BMP6-hepcidin response to oral and parenteral iron is impaired and tissue iron accumulation in animals fed iron-rich diet is increased compared to wild-type counterparts on the same diet (84). Conversely, pharmacological activation of NRF2 with the synthetic triterpenoid 2-cyano-3,12 dioxooleana-1,9 dien-28-imidazolide (CDDO-Im), which reacts with cysteine residues on KEAP1 (129) and potently induces cytoprotective genes in mouse liver (179), stimulates the BMP6-hepcidin axis. In *Hfe* knockout, a mouse model of haemochromatosis with increased dietary iron absorption and tissue deposition due to insufficient hepcidin production (2, 11, 183), sustained treatment with CDDO-Im over a 3-week period reduced serum iron and prevented hepatic iron accumulation (84).

Toxic iron also accumulates in tissues of non-transfusion-dependent thalassaemia, where aberrant EPO-induced erythroblast expression of ERFE suppresses hepcidin synthesis (69). As recently demonstrated, ERFE inhibits the induction of hepcidin by binding and inhibiting BMP6 (9). Treatment of *Hbb*(th3/+) mice, a model of β-thalassemia intermedia (177), with CDDO-Im counteracted the inhibition of BMP6 by ERFE, thus increasing hepcidin expression in the liver and decreasing serum iron (84) (Figure 5).

NRF2 regulates Bmp6 expression and is required for the induction of hepatic BMP6 in response to ‘toxic iron’ (with the toxic signal likely being iron-induced electron-seeking molecules or electrophiles in mitochondria) rather than iron itself. While hepatocytes readily detect increases in transferrin saturation via HFE and TFR2 to regulate hepcidin, the LSEC NRF2-BMP6-hepcidin axis is only activated by

potentially pathogenic iron levels. This represents an additional level of protection because, unlike HFE and TFR2, NRF2 also regulates antioxidant defences (Figure 5). Iron was shown to induce the expression of cytoprotective genes in murine hepatocytes and livers in a NRF2-dependent manner, and *Nrf2*-null hepatocytes are highly susceptible to iron-induced cell death (145). Likewise, *Nrf2*-null mice given dietary or parenteral iron display iron-related necroinflammatory lesions. These are likely caused by iron-induced mitochondrial oxidative damage, as they associate with injured hepatocyte and LSEC mitochondria, and are prevented by (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (mito-TEMPO), a mitochondria-targeted superoxide dismutase (SOD) mimetic (84, 145). Moreover, the genetic disruption of NRF2 prompted spontaneous necroinflammation that led to early-stage hepatic fibrosis in *Hfe*-knockout mice, a mouse model of hereditary haemochromatosis displaying only mildly elevated liver iron (41). The development of liver injury (including significant lipid peroxidation) in a double-knockout mouse for *Hfe* and *Nrf2* with mild hepatic iron overload illustrates the importance of the NRF2-coordinated ROS and electrophile detoxification pathway in preventing iron overload-induced injury.

Although not specifically addressed in any of the studies mentioned above, one mechanism through which NRF2 may preserve cell viability in the face of iron load is by decreasing sensitivity to ferroptosis, an iron-dependent non-apoptotic regulated form of cell death caused by the accumulation of lipid peroxides (178). Experimentally, ferroptosis is induced upon inhibition of glutathione peroxidase 4 (GPX4) or disruption of glutathione synthesis, exacerbated by iron, and prevented by iron chelators such as desferrioxamine (DFO) and by lipophilic antioxidants such as ferrostatin-1, liproxstatin-1, and endogenous vitamin E (35). NRF2 regulates a

number of genes that are expected to protect against ferroptosis, including GPX4, cystine/glutamate antiporter xCT (SLC7A11), γ-glutamylcysteine synthetase (GCS), the catalytic and modifier subunits of glutamate-cysteine ligase (GCLC/M), FTL and FTH1, and HMOX1 (36). Several studies report that activation of NRF2 is associated with resistance of different malignant cell lines to ferroptosis inducers (43, 85, 142, 149), whereas others reported that the stabilization of NRF2 only weakly protects from ferroptosis because of the GSH efflux that results from NRF2-mediated upregulation of ATP binding cassette (ABC)-family transporter multidrug resistance protein 1 (MRP1) (19). Many of the effects reported in the literature may thus be cell- or tissue-specific, and perhaps particular to malignant cells. While some ferroptosis features such as lipid peroxidation, mitochondrial disruption and iron dysregulation are also encountered in human iron overload disorders, the contribution of ferroptosis to the pathophysiology of iron overload remains elusive (37). Further studies are required to determine the role of NRF2 in ferroptotic cell death, particularly using relevant experimental models of severe iron overload in which ferroptosis may develop, such as *Hjv* knockout mice (168).

One yet unexplored question is whether the course of the interaction between the KEAP1/NRF2 pathway and iron homeostasis would be modified in the context of inflammation. Besides iron overload, hepcidin is also induced by acute and chronic inflammation, resulting in hypoferremia (110). A key mediator of hepcidin response to inflammatory stimuli is IL6, which stimulates hepcidin transcription through STAT3 (108). Compared to other hematopoietic cells, NRF2 mRNA expression is high in monocytes, granulocytes and lymphocytes (31). In keeping with its underlying role in maintaining tissue homeostasis, NRF2 is also reported to have a strong anti-inflammatory activity. This is partly explained by its modulation of redox metabolism,

which helps limiting the increased local and systemic ROS production that is known to be associated with inflammation (76). Another important explanation is the ability of the NRF2–KEAP1 axis to suppress nuclear factor-κB (NFkB) (30). Moreover, NRF2 has been shown to suppress pro-inflammatory genes. In macrophages, NRF2 binds to regulatory regions of genes encoding pro-inflammatory cytokines (IL6 and IL1b) and blocks their transcription (74). Also, macrophage activation in response to pro-inflammatory stimuli leads to increased production of itaconate, a mitochondrial metabolite that alkylates the critical KEAP1 cysteine residues, thereby activating NRF2 and suppressing transcription of pro-inflammatory cytokines, such as IL6 and IL1b (103). It can thus be hypothesized that, in inflammation, activation of NRF2 in immune cells may inhibit hepcidin.

## 5. The PHD/HIF oxygen-sensing pathway:

Whilst the need for oxygen to sustain life has been known for several centuries, the molecular basis of oxygen sensing and cellular adaptation to oxygen availability was only disclosed in the 1990's. An important milestone on this front was the identification of HIF1 as the transcription factor responsible for the hypoxic induction of EPO synthesis in hepatoma cells, through binding to a 3' hypoxia response element (HRE) on the *EPO* gene (140). This was followed rapidly by further seminal observations. First, that the cellular pathways required for hypoxic induction via the *EPO* 3' HRE were not restricted to *EPO*-expressing cell types (100), and second that HIF1 itself was widely expressed, and its transcriptional activity was not restricted to the *EPO* gene. The first non-*EPO* HIF target genes were identified in 1994 (46, 139),

and the HIF pathway is now recognised as essentially ubiquitous in mammalian cells, responsible for regulating the expression of >1000 genes during hypoxia (134).

