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Review Article

Frataxin and the molecular mechanism of mitochondrial iron-loading in Friedreich's ataxia

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Abstract

The mitochondrion is a major site for the metabolism of the transition metal, iron, which is necessary for metabolic processes critical for cell vitality. The enigmatic mitochondrial protein, frataxin, is known to play a significant role in both cellular and mitochondrial iron metabolism due to its iron-binding properties and its involvement in iron–sulfur cluster (ISC) and heme synthesis. The inherited neuro- and cardio-degenerative disease, Friedreich's ataxia (FA), is caused by the deficient expression of frataxin that leads to deleterious alterations in iron metabolism. These changes lead to the accumulation of inorganic iron aggregates in the mitochondrial matrix that are presumed to play a key role in the oxidative damage and subsequent degenerative features of this disease. Furthermore, the concurrent dys-regulation of cellular antioxidant defense, which coincides with frataxin deficiency, exacerbates oxidative stress. Hence, the pathogenesis of FA underscores the importance of the integrated homeostasis of cellular iron metabolism and the cytoplasmic and mitochondrial redox environments. This review focuses on describing the pathogenesis of the disease, the molecular mechanisms involved in mitochondrial iron-loading and the dys-regulation of cellular antioxidant defense due to frataxin deficiency. In turn, current and emerging therapeutic strategies are also discussed.

Key words: frataxin, iron dys-regulation, mitochondrial iron processing, nuclear factor erythroid 2-related factor 2 (Nrf2), oxidative stress.

INTRODUCTION

Friedreich's Ataxia (FA) is the most common hereditary autosomal recessive ataxia characterized by progressive cardio- and neuro-degeneration due to deficient expression of the mitochondrial protein, frataxin [1,2]. The disease affects both the central and peripheral nervous systems with neurological phenotypes that result from lesions in the dorsal root ganglia, corticospinal tracts, sensory peripheral nerves, and dentate nucleus in the cerebellum [3]. Major neurological symptoms include gait and limb ataxia, tendon areflexia, sensory loss, dysarthria, dysphagia, and pyramidal signs [4,5]. The heart, skeleton, and the endocrine pancreas are also affected in FA and this leads to the common non-neurological symptoms, namely: cardiomyopathy, skeletal abnormalities and diabetes mellitus [2,3,5].

The estimated frequency of asymptomatic carriers is from 1:50 to 1:100 in people of European, Middle Eastern, Indian, and North African descent, with approximately one individual in 50,000 being affected by the disease [1–3,6]. However, FA is significantly less prevalent among individuals of Asian, Saharan African, and American Indian decent [6,7]. Onset of the disease is typically around puberty with gradual progression of symptoms and shortened life-span, mostly due to cardiac complications rather than neurological causes [5]. Cardiomyopathy often develops after the onset of neurological features with a significant proportion of patients exhibiting cardiac hypertrophy leading to premature death [5,8].

In the majority of cases, the pathogenic mutation in FA consists of a homozygous GAA trinucleotide repeat hyper-expansion in the first intron of the *frataxin* gene (*FXN*) located on chromosome 9q13 [2,3,9]. Approximately 4% of cases are due to a heterozygous point mutation in the other allele [3]. The number of GAA repeats in healthy individuals ranges from 6 to 36, whereas FA patients have a range of 70–1700, most commonly between 600 and 900 repeats [10]. This pathogenic mutation causes an abnormal conformation of DNA, resulting in the transcriptional

Abbreviations: ALAS, aminolevulinate synthase; CIA, cytosolic iron–sulfur cluster assembly; DCYTB, duodenal cytochrome *b*; DMT1, divalent metal transporter-1; FA, Friedreich's ataxia; Fe(II), ferrous iron; Fe(III), ferric iron; FECH, ferrochelatase; FPN1, ferroportin 1; FTH1, ferritin heavy chain subunit 1; FTL, ferritin light chain; FTMT, mitochondrial ferritin; *FXN*, *frataxin* gene; GSH, glutathione; IRE, iron responsive element; IRP, iron regulatory protein; ISC, iron–sulfur cluster; ISCU, iron-sulfur cluster assembly enzyme; KO, knockout; LIP, labile iron pool; mAconitase, mitochondrial aconitase; MCK, muscle creatine kinase; MFRN1/2, mitoferrin 1 or 2; MRCKα, myotonic dystrophy kinase-related Cdc42-binding kinase α; NAC, *N*-acetylcysteine; NCOA4, nuclear receptor coactivator 4; NFS1, cysteine desulfurase; Nrf2, nuclear factor erythroid 2-related factor 2; PCBP, poly r(C)-binding protein; PPIX, protoporphyrin IX; ROS, reactive oxygen species; Tf, transferrin; TfR1, transferrin receptor 1.

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silencing of *FXN* [7]. This leads to a reduction in its mRNA expression and its encoded protein, frataxin [7]. The repression of *FXN* expression is due to the arrested RNA polymerase II progression and the formation of heterochromatin-like structures proximal to the region of the expansion [4,11]. This prevents the efficient transition from initiation to elongation during *FXN* transcription [4,11]. Furthermore, the expansion causes a decrease in splicing efficiency of the first intron, whereby GAA repeats bind to splicing factors that affect the turnover of intronic RNA in pre-mRNA processing, decreasing the generation of mature mRNA [12]. The overall consequence is a decrease in the expression of frataxin, which is a mitochondrial protein known to play a significant role in mitochondrial iron metabolism particularly, iron–sulfur cluster (ISC) biogenesis and heme synthesis [10,13,14]. In FA, frataxin deficiency plays a critical role in the pathology of the disease in which redox-active iron accumulates in the mitochondria, disrupting iron metabolism and causing oxidative stress [2,4].

This review will discuss the molecular mechanisms of mitochondrial iron-loading involved in the pathogenesis of FA. Since frataxin plays a major role in regulating iron metabolism and its deficiency is a primary pathological feature of the disease, it is necessary to first review the processes of cellular and mitochondrial iron metabolism in order to understand the deleterious consequences of frataxin deficiency.

GENERAL IRON METABOLISM AND HOMEOSTASIS

Iron plays an essential role in many important cellular processes responsible for cell growth, proliferation and death [15]. Such cellular processes include energy production, cellular respiration, electron and oxygen transport, mitochondrial energy metabolism and DNA synthesis [15–17]. Consequently, depletion of iron can lead to cellular death [18,19]. However, excessive cellular iron can also cause cytotoxicity as a result of the iron-mediated generation of reactive oxygen species (ROS) [20,21]. In the presence of oxygen, ferrous iron [Fe(II)] catalyses the production of cytotoxic hydroxyl radicals via the Fenton and Haber–Weiss-like reactions [22,23]. Therefore, iron homeostasis is tightly controlled to ensure cell survival [24]. Considering that many diseases, including FA, attribute their pathogenesis to the dys-regulation of iron homeostasis, it is imperative to first review iron metabolism under physiological conditions in order to understand its defective alterations.

