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Review

Cellular iron uptake, trafficking and metabolism: Key molecules and mechanisms and their roles in disease

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ABSTRACT

Iron is a crucial transition metal for virtually all life. Two major destinations of iron within mammalian cells are the cytosolic iron-storage protein, ferritin, and mitochondria. In mitochondria, iron is utilized in critical anabolic pathways, including: iron-storage in mitochondrial ferritin, heme synthesis, and iron–sulfur cluster (ISC) biogenesis. Although the pathways involved in ISC synthesis in the mitochondria and cytosol have begun to be characterized, many crucial details remain unknown. In this review, we discuss major aspects of the journey of iron from its initial cellular uptake, its modes of trafficking within cells, to an overview of its downstream utilization in the cytoplasm and within mitochondria. The understanding of mitochondrial iron processing and its communication with other organelles/subcellular locations, such as the cytosol, has been elucidated by the analysis of certain diseases *e.g.*, Friedreich's ataxia. Increased knowledge of the molecules and their mechanisms of action in iron processing pathways (*e.g.*, ISC biogenesis) will shape the investigation of iron metabolism in human health and disease.

1. Overview of mammalian iron metabolism

Iron is vital for cellular survival, as demonstrated by cell death following excessive iron depletion [1–3]. Adult humans contain ~3–5 g of iron, up to 80% of which is found in erythrocyte hemoglobin, while a further ~20% is stored within macrophages and hepatocytes [2,4]. Cellular iron storage typically occurs within protein nanocages created by ferritin [5]. The remainder of the iron is present in other heme-containing proteins (*e.g.*, cytochromes), iron–sulfur cluster (ISC)-containing proteins (*e.g.*, succinate dehydrogenase) [6,7] and non-heme/non-ISC iron-containing proteins (*e.g.*, iron- and 2-oxoglutarate-dependent dioxygenases) [8,9].

Improperly sequestered iron catalyzes toxic reactive oxygen species (ROS) through Fenton and Haber-Weiss-type reactions [2]. As too much or too little iron is detrimental, iron homeostasis is tightly controlled through the regulation of its import, storage and efflux [2,10–12].

1.1. Two major routes of cellular iron uptake

Non-heme iron is transported into cells by two mechanisms: transferrin (Tf)-bound iron uptake and non-Tf-bound iron (NTBI) uptake (Fig. 1). Under physiological conditions, almost all iron in the circulation is bound to Tf [2,11]. However, in diseases resulting in iron overload, Tf becomes saturated with iron, with excess plasma iron occurring as NTBI [2]. The exact uptake route(s) for NTBI remains unclear, but appears to involve one or more cell surface ferrireductases (*e.g.*, duodenal cytochrome *b*, DCYTB [13]) or the release of cellular reductants, such as ascorbate [14–17] (Fig. 1). These enzymes reduce ferric NTBI to its ferrous state that can then be imported by transporters such as the transmembrane protein, divalent metal transporter 1 (DMT1) [18], or the ZRT/IRT-like proteins (ZIPs), ZIP14 or ZIP8 [19].

Under physiological circumstances, the major iron uptake route utilized by most cells involves Tf-bound iron, which is internalized by receptor-mediated endocytosis after binding to transferrin receptor 1 (TfR1) [11,12] (Fig. 1). Ferric iron is released from Tf within the endosome after its acidification and is then reduced by an endosomal ferrireductase (*e.g.*, six transmembrane epithelial antigen of the prostate 3 [STEAP3] [20]) [12], or by an novel mechanism involving cellular

Abbreviations: ABC, ATP-binding cassette; ALAS, δ-aminoleuvulinate synthase; CIA, cytosolic ISC protein assembly; DCYTB, duodenal cytochrome *b*; DMT1, divalent metal transporter 1; FBXL5, F-box and Leucine-rich Repeat Protein 5; FIH1, factor inhibiting HIF 1; FPN1, ferroportin 1; FTL, L-ferritin; FTH1, H-ferritin; HIF, hypoxia-inducible factor; FDXR, ferredoxin reductase; FDX1/2, ferredoxins 1 and 2; FRDA, Friedreich's ataxia; FTMT, mitochondrial-specific ferritin; GLRX5, glutaredoxin 5; IMM, inner mitochondrial membrane; IRP, iron regulatory protein; IRE, iron responsive element; ISC, iron–sulfur cluster; ISCU, ISC assembly enzyme; KH, K homology; LIP, labile iron pool; LYR, leucine–tyrosine–arginine; LYRM, LYR motif; LYRM4, LYR motif-containing 4; MFRN, mitoferrin; NCOA4, nuclear receptor coactivator 4; NFS1, NFS1 cysteine desulfurase; NTBI, non-transferrin-bound iron; OMM, outer mitochondrial membrane; PCBP, poly (rC)-binding protein; PHD, prolyl-4-hydroxylase domain-containing iron-dependent prolyl hydroxylase; PPIX, protoporphyrin IX; ROS, reactive oxygen species; SDHAF1, succinate dehydrogenase assembly factor 1; SDHB, succinate dehydrogenase subunit B; STEAP3, six trans-membrane epithelial antigen of the prostate 3; Tf, transferrin; TfR1, transferrin receptor 1; UTR, untranslated region; VDAC, voltage-dependent anion channel; VHL, von Hippel–Lindau protein; ZIP, ZRT/IRT-like protein

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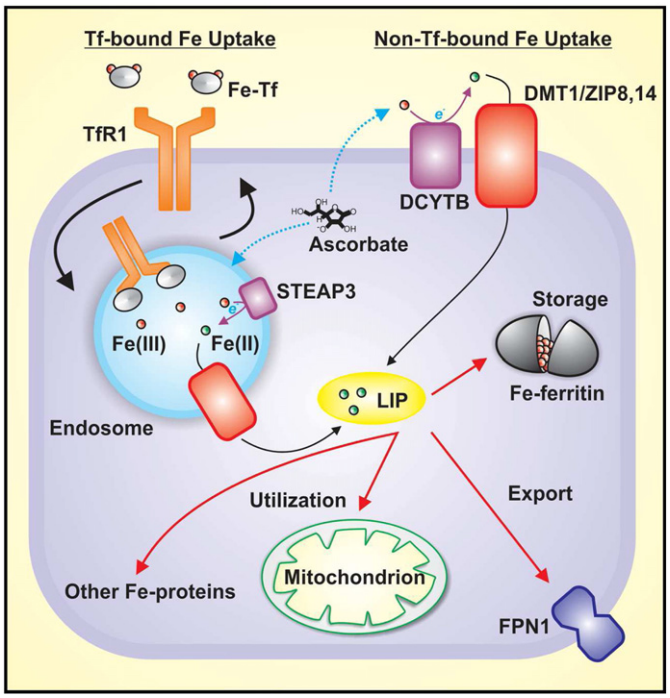

Fig. 1. Two major cell iron (Fe) uptake pathways, namely transferrin (Tf)-bound Fe uptake and non-Tf-bound Fe (NTBI) uptake. Under physiological conditions, most Fe is bound to Tf which binds to the transferrin receptor 1 (TfR1) on the cell surface that is then involved in receptor-mediated endocytosis with the Fe being released from Tf by a decrease in endosomal pH and reduction by an endosomal reductase [*e.g.*, six transmembrane epithelial antigen of the prostate 3 (STEAP3)] or, potentially, by ascorbate. The Fe(II) is then transported across the endosomal membrane by divalent metal transporter 1 (DMT1) where it then becomes part of the poorly characterized labile iron pool (LIP) in the cytosol. Iron in the LIP acts as an intermediate and can be utilized for storage in the iron storage protein, ferritin, or used for synthesis of heme and iron-sulfur clusters in the mitochondrion or cytosol. Iron can also be exported from the cell by ferroportin 1 (FPN1). In conditions of iron overload, NTBI exists in the blood, and may be taken up by processes that include cell surface reduction by ferrireductases such as duodenal cytochrome *b* (DCYTB), or by effluxed reductants, such as ascorbate. Intracellular ascorbate can also supply electrons for DCYTB-dependent ferrireduction. Such enzymes reduce ferric NTBI to its ferrous state, which can be imported from the plasma membrane by transporters such as DMT1.

ascorbate [14,21,22]. Similarly to NTBI uptake, this ferrireduction is followed by transport of the resulting ferrous iron across the endosomal membrane by DMT1 [18] or ZIP14 [19]. This nascent cytosolic iron then becomes part of a poorly characterized chelatable or labile iron pool (LIP) and can be utilized for metabolism, stored in ferritin or released back to the extracellular space [11,12] (Fig. 1).

1.2. Cytosolic iron trafficking

Tf-bound iron and NTBI entering the transitory LIP is either: *(i)* stored in ferritin; *(ii)* utilized by downstream metabolic pathways (*e.g.*, imported into mitochondria for usage in ISC and heme synthesis, and/or incorporated in cytoplasmic iron-requiring proteins); or *(iii)* released from the cell by the ferrous iron exporter, ferroportin 1 (FPN1) (Fig. 1).

In non-erythroid cells, the majority (*i.e.*, 70–80%) of this newly imported iron is incorporated into ferritin [5]. Ferritin is a multimeric protein composed of 24 subunits that forms a hollow sphere capable of storing ~4,500 iron atoms as a mineralized ferric, phosphate and hydroxide core [5,23,24]. In mammals, there are two ferritin subunits: H-ferritin (heavy subunit, encoded by *FTH1*) and L-ferritin (light subunit, encoded by *FTL*), which hetero-polymerize to form different isoferritins with tissue specific distributions [5,23,24]. Iron(II) entering ferritin is oxidized to iron(III) by the ferroxidase activity of H-ferritin in an oxygen-dependent manner [5,23,24]. Subsequently, iron(III) is transported to the protein cavity, where core formation commences at carboxyl groups on glutamates of L-ferritin, which is devoid of ferroxidase activity [5,23,24]. This enclosure and sequestration of iron as ferrihydrite is vital, as it prevents toxic redox reactions from occurring [5,23,24].

Ferritin typically releases iron in a controlled manner under *in vivo* conditions by targeted autolysosomal proteolysis, although proteasomal degradation of the protein can occur under specific conditions [5,25]. The targeting of ferritin for autophagic turnover (*i.e.*, ferritinophagy) has recently been shown to involve nuclear receptor coactivator 4 (NCOA4), which binds to ATG8 proteins on forming autophagosomes and recruits ferritin as a cargo molecule [26]. Considering that autophagocytosed macromolecules and organelles, such as ferritin and mitochondria, contain relatively high levels of iron, active lysosomal compartments are rich in this metal [27]. Interestingly, studies have suggested the possibility that the lysosome may also be involved in iron storage, due to the expression of various membrane metal transport proteins on lysosome-like organelles [28].

1.3. Intracellular iron transport: low-molecular weight (M_r) iron pool or directed protein–protein transfer?

While the nature of the LIP may consist of low-M_r iron complexes, accumulating evidence supports the hypothesis that iron is typically transported in a direct manner *via* a complex of interacting proteins, in which the binding of iron to each component acts as an intermediate that minimizes cytotoxic redox chemistry within the oxygen-rich environment of the cell [3,29,30]. Indeed, a putative protein-containing high-M_r intermediate that appeared to donate its iron to ferritin has been observed after incubation of K562 cells with Tf-^{59}Fe [30].