In 1995, the cloning of HIF1 revealed the transcription factor to be a basic-helix-loop-helix PAS heterodimer, consisting of a constitutively-expressed β subunit (HIF1b, encoded by the aryl hydrocarbon nuclear translocator (*ARNT*) gene) and a hypoxia-inducible α subunit (165, 167). Three isoforms of the HIFa subunit (HIF1a, HIF2a and HIF3a) have subsequently been described, with differing tissue distributions and overlapping but distinct sets of target genes (62, 159, 172). HIF1a is the most widely expressed, but HIF2a has a number of defined tissue-specific roles, including the regulation of renal EPO expression (169).

Under normoxic conditions, the HIFa isoforms are constitutively expressed but rapidly degraded. The key oxygen-dependent step in this degradation pathway is the hydroxylation of two specific proline residues on HIFa, promoting its association with the VHL ubiquitin E3 ligase complex and subsequent destruction via the ubiquitin-proteasomal pathway (64, 65). Under hypoxic conditions, when hydroxylation is inhibited, HIFa accumulates, translocates to the nucleus, and forms a complex with HIF1b and co-activators, leading to activation of target genes (Figure 1). An early clue as to the nature of the enzyme catalysing the hydroxylation reactions was the observation that the iron chelator DFO stabilises HIF in cell culture (166). Three iron- and oxygen-dependent prolyl hydroxylase domain enzymes (PHD1, PHD2, PHD3) were subsequently identified as the key oxygen sensors in the HIF pathway (15, 42). These enzymes, which are members of the 2-oxoglutarate (2-OG) dependent dioxygenase superfamily, are oxygen dependent by virtue of the requirement for molecular oxygen in the prolyl hydroxylation reaction, and iron dependent through the labile binding of iron ($Fe^{2+}$) in the catalytic site, with ascorbate keeping iron in the

reduced state. The PHD isoforms are differentially expressed across cell types, with PHD2 being the most abundant, and display a degree of HIF isoform specificity (7, 12).

A fourth 2-OG dependent oxygenase has also been described in the regulation of the HIF pathway (90). Factor inhibiting HIF (FIH) catalyses the hydroxylation of specific asparagine residues in HIF1a and HIF2a, disrupting their interaction with the p300/CBP transcriptional co-activator complex and impeding activation of HIF-target genes (58, 81). For both FIH and the PHDs, there is also considerable interest in the potential for non-HIF hydroxylation targets, an issue of particular importance in the light of recent pharmacological targeting of the HIF hydroxylases.

## 6. HIFs and iron homeostasis:

Exposure to low atmospheric oxygen leads to HIF2a-mediated EPO synthesis and increased RBC production. This, in turn, increases the demand for iron, which is required for haemoglobin synthesis, in the bone marrow. Since iron availability is the most common limiting factor in erythropoiesis, regulation of hypoxic responses and of iron metabolism needs to be coordinated. In fact, iron levels are regulated by hypoxia. Systemic HIF activation causes marked suppression of hepcidin production in the liver, enhancing iron uptake and mobilization (54, 110). Initial studies with liver-specific *Hif1a* knockout or *Vhl* knockout (stabilizing both HIF1a and HIF2a) suggested that HIF1 could directly suppress hepcidin transcription via a HRE within its promoter (117). However, studies with mouse models with global or liver-specific HIF activation/inactivation have disclosed that hepcidin suppression was instead an indirect effect of HIF2a-dependent, EPO-mediated stimulation of erythropoietic

activity (86, 96). Mechanisms explaining hepcidin repression EPO-stimulated erythropoiesis include the production of ERFE by erythroblasts (69) and its inhibitory effect on hepatic BMP/SMAD signalling (9) (Figure 6).

*In vitro* studies suggested that HIFs may also indirectly regulate hepcidin independently of EPO by acting upon HJV. During hypoxia, hepatocyte HIF1 and HIF2 increase the expression of Matriptase-2 (TMPRSS6), a type II transmembrane serine proteinase that antagonizes hepcidin induction by BMPs by cleaving HJV from the cell membrane (80, 98). Moreover, in hypoxic skeletal muscle cells, HIF1 up-regulates furin, a proprotein convertase that increases soluble HJV, which also reduces the expression of hepcidin by competitively interfering with BMP signalling (146).

Besides hepcidin, systemic iron homeostasis is also dictated by local iron handling mechanisms in the gut. Iron absorption in the enterocyte involves reduction of $Fe(^{3+})$ to $Fe(^{2+})$ by the apical ferric reductase duodenal cytochrome b (DCYTB), followed by uptake by the apical iron transporter divalent metal transporter-1 (DMT1). Iron is eventually exported from the enterocyte into the bloodstream via FPN1, the only known cellular iron exporter. HIF2a increases the transcription of DCYTB (141), DMT1 (95, 141) and FPN1 (155) in the enterocyte, thus promoting iron uptake (Figure 6). In addition to regulating normal absorption rates at basal level, intestinal HIF2a is essential to mediate the adaptive increase in iron absorption during systemic iron deficiency (141) and increased erythropoiesis (4, 123, 155). HIF2a also mediates the increased absorption of dietary iron leading to systemic iron overload in haemochromatosis (94), b-thalassemia (5), and sickle cell disease mouse models (33). Recently, Schwartz et al. (137) demonstrated that HIF2a-mediated iron hyperabsorption in both anaemia and iron overload is actually linked to the well-

characterized hepcidin/FPN1 axis. FPN1 stabilization following a transient fall in hepcidin production leads to a decrease in intracellular iron in the duodenal enterocytes, which stabilizes HIF2a and induces transcription of the iron absorption genes (*DMT1*, *DCYTB*, *FPN1*) in a cell-autonomous manner (137). As noted above, HIF activation under low cellular iron concentrations occurs through regulation of PHD activity. PHDs are $Fe(^{2+})$/2-OG-dependent oxygenases (59) and iron chelators were shown to inhibit hydroxylase activity and increase stabilization of HIFa, thus mimicking hypoxia (42, 81). Ascorbate, in turn, is required for maximum enzymatic activity, presumably by keeping the iron atom in its reduced, catalytic state (47).

Notably, HIF2a contains an IRE in its 5' UTR, which binds IRPs (131). Under low intracellular iron, IRPs bind to the HIF2a 5' UTR, which has been shown to inhibit HIF2a translation (5, 52, 173). It seems reasonable to speculate that the control of HIF2a translation by IRP activity may serve to attenuate its activation by hepcidin/FPN1/PHD during increased systemic iron demand (137).

Finally, HIF1 has also been shown to regulate the transcription of genes involved in iron utilization, including: transferrin (127), a liver-derived protein that transports iron in its ferric form ($Fe^{3+}$) in blood, and the TFR1 (88, 152); ceruloplasmin (107), which oxidizes $Fe^{2+}$ to $Fe^{3+}$ allowing its incorporation into transferrin; HMOX1 (83), which is involved in iron recycling in erythrophagocytosis; and FECH (87), the last enzyme on the haem synthetic pathway. It is worth noting that, while several HIF (including HIF1 and HIF2) target genes are known to contribute to erythrophagocytosis (e.g. *DMT1*, *HMOX1*, *FPN1*), Mathieu et al. have demonstrated that deletion of macrophage HIF1 or HIF2 does not have an impact on iron recycling *in vivo* or *in vitro* (97).