IRON UPTAKE

Dietary and cellular iron uptake

In mammals, dietary non-heme iron is reduced from the ferric form, Fe(III), to the ferrous form, Fe(II), before being exported to the circulation through the enterocytes of the duodenal and upper jejuna epithelia [25]. This reduction of iron occurs at the apical surface of these enterocytes, which appears to be mediated by enterocytic ferrireductases [23,25] and secreted ascorbate [26]. The cytochrome b_{561} protein, duodenal cytochrome *b* 4(DCYTB/CBRD1/CYB561A2), is an ascorbate-dependent oxidoreductase that is thought to be the most likely ferrireductase to be involved in the reduction of dietary iron at the brush-border of the duodenal enterocyte [23,27,28]. However, the importance of DCYTB in duodenal iron absorption is unclear, as it has been found to be non-essential for dietary iron uptake [23,29], suggesting that other enzymatic or non-enzymatic reductants (e.g. secreted ascorbate) may also be involved [29]. Interestingly, studies have demonstrated that non-enzymatic ferrireduction can be achieved by ascorbate that is actively secreted from cells, which can contribute up to 50% of the reduction of low-M_r iron (e.g. non-heme iron) [30–32].

Once iron is reduced to Fe(II), it is transported into the enterocyte via the divalent metal transporter-1 (DMT1) [33], which is expressed at the apical surface for intestinal uptake [25]. Subsequently, Fe(II) is often thought to enter the 'labile iron pool' (LIP), which is a methodologically-defined chelatable-iron compartment in the cytosol that remains poorly characterized [25]. The Fe(II) is then exported to the plasma through the basolateral membrane of the enterocyte through the iron exporter ferroportin 1 (FPN1) [23,34]. During its efflux, Fe(II) is subsequently oxidized to Fe(III) by the transmembrane, copper-dependent ferroxidase, hephaestin, which suggests that iron export and re-oxidation are coupled [23,35]. The soluble plasma proteins, ceruloplasmin and transferrin (Tf), are also thought to play a role in oxidizing plasma Fe(II) to Fe(III) to enhance iron efflux from cells [36]. The released Fe(III) in the plasma then binds to apo-Tf to form holo-Tf, which is the major plasma iron transport protein that contains two high affinity Fe(III) sites (for reviews see: [16,17,23,37]). Tf functions to maintain the redox-inert state of iron in the circulation and facilitates its systemic distribution to tissues and cells [38,39]. The cellular uptake of iron involves the binding of mono- and di-ferric Tf to transferrin receptor 1 (TfR1) on the plasma membrane, with this complex subsequently being internalized via receptor-mediated endocytosis [13] (Figure 1).

A vacuolar-type proton-pumping ATPase instigates the acidic environment of the endosome, resulting in the reduction of the affinity of Tf for Fe(III) [13,25]. The NAD(P)H-dependent ferrireductase, STEAP3, is thought to play an important role in the reduction of this intra-endosomal iron from Fe(III) into Fe(II) prior to its export into the cytosol by the Fe(II)-selective proton-dependent DMT1 at the endosomal membrane [23,40,41]. This nascent cellular iron can then be utilized for cellular and mitochondrial iron metabolism. The endosomes containing iron-free Tf (apo-Tf) and TfR1 are then recycled to the cell surface whereby apo-Tf dissociates from TfR1 at pH values close to neutrality and returns to the circulation, resuming iron transport and uptake [13,37,42] (Figure 1).

CELLULAR IRON METABOLISM

There are a number of proposed destinations for iron upon its exportation from the endosome to the cytosol (Figure 1). In both

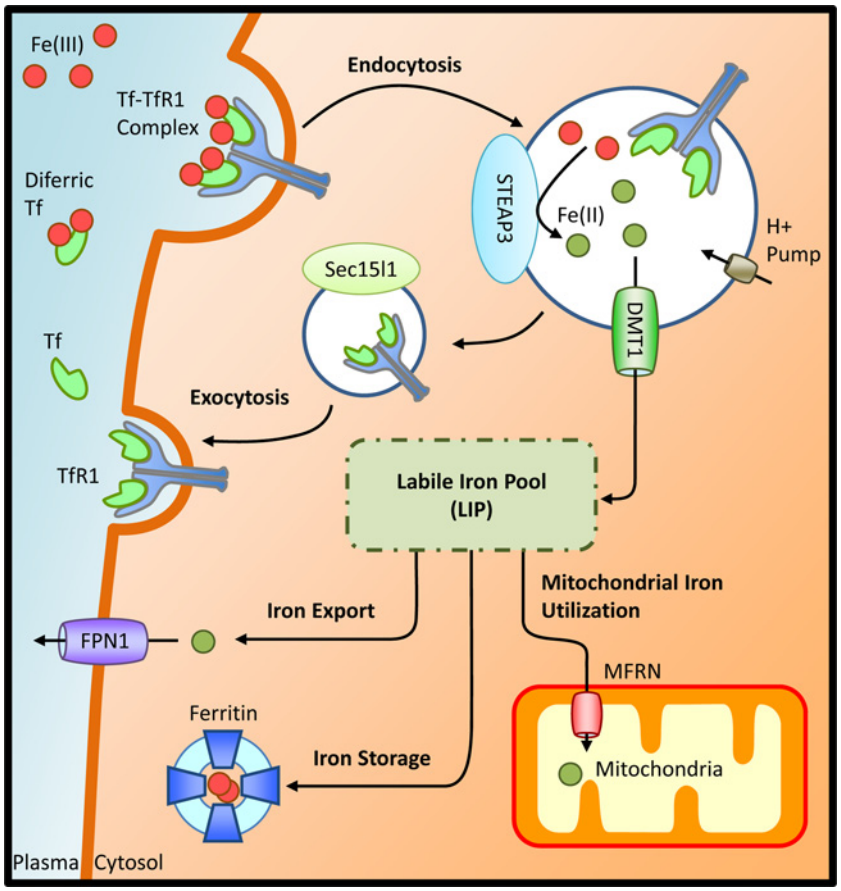

Figure 1 **Schematic model of cellular iron uptake and trafficking**

Fe(III) binds to Tf, which then binds with the TfR1 and enters the cytosol to form an endosome via receptor-mediated endocytosis. The exocyst complex protein, Sec15l1, has a role in the trafficking of the Tf-containing endosome and iron uptake. Within the endosome, Fe(III) is released from Tf via the acidic microenvironment, and is then reduced to Fe(II) by the enzyme, STEAP3. The Fe(II) is then transported to the cytosol via DMT1. Once in the cytosol, Fe(II) transiently resides in the LIP before being either stored in ferritin, exported by FPN1, or taken up into the mitochondrion by the mitochondrial transporter, MFRN.

erythroid and non-erythroid cells, cytosolic iron enters a low M_r LIP within the cytosol before entering downstream metabolic processes [43,44]. However, the nature of the LIP remains unclear with very little low M_r iron being found in actively metabolizing reticulocytes [45]. It has been proposed that the LIP is composed of redox-active and chelatable Fe(II) that may be exchanged between metal-binding ligands for cellular processes [46,47].