More recently, studies have demonstrated a chaperone role for human poly (rC)-binding proteins 1-4 (PCBPs 1-4) in iron transport to ferritin and other non-heme iron requiring proteins [9,31–33]. The PCBPs are members of the heterogenous nuclear ribonucleoprotein family, and is comprised of PCBPs 1-4. These proteins are classical RNA/DNA-binding proteins involved in diverse processes such as splicing, transcript stabilization and translational regulation [34]. In 2008, PCBP1 was identified as being crucially involved in ferritin iron mineralization through functional screening of a human liver cDNA library in yeast [35]. In this assay, it was identified that PCBP1 and human ferritin coexpression in naturally ferritin deficient yeast cells caused an iron deficiency response, indicating PCBP1-dependent ferritin iron loading [35]. Further biochemical, biophysical and genetics experiments have provided support for the role of PCBP1 as an iron chaperone for ferritin [31,35].

As novel iron chaperones, the PCBPs 1-4 can assist in the delivery of iron to the cytosolic iron storage protein, ferritin, in mammalian cells [31,35]. However, while PCBPs 1-3 can directly bind ferritin, PCBP4 appears to act indirectly, perhaps as an intracellular iron buffer for the other PCBPs [8,31]. Indeed, iron-loaded PCBP1 is able to bind to ferritin *in vivo* and *in vitro* in multiple molar equivalents with low micromolar affinity, as was determined from isothermal titration calorimetric studies [31,36]. This binding affinity of iron-PCBP1 for ferritin is much higher than the binding affinity of iron(II) for ferritin or the affinity of apo-PBCP1 for ferritin [31,35]. The binding of iron-loaded PCBP1/2 to ferritin may act to increase the efficiency of iron loading into nascent ferritin polymers [31].

PCBP 1, and probably PCBP2 [8], appears to be able bind Fe(II) in a six coordinate oxygen/nitrogen ligand environment [36]. PCBP1 can bind a total of three iron atoms, in which the first iron atom has a dissociation constant (K_d) of 0.9 ± 0.1 μM and a mean K_d of 5.8 ± 0.3 μM for the remaining two iron atoms [9,32,35]. Iron-loaded PCBP1 exhibits a stoichiometry of binding of approximately nine iron-PCBP1 units per ferritin oligomer [31]. Given that the number of putative iron delivery channels is eight per ferritin polymer of 24 subunits, this stoichiometry of PCBP1 binding to ferritin supports a model of iron delivery *via* direct binding of iron-PCBP1 molecules near the iron delivery channels formed by the

three-fold axes of symmetry [8]. Interestingly, iron-PCBP2 binds to ferritin in a lower stoichiometric ratio of 4:1 iron-PCBP2:ferritin [31].

The functions of PCBPs 1 and 2 appear to be non-redundant in the delivery of iron to ferritin [8]. PCBP1 and PCPB2 can self-associate as well as form complexes with other PCBPs and other RNA-binding proteins [9,32]. Although PCBP1 and PCBP2 can be detected as a complex in human cells, their association appears to be relatively unstable [31,37]. Importantly, both PCBP1 and PCBP2 are essential for the formation of a stable hetero-oligomeric complex with ferritin [31]. This has been demonstrated in studies in which the interaction between ferritin and PCBP1 or PCBP2 is reduced upon PCBP2 or PCBP1 depletion, respectively [8,31,37]. That is, depletion of either one of the PCBPs reduces the binding of the other paralog to ferritin, suggesting that the hetero-oligomer of PCBP1 and PCBP2 can more stably bind ferritin compared to either the homo-oligomer or monomeric PCBPs [31].

The requirement for both PCBPs in binding to ferritin and delivery of iron to this protein may relate to the recent finding that PCBP2 is a DMT1-binding partner that regulates iron influx from Tf-iron across the endosomal membrane to the cytosol [33]. As PCBP2 binds iron and associates with ferritin to deliver iron [8,31], this may be the first direct evidence of an iron-transport complex. Further studies are required to assess this possibility.

PCBPs 1 and 2 also appear to mediate iron acquisition by several members of a class of iron- and 2-oxoglutarate (2-OG)-dependent dioxygenases, which form a large family of non-heme iron enzymes that depend on the insertion of a single iron atom into their active site [8]. The enzymes identified as putative recipients for PCBP-mediated iron delivery are the mono-nuclear iron-center hypoxia inducible factor 1α (HIF1α) prolyl and asparaginyl hydroxylases, prolyl hydroxylase 2 and factor inhibiting HIF, respectively, as well as the dinuclear iron center of deoxyhypusine hydroxylase [9,31,32]. For a recent review see Philpott and Ryu [8].

1.4. Control of cellular iron homeostasis

Cellular iron homeostasis is tightly regulated by a post-transcriptional mechanism that modulates the synthesis of key iron metabolism proteins involved in iron uptake, storage and release [1,10]. Specifically, the iron regulatory protein (IRP)-iron responsive element (IRE) system is responsible for this post-transcriptional regulation, and allows for rapid alterations in the synthesis of key iron metabolism proteins in response to intracellular iron levels [1,10,38]. This system depends on the mRNA-binding proteins, IRPs 1 and 2, which post-transcriptionally control the expression of mRNAs possessing IREs [1,10,38] (Fig. 2A). IRPs bind to IREs in the 5′- or 3′ untranslated regions (UTRs) of key mRNAs involved in iron metabolism with high affinity in iron-depleted cells, either suppressing the translation of the mRNA (*i.e.*, mRNAs in which the IRE is located in the 5′ UTR; *e.g.*, *FTH1*, *FTL*, *FPN1 etc.*), or by enhancing mRNA stability against nuclease attack

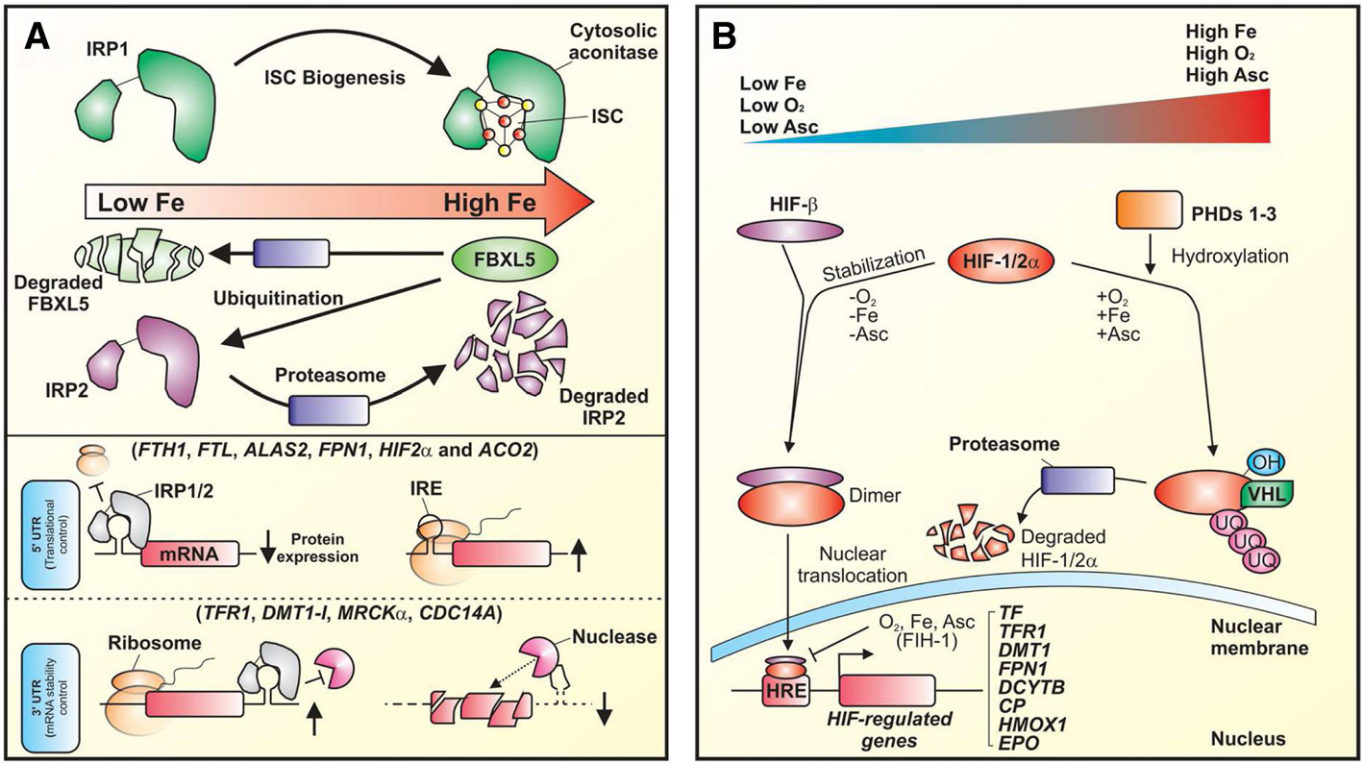

Fig. 2. Cellular iron homeostasis. There are two major regulatory mechanisms mediated by: (A) iron regulatory proteins 1 and 2 (IRPs 1 and 2); and (B) hypoxia inducible factor 1 and 2 (HIF1 and 2). (A) The IRP/iron responsive element (IRE) system regulates the translation of proteins involved in iron storage [H-ferritin (FTH1) and L-ferritin (FTL)], iron export (FPN1), iron uptake (TfR1, DMT1, MRCKα), the mitochondrial citric acid cycle (ACO2), mitochondrial heme synthesis (ALAS2), oxygen sensing (HIF2α) and cell cycle control (CDC14A). Under conditions of low iron, the IRPs are in their IRE-binding forms. Under conditions of high iron, the IRE-binding activity of the IRPs is decreased. IRP1 acquires an iron-sulfur cluster (ISC), which converts it into a cytosolic aconitase incapable of binding IREs, while IRP2 is targeted for degradation by the proteasome by the FBXL5 E3 ligase complex. When intracellular iron is low, the binding of IRP1 or IRP2 to IREs in the 5′-UTR of select mRNAs inhibits translation (↓) of the mRNA, while the binding of IRPs to IREs in the 3′-UTRs protects the transcripts against nuclease-mediated degradation, leading to increased translation (↑). When cellular iron is high, there is decreased IRP–IRE binding, and the translation of mRNAs possessing 5′ IREs is increased (↑), while mRNAs possessing 3′ IREs are degraded faster, leading to decreased protein translation (↓). (B) Cellular iron metabolism is also regulated at the transcriptional level by the hypoxia-inducible factor (HIF) system. Under conditions of high iron, O_2 and ascorbate, HIF1α and HIF2α are hydroxylated at specific proline residues by a class of prolyl hydroxylase domain proteins (PHDs 1–3). Prolyl hydroxylation targets HIF1/2α proteins for ubiquitination by the E3 ubiquitin ligase, von Hippel Lindau protein (VHL). Under conditions of low iron, O_2 and ascorbate concentrations, HIF1/2α proteins are stabilized and form hetero-dimers with HIF1β that translocate to the nucleus and activate the transcription of specific genes possessing hypoxia response elements (HREs). Additionally, high iron, O_2 and ascorbate levels increase hydroxylation activity of the asparaginyl hydroxylase known as factor inhibiting HIF 1 (FIH1), which inhibits HIF transcriptional activity.
Modified from (Lawen and Lane) [2].