## 7. Clinical and therapeutic implications:

The realization that redox-sensitive transcription factors including HIF and NRF2 play key roles in systemic iron regulation may highlight novel therapeutic approaches. A number of common disorders are characterised by disturbances in HIF or NRF2-regulated gene networks. In conditions associated with tissue or systemic hypoxia, upregulation of HIF will often be the predominant cellular response, which may be adaptive, for example in chronic anaemia, or maladaptive, for example in the development of pulmonary hypertension in chronic lung disease- however iron also plays a role in these disorders. Where iron overload appears to be the predominant pathology, NRF2 activation represents an important line of defence against oxidative stress. Therapeutic approaches targeting either the HIF or the NRF2 systems are becoming available, but their effective deployment may depend on a full appreciation on how the iron and oxygen sensing mechanisms interact.

By responding to toxic insults and controlling the expression of a comprehensive battery of antioxidant, detoxification and anti-inflammatory proteins, the NRF2–KEAP1 axis may protect against a variety of diseases in which oxidative stress and inflammation are important drivers of pathology. Mouse studies have supported the idea that NRF2 provides protection against a variety of chronic diseases of the lung and gastrointestinal tract; autoimmune, neurodegenerative and metabolic disorders (29, 31). Likewise, association studies between functional genetic variations of *NFE2L2* and disease risk support the notion that NRF2 is a determinant of human susceptibility to chronic disease (25). In recent years, this has raised interest from biopharmaceutical companies, which resulted in substantial investment in the clinical development of NRF2 modulators, including several electrophilic modifiers of the cysteine-based sensor KEAP1 (31). Among these, dimethyl fumarate (Tecfidera) is

currently in clinical use to treat psoriasis and remitting-relapsing multiple sclerosis. A number of other NRF2 activators targeting the KEAP1 cysteines are at various stages of clinical development, including different fumaric acid derivatives, the isothiocyanate sulforaphane, or cyano enone triterpenoids. Additional drug candidates include non-electrophilic small molecules that inhibit the direct protein-protein interaction between KEAP1 and NRF2 (114, 133), or the binding of BACH1 to some ARE-driven genes like *HMOX1* independently of KEAP1 (10), and manganese porphyrins are in clinical trials for their radioprotective properties, which appear to result at least in part from oxidation of KEAP1 and subsequent detachment from the KEAP1-NRF2 complex and activation of NRF2 (143). Readers requesting more extensive information on the clinical development of NRF2 activators are referred to a recent review (31).

Inappropriately low hepcidin expression is a feature of both mouse models and human patients with HFE-related haemochromatosis (2, 14, 111, 119). Recent studies have shown that the BMP-SMAD signalling pathway is impaired in *Hfe* knockout mice (26, 68). Notably, supraphysiological doses of recombinant BMP6 increase hepcidin and reduce serum iron, thus compensating for the molecular defect underlying HFE-related haemochromatosis (27). Increasing hepcidin in β-thalassaemia is also therapeutic. Treatment of *Hbb*(th3/+) mice with minihepcidins, which are hepcidin agonists known to prevent iron overload in murine models of haemochromatosis (120, 124), improves ineffective erythropoiesis, anaemia, and iron overload (21). Lim et al. (84) activated NRF2 with CDDO-Im in *Hfe* knockout mice and th3/+ mice and observed robust upregulation of *Bmp6* and hepcidin expression, and decreased serum iron accumulation. This was accompanied by the activation of genes involved in the antioxidant response; in *Hfe* knockout mice,

hepatic lipid peroxidation was decreased. Pharmacological NRF2 activation is thus likely to be beneficial in the treatment of iron overload disorders (e.g. haemochromatosis, thalassaemia) for stimulating the BMP6–hepcidin axis while simultaneously boosting the capacity to detoxify iron-induced ROS. In addition, this dual protective action of NRF2 agonists may be relevant in a range of chronic diseases where deregulated iron homeostasis is implicated, including neurodegenerative and metabolic disorders.

The pharmacological manipulation of the HIF/PHD signalling pathway has been a field of intense research in recent years. The major focus has been the treatment of chronic kidney disease (CKD), in which insufficient renal EPO production leads to reduced erythropoiesis, causing both anaemia and hyperferremia. Notably, the ensuing tissue iron accumulation may further decrease EPO production by reducing HIF2a concentration in renal interstitial fibroblasts (150). The identification of 2-OG-dependent PHDs as the oxygen sensors in the HIF pathway and hence the key regulators of erythropoiesis has fuelled the development of a variety of PHD inhibitors that stabilize HIF and stimulate the expression of HIF target genes, including EPO, for treatment of diseases including renal anaemia, ischaemic stroke, and wound healing (34, 99, 135). Currently, four HIF-PHD inhibitors have entered clinical trials for treating anaemia in CKD, all of which stabilize both HIF1a and HIF2a: FG4592 (Roxadustat) from FibroGen, GSK1278863 (Daprodustat) from GlaxoSmithKline, Bay85-3934 (Molidustat) from Bayer, and AKB-6548 (Vadadustat) from Akebia (23, 180). Results from the trials showed that, in addition to improving anaemia, HIF-PHD inhibitors could also normalize iron metabolism, as evidenced by increased total iron binding capacity (TIBC) and decreased hepcidin in serum (55).

The efficacy and safety of HIF-PHD inhibitors is a subject that warrants future studies.

HIFs are markedly upregulated in many types of cancer. Hence, there is great interest in applying inhibitors of HIF as anticancer therapeutics. Loss of the VHL tumor suppressor protein leading to HIFa accumulation is a key oncogenic event in patients with clear cell renal cell carcinoma (ccRCC). Pharmacological inhibitors of HIF2 that selectively disrupt the heterodimerization of HIF2a with HIF1b have been developed to treat ccRCC (93). One of these HIF2a antagonists, PT2385, which proved efficient in the treatment of Von Hippel-Lindau disease-associated ccRCC in pre-clinical models (163) and in patients (28), is currently in clinical phase II trials. Interestingly, in the early phase trial a possible unexpected effect of this drug in some patients was systemic hypoxia, which may reflect physiological effects of HIF2a antagonism on ventilatory or pulmonary vascular control (28). In keeping with this, PT2385 was shown to affect ventilatory responses to hypoxia at relevant doses in the mouse (24) and also to reverse systemic iron accumulation in hepcidin-deficient mice (137), suggesting that intestinal HIF2a could be a viable target for the treatment of human iron overload diseases characterized by dysfunction of the hepcidin/FPN1 axis and intestinal iron hyperabsorption.