Importantly, ROS generation in cells is dependent on the level and form of iron, which is mediated by changes in iron uptake and export, as well as iron chelation and storage in the cytosol [47]. Cytosolic iron can be stored within nano-cages created by hetero-polymers of ferritin heavy chain (FTH1) and ferritin light chain (FTL) [48,49]. Recently, the iron chaperones, poly r(C)-binding protein (PCBP) 1 and 2, have been shown to mediate the uptake of iron by ferritin [50]. Moreover, PCBP2 has been demonstrated to bind to the cytoplasmic domain of endosomal DMT1, suggesting its role in the direct protein-to-protein transport of iron from the endosome to ferritin for storage [51]. The labile iron-bound to ferritin is catalytically mineralized to become the iron core of the ferritin protein [48]. FTH1, but not FTL, has intrinsic ferroxidase activity, and the storage of iron within a redox-inert form within ferritin prevents the iron from undergoing Fenton reactions that would otherwise produce toxic free radicals [48]. Importantly, stored iron can be released from ferritin through lysosomal proteolysis (i.e. ferritinophagy), involving nuclear receptor coactivator 4 (NCOA4) and ATG8 proteins that recruit cargo–receptor complexes into autophagosomes [52]. NCOA4 is down-regulated by high cellular iron levels in a HERC2 ubiquitin ligase and proteasome-dependent manner, providing a partial explanation for how NCOA4 regulates ferritinophagy [53].

Furthermore, cytosolic iron can also be exported from the cell via the iron exporter FPN1, which also plays a crucial role in maintaining systemic iron homeostasis [34,40,54] (Figure 1). Due to the necessity of iron in so many metabolic processes and the double-edged nature of iron where a deficiency or excess can be toxic, its utilization is closely monitored through a number of regulatory systems that are discussed below.

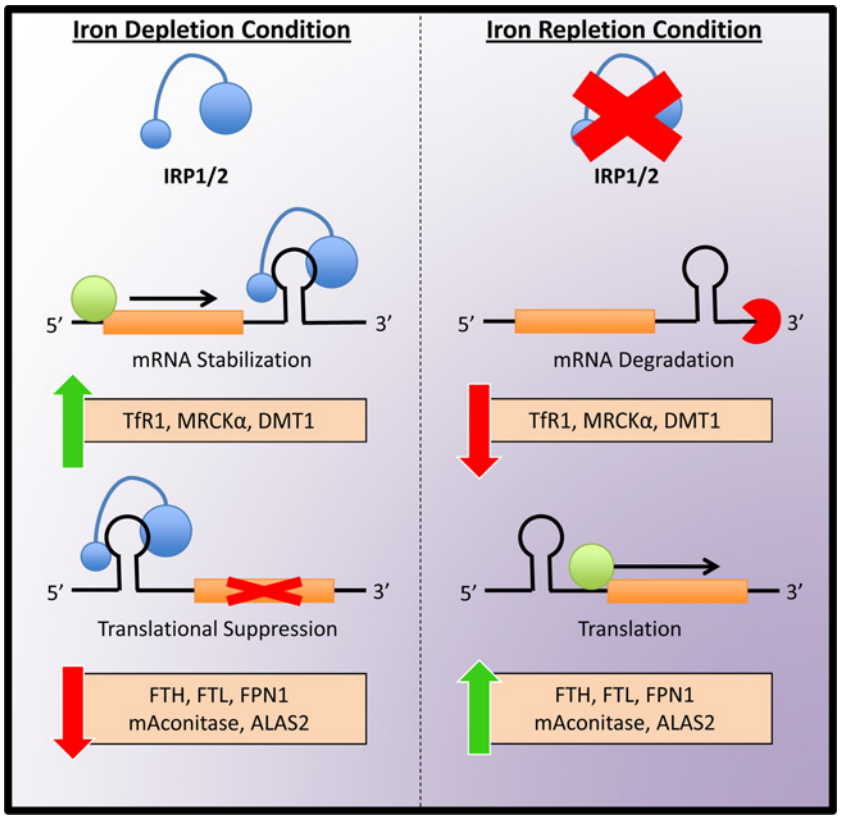

Figure 2 **Regulation of cellular iron metabolism by IRPs**

In iron deplete conditions, IRPs bind to the IREs at either the 5′ or 3′ UTR of the targeted mRNA. Binding of IRPs to 3′ IREs stabilizes mRNA for translation, whereas binding of IRPs to 5′ IREs inhibits translation. When iron status is replete, IRP1 has cytosolic aconitase activity through the assembly of an ISC and is thus unable to bind IREs, whereas IRP2 undergoes proteasomal degradation. The 5′ IRE-containing mRNAs include FTH and FTL, FPN1, erythroid-specific δ-aminoleuvulinate synthase 2 (ALAS2) and mAconitase, whereas 3′ IRE-containing mRNAs include TfR1, DMT1 and MRCKα. The translational activity of mRNAs is dependent on the IRE-binding activity of IRP1 and IRP2, which is dependent on cellular iron levels.

REGULATION OF IRON METABOLISM

The regulation of iron metabolism prevents the toxicity associated with iron deficiency, but also prevents oxidative damage by directly regulating the availability of ROS-generating redox-active iron [17]. To this end, cellular iron levels are monitored and regulated by cytosolic iron regulatory proteins (IRPs) 1 and 2 that affect the expression of proteins involved in iron uptake, transport, and storage in the cytosol [47]. The IRPs are RNA-binding proteins that bind to iron responsive elements (IREs), which are specific sequences in the 5′ or 3′ UTRs of mRNAs that encode iron homeostatic proteins. The binding of IRPs to IREs directly controls the stability or translation of their targeted mRNA, thereby regulating control over the synthesis of iron metabolism proteins [16,47] (Figure 2).

The binding of IRPs to mRNAs containing 5′ IREs leads to translational repression, which rapidly reduces the translation of proteins responsible for iron storage (FTH1 and FTL), cellular respiration [mitochondrial aconitase (mAconitase)], heme synthesis (erythroid 5-aminolevulinate synthase) and iron export (FPN1) [22,55,56]. On the other hand, the binding of IRPs to mRNAs containing a 3′ IRE increases their stability, leading to increased translation of encoded proteins, such as TfR1 (cellular iron uptake), DMT1 (endosomal transporter of iron), and myotonic dystrophy kinase-related Cdc42-binding kinase α (MRCKα; cytoskeletal re-organization) [57].