(*i.e.*, mRNAs in which the IRE is located in the 3′-UTR; *e.g.*, *TfR1*, *DMT1-I etc.*) [10,12].

Under conditions of increased cellular iron, which can be potentiated by endogenous reductants such as ascorbate [14], IRP1 loses its IRE-binding activity by acquiring an ISC (4Fe–4S cluster) [38]. The acquisition of this 4Fe–4S cluster converts IRP1 into a cytosolic aconitase (Fig. 2A). In the case of IRP2, the iron-dependent, proteasomal degradation is the major regulatory mechanism [39]. On the other hand, IRP2, which is unable to acquire an ISC in response to increased cellular iron, is post-translationally regulated by proteasomal degradation [39] (Fig. 2A).

The major mechanism by which iron regulates proteasomal degradation of IRP2 is by targeting of IRP2 to a subunit of the SKP1-Cullin-1-F-box E3 ubiquitin ligase complex, namely F-box and Leucine-rich Repeat Protein 5 (FBXL5), which is post-translationally regulated by iron and oxygen [40,41]. FBXL5 contains a hemerythrin-like domain that is capable of binding iron, leading to stabilization of the protein, preventing proteasomal degradation [40,41]. This leads to a consequent loss of IRP2 due to increased ubiquitination and subsequent degradation of IRP2 [40,41] (Fig. 2A).

Regulation of cellular iron homeostasis is also controlled at the level of the transcription of iron metabolism genes. A major regulator of these changes in transcription is the HIF system, which includes the oxygen- and iron-regulated proteins, HIF1α and HIF2α [42] (Fig. 2B). Low oxygen tensions (*i.e.*, hypoxia), as well as low intracellular iron concentrations, activate HIF1 and HIF2-regulated transcription by the increased formation of heterodimers of HIF1α or HIF2α with the constitutively expressed HIF1β subunit [42]. HIF1α is ubiquitously expressed, while HIF2α has a more restricted tissue distribution [12,43a]. The HIFα/β heterodimers form transcription factors that regulate a range of genes encoding proteins important for cellular oxygen homeostasis and the response to hypoxia [42] (Fig. 2B).

Both HIF1α and HIF2α are post-translationally regulated through protein degradation or inhibition of their transcriptional activity in an oxygen- and iron-dependent manner [42] (Fig. 2B). This occurs by a specific class of 2-oxoglutarate-dependent dioxygenases: the prolyl-4-hydroxylase domain-containing iron-dependent prolyl hydroxylases (PHDs) 1-3, which regulate HIF1/2α degradation, and the asparaginyl hydroxylase, factor inhibiting HIF1 (FIH1), which inhibits HIF1α-complex transcriptional activity [43a]. Under conditions of iron repletion, these hydroxylases are fully active at the oxygen tensions found under typical cell-culture conditions (*i.e.*, 21%) [43a]. PHDs hydroxylate HIF1/2α proteins at specific proline residues, leading to proteasomal degradation, while FIH1 hydroxylates a conserved asparagine residue, resulting in inactivation of transcriptional activity [43a] (Fig. 2B). Importantly, it is the strict dependence of these hydroxylases on iron that allows cellular iron to modulate HIF-regulated gene expression [43a]. The hydroxylated α-subunits of HIF are targeted for ubiquitination by the elongin B/elongin C/cullin 2/von Hippel–Lindau (VHL) E3 ubquitin ligase (also known as CRL-2) subunit, VHL, which directs the proteins to be degraded by the proteasome (Fig. 2B) [42]. Importantly, HIF2α contains an IRE in its 5'-UTR, and is regulated by IRP1. The IRP1-HIF2 axis is an important iron- and hypoxia-dependent regulator of key genes that contribute to iron absorption from the gut (i.e., *DCYTB*, *DMT1* and *FPN1*) and to erythropoiesis (i.e., EPO). For a recent review, see Shah et al. [43b].

2. Mitochondrial iron metabolism

Mitochondria play a vital role in cellular iron metabolism [3,44]. These organelles are the sole site of heme synthesis (Fig. 3) and the major site for ISC biogenesis [7,44,45]. As many of the proteins depending on these iron-containing groups play vital roles in cellular metabolism, the dysregulation of mitochondrial iron metabolism often leads to severe diseases [7,46,47].

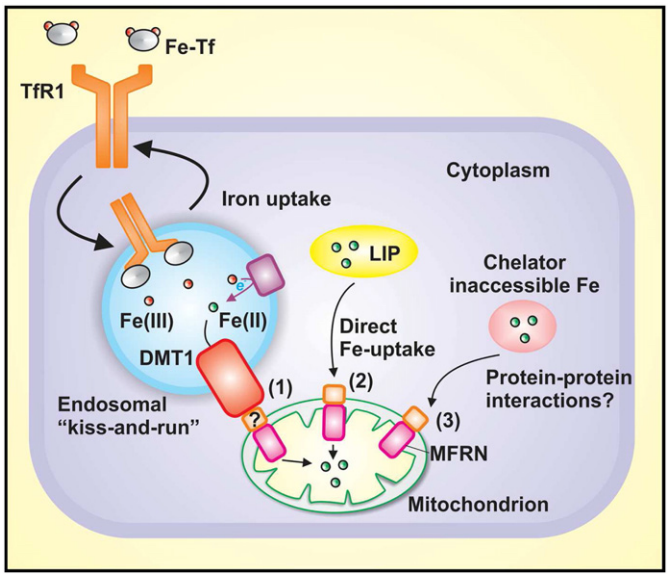

Fig. 3. Mitochondrial iron import pathways. There are three potential iron uptake pathways that provide the metal for transport across the outer mitochondrial membrane *via* an unknown transporter (designated "?"), and into the mitochondrion, all of which may involve mitoferrin isoforms (MFRN) in the inner mitochondrial membrane. These are: (1) the hypothesized endosomal "kiss-and-run" mechanism, which involves the direct interaction of the endosome with the mitochondrion; (2) direct iron uptake by the mitochondrion from the LIP (potentially chelator-accessible iron); and (3) the donation of iron to the mitochondrion by protein–protein interactions that are potentially chelator-inaccessible, including specific chaperone molecules that transport and deliver iron to the mitochondrion.

2.1. Mitochondrial iron import

While the pathways involved in heme and ISCs synthesis are largely known (see below), only more recently have some of the molecular players involved in mitochondrial iron transport been identified. Iron may be imported from the cytosol across the outer mitochondrial membrane (OMM) by one or more of the following mechanisms (Fig. 3): (*i*) by a putative endosomal "kiss-and-run" or direct protein–protein transfer mechanism, at least in hemoglobin-producing erythroid precursors, in which Tf-containing endosomes make a transient contact with the OMM [29]; (*ii*) direct uptake of iron(II) from the cytosol, driven by the mitochondrial membrane potential ($\Delta\Psi_m$) [2]; and/or (*iii*) uptake of a "chelator-inaccessible" iron pool from the cytosol, which may involve directed transfer by protein–protein contacts [2].

In order for iron to enter the mitochondrial matrix to be processed by the heme and ISC synthetic pathways, iron must traverse the OMM and inner mitochondrial membrane (IMM). The passage of iron across the OMM, which has high metabolite-permeability relative to the IMM, is due largely to the presence of voltage-dependent anion channels (VDACs), remains ill-defined. Indeed, VDACs are the most singly abundant proteins in the OMM, and are responsible for the shuttling of almost all energy-related metabolites, including pyruvate, ATP, *etc.* between the cytosol and the mitochondria [48]. VDACs also have cationic conductance and may be responsible for allowing the trans-OMM flux of divalent cations such as Ca^{2+} and Mg^{2+} [48]. An untested possibility is that VDACs may facilitate the passage of ferrous iron across the OMM. Alternatively, a recent study suggested that mitochondrial isoforms of DMT1 may be involved [49a]. As PCBP2 can bind to DMT1 to facilitate iron efflux from the endosome [33], PCBP2 may similarly promote iron influx into mitochondria through DMT1. Further studies are needed to test these hypotheses.

In contrast, the iron import pathway across the IMM is more comprehensively characterized. In budding yeast, two homologous, high affinity iron transporters, Mrs3 and Mrs4, are involved in mitochondrial iron uptake across the IMM [49b,c]. Orthologs of these proteins, known as mitoferrins (MRFNs), exist in all eukaryotes. In mammalian

cells, it was demonstrated that iron traverses the IMM predominantly by the action of one of two MFRN isoforms: MFRN1 or MFRN2 [50,51]. The levels of MFRNs control mitochondrial iron levels, and this regulation occurs at the post-translational level due to alterations in protein half-life [50]. In the mitochondria of differentiating erythroid cells, MFRN1 (also known as SLC25A37) accumulates due to an extended protein half-life relative to MFRN2 [50], and is responsible for iron import across the IMM in these red blood cell precursors [51]. As might be expected, the loss of this protein leads to profound anemia and disruptions in ISC biogenesis [51]. On the other hand, MFRN2 (also known as SLC25A28) is ubiquitously expressed in different tissues, and, unlike MFRN1, its expression does not increase during erythroid differentiation [50,51].

Although the precise biochemistry of MFRN1-mediated iron import by mitochondria remains unknown, MFRN1 interacts with the IMM ATP-binding cassette (ABC) transporter 10, which is highly expressed in erythroid mitochondria, and increases MFRN1 stability and mitochondrial iron import [52]. Since a complex consisting of MFRN1, ABCB10 and ferrochelatase has since been discovered [53], this association may promote efficient heme synthesis during erythroid development by stabilizing MFRN1 and funneling iron directly to ferrochelatase, the enzyme which inserts iron into protoporphyrin IX (PPIX) to form heme [54]. Interestingly, the identity of the transported substrate of ABCB10 remains unknown, and recent evidence suggests that ABCB10 plays a role in protection from oxidative stress during normal erythropoiesis and cardiac recovery [55]. Moreover, a very recent study has determined that ABCB10 is critical for heme synthesis, and that *Abcb10*-knockout mice demonstrate anemia with PPIX accumulation [56]. Taken together, these data suggest the role of ABCB10 in erythropoiesis may be to increase MFRN1 stability and complexation with ferrochelatase in order to direct the large iron influx from Tf into the heme synthesis pathway.

Interestingly, recent studies using Ru360, a specific inhibitor of the mitochondrial calcium uniporter (MCU), also suggests the involvement of the MCU in mitochondrial uptake of Fe(II), possibility *via* an interaction with the ubiquitously expressed MFRN2 [57,58]. Indeed, blockade of MCU with Ru360 attenuated mitochondrial dysfunction caused by iron overload [58]. However, the specific mechanisms involved in this process require further elucidation.