Regulation of HIF2a stability by the IRP/IRE system is another potential drug target. Chuvash polycythemia is an inherited disease caused by a homozygous germline $VHLR^{200W}$ mutation, and is associated with elevated levels of HIF2 and higher levels of serum EPO. VHL promotes the degradation of HIF2a under normoxic conditions. However, a mutant form of the protein, $Vhl^{R200W}$, fails to promote HIF2a degradation and results in increased EPO production. HIF2a is also translationally regulated by the IRP/IRE regulatory system. *Irp1*-knockout mice develop

erythrocytosis/polycythemia via translational derepression of HIF2a (6, 52, 173). Recently, Ghosh et al. (53) demonstrated that repression of HIF2 translation with Tempol, which increases the IRE-binding activity of IRP1, decreases EPO expression and reduces erythrocytosis/polycythemia in a mouse model of Chuvash polycythemia (*Vhl*$^{R200W}$).

In summary, the increasing availability of targeted therapies against the HIF or NRF2 pathways represents an exciting therapeutic opportunity across a range of clinical disorders of iron or oxygen homeostasis. However, to fully exploit this opportunity, it will be important to appreciate the complexity of the interaction between these pathways. For example, in chronic lung disorders involving defects in oxygen homeostasis and HIF signalling, disturbance of iron homeostasis may also be a feature of (or a contributor to) the primary condition (51, 122), and its treatment – and this may then influence NRF2 activity. In haemochromatosis or thalassaemia, aberrant HIF activation appears to contribute to iron overload by enhancing intestinal iron uptake (5, 94), and so needs to be taken into account in addition to NRF2, which defends against iron toxicity, and hepcidin, which impairs iron absorption. The interconnected nature of HIF and NRF2 signalling and the effects of targeting these transcription factors on cellular and systemic iron homeostasis represent important considerations in the development of therapeutic strategies.

## 8. Conclusions:

Both oxygen and iron play significant roles in human health. Iron-oxygen interactions are fundamental to life but potentially toxic if not carefully regulated. PHD/HIF and KEAP1/NRF2 are critically important oxygen-sensing pathways that mediate tissue

adaptation to low and high oxygen, respectively, primarily through the transcriptional activation of gene expression. NRF2 and HIFs are constitutively expressed by cells, and subject to proteasomal protein degradation in homeostatic conditions. Notably, NRF2 or HIF proteins are rapidly stabilized by oxidative stress or hypoxia, respectively, thus ensuring their prompt protective response to alterations in the cellular redox state. NRF2 plays an essential sensing role linking cellular health to systemic iron homeostasis to constrain the toxic effects of iron-induced oxidative stress. Under hypoxia, HIF2a is the major regulator of erythropoiesis and cellular iron metabolism. It controls hepatic hepcidin production through EPO, and regulates intestinal dietary iron uptake and mobilization. The realization that redox-sensitive transcription factors act as both iron sensors and iron homeostasis regulators suggests new therapeutic manipulations for iron metabolism disorders.

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List of Abbreviations

| | |
|---|---|
| 2-OG | 2-oxoglutarate |
| ARE | antioxidant response elements |
| ABCB6 | ATP binding cassette subfamily B member 6 |
| BMP | bone morphogenetic protein |
| BACH1 | BTB domain and CNC homolog 1 |
| BLVRB | biliverdin reductase B |
| CDDO-Im | 2-cyano-3,12 dioxooleana-1,9 dien-28-imidazolide |
| CKD | chronic kidney disease |
| CP | ceruloplasmin |
| CPgen III | coproporphyrinogen III |
| Cul2/3 | cullin-based E3 ligase 2/3 |
| DCYTB | duodenal cytochrome B |
| E2 | E2 ubiquitin-conjugating enzyme |
| EPO | erythropoietin |
| ERFE | erythroferrone |
| FBXL5 | Fbox/LRR-repeat protein 5 |
| FECH | ferrochelatase |
| FIH | factor inhibiting HIF |
| FTH1 | ferritin heavy chain |
| FTL | ferritin light chain |
| Ga | billion years ago |
| GLUT1/3 | glucose transporter 1/3 |
| GPX4 | glutathione peroxidase 4 |
| GST | glutathione S-transferase |
| HEPH | hephaestin |
| HFE | homeostatic iron regulator |
| HIF | hypoxia-inducible factor |
| HMOX1 | haem oxygenase 1 |
| HRE | hypoxia response elements |

| | |
|---|---|
| HRG1 | haem transporter (SLC48A1) |
| IRE | iron responsive elements |
| IRP | iron regulatory proteins |
| KEAP1 | Kelch-like ECH-associated protein 1 |
| LSEC | liver sinusoid endothelial cell |
| MAF | musculoaponeurotic fibrosarcoma |
| MRP | multidrug resistance-associated protein |
| NQO1 | NAD(P)H quinone dehydrogenase 1 |
| NRF2 | NF-E2 p45-related factor 2 |
| NTBI | non-transferrin-bound iron |
| PDK1 | pyruvate dehydrogenase kinase 1 |
| PHD | prolyl hydroxylase domain |
| PPIX | protoporphyrin IX |
| Pro | prolyl residue |
| RBC | red blood cells |
| RLS | reactive lipid species |
| ROS | reactive oxygen species |
| SMAD | Small Mothers Against Decapentaplegic |
| Tf | transferrin |
| TFR | transferrin receptor |
| UTR | untranslated regions |
| VEGF | vascular endothelial growth factor |
| VHL | von Hippel-Lindau |
| ZIP14 | ZRT/IRT-like protein |

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Figure legends

Figure 1. Redox regulation of the NRF2 and HIF signalling pathways. Although NRF2 and HIF1/2a are constitutively synthesized, they are rapidly degraded through the proteasome protein degradation pathway under normoxia/unstressed conditions. According to the 'hinge and latch' regulation model, the KEAP1 homodimer binds NRF2 at two different sites. The two-site binding prompts the formation of a KEAP1-Cul3 E3 ubiquitin ligase complex, which is responsible for the polyubituitination and protasomal degradation of NRF2. Proteasomal degradation of HIFa requires HIFa prolyl-4-hydroxylation by oxygen- and iron-dependent prolyl hydroxylase domain (PHD) dioxygenases (PHD1-3). The von Hippel-Lindau (VHL) protein binds to hydroxylated HIFa containing Pro–OH and recruits an ubiquitin E3 ligase. The VHL-Cul2 E3 ubiquitin ligase promotes the polyubiquitination of HIFa, prompting its degradation by the 26S proteasome.

Under oxidative/electrophilic stresses, reactive cysteine residues on KEAP1 are covalently modified by reactive oxygen species (ROS) or reactive lipid species (RLS), which induces conformational changes that lead to detachment of the weak-binding motif (latch) from KEAP1, while the strong-binding motif (hinge), remains attached. This allows NRF2 to escape polyubiquitylation and to translocate to the nucleus, where it dimerizes with one of the small musculoaponeurotic fibrosarcoma (MAF) proteins to promote the transcription of cytoprotective genes containing antioxidant response elements (ARE) in the promoter region (e.g. glutamate-cysteine ligase catalytic/modifier subunits, *GCLC/M*; glutathione S-transferase, *GST*;

multidrug resistance-associated proteins, *MRP*; NAD(P)H quinone dehydrogenase 1, *NQO1*).