Cellular iron levels regulate the IRE-binding activity of IRPs, which in turn regulates the translational activity of targeted mRNAs [22]. Under conditions of low cellular iron, IRP1- and IRP2-binding to IREs is increased [22] (Figure 2). This promotes stability and translation of mRNAs containing 3′ IREs, which up-regulates their encoded proteins, whereas mRNAs containing 5′ IREs are translationally suppressed, leading to down-regulation of their encoded proteins [22,58]. As a result, iron homeostasis is restored with reduced iron loss, enhanced iron uptake and increased iron availability for cellular metabolism [59].

The IRP-dependent IRE-binding activity of IRPs is significantly decreased under conditions of high cellular iron levels [22]. This occurs by two different mechanisms, depending on the IRP involved. The IRE-binding activity of IRP1 is inactivated due to acquisition of an ISC, which converts the protein into a cytosolic aconitase [22]. In the case of IRP2, IRE-binding capacity is lost due to its degradation by iron-dependent ubiquitination [60–62] (Figure 2). Hence, in iron-replete cells, the translation of the

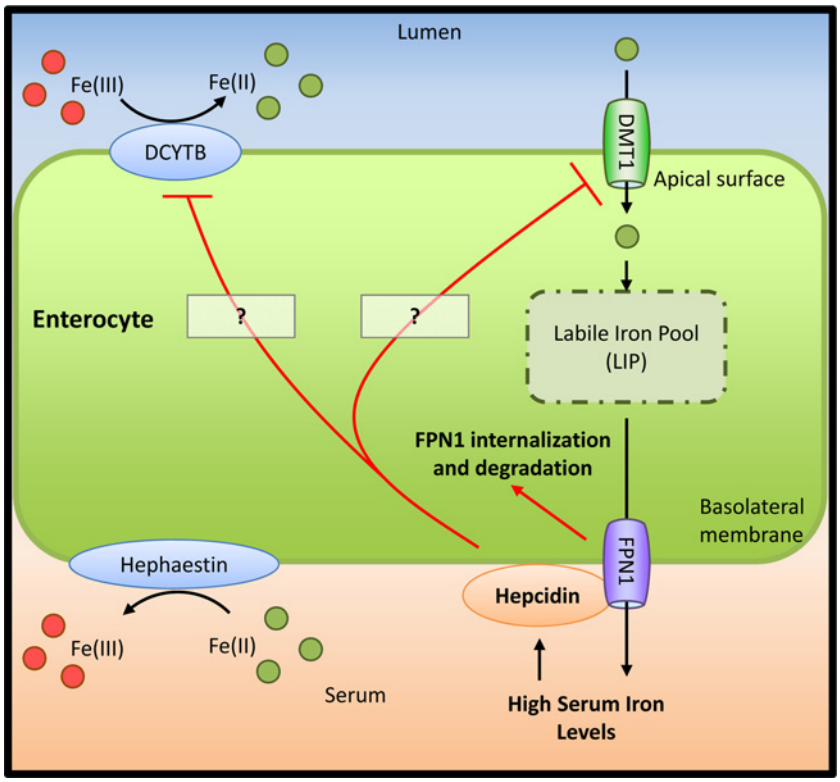

Figure 3 **The role of hepcidin in cellular iron uptake in response to increased systemic iron levels**
Tissue-iron overload causes the liver to secrete hepcidin into the circulation, which binds to FPN1 and causes its internalization for degradation in enterocytes, hepatocytes and macrophages. This prevents iron export from the liver and reduces serum iron availability to other tissues. Hepcidin also negatively regulates the iron transporter, DMT1, and the ferrireductase, DCYTB, via unknown mechanisms, leading to reduced dietary iron uptake.

mRNA encoding for TfR1 decrease, whereas mRNAs encoding ferritin subunits and FPN1 are more rapidly translated. This response in the iron replete state maintains iron homeostasis and prevents cytotoxicity by decreasing iron uptake, and increasing iron storage and export (for reviews see: [16,22]).

Iron homeostasis involves the key role of the iron exporter, FPN1, which is a major target of a systemic iron regulatory system, in addition to the post-transcriptional IRP–IRE regulatory system [17]. Systemic iron levels (e.g. plasma Tf saturation and hepatic iron levels) are predominantly 'sensed' by hepatocytes of the liver, which promotes the transcription and secretion of the first described 'hormone of iron metabolism', hepcidin [63]. Notably, hepcidin is a peptide that plays a major role in regulating the post-translational expression of FPN1 [63]. This is thought to occur by the binding of hepcidin to FPN1, causing its cytoplasmic phosphorylation by JAK2 kinase followed by its internalization by endocytosis and subsequent degradation in the lysosome, which reduces cellular iron export [34,63–65]. This occurs in cells such as enterocytes, hepatocytes and macrophages and effectively limits serum iron availability [63,66].

In mammals, iron overload induces the secretion of hepcidin from the liver, which binds to FPN1 and down-regulates its expression on the plasma membrane, thereby reducing iron export from enterocytes or iron-storage cells [63,67] (Figure 3). It is also known that hepcidin negatively regulates iron transport through the inhibition of apical iron uptake via the induction of DMT1 degradation [25,67,68] (Figure 3). Therefore, hepcidin functions in response to changes in systemic iron levels to regulate iron uptake and maintain iron homeostasis [69]. Conversely, during iron deficiency, hepcidin synthesis is repressed, and DMT1 expression is induced at the transcriptional level to increase systemic iron uptake [68]. Thus, overall, during systemic iron deficiency, iron homeostasis is maintained with enhanced iron absorption and accumulation, and decreased cellular iron efflux.

MITOCHONDRIAL IRON UPTAKE AND METABOLISM

Iron uptake by the mitochondrion

The mitochondrion is a critical organelle for intracellular iron processing [70,71]. There are a number of hypotheses that explain the intracellular mechanism of iron trafficking and uptake by the mitochondrion [13].