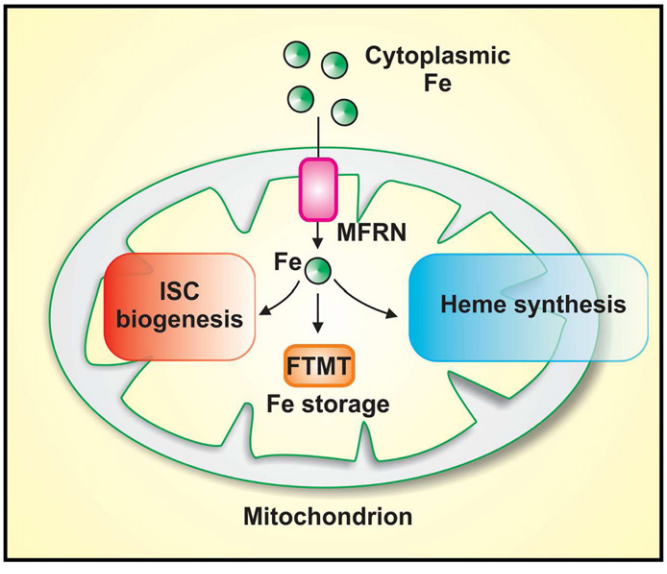

Fig. 4. There are three major mitochondrial iron metabolic pathways in mitochondria. Cytoplasmic iron is delivered to the mitochondrion by three possible mechanisms (see Fig. 4) with transport occurring across the outer mitochondrial membrane (see text for details). The iron is then transported across the inner mitochondrial membrane *via* mitoferrin-1 or -2 (MFRN) and then utilized for: (1) ISC biogenesis; (2) iron storage in mitochondrial ferritin (FTMT); and/or (3) heme synthesis.

Once imported by the mitochondrion, there are essentially three major metabolic pathways for iron: (*i*) mitochondrial iron storage; (*ii*) heme synthesis; and (*iii*) ISC biogenesis (Fig. 4). We provide a brief overview of the former two pathways (see below), while the remainder of this review will focus on some recent advances and controversies in ISC biogenesis and delivery.

2.2. Mitochondrial iron storage

Some mammalian tissues express a mitochondrial-specific ferritin (FTMT) that has a high level of sequence identity with H-ferritin. FTMT stores iron within a homopolymer and demonstrates distinct iron oxidation and hydrolysis chemistry to H-ferritin [5]. Interestingly, the highest expression of FTMT is observed in the testis (spermatozoa), while it appears to be completely absent from typical iron-storage tissues such as liver and spleen [59]. High levels of FTMT are also expressed in sideroblasts (*i.e.*, erythroblasts with iron granules) of patients affected by sideroblastic anemia [59,60a].

2.3. Heme synthesis

We only provide a very brief overview of the heme synthesis pathway here, and direct readers to a recent and comprehensive review on the topic [60b]. Heme synthesis involves the sequential catalytic activity of eight enzymes and is dependent on the mitochondrion [54]. Four of the intermediate enzymatic steps are within the cytosol [54]. The biosynthetic pathway spans all mitochondrial compartments, as well as the cytoplasm. The first enzyme in the pathway is δ-aminoleuvulinate synthase (ALAS), which catalyzes the condensation of glycine and succinyl-CoA to form δ-aminolevulinic acid [54]. Two variants of ALAS exist: ALAS2 is expressed specifically in erythroid cells, while ALAS1 is expressed in other cell-types. Unlike *ALAS1*, the *ALAS2* mRNA possesses an IRE in its 5′-UTR, and is subject to regulation *via* the IRP–IRE system (Fig. 2A). It is due to this difference that in erythroid cells expressing ALAS2, the rate of heme synthesis may be governed by cytosolic iron availability [54].

Interestingly, MFRNs appear capable of regulating the heme synthesis pathway *via* the IRP–IRE system. Indeed, a recent report determined the mechanism behind the lack of porphyria (*i.e.*, an accumulation of PPIX) in animals lacking MFRN1 [61]. Due to the loss of MFRN1-dependent mitochondrial iron import, such mutant animals are unable to synthesize appropriate heme levels during erythroid differentiation [61]. The mechanism appears to involve an increase in the IRE-binding form of IRP1 (Fig. 2A), presumably resulting from impaired and iron-limited ISC biogenesis, that leads to attenuated translation of *ALAS2* mRNA (which contains an IRE in its 5′-UTR), decreased ALAS2 protein synthesis and decreased production of protoporphyrin [61].

The final enzyme in the heme synthesis pathway is ferrochelatase, which inserts iron(II) into the heme precursor, PPIX, to form heme [54]. Since mammalian ferrochelatase is an ISC-containing enzyme, any disruption in ISC synthesis may deregulate heme synthesis, as shown in disease models [62a].

3. Iron–sulfur cluster biogenesis: an overview

3.1. Basic ISC biochemistry and function

ISCs are ancient biochemical structures that are composed of non-heme iron and inorganic sulfide [7]. Although a range of configurations exist, ISCs are mostly found in the cubane form (*i.e.*, [4Fe-4S]). These prosthetic groups are ligated to cysteine residues of proteins and are necessary for a wide variety of protein functions including: (*i*) electron transfers, which are almost all mono-electronic (*e.g.*, as occurs in the ISC-containing complexes I-III of the respiratory chain); (*ii*) forming substrate-binding sites and promoting enzymatic catalysis (*e.g.*, cytosolic and mitochondrial aconitases); and/or (*iii*) metabolic sensors (*e.g.*, IRP1/cytosolic aconitase and the regulation of cellular iron metabolism) [1,7].

The biogenesis of ISCs is conserved essentially in all organisms (for an excellent detailed review, see Lill [6], and for a recent bacteria-specific review, see Blanc *et al.* [62b]). The remainder of this review will concentrate on the mammalian systems, with reference to other organisms where necessary. Mammalian ISC biogenesis occurs in two different, but connected systems corresponding to different cellular compartments: the "ISC" assembly apparatus in mitochondria (the focus of this section) and the cytosolic ISC biogenesis (CIA) system (overviewed below). Genetic disruptions affecting key proteins involved in ISC cluster biogenesis are recognized as being involved in the development of an increasing number of human diseases [7]. In the case of mitochondrial ISC biogenesis, such diseases include Friedreich's ataxia (FRDA), ISC assembly enzyme (ISCU) myopathy, a rare sideroblastic-like microcytic anemia, hereditary myopathy with lactic acidosis, X-linked sideroblastic anemia with cerebellar ataxia and an encephalomyopathy caused by a deficiency in ISC-dependent complex I activity in the IMM respiratory chain [7,46,47].

3.2. The core mitochondrial ISC biogenesis pathway

Certain apo-proteins (*e.g.*, some ferredoxins) can acquire their ISCs following simple addition of S^{2-} and $Fe^{2+/3+}$ to solution *in vitro* [63]. While this led to the long-held belief that ISCs could be generated and incorporated into their apo-proteins spontaneously, this view was not compatible with the known toxicities of free iron and sulfur in biological systems [63]. It is now known that both iron and sulfur are coordinately delivered in a minimally toxic form to apo-proteins by carrier proteins, chaperones and scaffolds [7,46,64].

The process of ISC biogenesis, in general, can be envisaged as occurring in two key phases: (1) the transient synthesis of the ISC on a scaffold assembly protein and (2) the transfer of this *de novo* ISC to target apo-proteins [65] (in the case of mitochondrial ISC biogenesis, see Fig. 5).

In humans, the core ISC assembly machinery includes the dimeric NFS1 cysteine desulfurase (NFS1; called IscS in *Escherichia coli* and Nfs1 in *Saccharomyces cerevisiae*), which is required for abstraction of sulfur from cysteine, leading to alanine (Fig. 5) [66]. In eukaryotes, two monomers of a small accessory protein, ISD11 (also known as LYR motif-containing 4, LYRM4), are bound to the NFS1 dimer. The interaction of ISD11 with NFS1 promotes NFS1 stability [67] and causes a conformational change in the enzyme allowing the bound cysteine substrate and active site cysteine to be brought into close proximity [68]. In the presence of ISD11, NFS1 binds the substrate cysteine at the pyridoxal phosphate-containing active site, and a persulfide intermediate is formed on the active site cysteine. The abstracted sulfur is then transferred to one of two monomers of the dedicated scaffold protein, ISCU (called IscU in bacteria, Isu1 or Isu2 in yeast), which bind at either end of the NFS1/ISD11 core [69], but at a site distal to ISD11 (Fig. 5), with iron being supplied by an unknown source (perhaps frataxin; reviewed further below).

ISCU is crucial as it provides a protein-scaffolding, consisting of a polypeptide backbone structure and cysteine ligands, on which the nascent ISC can be synthesized [6,46]. In mammals, ISCU is found in both the mitochondria and cytosol, and may contribute to ISC biogenesis in both [70a]. Assembly of the nascent ISCs on ISCU relies on a source of electrons in order to achieve the appropriate electronic configurations for the given ISC [7] (Fig. 5). In yeast, the ferredoxin reductase, Arh1, and ferredoxin, Yah1, may utilize NAD(P)H as an electron donor [65,70b,70c]. In mammals, the ferredoxin reductase (FDXR) and ferredoxins 1 and 2 (FDX1/2) appear to be involved in this activity [70b,c] (Fig. 5).

Currently, the source of iron necessary for ISC biogenesis is unknown. In the case of mitochondrial ISC biogenesis, it is likely that iron is delivered to ISCU by an as yet undetermined chaperone, such as the mitochondrial protein, frataxin [3,71]. Frataxin is critical for ISC biogenesis and can bind multiple iron(II) ions on exposed acidic patches [3,71,72]. Alternatively, this protein may perhaps act as a crucial regulator of ISC formation [7,73], *via* allosteric regulation of the cysteine desulfurase activity of NFS1 [74] and/or regulation of ISCU [75]. Another potential source of iron is the iron-binding complex of glutathione and glutaredoxin that can tether an ISC [76].

Once synthesized, nascent and labile ISCs must then be transferred from ISCU to recipient apo-proteins, thereby converting them to their holo-form (Fig. 5). The complex logistics of this directed transfer of ISCs to specific apo-proteins occurs by the concerted action of cluster-transfer proteins [6]. These proteins work to dissociate the nascent ISC from the ISCU and ensure accurate and specific transfer to the correct apo-proteins. They also assist in the correct assembly and integration of the ISC at the acceptor site [6,7,65] (see below).