Under hypoxic conditions, prolyl hydroxylation of HIF is inhibited, and HIFa rapidly accumulates and translocates to the nucleus, where it forms a heterodimer with HIFb, recruits the coactivator protein p300 and binds to hypoxia response elements (HRE). This activates the transcription by RNA polymerase II of target genes involved in processes such as erythropoiesis (e.g. erythropoietin, *EPO*), angiogenesis (e.g. vascular endothelial growth factor, *VEGF*), and glucose metabolism (e.g. glucose transporter 1/3, *GLUT1/3*; pyruvate dehydrogenase kinase 1, *PDK1*). Cul2/3, cullin-based E3 ligase 2/3; E2, E2 ubiquitin-conjugating enzyme; Pro, prolyl residue.

Figure 2. Regulation of cellular iron homeostasis by iron regulatory proteins (IRPs). IRP1 and IRP2 bind to stem loop structures called iron responsive element (IREs) present in either the 5' untranslated regions (UTR) or 3' UTR of mRNAs, thus regulating their translation or stability, respectively. When cellular iron is sufficient, IRP1 functions a cytosolic aconitase with no IRE-binding activity, and IRP2 is degraded by the proteasome. The lack of IRP binding to IREs favours the translation of mRNAs containing an IRE in the 5' UTR, while mRNAs containing IREs in the 3' UTR remain unstable. In conditions of iron depletion, loss of an iron-sulfur cluster causes a structural change in IRP1, which becomes and IRE-binding protein, and IRP2 accumulates. IRP binding to IREs in the 3' UTR stabilizes mRNAs, thus increasing translation of proteins involved in iron uptake such as transferrin receptor 1 (TFR1) and divalent metal transporter 1 (DMT1); on the other

hand, IRP binding to an IRE located in the 5' UTR of mRNAs represses translation of ferritin heavy and light chains (FTH1 and FTL) or ferroportin (FPN1), which favours iron release from intracellular stores and its retention within the cell. Hypoxia-inducible factor-2α (HIF2a) is also subject to regulation by the IRE/IRP system.

Figure 3. Overview of systemic iron homeostasis. Since iron is not actively excreted, iron homeostasis is dictated by a balance between absorption, utilization, recycling and storage. Dietary iron absorption occurs at the brush-border membrane of duodenal enterocytes. Elemental iron is transported across the membrane via divalent metal transporter 1 (DMT1) after reduction of ferric iron (Fe3+) to ferrous iron (Fe2+) by duodenal cytochrome B (DCYTB) at the apical membrane. Dietary haem is taken up via an unknown transport system, after which iron is released inside the cell by haem oxygenase 1 (HMOX1). Iron is exported into the bloodstream via ferroportin (FPN1) and its loading onto transferrin (Tf) requires oxidation of Fe2+ to Fe3+ by hephaestin (HEPH) at the basolateral side. The other major suppliers of iron to the plasma are splenic reticulo-endothelial macrophages, which recycle iron from senescent red blood cells (RBC). Haem iron is released by HMOX1 and it can be either stored in ferritin or exported back to the circulation via FPN1, a process that is coupled with the re-oxidation of the metal mediated by ceruloplasmin (CP). Plasma Tf delivers iron to tissues. Diferric transferrin (Tf-Fe2) binds to transferrin receptor 1 (TFR1) and the complex is endocytosed into the cell. Erythropoiesis is the major consumer of iron, which is required for the hemoglobinization of new RBCs. Iron storage

occurs primarily in the liver. Hepatocytes take up both Tf-Fe2 and non-transferrin-bound iron (NTBI) via TFR1 and ZRT/IRT-like protein (ZIP14), respectively. Iron surplus is stored in ferritin; when iron demand increases, the metal can be exported through FPN1. Hepatocytes are also important sensors of both tissue iron, via the binding of bone morphogenic proteins (BMPs) synthesized by liver sinusoidal endothelial cells to BMP receptors in the hepatocyte membrane, as well as plasma iron. This involves the formation of an iron-sensing complex including hemojuvelin (HJV), neogenin (NEO), transferrin receptor 2 (TFR2), and iron homeostatic protein (HFE), which is displaced from TFR1 upon binding of Tf-Fe2. The activation of the iron-sensing complex causes phosphorylation of SMAD1/5/8, which binds to SMAD4 and translocates to the nucleus to induce hepcidin (*HAMP*) transcription. Hepcidin, which is also activated by inflammation via the Janus kinase (JAK)/Signal transducer and transcription activator 3 (STAT3) pathway (not depicted here), binds to FPN1 and promotes its internalization and degradation. As a result, dietary iron absorption is inhibited and iron is retained within reticuloendothelial macrophages, which decreases circulating iron levels.

Figure 4. NRF2-mediated regulation of haem synthesis and haem-iron recycling. Senescent erythrocytes are engulfed by reticuloendothelial macrophages. Haem is released within the phagolysosome and exported into the cytoplasm by the haem transporter HRG-1 (SLC48A1, HRG1). In the cytosol, haem oxygenase 1 (HMOX1) converts haem into biliverdin, which releases iron and carbon monoxide. Haem-derived iron is either stored in a

ferritin shell or exported into the blood circulation via FPN1. Biliverdin reductase B (BLVRB) converts biliverdin to bilirubin, which is exported into the bloodstream and eventually excreted in bile and urine. The expression of *HMOX1* and *FPN1* is transcriptionally regulated by NRF2, upon haem-induced inactivation of the repressor protein BTB domain and CNC homolog 1 (BACH1). *HRG1*, *BLVRDB*, and ferritin heavy (*FTH1*) and light (*FTL*) chains are also transcriptional targets of NRF2. In hepatocytes, NRF2 regulates the transcription of genes involved in the haem biosynthetic pathway, including: ATP binding cassette subfamily B member 6 (*ABCB6*), a membrane-associated protein that transports coproporphyrinogen III (CPgen III) from the cytosol to the mitochondrial intermembrane space; and ferrochelatase (*FECH*), an enzyme located in the mitochondrial intermembrane space, which is responsible for the incorporation of ferrous iron into the protoporphyrin IX (PPIX) ring in the last step of haem synthesis. TFR1, transferrin receptor 1.

Figure 5. NRF2-mediated regulation of iron homeostasis via BMP6 and hepcidin. Systemic iron overload leads to hepatic iron accumulation and production of reactive oxygen species (ROS) in the mitochondria of hepatocytes and liver sinusoid endothelial cells (LSECs). Iron-induced, mitochondria-derived reactive lipid species (RLS) are responsible for NRF2 activation and nuclear translocation. In LSECs, NRF2 activates the expression of Bone Morphogenetic Protein 6 (BMP6), which is secreted and induces hepcidin expression in neighbouring hepatocytes through the Small Mothers Against Decapentaplegic (SMAD) pathway. Increased hepcidin synthesis inhibits dietary iron absorption and the release of iron from

erythrophagocytosing macrophages into the bloodstream. In hypoxia/anaemia (e.g. non-transfusion-dependent thalassaemia), hypoxia-inducible factor-2α (HIF2a) stabilization causes erythropoietin (EPO)-induced erythroblast expression of erythroferrone (ERFE) and concomitant suppression of hepcidin synthesis; NRF2 activation counteracts the inhibition of BMPs (including BMP6) by ERFE, thus increasing hepcidin expression in the liver and decreasing serum iron. In hepatocytes, NRF2 activation increases the transcription of cytoprotective genes such as NAD(P)H:quinone Oxidoreductase 1 (*NQO1*), Glutamate-Cysteine Ligase Catalytic Subunit (*GCLC*) or Glutathione S-Transferase Alpha 1 (*GSTA1*), which increase the cell capacity to deal with iron-induced ROS and RLS.