Studies in erythroid cells indicated that there was very little low M_r iron, with the kinetics of iron uptake into such fractions having the properties of an end product rather than an intermediate in metabolism [45]. This observation prompted the hypothesis that intracellular iron was transported from one protein to another in their hydrophobic environments preventing the

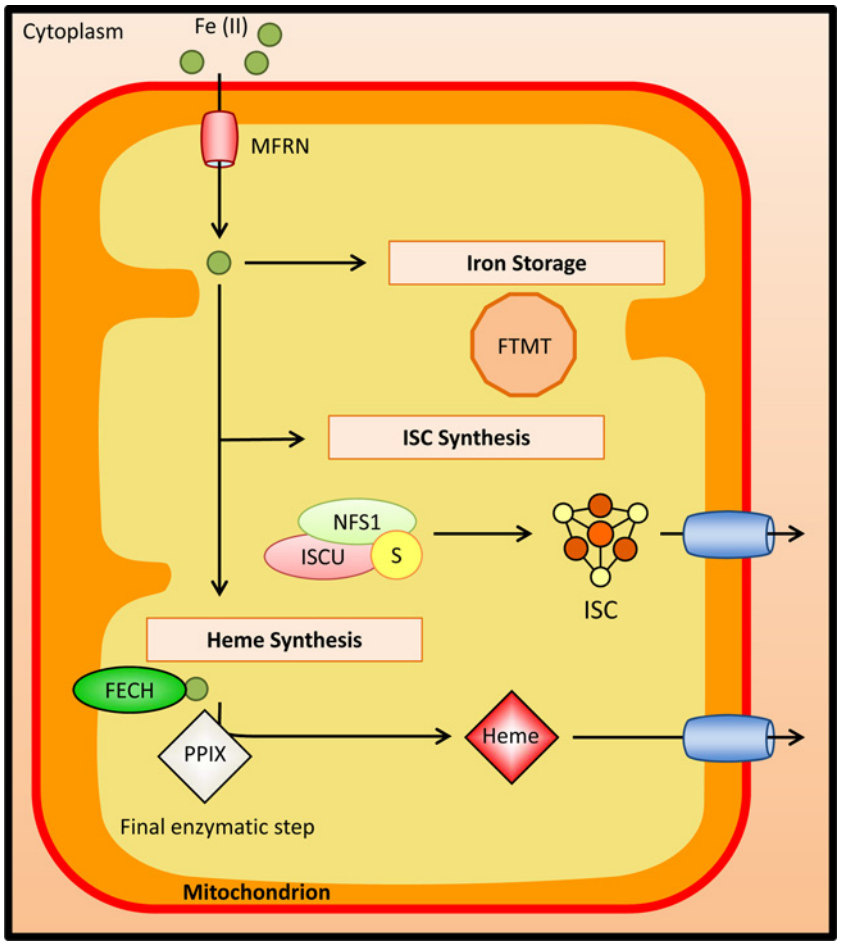

Figure 4 Mitochondrial iron uptake and utilization
Cytosolic iron Fe(II) is taken up into the mitochondrion by crossing the inner mitochondrial membrane through the mitochondrial iron importer, MFRN. Iron in the mitochondrion can be processed by three main pathways: (1) iron storage via sequestration by FTMT; (2) iron–sulfur (S) cluster (ISC) synthesis mediated by cysteine desulfurase (NFS1) and scaffolding protein iron-sulfur cluster assembly enzyme (ISCU) and (3) heme synthesis via the final enzymatic step of iron insertion by FECH into PPIX.

reaction with oxygen and generation of ROS [45]. Such transport proteins could include chaperone molecules such as the previously described members of the PCBP class [50].

Later studies suggested a 'kiss-and-run' hypothesis which describes the direct transport of iron from the endosome to the mitochondria in erythroid cells [44]. With this model, the endosome transiently docks at the mitochondrion to deliver iron, avoiding the cytosolic LIP [13,44]. However, the molecular mechanisms that are involved regarding this potential process remain unclear. It has also been suggested that cytosolic iron may be taken up directly by the mitochondria as 'free' Fe(II), which is dependent on the mitochondrial membrane potential [72]. This would involve the passage of iron across the outer and inner mitochondrial membranes. The mitochondrial-specific iron transport protein, mitoferrin (MFRN), facilitates the transport of iron across the inner mitochondrial membrane [73] (Figure 4). Interestingly, MFRN exists as two homologues, MFRN1 (SLC25A37) and MFRN2 (SLC25A28) that are vital for mitochondrial iron uptake in erythroid and non-erythroid cells respectively [73,74]. MFRN1 interacts with the inner mitochondrial membrane ATP-binding cassette transporter, ABCB10, to enhance its stability and efficiency for erythroid mitochondrial iron import [23,75].

MITOCHONDRIAL IRON METABOLISM

ISC biogenesis

The mitochondrion is also a major site of cellular iron utilization. Elemental iron is utilized by the mitochondrion for three major metabolic pathways: (i) mitochondrial ISC biogenesis; (ii) heme biosynthesis; and (iii) mitochondrial iron storage (Figure 4) [17,23,71]. These metabolic processes are crucial for the vitality of the cell [17,23,71]. ISCs are ancient and vital protein-bound prosthetic groups that are found in a wide range of proteins found in the mitochondria, cytosol and nucleus. Many of these proteins play major roles in metabolism, such as metabolic catalysis, electron-transfer in redox reactions and the regulation of gene expression [76,77]. Although ISCs come in different

configurations, the most common in mammals are the [2Fe-2S] and [4Fe-4S] clusters in which iron ions are coordinated with cysteine residues [76,78].

Importantly, mammalian ISC biogenesis occurs in two different, but functionally connected compartments: the 'ISC' assembly apparatus in mitochondria and the more recently-characterized cytosolic ISC assembly (CIA) system (overviewed below). The biogenesis of ISCs, in general, can occur in two phases: (1) the transient synthesis of the ISC on a scaffold assembly protein and (2) the transfer of these ISCs to target apo-proteins [21,79].

In the mitochondrial ISC system, the ISC core is assembled with the cysteine desulfurase (NFS1) cysteine desulfurase, which abstracts sulfur from cysteine residues with the aid of the cofactor, pyridoxal phosphate [80,81]. In eukaryotes, the small accessory protein, ISD11, and two monomers of the dedicated scaffold protein, iron-sulfur cluster assembly enzyme (ISCU), bind NFS1 [81,82]. It is known that ISD11 functions to stabilize the cysteine desulfurase, whereas ISCU provides the backbone structure for the formation of new clusters containing covalently bound iron and inorganic sulfur [81–83]. The binding of Fe(II) to the assembly contributes to the formation of ISCU, which allows for the synthesis of ISCs [13,84,85].

Of note, ISCU is found in both the mitochondria and the cytosol of mammalian cells, suggesting that this scaffolding protein may contribute to both mitochondrial and CIA-dependent ISC biogenesis [21,86]. Furthermore, the assembly of nascent clusters relies on a source of electrons to achieve the appropriate electron configurations [81]. In mammals, ferredoxins 1/2 and ferredoxin reductase have been implicated to be responsible for this activity [87,88]. Newly synthesized ISCs are transferred from the ISCU to recipient proteins in which the ISC functions as a cofactor for other cellular metabolic processes, such as the respiratory chain and the citric acid cycle [17,21,81].

The complex logistics of this directed transfer of nascent ISCs to specific apo-proteins occurs by the concerted action of cluster-transfer proteins [78]. These proteins dissociate the nascent ISC from ISCU and ensure accurate and specific transfer to the correct apo-proteins, as well as assisting in the correct assembly and integration of the ISC at the acceptor site [78,79,81]. A detailed discussion of this aspect of mitochondrial ISC biogenesis is beyond the scope of this article. As such, we refer readers to ISC-focused reviews [78,79,81].