3.3. The core cytosolic ISC biogenesis and delivery (CIA) pathway

In addition to the classical mitochondrial ISC proteins, eukaryotic cells contain numerous cytosolic and nuclear ISC proteins that are

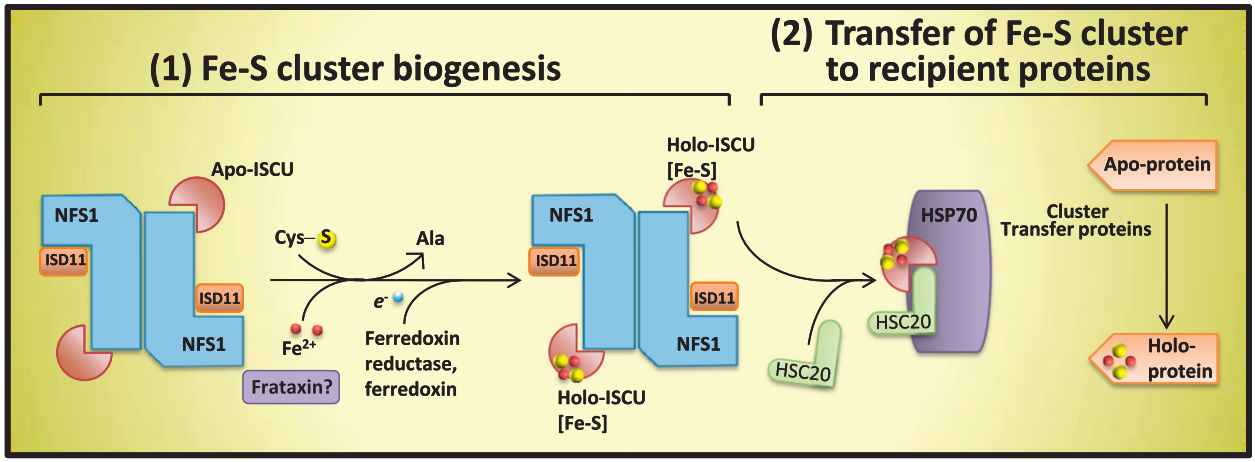

Fig. 5. Iron sulfur cluster (ISC) biogenesis. A general schematic of ISC biogenesis in the mitochondria of human cells. ISCs are assembled on the ISC assembly enzyme (ISCU). ISCU binds to the cysteine desulfurase, NFS1, which forms a homo-dimer, and removes sulfur from cysteine (Cys), providing the sulfur necessary for ISC synthesis. It is not clear how Fe is delivered to the ISCU, although frataxin may be involved. Moreover, ferredoxin reductase and ferredoxins 1 and 2 utilize electrons from NADPH to reduce sulfane (S^0), which occurs in cysteine, to sulfide (S^{2-}), which occurs in ISCs [7]. After ISCs are assembled on the scaffold, the DNAJ-type co-chaperone HSC20 binds to ISCU and, in turn, this complex binds an ATP-dependent HSPA9 chaperone. The newly synthesized ISCs are then transferred from ISCU to recipient apo-proteins to convert them to their holo-form with the help of various chaperones and/or accessory proteins.

critical to metabolic catalysis, regulation of iron metabolism, DNA synthesis, and DNA repair. As such, the synthesis of ISCs for incorporation into the respective proteins in the cytosol and nucleus are vital for cellular viability. The major process involved in the synthesis of these extra-mitochondrial ISCs occurs in two major steps of a highly conserved eukaryotic pathway, known as the cytosolic ISC protein assembly (CIA) pathway, that involves nine known proteins (for a recent excellent review, see: [77a]). The below overview will describe the major components of the budding yeast pathway, but the mechanisms and proteins involved are highly conserved in mammals [77b]. In the first step of the CIA pathway in yeast, a [4Fe-4S] cluster is assembled on a scaffolding hetero-tetrameric complex composed of the P-loop NTPases, Nbp35 and Cfd1 (NUBP1 and NUBP2 in humans, respectively), which requires a currently unknown source of sulfur (designated "X-S") that appears to be exported from mitochondria by Atm1 (ABCB7 in humans). Additionally, an intermembrane space sulfhydryl oxidase, Erv1 (GFER in humans), is thought to be involved in disulfide formation and the facilitation of export of this unknown intermediate to the cytosol [77c,d]. In similarity to the mitochondrial ISC system, in order to generate the appropriate electronic configuration of the cluster on the Nbp35-Cfd1 complex, a source of reducing equivalents is required, which utilizes an NADPH-dependent electron transfer chain involving the FAD- and FMN-containing flavoprotein, Tah18 (NDOR1 in humans), that transfers electrons to the ISC of Dre2 (CIAPIN1 in humans) [77a,77e,f].

In the second step, the nascent ISCs are transferred from the Nbp35-Cfd1 scaffolding complex to specific apo-proteins by the concerted actions of the iron-hydrogenase-like protein, Nar1 (IOP1 in humans), and the CIA targeting complex, which is a hetero-trimeric complex consisting of Cia1 (CIA1/CIAO1 in humans), Cia2 (CIA2A/FAM96A or CIA2B/FAM96B in humans, which target different sets of apo-acceptors) and Mms19 (MMS19 in humans) (reviewed in depth in [77a]). The latter three proteins are crucial for interacting with target apo-proteins and ensuring specific and efficient transfer of ISCs [78a]. The glutaredoxins, Grx3 and Grx4, which are capable of binding a GSH-coordinated, bridging [2Fe-2S] cluster, are also known to be involved in the CIA pathway, but at an as-yet-unknown step [77a,78a].

In the human CIA pathway, there are several crucial differences that are worth noting. First, while the components and biochemical mechanisms of the human CIA pathway are similar to those in yeast, there is a crucial difference in that, human cells express two isoforms of CIA2 (*i.e.*, CIA2A and CIA2B), both of which bind to CIA1. Importantly, CIA2B is the functional ortholog of Cia2 in yeast, and is involved in canonical ISC assembly in cytosolic and nuclear ISC proteins. However, CIA2A is known to be specifically involved in the ISC assembly for IRP1 [78b], which is a crucial regulator of cellular iron homeostasis in

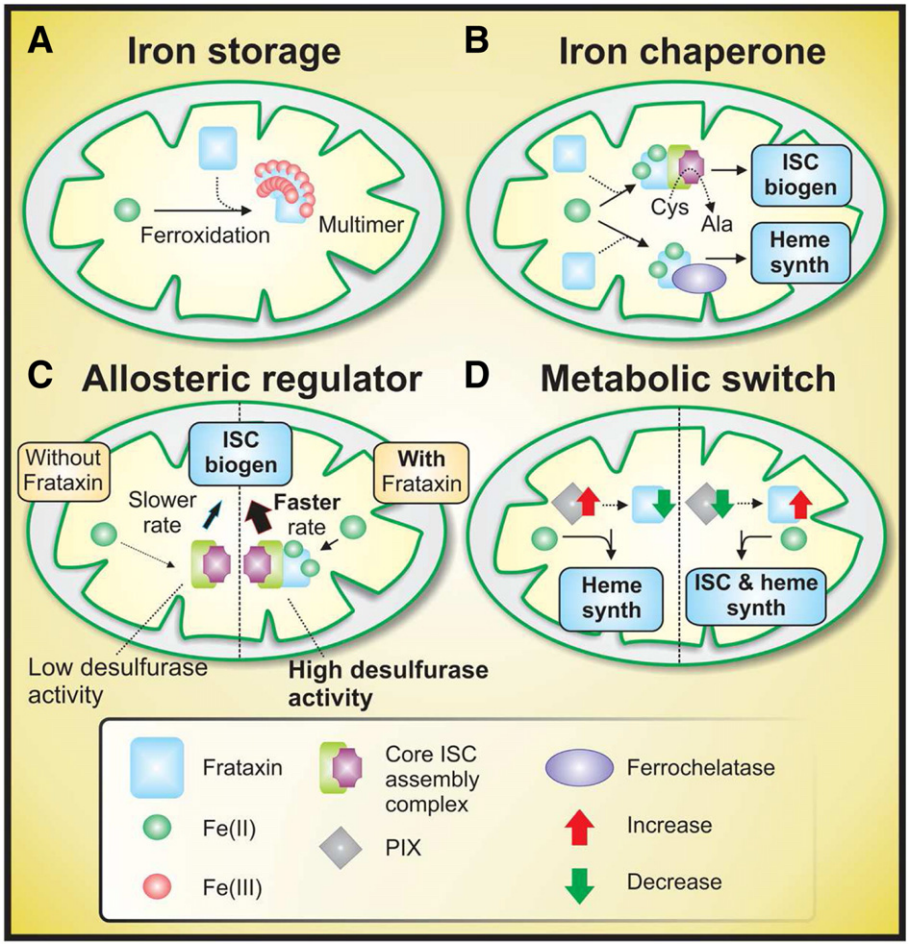

Fig. 6. Possible roles for mitochondrial frataxin. There have been four major roles hypothesized for the function of frataxin in mitochondrial iron metabolism in eukaryotic cells: (A) iron storage; (B) iron chaperone; (C) allosteric regulator; and (D) metabolic switch. Each of these roles may be complementary and not exclusive of each other. (A) It has been proposed that frataxin, at least in yeast, may perform an iron-storage function within the mitochondrial matrix. (B) Frataxin may function as an iron chaperone by binding iron in the mitochondrial matrix and then delivering it to either the ISC assembly complex of the ISC biosynthetic pathway, or ferrochelatase of the heme biosynthetic pathway. (C) Recent evidence suggests that frataxin may play the role of an allosteric regulator of desulfurase activity of the NFS1-ISD11-ISCU complex such that by binding to the complex, frataxin increases the rate of cysteine desulfuration. (D) Frataxin may function as a type of 'metabolic switch' that allows the mitochondrion to favor heme or ISC/heme syntheses, depending on frataxin levels. A possible mechanism behind this 'switching' may be the relative affinities of frataxin for ferrochelatase (*i.e.*, 17 nM) *vs* ISCU (*i.e.*, 480 nM). This may result in preferential binding of frataxin to ferrochelatase over ISCU at relatively lower frataxin levels.
Modified from (Richardson et al.) [3].

mammals, but is not present in yeast (see above) (for a recent review, see [77a]). Intriguingly, although IRP2 does not possess an ISC, IRP2 is still bound, and stabilized, by CIA2A [78b]. This is important in considering the possible mechanisms by which cellular ISC biogenesis may intersect with the classical regulatory pathways for the IRPs described above.

4. Further insights into mitochondrial ISC biogenesis and delivery: some key players and mechanisms

While there remain numerous molecular and biochemical details of the mitochondrial ISC biogenesis pathway that have not been fully characterized, several discoveries have revealed important insights into the process. Only an overview of some these discoveries and mechanistic insights is detailed below, as this topic has been recently reviewed [78a].

4.1. Frataxin and ISC biogenesis

Frataxin is essential for cellular iron homeostasis [79], and it is involved in regulating systemic iron homeostasis by an as-yet-unknown mechanism [3,62,79,80]. In addition, evidence supports its role as an essential factor in ISC biogenesis [79]. Crucially, frataxin-deficiency causes the rare, but severe neuro- and cardio-degenerative disease, FRDA, which is due to a GAA-repeat expansion in intron 1 of *FRDA* gene that encodes for frataxin [81]. Frataxin is a vital protein that is highly expressed in tissues rich in mitochondria *e.g.,* heart and neurons [82], with total deletion being embryonically lethal [83].

Most suggested functions for frataxin, a mitochondrial matrix protein [79,81], relate to iron-binding and maintenance of mitochondrial iron metabolism (Fig. 6). These proposed functions include mitochondrial iron storage (for a recent review see [79]) (Fig. 6A), which is considered an unlikely role in mammalian cells, because of the presence of FTMT, which plays an iron storage role in the mitochondria of some cell types; as well as the regulation of ISC biogenesis (Fig. 6B, C and D) and heme synthesis (Fig. 6B and D) [84]. However, most evidence suggests frataxin is involved in the maintenance of mitochondrial ISC biogenesis and/or heme synthesis [79] and/or allosteric activation of the NFS1-ISD11-ISCU complex (see below).