Figure 6. HIF2a-mediated regulation of iron homeostasis. Although HIF2a is constitutively synthesized in peritubular fibroblasts present in the renal cortex and in duodenal enterocytes, it is rapidly degraded under normoxic conditions. The degradation is mediated by oxygen- and iron-dependent prolyl hydroxylases (PHDs). Under hypoxia and/or iron deficiency, PHDs fail to hydroxylate HIF2a, which accumulates in kidney cells. As a result, erythropoietin (EPO) synthesis increases, which stimulates erythropoiesis in the bone marrow. Erythroid precursor cells produce increased amounts of erythroferrone (ERFE), which binds to bone morphogenetic proteins (BMPs) and represses hepcidin synthesis by the liver. Reduced hepcidin levels result in increased dietary iron absorption and its release from iron-recycling macrophages in spleen. Notably, HIF2a activation is further adjusted to iron availability: if iron is insufficient, the iron regulatory protein-1 (IRP1) binds to

an iron-responsive element (IRE) in the HIF2a mRNA 5' untranslated region (UTR), which inhibits HIF2a protein translation. Likewise, tissue hypoxia or iron deficiency also increase iron absorption in the intestine. As for the kidney, loss of PHD activity results in HIF2a stabilization in duodenal enterocytes. This increases transcription of HIF2a target genes, including the ferrireductase duodenal cytochrome b (*DCYTB*) and the divalent metal transporter 1 (*DMT1*), which are involved in the uptake of elemental iron at the intestinal brush border, and ferroportin (*FPN1*), which mediates the export of iron into the bloodstream. In circulation, iron binds transferrin, which is also a HIF-regulated gene. Intestinal HIF2a is regulated by the hepcidin/ferroportin axis, thus controlling dietary iron absorption. In iron overload conditions associated with deficient hepcidin production (e.g. haemochromatosis), FPN1 is highly expressed by duodenal enterocytes. This causes an intracellular iron deficiency, which inhibits PHDs and stabilizes HIF2a. Increased expression of *DCYTB*, *DMT1* and *FPN1* is responsible for increased dietary iron uptake. Noteworthy, HIF2a over-activation is prevented by the binding of IRP-1 to the IRE in its 5' UTR.

Figure 1. Redox regulation of the NRF2 and HIF signalling pathways.

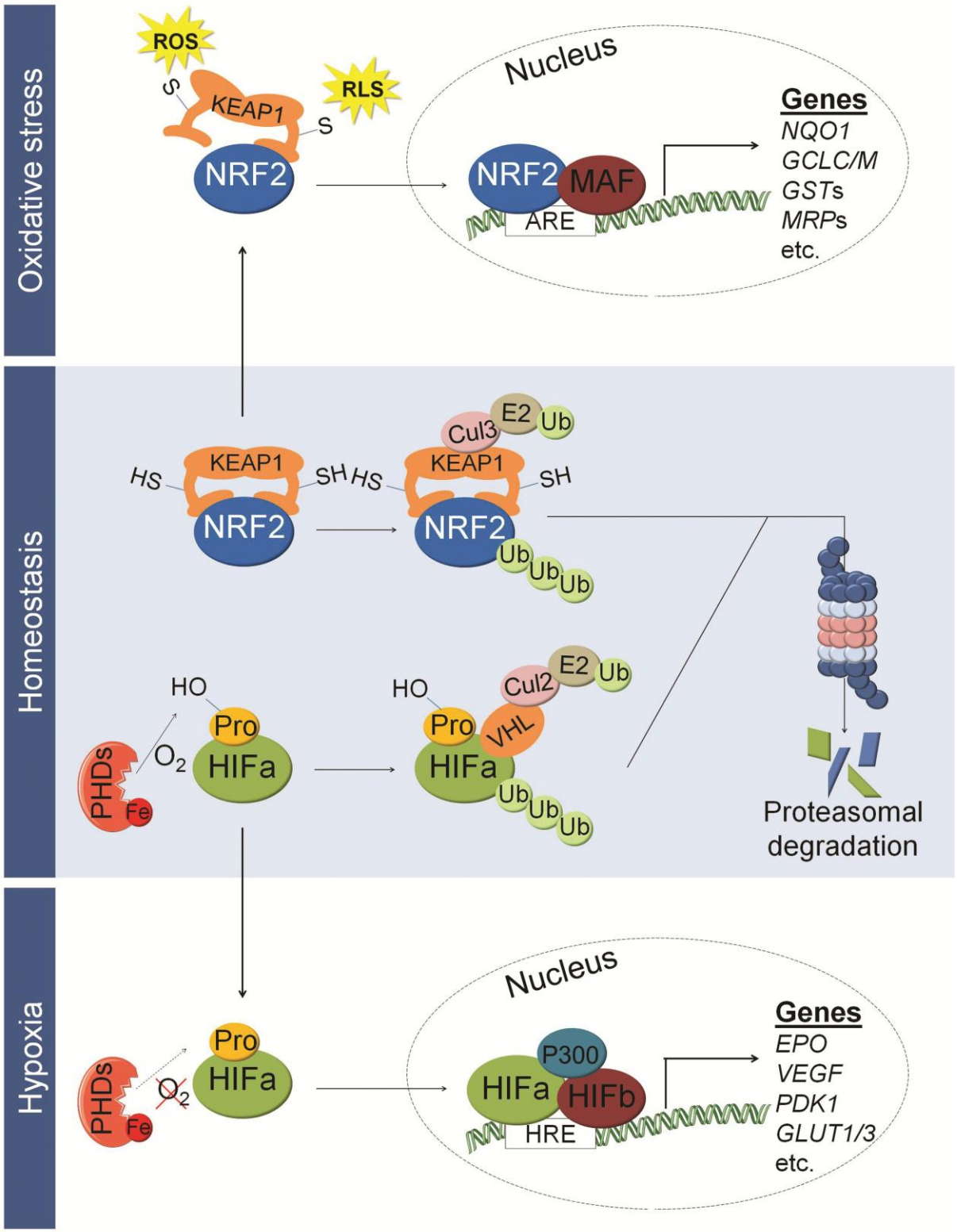

Figure 2. Regulation of cellular iron homeostasis by iron regulatory proteins (IRPs).