As alluded to above, the biogenesis of extra-mitochondrial ISCs occurs in the CIA pathway. The CIA pathway involves distinct molecular machineries and, like mitochondrial ISC biogenesis, occurs in two major steps that are highly conserved throughout the eukaryotes involving nine known proteins [89–92]. Importantly, the CIA pathway has a dependence on mitochondrial sulfur metabolism [93]. Indeed, in the first step of the mammalian CIA pathway, a [4Fe-4S] cluster is assembled on a scaffolding hetero-tetrameric complex composed of the P-loop NTPases, NUBP1 and NUBP2 [90,94,95]. This process requires a currently unknown source of sulfur (designated 'X-S'), which appears to be exported from the mitochondrion by ABCB7 [96]. Additionally, an intermembrane space sulfhydryl oxidase, GFER, may play a role in disulfide formation and the facilitation of export of this unknown intermediate to the cytosol [97,98]. As a detailed discussion of the CIA pathway is outside the scope of the current article, we refer readers to the following excellent reviews [92,99].

Heme synthesis

The mitochondrion can also utilize iron for the synthesis of heme (Figure 4), which is an essential cofactor of important proteins such as hemoglobin and myoglobin [13,100]. There are eight enzymes in the heme biosynthetic pathway that involves all compartments of the mitochondrion, as well as four intermediate steps in the cytosol [100]. The first and rate-limiting enzyme of the pathway, aminolevulinate synthase (ALAS), has two variants, namely ALAS1 that is expressed ubiquitously, and ALAS2 expressed in erythroid cells [17]. Notably, ALAS2 can be regulated by the IRP system due to its possession of an IRE in its 5′ UTR [101]. This indicates that the rate of heme synthesis is regulated by the levels of cytosolic iron in erythroid cells [100]. The final enzyme for heme synthesis is ferrochelatase (FECH) that functions to insert iron into protoporphyrin IX (PPIX), a heme precursor, to generate heme [70,100] (Figure 4). For a detailed review of heme metabolism, we refer readers to the following reviews [100,102].

Mitochondrial iron storage

Iron can be stored in the mitochondrion via its sequestration in mitochondrial ferritin (FTMT) [48,103] (Figure 4). The storage of iron in FTMT prevents redox reactions that generate ROS which induce mitochondrial oxidative damage [48]. FTMT has ferroxidase activity that inhibits the production of free radicals, thus preventing iron toxicity [48]. FTMT is expressed in cells with high-energy requirements and consumption, such as the brain, heart, thymus, kidney, smooth muscle and testis [103,104]. Consequently, FTMT is not expressed in tissues with iron storage functions, such as the liver and spleen [105]. This suggests that the level of FTMT expression is associated with oxidative metabolic activity [105]. Furthermore, FTMT may allow the mobility and trafficking of iron within the mitochondrion so as to prevent the formation of ROS [48].

The three major pathways involved in mitochondrial iron metabolism described above namely, heme synthesis, ISC synthesis and mitochondrial iron storage, are affected by the deletion of the mitochondrial protein, frataxin [106,107]. Deficient expression of frataxin results in the devastating neuro-degenerative and cardio-degenerative disease, FA [108]. Hence, the role of these molecules in mitochondrial iron metabolism is critical to understand and is described in detail below.

FRATAXIN AND ITS METABOLIC ROLE

Frataxin is an evolutionarily-conserved mitochondrial protein made of 210 amino acids and it is highly expressed in tissues that are rich in mitochondria such as the heart, skeletal muscle, liver and neurons [1,13,71]. Frataxin is encoded by the nuclear gene *FXN* and is synthesized as a precursor before being

transported into the mitochondria, where it is processed into its mature form [109,110].

The precise role of frataxin is unclear, but many studies have indicated an important role in iron metabolism [10,13,17]. There are indications for its involvement in regulating iron utilization by the mitochondria for ISC and heme biogenesis, and iron storage [71]. Frataxin was initially found to be associated with the inner mitochondrial membrane and crests, which led some investigators to suggest that frataxin could potentially regulate iron transport [111]. However, frataxin has no apparent structural features that would enable its anchorage at the mitochondrial membrane. Thus, it is possible that frataxin associates with inner mitochondrial membrane-bound mitochondrial proteins, such as FECH, to form complexes that regulate iron metabolism [71,112]. Many studies have suggested other possible roles for frataxin, including its function as an iron chaperone, a regulator of iron metabolism, or as an iron storage protein, as discussed further below [10].

Frataxin and ISC biogenesis

As discussed above, ISCs are prosthetic groups that are ligated to cysteine residues of proteins that are involved in a number of essential cellular processes [81]. They are composed of non-heme iron and inorganic sulfide and are predominately found in the cubane form of four iron and four sulfur atoms, i.e. [4Fe-4S] [21,81]. In addition to the functions discussed above, ISCs generated in the CIA pathway, such as those found in mammalian IRP1, enable this protein to function as a metabolic sensor that helps control cellular iron homeostasis [21,59,81] (also see above).

Frataxin has been suggested to act as a chaperone protein due in part to its ability to bind multiple Fe(II) ions on exposed acidic patches [21,71,113]. Previous studies using knockout (KO) mice [106,114] and yeast [115,116] models have shown that frataxin deficiency is associated with ISC deficiency, suggesting a possible role for frataxin in ISC biosynthesis [117–119]. Furthermore, multiple lines of evidence in yeast and human cells have shown that frataxin interacts with the core ISC assembly proteins, NFS1, ISD11 and ISCU [116,120–123]. Moreover, the biophysical interaction between purified human frataxin and ISCU1 appears to be iron-dependent [124,125]. However, whether frataxin plays a role in supplying iron for ISC biogenesis in the CIA pathway remains to be determined.

Frataxin and heme biosynthesis

The mitochondrion is the site for heme biosynthesis and this occurs in all cells, particularly hepatocytes and erythroid cells [100]. The final step of the biosynthesis pathway involves the ISC-containing enzyme, FECH, which is an ISC-containing enzyme in humans [126], that catalyses the insertion of Fe(II) into PPIX to generate heme [106,127]. Studies have suggested that frataxin plays a role in heme biosynthesis, as it has a high binding affinity for FECH, and thus, may be capable of mediating the final step of this pathway [112,127]. This was demonstrated in a study examining the yeast frataxin homologue, Yfh1, where endogenous yeast FECH was down-regulated when frataxin was depleted [128]. This was further supported by the observation of decreased heme levels in the frataxin deficient yeast and mouse model [24,106,128]. It could be argued that frataxin deficiency indirectly caused this effect due to the requirement of FECH for the ISC moiety [17]. However, as yeast FECH is not an ISC-containing protein, the down-regulation of FECH upon deletion of frataxin homologue in yeast [128] supports the direct involvement of frataxin in heme biosynthesis [17].