Many insights regarding frataxin function have come from studies of frataxin orthologs in yeast (*i.e.*, Yfh1) and bacteria (*i.e.*, CyaY) [85]. A consensus exists that the functions of frataxin share a requirement for iron-binding [85]. The major proposed roles for frataxin in ISC biogenesis are discussed below.

4.2. Frataxin as an iron chaperone

It has long been suspected that frataxin is an iron chaperone for ISC biogenesis and/or heme biosynthesis (Fig. 6B). Frataxin has been observed to interact with, and presumably donate iron to, iron-dependent proteins involved in ISC biogenesis and/or heme synthesis [86,87]. For example, yeast frataxin, Yfh1, can interact with the core ISC assembly complex comprising the scaffolding protein, Isu, and the cysteine desulfurase, Nfs1, which is enhanced by iron(II) [86,88].

In a similar manner, human frataxin also interacts with ferrochelatase for heme synthesis [87,89]. Importantly, the interaction of frataxin with either ISCU or ferrochelatase appears to increase the rate of ISC synthesis [86], or the ferrochelatase-catalyzed insertion of iron(II) into PPIX [87], respectively. These observations suggest frataxin donates iron to ISCU and/or ferrochelatase.

Intriguingly, accumulating data suggests the role of frataxin in ISC biogenesis may be more complex than just iron-donation. As discussed further below, the interaction of frataxin with the core ISC biogenesis apparatus, and perhaps ferrochelatase during heme synthesis, may serve to kinetically regulate key reactions.

4.3. Frataxin as an iron-sensing, allosteric regulator

A 2009 study suggested that the bacterial frataxin ortholog, CyaY, may act as an "iron-sensor" and kinetic regulator that negatively regulates ISC biosynthesis under conditions of high iron and low ISC apo-acceptor availabilities [90] (Fig. 6C). This is dependent on the binding of frataxin to IscS (the bacterial equivalent of NFS1) [73,90,91].

Although CyaY does not inhibit the desulfurase activity of IscS, it slows down the rate of ISC formation in an iron-dependent manner *via* a CyaY-dependent increase in the affinity of IscS for the scaffold protein, IscU [91,92]. This increase in affinity between the two proteins may inhibit the release of the scaffold protein from the cysteine desulfurase during the delivery of the nascent ISC. Thus, in prokaryotic systems, CyaY appears to regulate ISC biogenesis by acting as an iron-dependent inhibitor of the IscS-IscU core complex.

Similar studies in eukaryotic systems indicate that, rather than acting as an inhibitor, frataxin acts as an activator of ISC biogenesis [73,93,94a]. Indeed, in 2010, Tsai and Barondeau demonstrated that human frataxin acts as an iron-dependent allosteric switch that activates the core ISC biogenesis machinery by markedly increasing the cysteine desulfurase K_M and catalytic efficiency (k_{cat}/K_M) [94a]. Moreover, a recent study by Pandey and colleagues indicates that yeast frataxin (*i.e.*, Yfh1) directly stimulates the cysteine desulfurase of Nfs1 activity by binding to the enzyme and exposing substrate-binding sites [74]. Intriguingly, a mutant form of the scaffold protein, Isu1, which can operate independently of Yfh1, causes a similar increase Nfs1 activity by the same mechanism [74]. Bridwell-Rabb and colleagues [94b] have provided evidence supporting this allosteric activation model and have proposed that frataxin accelerates persulfide formation on NFS1 and favors a helix-to-coil interconversion on ISCU2 that helps drive the transfer of sulfur from the NFS1-ISD11 complex to ISCU2. Moreover, a very recent study by Parent and colleagues [94c] has indicated that mammalian frataxin directly enhances the rate of two similar reactions, including NFS1 persulfide transfer to thiols on ISCU and small thiols such as L-cysteine and GSH. Thus, eukaryotic frataxin appears to be an allosteric activator of the persulfide-forming activity of the NFS1-ISD11-ISCU complex (Fig. 5).

Interestingly, the apparent opposing effect in the regulation of ISC biogenesis by the prokaryotic and eukaryotic frataxin orthologs (*i.e.*, prokaryotic frataxin appears to be an inhibitor, while eukaryotic appears to be an activator) seems to be dependent on the identity of the cysteine desulfurase, rather than the frataxin ortholog [95].

4.4. Is frataxin a "metabolic switch" between ISC biogenesis and heme synthesis?

An extension of the allosteric regulator model of frataxin in ISC biogenesis may also be applicable to frataxin's ability to regulate heme biosynthesis and act as a metabolic switch (Fig. 6D). As there is a decline in frataxin levels during erythroid differentiation, and since frataxin expression is markedly decreased during Friend cell hemoglobinization [96], it has been proposed that frataxin is down-regulated during erythroid differentiation to allow for higher rates of heme synthesis, potentially at the expense of decreased levels of ISC synthesis [3,79,84,96]. This hypothesis is supported by the observation that the immediate precursor for heme synthesis, PPIX, down-regulates frataxin expression [96]. Hence, increased PPIX levels, which indicate a requirement for heme synthesis, lead to decreased frataxin expression and a diversion of iron from other mitochondrial pathways (*i.e.*, ISC synthesis and/or iron storage) to heme biogenesis [96].

This latter hypothesis is supported by the observation that an increase in frataxin levels relative to ferrochelatase (*i.e.*, above a molar ratio of 1:1 frataxin:ferrochelatase dimer) results in decreased rates of heme synthesis *in vitro* [87]. It has also been observed that iron-bound human frataxin has a higher putative binding affinity for ferrochelatase (17 nM) than Isu (480 nM) [87]. These observations provide a basic

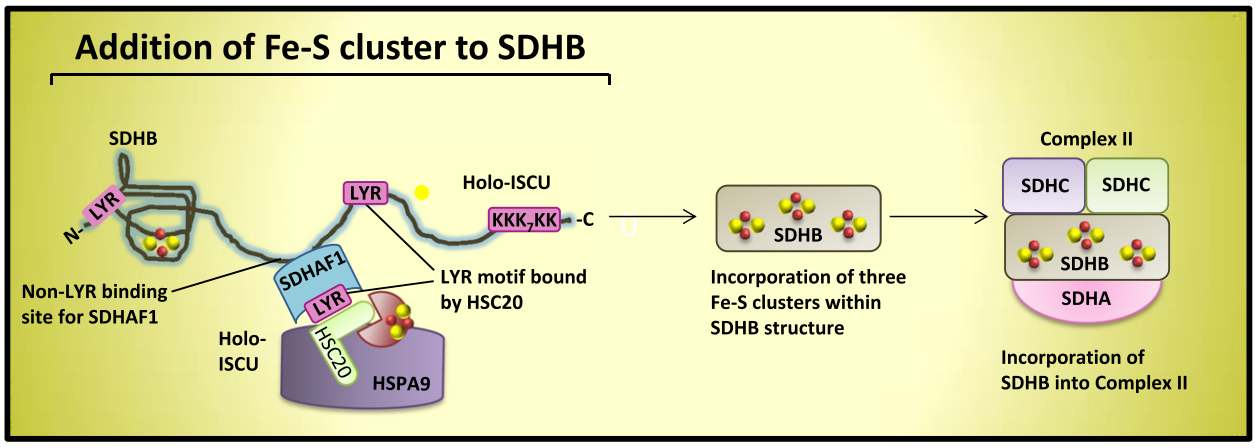

Fig. 7. Iron sulfur cluster (ISC) delivery. Addition of ISCs to succinate dehydrogenase subunit B, SDHB. ISCs assembled on the ISCU are transferred to the ISC protein, SDHB, by a chaperone-co-chaperone system. In the SDHB protein sequence, two LYR motifs and the KKX_7KK consensus sequence bind to HSC20 to engage the ISCU-HSC20-HSPA9-complex and aid the incorporation of three ISCs. Moreover, the accessory protein, SDHAF1, binds both HSC20 at a LYR motif and SDHB at a non-LYR binding site, facilitating the delivery of the ISC from ISCU to SDHB. Upon acquisition of three ISC centers, SDHB forms one of the four subunits in complex II (SDH).
Modified from (Lane et al.) [130].

mechanism that supports the hypothesis that frataxin expression levels may allow metabolic switch between the ISC biogenesis and heme synthesis pathways, depending on expression levels relative to those of Isu and ferrochelatase [87,96]. That is, in analogy to the allosteric regulation of the Nfs1-Isd11-Isu1 complex, frataxin may also exert kinetic regulation of heme synthesis by binding to and inhibiting ferrochelatase at frataxin/ferrochelatase ratios above parity, a scenario in which ferrochelatase is progressively inhibited by frataxin [87]. Similarly, a decrease in the frataxin/ferrochelatase ratio below parity may also be inhibitory to heme synthesis, as frataxin-deficient murine heart tissue shows increased PPIX levels and decreased heme production [62,97]. Importantly, a decrease in the frataxin/ferrochelatase ratio, as in the case of frataxin-deficiency, affects both the transcriptome and proteome of enzymes in the heme synthetic pathway [62,97].

Taken together, the emerging view of frataxin function in mitochondrial iron metabolism is one of an allosteric modulator and/or metabolic switch between two key pathways: ISC biogenesis and heme synthesis (Fig. 6D). Whether frataxin is the elusive source of iron for ISC biogenesis, or whether iron-binding to frataxin is merely a means of regulating its allosteric activity in an iron-dependent manner, is yet to be determined. Furthermore, some studies indicate that frataxin may also have a role in facilitating ISC delivery (see below).

5. HSP70, HSC20 and the logistics of mitochondrial ISC delivery

One key unresolved question in the ISC biogenesis and delivery field has been determining how specific ISCs are directed to cognate target proteins. In fact, although the delivery pathway for nascent ISCs is still relatively uncharacterized, as with the CIA pathway (see above), key players and their mechanisms of action are emerging. We here provide only an overview of some these discoveries and mechanistic insights with respect to mitochondrial ISC biogenesis, as this topic has been recently reviewed in depth [78a].

The targeted delivery of nascent ISCs to specific apo-acceptors involves a dedicated HSP70-chaperone–HSC20-co-chaperone pair of proteins that transfers the ISCs from the primary scaffold (referred to herein as "ISCU") to specific recipient proteins [7] (Fig. 7). In bacteria and yeast, holo-ISCU (*i.e.*, harboring a nascent ISC) must dissociate from the cysteine desulfurase (NFS1) and bind to an HSC20 J-protein (HscB in bacteria, Jac1 in yeast) through hydrophobic contacts [7,98]. ISCU must also bind to an HSP70 chaperone (HscA in bacteria, Ssq1 in yeast, possibly HSPA9 in mammals) *via* a conserved LPPVK-motif in ISCU [98]. Binding of the J-domain of the HSC20 protein (specifically a His-Pro-Asp motif) to HSP70 leads to activation of the latter's intrinsic ATPase activity, which hydrolyzes ATP and causes a conformational change in the ISCU-binding domain of HSP70 [98]. This is thought to facilitate extrusion of the LPPVK-motif of ISCU, promoting a conformational change in ISCU that may allow for transfer of the ISC to recipient apo-proteins or to secondary scaffolds [98] (Fig. 7).