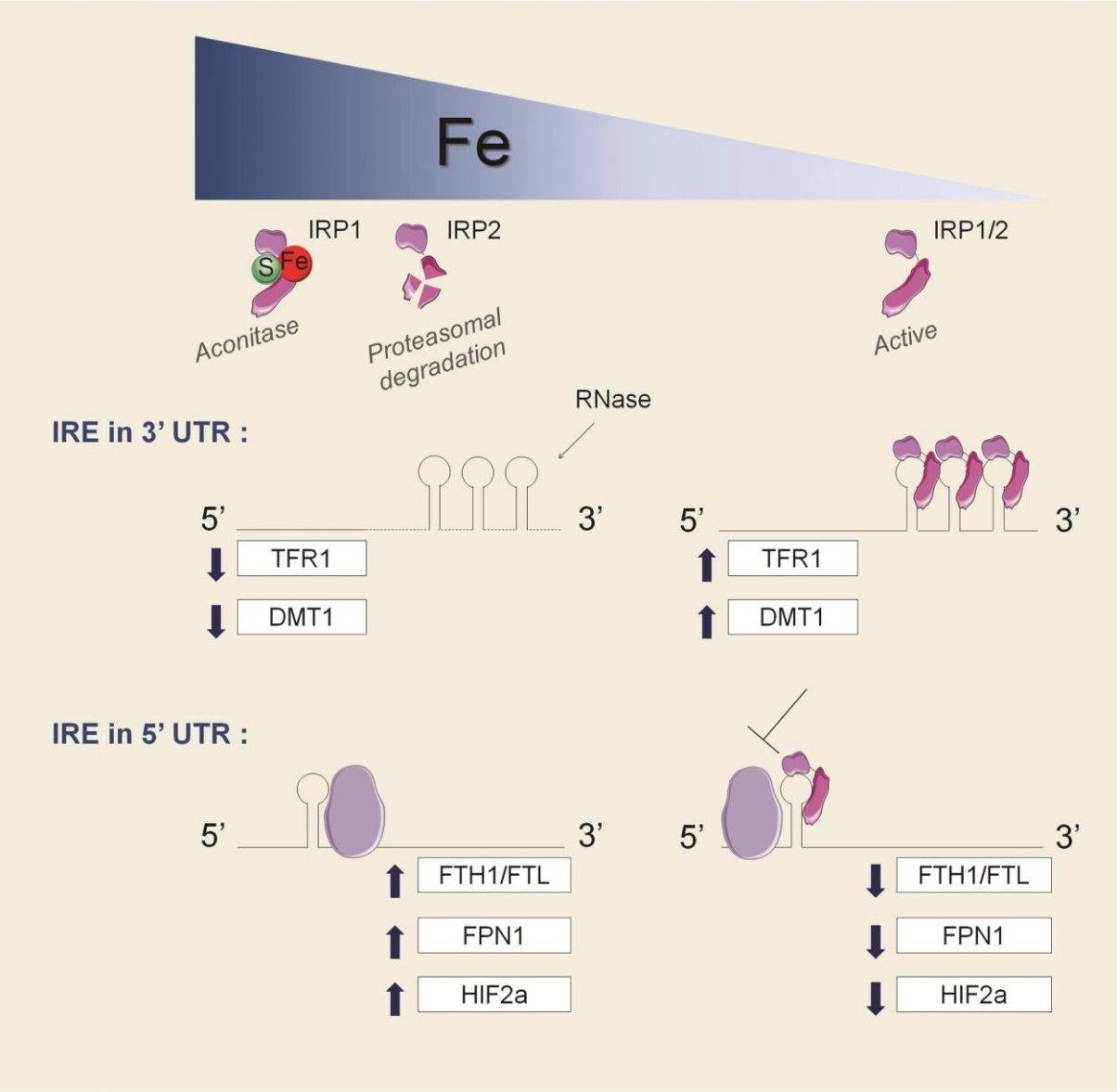

Figure 3. Overview of systemic iron homeostasis.

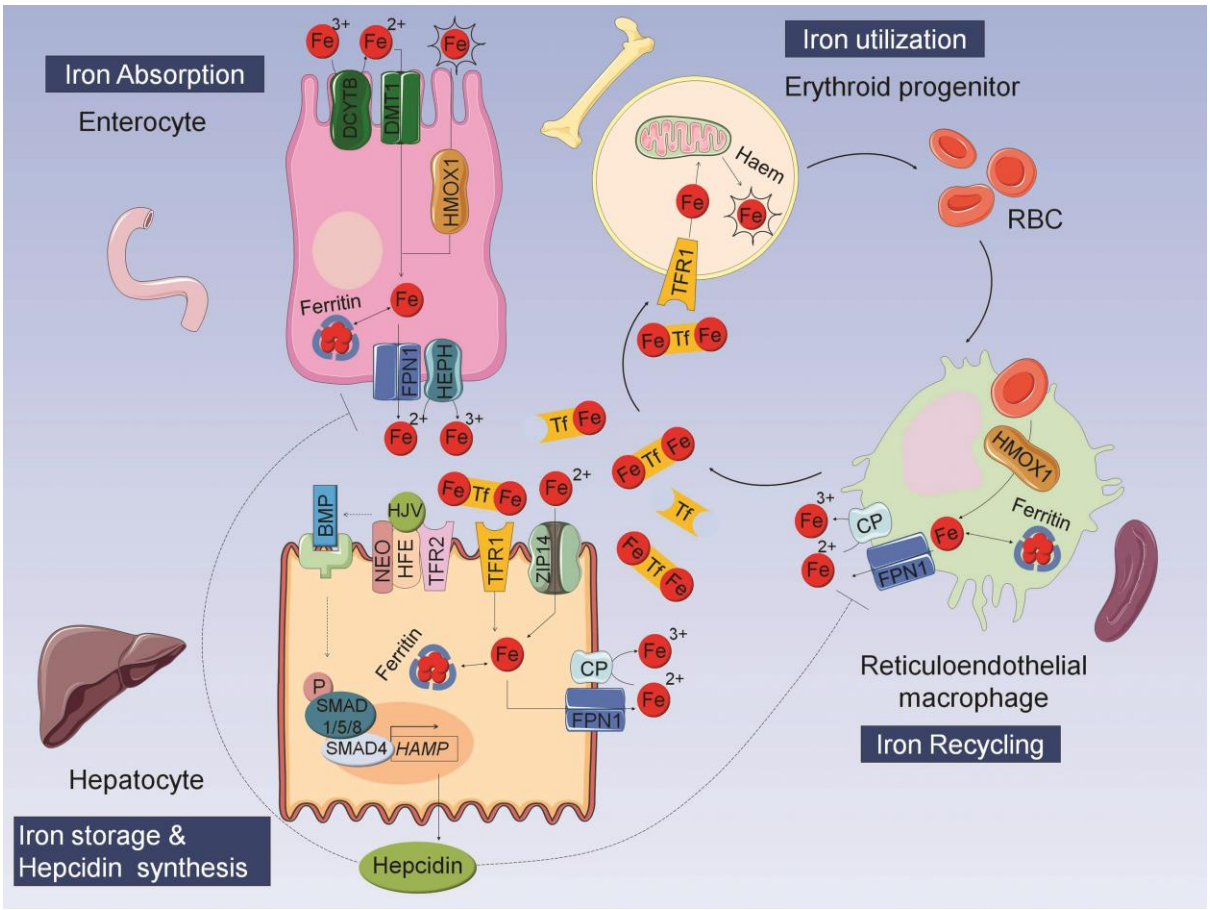

Figure 4. NRF2-mediated regulation of haem synthesis and haem-iron recycling.