Frataxin as an iron chaperone

It was suggested that frataxin functions as an iron chaperone because it facilitates the formation of ISCs [113]. The synthesis of ISC requires the assembly of the scaffold protein ISCU that acquires sulfur and iron, via the activity of NFS1 and an unidentified donor respectively [13]. Therefore, frataxin has been proposed as the iron donor that presents iron to the complex (Figure 5A). This was validated by structural studies on the protein, suggesting its role as an iron donor [4,124,129,130]. Comparisons between bacterial frataxin, CyaY, and known chaperone proteins, such as hscA and hscB, that are involved in ISC synthesis, show significant similarities [131,132], which further supports the hypothesis that frataxin could be an iron chaperone. This role could also be relevant to heme synthesis, where frataxin has been suggested to bind to FECH to present its iron for heme synthesis [112].

The role of frataxin in iron storage

The hypothesis that frataxin functions as an iron storage protein was proposed due to the oligomerization of frataxin in yeast, forming frataxin oligomers that have ferroxidase activity (Figure 5B) [17,133]. The iron-binding capacity of yeast frataxin is high in aggregates, which are able to maintain iron in a redox inactive state, while still retaining the ability to release iron for biosynthetic pathways [133]. However, human frataxin has a lower iron-binding capacity than yeast frataxin, even under conditions of iron accumulation in the mitochondria [134]. This discrepancy between the different organisms may be due to the fact that yeast, unlike higher organisms, do not express ferritin [48]. Along with the discovery of FTMT in human tissue [135], the role of frataxin for iron storage becomes redundant and improbable [17]. Furthermore, intracellular iron levels do not influence frataxin expression in erythroid cells, which again contradicts the hypothesis that the activity of an iron storage protein should be regulated by the availability of iron in the cell [136].

Frataxin as a metabolic switch

Other than its proposed roles as an iron chaperone and storage protein, frataxin has also been suggested to regulate iron metabolism by functioning as a metabolic switch (Figure 6A). A previous study has demonstrated this role of frataxin in differentiating erythroid cells, in which frataxin was able to distribute iron between major mitochondrial metabolic pathways [136]. Considering the increase in mitochondrial iron uptake or decrease in iron export as a result of frataxin deficiency, it was hypothesized that decreased frataxin expression could potentially trigger a higher rate of heme synthesis at the expense of ISC synthesis [136]. Therefore, frataxin could act as a metabolic switch that allocates and divides the utilization of iron between metabolic pathways in the mitochondria [136].

This hypothesis is supported by the observation that an increased level of the heme intermediate, PPIX, leads to the

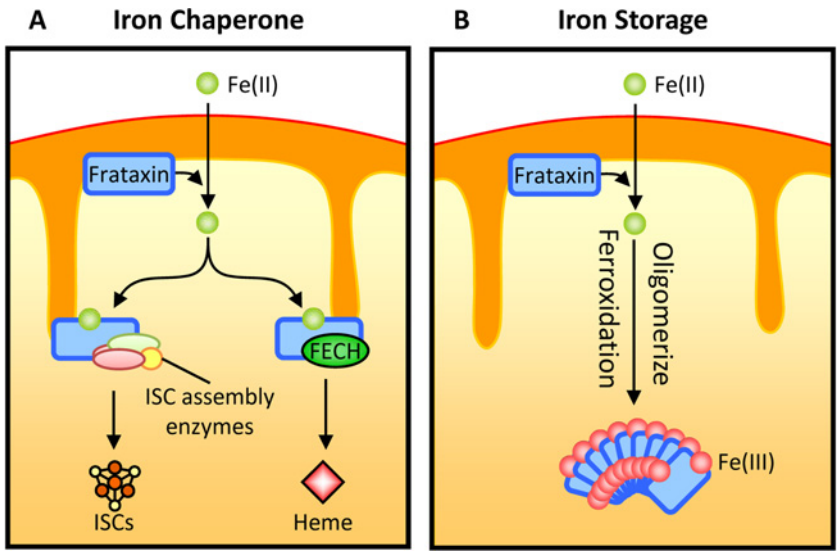

Figure 5 **Proposed roles of frataxin**
Frataxin (**A**) as an iron chaperone that facilitates: (i) the biosynthesis of ISCs by presenting iron to the ISC assembly machinery; and (ii) heme via presenting iron to FECH; and (**B**) as a protein for iron storage that oligomerizes and possesses ferroxidase activity.

down-regulation of frataxin expression and potentially a diversion of iron from metabolic pathways, such as ISC synthesis, towards heme synthesis [136]. In addition, the molar ratio between frataxin and FECH affects the rate of heme synthesis [112]. A decrease in FECH activity is observed when frataxin levels are higher than a ratio of 1 frataxin to 1 FECH molecule, which leads to a decrease in the rate of heme synthesis [112]. Overall, these findings suggested that frataxin may function as a metabolic switch in regulating mitochondrial iron metabolism, which is dependent on the expression levels of its binding ligand, such as PPIX and FECH. Further studies are clearly required to delineate this.

Frataxin as an iron sensor

It has also been hypothesized that frataxin may act as an iron sensor, particular in regulating and fine-tuning ISC synthesis (Figure 6B) [132]. Studies examining the bacterial orthologue of frataxin, CyaY, have shown that it negatively regulates ISC synthesis when there is an excess of iron relative to the amount of available ISC apo-acceptor protein [132]. Considering this, without sufficient acceptor proteins, ISCs degrade rapidly, and hence, there is a need to regulate the balance between ISC synthesis and available apo-proteins (Figure 6B). In the absence of a negative regulator such as CyaY, a high supply of iron with low ISC apo-acceptor availability will lead to excessive generation of unbound ISCs [132]. As a result, the degradation of these ISCs will generate free iron that is capable of producing toxic ROS and can lead to oxidative damage [132]. Based on these findings, the presence of a negative regulator is essential for monitoring ISC synthesis and balancing it with apo-protein availability. A surplus of iron would cause the binding of CyaY to the bacterial homologue of NFS1, IscS, and lead to the suppression of ISC assembly [132]. In addition, the same study also proposed that the formation of iron aggregates under frataxin deficiency in FA is caused by an excess of ISCs relative to its apo-protein due to the dys-regulation of ISC synthesis [132]. However, this observation in bacteria contradicts the finding of low ISC protein levels in FA patients and animal models [106,114,137,138], thus arguing against this role of frataxin as a negative regulator of ISC synthesis in higher eukaryotes.

Despite the uncertainty regarding the exact function of frataxin, it is evident that it plays a significant role in mitochondrial iron metabolism.