5.1. The HSP70 protein

Unlike bacterial and yeast systems, most eukaryotic mitochondria do not appear to have a HSP70 member that is dedicated to ISC assembly and transfer [45]. Although it is yet to be conclusively demonstrated, the available evidence suggests that a multi-functional mitochondrial HSP70, namely HSPA9/mortalin/mtHSP70/GRP75/PBP74, is capable of "moon-lighting" in this role [99–101]. Interestingly, HSPA9/mortalin is involved in many cellular processes including facilitation of protein-folding and import, intracellular trafficking, the cellular stress response, hematopoiesis, control of cell proliferation and tumor progression [102]. Further genetic studies are needed to demonstrate the role of HSPA9/mortalin in vertebrate ISC biogenesis and delivery.

5.2. The HSC20 protein

The protein now known as HSC20 was originally predicted to be involved in ISC biogenesis due to its similar phylogenetic distribution to frataxin and its high levels of expression in mitochondria [103]. More recently, several studies have implicated this J-protein type III co-chaperone in ISC synthesis [100,101,104] (Fig. 7). Indeed, human HSC20 can be substituted for its yeast counterpart, Jac1, and has been shown to directly interact with ISCU and HSPA9/mortalin [104]. RNAi-depletion of HSC20 resulted in a deficit of mitochondrial and cytosolic ISC proteins [100,104]. Moreover, HSC20 possesses a cysteine-rich *N*-terminal domain, which differs from its counterparts in bacteria and yeast, which may be important in protecting nascent ISCs from oxidative insults [104]. While HSC20 is located predominantly in mitochondria, some of the protein is also found in the cytosol, where it may contribute to cytosolic ISC-biogenesis and delivery [104].

Intriguingly, frataxin has been found to interact with HSC20 in an iron-dependent manner, and HSC20 levels are regulated by frataxin levels [100]. If frataxin is involved in regulating the kinetics of the cysteine desulfurase complex, why does the protein also interact with HSC20,

which is involved in the delivery phase of the ISC assembly line? Although many possibilities exist, the interaction of frataxin with HSC20 may indicate that frataxin allows fine-tuning of ISC biosynthetic rates, through protein–protein interactions with key players, in response to ISC apo-acceptor availability. This scenario would be reminiscent of the proposed ability of the bacterial frataxin ortholog, CyaY, to down-regulate ISC biogenesis in response to low apo-acceptor availability [90].

5.3. The role of LYR motifs in HSC20 client discrimination

Type III J-proteins, such as HSC20, do not typically act alone as molecular chaperones, and are known to interact selectively with a small subset or even a single substrate [105]. Until very recently, the answer to the question of how specific ISCs are directed to target proteins was unknown.

Interestingly, a recent study from Rouault's group has revealed basic, but crucial, insights into the molecular logistics of HSC20-mediated client discrimination in the transfer of ISCs to discrete subsets of recipient proteins [101]. Maio and colleagues have shown that HSC20 guides nascent ISCs to their cognate apo-acceptors based, in part, on a highly conserved "leucine–tyrosine–arginine" (LYR) motif in target proteins [101] (Fig. 7). These authors discovered that HSC20 binds the succinate dehydrogenase subunit B (SDHB; the ISC-containing subunit of respiratory chain complex II), and demonstrated that both HSC20 and HSPA9/mortalin were required for complex II assembly and activity [101]. When these authors examined the SDHB sequence more closely, they identified two LYR motifs [101] that are highly conserved within eukaryotes and prokaryotes [106].

The LYR motif (LYRM) family of mitochondrial proteins consists of proteins of diverse functions that typically carry this tri-peptide motif in the *N*-terminal region [106]. These proteins appear to function as adaptor-like or accessory factors in the control of mitochondrial homeostasis, particularly ISC biogenesis and delivery and fatty acid synthesis [106]. Maio and colleagues were able to determine that the LYRMs in SDHB are responsible for binding the *C*-terminal domain of HSC20 and thereby engage the ISC transfer apparatus (Fig. 7). A third binding site for HSC20 was also discovered in the SDHB sequence. This binding site comprises a KKX_7KK sequence and is located at the *C*-terminal end of SDHB (Fig. 7). While the functional significance of this HSC20-binding site is unclear, similar sequences are present in the *C*-terminal ends of two other proteins (*i.e.*, glutaredoxin 5 and SUCLG2) that were further identified as HSC20-binding partners [101].

An additional significant finding in the Maio et al. study was that succinate dehydrogenase assembly factor 1 (SDHAF1), which participates in complex II assembly, binds to SDHB through a non-LYR site [101]. This is significant, as the authors observed that HSC20 bound to SDHAF1, probably through the LYR site in SDHAF1, suggesting that SDHAF1 may initially dock with SDHB, which may allow subsequent HSC20-binding to SDHB and/or perhaps other ISC transfer complexes that are bound at the LYR binding sites on SDHB (Fig. 7). These seminal data indicate that the LYRM may represent a marker that allows for HSC20-dependent client discrimination in the targeted delivery of ISCs to recipient proteins.

The finding that HSC20 is guided, at least in part, by LYRMs in target proteins is important as other LYRM proteins are known to play a role in ISC biogenesis and delivery from complexes I-III [106]. These include ISD11, the obligate NFS1-binding partner [107], SDHAF1 and SDHAF3, the latter two of which were recently shown to be involved in mediating the maturation of the SDHB [108]. Additionally, Winge's group has also shown that the LYRM protein, Mzm1, functions in the insertion and/or stabilization of the ISC-containing subunit of complex III [109a]. Taken together, these recent findings suggest that these proteins are guided by their LYRMs for ISC biogenesis and the targeted delivery of ISCs to proteins. As the human genome encodes at least 11 LYRM proteins [106], it will be crucial for further studies to consider the possible interaction between HSC20 and these proteins in ISC biogenesis and delivery.

5.4. Other proteins associated with mitochondrial ISC delivery

In addition to the proteins reviewed above, other mitochondrial proteins have been found to play a role in delivery of ISCs to specific apoproteins. These include, human NFU1, which can assemble a [4Fe-4S] cluster per two NFU1 monomers and appears to be a late acting factor that is specifically required for the maturation of lipoic acid synthase, with mutations in the NFU1 gene being associated with a disruption of lipoic acid metabolism [109b]. Another group of proteins, which were initially suspected to act as alternative scaffolds in mitochondrial ISC biogenesis, but have recently been shown to be involved in the maturation of a subset of ISC-containing apoproteins, are the A-type proteins. In mammals, ISCA1, ISCA2 and IBA57 appear to be additional specific late acting factors in the mitochondrial ISC assembly pathway that facilitate the maturation of [4Fe-4S] clusters on target apoproteins [109c]. For further information, as well as the roles of glutaredoxins and BOLA proteins, see [78a] and [109d] for excellent recent reviews.

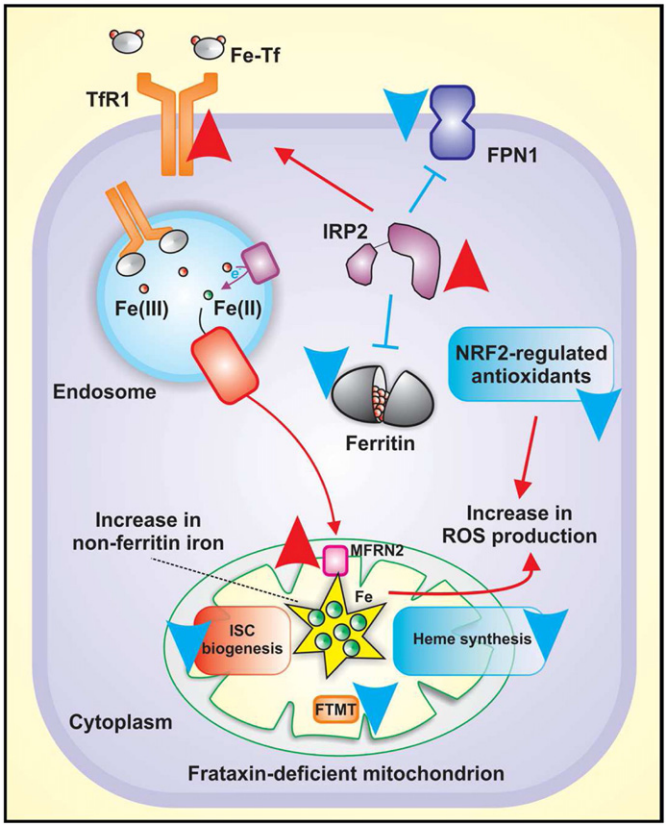

Fig. 8. Some major alterations in cellular iron metabolism in the heart of frataxin-deficient mice. Frataxin deficiency leads to an increase in mitochondrial iron-uptake and a consequent cytosolic iron deficiency, which is facilitated by (i) TfR1 upregulation, increased Tf-dependent iron uptake; (ii) FPN1 down-regulation, preventing iron release; and (iii) ferritin down-regulation, preventing cytosolic iron storage. These changes presumably result from a decrease in cytosolic iron leading to an increase in IRP2 levels. Iron is taken up avidly by the mitochondrion, in part, *via* an increase in MFRN2, and there is a down-regulation of the three major pathways of mitochondrial iron utilization, namely: ISC biogenesis, heme synthesis, and mitochondrial iron storage (FTMT). The decreased iron utilization in these pathways reduces iron export from the mitochondrion as heme and ISCs, which is coupled with an increase in mitochondrial non-ferritin and redox-active iron deposits as inorganic crystallites. The latter lead to an increase in reactive oxygen species (ROS) production, which is potentiated by a decrease in NRF2-regulated anti-oxidant enzymes.

6. Why does defective ISC biogenesis cause mitochondrial iron-loading and cytosolic iron-depletion?

One of the most common phenotypes of defective ISC biogenesis is the development of mitochondrial iron-loading, with subsequent mitochondrial damage resulting, to some degree, from oxidative stress [3,7,47,84]. Indeed, disruptions to mitochondrial ISC biogenesis cause cytosolic iron-depletion and/or mitochondrial iron-loading [3,7,47,84]. For example, mitochondrial iron-loading occurs in: (*i*) erythroid progenitors of a sideroblastic anemia patient resulting from a GLRX5-deficiency [110]; (*ii*) skeletal myocytes of individuals with ISCU myopathy [111]; *Abcb10*-knockout mice, which causes anemia due to ineffective erythropoiesis in erythroid progenitors [56] (*iii*) cardiomyocytes and some neurons in FRDA and FRDA models [62,80,84,112,113].

6.1. Lessons learnt from frataxin deficiency

In the case of frataxin deficiency, the iron accumulation in affected cells occurs as redox-active, biomineral iron aggregates that are biochemically distinct from holo-ferritin molecules [80]. Mitochondrial iron accumulation has been detected in the heart, liver, and spleen, but not in the dorsal root ganglion, spinal cord, cerebellum, peripheral nerves, skeletal muscle, or pancreas of FRDA patients [112]. Interestingly, in the MCK transgenic mice harboring frataxin deficiency in the heart, diminished ISC enzyme activity was observed prior to the onset of mitochondrial iron accumulation [114], suggesting that, in frataxin deficiency, dysregulation in ISC biogenesis/metabolism precedes the precipitation of microcrystalline iron aggregates in the mitochondria [80].