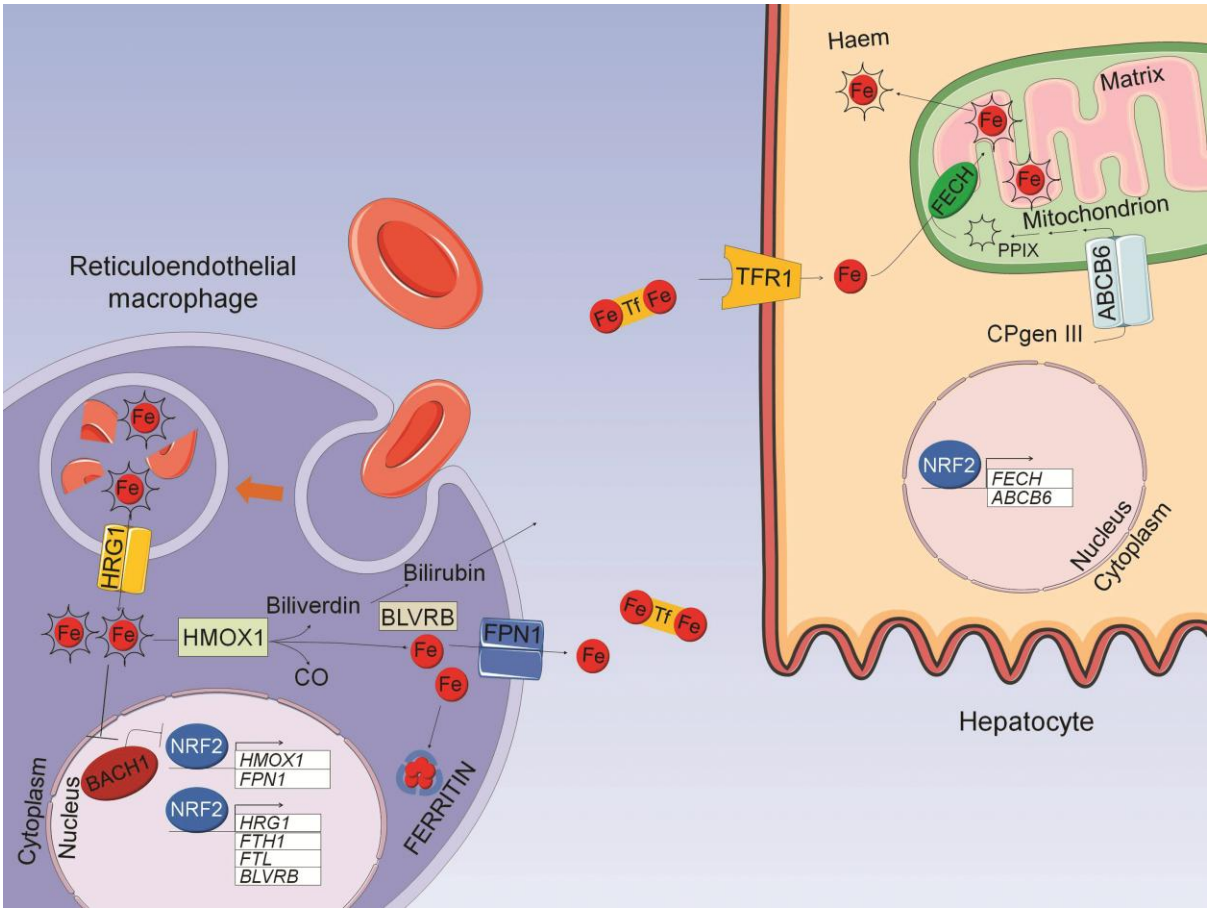

Figure 5. NRF2-mediated regulation of iron homeostasis via BMP6 and hepcidin.

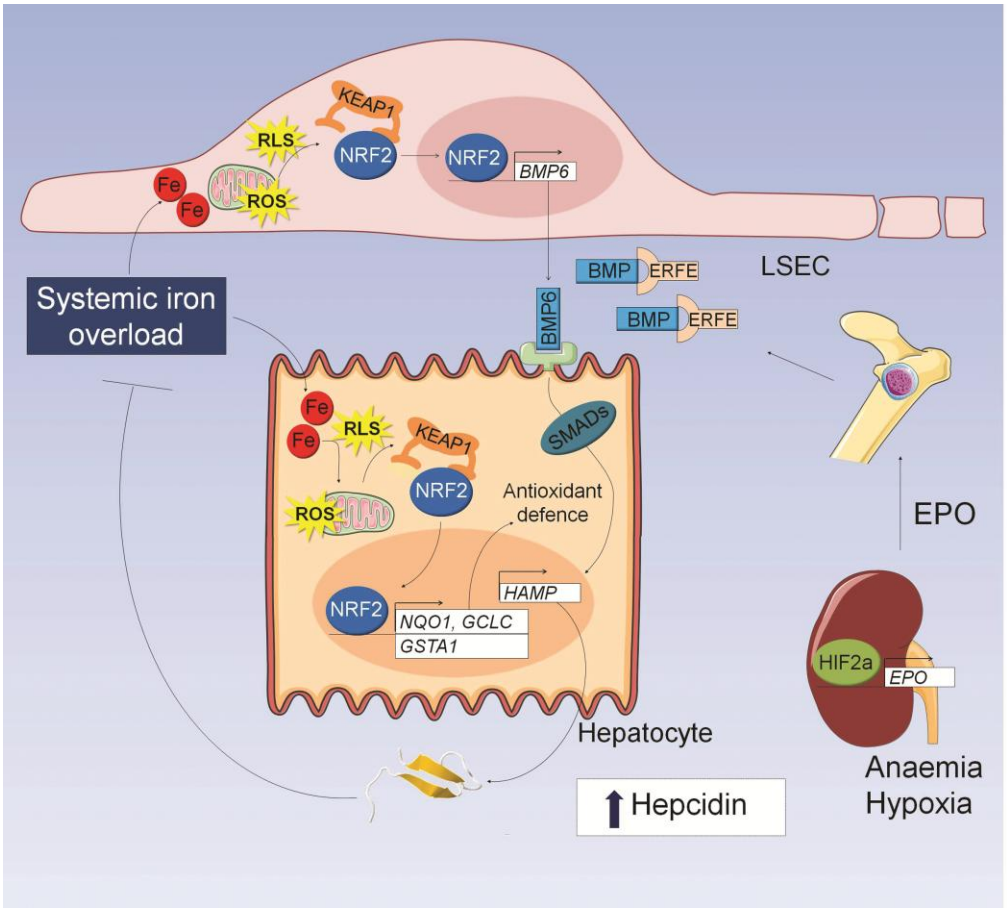

Figure 6. HIF2a-mediated regulation of iron homeostasis.