Analysis of gene and protein expression in the MCK frataxin knockout mice identified a mechanism of iron redistribution characterized by an upregulation of genes involved in cellular and mitochondrial iron uptake (*i.e.*, TfR1 and Mfrn2, respectively), and down-regulation of genes encoding proteins involved in cytosolic iron storage (*i.e.*, ferritin-H and -L chains), cellular iron efflux (*i.e.*, Fpn1), mitochondrial heme synthesis (*i.e.*, δ-aminolevulinate dehydratase, uroporphyrinogen III synthase and ferrochelatase), and ISC biogenesis (Iscu 1/2 and Nfs1) [62]. In addition, it was shown *in vivo* in MCK frataxin-knockout mice that ^{59}Fe uptake from radiolabeled holo-transferrin was markedly increased in frataxin deficient hearts relative to wild-type mice, and further, that redistribution of ^{59}Fe from the cytosol to the mitochondria occurred [113]. These data suggest increased iron uptake occurred *via* the marked elevation in TfR1, and that the increased mitochondrial iron accumulation results from increased mitochondrial iron uptake [62,113].

In fact, in the MCK frataxin knockout heart, mitochondrial iron-loading resulted from up-regulated iron uptake, decreased cytosolic iron storage and increased mitochondrial iron accumulation, coupled with defective iron incorporation into heme and ISCs [62,113]. In the absence of frataxin, the marked iron uptake into the frataxin deficient mitochondria is not effectively incorporated into heme or ISCs, nor is it effectively incorporated into FTMT, as its expression is reduced [62,

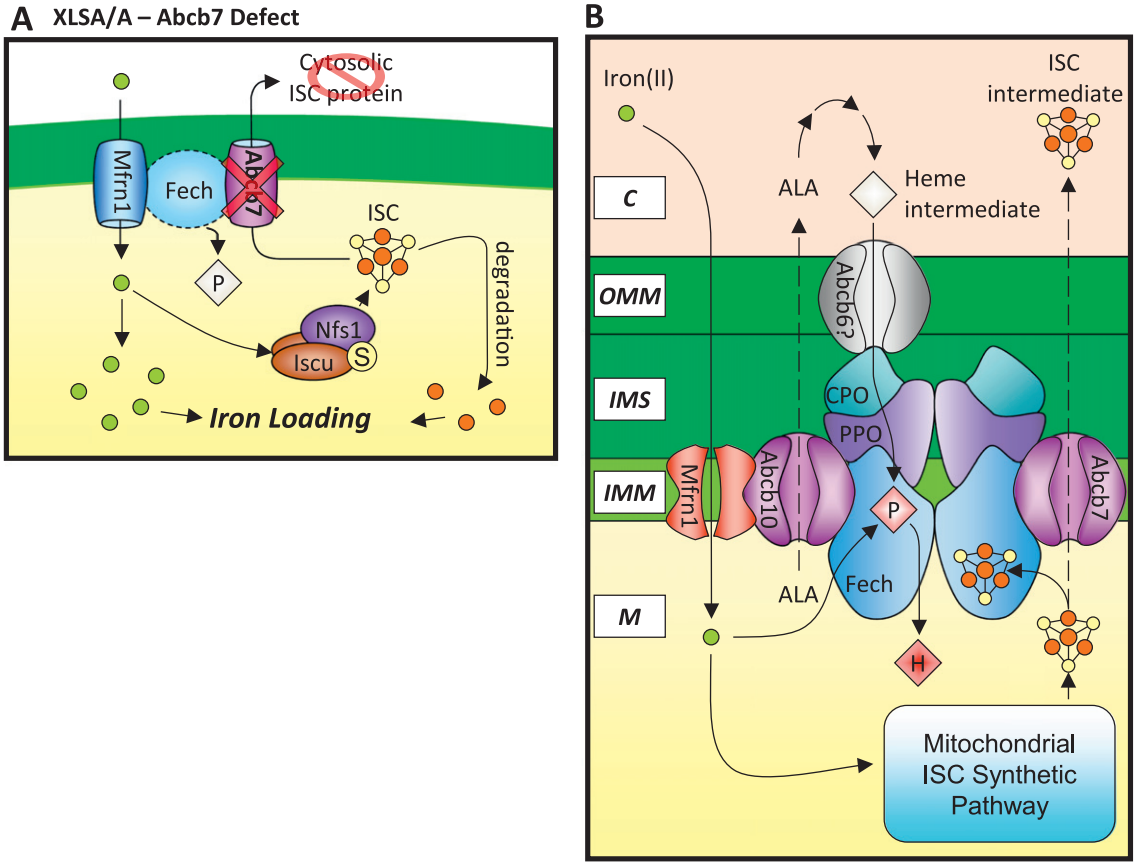

Fig. 9. X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A) and schematic of a possible mitochondrial iron-transport complex in the developing erythron. (A) X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A). XLSA/A is caused by mutation in the mitochondrial transporter Abcb7, which has been suggested to be the exporter of mitochondrial ISCs. This mutation leads to elevated PPIX levels and decreased Fech activity, possibly due to the requirement of Abcb7 for Fech's activity, and ultimately results in the accumulation of mitochondrial iron. (B) Recent studies have suggested the existence of an interaction between the mitochondrial transporters and the terminal enzymes involved in heme synthesis. This interaction involves the formation of a higher-order multimeric complex that enhances the efficiency of the activities of each of the component proteins, such as Mfrn1 and Fech. The formation of this multimeric complex is likely to facilitate the efficient mobilization of metabolites and intermediates for the synthesis of heme during erythroid differentiation. C, cytosol; OM, outer mitochondrial membrane; IMS, inter-mitochondrial membrane space; IM, inner mitochondrial membrane; M, mitochondrial matrix; H, heme; P, PPIX; ISC, iron–sulfur cluster; CPO, coproporphyrinogen III oxidase; PPO, protoporphyrinogen III oxidase.
Modified from (Huang et al.) [47].

80]. Hence, the increased iron uptake and decreased iron release by the mitochondrion leads to toxic mitochondrial iron-loading that promotes oxidative stress (Fig. 8).

Importantly, the oxidative stress in frataxin deficient cells is potentiated by a decrease in the antioxidant nuclear factor-erythroid 2-related factor 2 (NRF2) signaling pathway [115–117] (Fig. 8). This pathway drives a major antioxidant cellular response to oxidative stress by up-regulating the expression of genes possessing a *cis*-acting *anti-oxidant response element* (*ARE*) [118,119], leading to increased levels of major antioxidant enzymes, such as the superoxide dismutases, catalase, glutathione reductase, glutathione-S-transferase, the glutamate-cysteine ligase catalytic subunit, and NADH quinone oxidoreductase [118,120]. The down-regulation of the NRF2 signaling pathway by frataxin depletion appears to be due to a decrease in NRF2 expression and/or a decrease in NRF2 translocation to the nucleus [115–117], the latter of which is required for increased transcription of *ARE*-containing genes [118,119]. The molecular mechanism(s) leading to a down-regulation of NRF2 may be an important aspect of the FRDA pathogenesis that warrants further exploration.

6.2. Hypotheses for regulation of cellular iron metabolism by ISC biogenesis

How does ISC biogenesis regulate cellular iron metabolism? While it was initially suggested that iron could only exit the mitochondrion following incorporation into an ISC [121], this view was later reformulated to suggest ABCB7, a mitochondrial half-transporter located in the IMM, had a role in the mitochondrial export of a sulfur-containing moiety required for cytosolic ISC biogenesis [6]. Whether iron is also transported by ABCB7 is unknown and the identity of the ABCB7 export substrate remains unclear [6]. Importantly, ABCB7 appears to be required for cytosolic ISC biogenesis. As might be expected, mitochondrial iron-loading occurs in X-linked sideroblastic anemia with ataxia, which is caused by mutations in ABCB7 (Fig. 9A) [122]. Indeed, studies in developing erythrons suggest that mitochondrial iron utilization for ISC and heme synthesis is a tightly coupled process involving a metabolon of physically-interacting mitochondrial membrane transporters under conditions of an increased requirement for mitochondrial iron [123–126] (Fig. 9B).

One hypothesis for ISC biogenesis-mediated control over cellular iron metabolism relates to IRP1. Cellular iron metabolism is controlled by IRP1 and 2, and under normal *in vivo* conditions, IRP2 is the major contributor to iron-dependent regulation of the IRP–IRE system [127]. However, there is typically a large pool of IRP1 in the ISC-containing aconitase form [38,128]. A deficit in ISC biogenesis in the mitochondria and/or cytosol would be expected to lead to a decrease in the ISC-containing form of IRP1 and a corresponding increase in the IRP1-IRE-binding form, which would lead to increased TfR1 and decreased ferritin. It is known that the depletion of numerous components of the ISC biogenesis pathway leads to activation of the IRE-binding activity of IRP1, leading to increased TfR1 and decreased ferritin [see [45]]. For example, suppression of ISCU by RNAi inactivates mitochondrial and cytosolic aconitases and leads to inappropriate upregulation of IRP1-IRE-binding activity, which leads to disrupted iron homeostasis [70a].

In some model systems, frataxin deficiency does lead to IRP1-activation [129]. Indeed, a very recent study suggests that frataxin deficiency in mice leads to mitochondrial iron overload through a combination of increased Irp1 activation, resulting from defective ISC biogenesis, and heme deficiency-dependent upregulation of Mfrn2 [131]. However, there is no change in the IRE-binding activity of IRP1 in the heart of MCK frataxin knockout mice relative to their wild-type counterparts, which was fully activated from 4 weeks-old [113]. Nevertheless, there is a significant increase in IRP2-IRE-binding activity in the frataxin knockout mice [113]. Moreover, RNAi-mediated depletion of HSC20 resulted in up-regulation of TfR1 and IRP2, suggesting a deficit in the J-protein co-chaperone also leads to a state of cytosolic iron deficiency and mitochondrial iron-loading [100]. This suggests IRP2-activation can have primacy over IRP1-activation in terms of responding to iron levels in some settings, although the mechanism is unclear.

7. Conclusions and perspectives

The list of diseases with a defect in ISC biogenesis and delivery is growing. In this review, we have discussed the field of iron- and ISC-biogenesis from the initial uptake of iron by the cell to its utilization in ISC biogenesis. We have discussed the major players involved in their biogenesis and delivery to target proteins, as well as highlighting new players and mechanisms that have provided insight into the processes involved and how these connected processes are regulated. We have further suggested ways in which mitochondrial ISC biogenesis may be linked with cellular iron processing; a hypothesis clarified by analysis of diseases of ISC assembly (*e.g.*, FRDA). Increased knowledge of mitochondrial "ins-and-outs" of iron-cofactor processing pathways, and their ability to communicate with the other compartments (*e.g.*, the cytosol), will help shape the investigation of iron metabolism in health and disease.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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