EFFECT OF FRATAXIN DEFICIENCY

Dys-regulated iron metabolism

The pathogenesis of FA is caused by frataxin deficiency that leads to severe alterations in iron metabolism [139]. Frataxin deficiency leads to increased cellular and mitochondrial iron uptake, possibly as a consequence of suppressed utilization of mitochondrial iron in metabolic processes such as ISC and heme biosynthesis [106,138].

Previous studies have shown in the muscle creatine kinase (MCK) frataxin KO mice model of FA, which exhibits targeted frataxin-deletion in the heart and skeletal muscle [114], that TfR1 expression is up-regulated, whereas FPN1 and ferritin expression is down-regulated due to the up-regulation of IRP2 (Figure 7) [106,138]. As a result, there is a marked influx of iron into the cardiomyocyte and a decrease in iron release and iron storage [106,138]. Notably, the expression of the exocyst complex protein, Sec15l1, is up-regulated [106,140], which could potentially aid cellular iron uptake through the intracellular cycling of Tf-containing endosomes [106,140] (Figure 7).

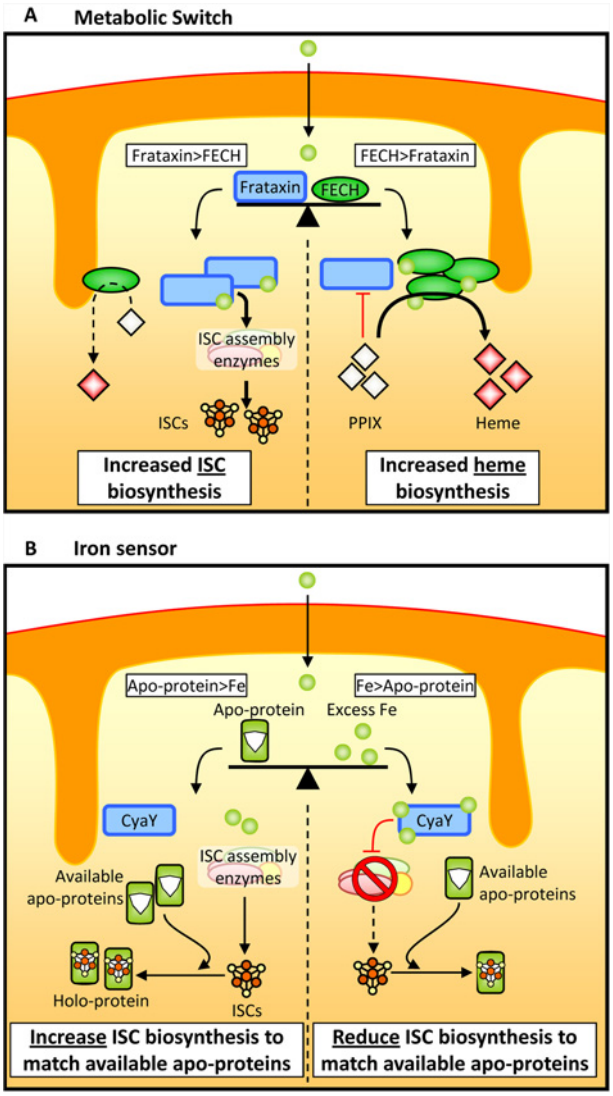

Figure 6 Proposed roles of (A) frataxin and (B) the bacterial frataxin orthologue, CyaY

(**A**) Frataxin acting as a metabolic switch that allocates iron utilization between mitochondrial metabolic processes, namely ISC synthesis and heme synthesis. (**B**) CyaY acting as an iron sensor that regulates ISC synthesis depending on the availability of apo-ISC proteins relative to free iron. This would balance ISC synthesis and the availability of apo-ISC proteins to minimize the formation of unstable proteins.

This influx of iron into the cell leads to an increase in its uptake by the mitochondria via an increase in the mitochondrial transporter, MFRN2, in the MCK frataxin KO mouse [73,106] (Figure 7). Furthermore, frataxin-deficiency in this model leads to the down-regulation of all three major iron-dependent metabolic pathways of the mitochondrion, namely: ISC synthesis, heme synthesis and mitochondrial iron storage [106,138] (Figure 7). For ISC synthesis, both the mRNA and protein expression of NFS1 and ISCU1/2 were found to be down-regulated in the hearts of the MCK frataxin KO model [106] (Figure 7). This is consistent with depressed expression of ISC-dependent proteins, i.e. SDHA and FECH [106,141], and supports the hypothesis of the requirement of frataxin in ISC synthesis [117–119]. Additionally, down-regulation of a number of key heme synthesis enzymes as well as decreased intracardiac heme levels were observed in the MCK KO model [106,142] (Figure 7). Frataxin deficiency also resulted in the down-regulation of FTMT, thereby limiting mitochondrial iron storage (Figure 7) [106].

Of interest, decreased frataxin expression in FA patients does not result in marked defects in heme synthesis in erythroid cells [143]. The reason for this interesting finding could be that it has been demonstrated that erythroid differentiation leads to a reduction in frataxin expression [136]. Since a decrease in frataxin expression occurs concurrently with an increase in iron uptake, heme synthesis, and TfR1 and β-globin expression [33,100,129,130,144–146], this decrease in frataxin levels may facilitate these latter processes. Indeed, in erythroid cells the limiting factor for heme synthesis is iron assimilation [100,129]. Thus, in erythroid cells, the reduction in frataxin expression appears coupled to the marked increase in heme synthesis that is then exported from the mitochondrion preventing toxic iron-loading [136]. In contrast, in non-erythroid cells, the decreased frataxin levels lead to mitochondrial iron-loading. However, since non-erythroid cells have minimal heme production rates, the increased iron uptake is not matched by a similar rise in heme synthesis that is critical for iron uptake into PPIX and its transport out of the mitochondrion as heme [136]. Under these conditions in non-erythroid cells, iron accumulates within the mitochondrion rather than being effectively assimilated by rapid heme synthesis.

In summary, frataxin-deficiency results in mitochondrial-targeted iron trafficking and inhibition of mitochondrial iron utilization [106]. In the absence of known mitochondrial iron exporting molecules, these alterations in metabolism culminate in the accumulation of excess free iron, which cannot be exported out of the mitochondria in the form of iron metabolites, namely ISCs and heme (Figure 7) [106]. This leads to mitochondrial iron-loading and the formation of inorganic iron crystallite aggregates that contributes to the pathogenesis of FA (Figure 7) [106,107].

Cellular antioxidant defense and FA

In addition to the deleterious effects on iron metabolism caused by frataxin deficiency, the ensuing oxidative stress is a feature of FA [147–149]. This is evident by the leakage of electrons due to the respiratory chain impairment and subsequent increased formation of ROS [150]. Mitochondrial iron accumulation in the form of an inorganic iron crystallite without a protective protein shell could be responsible, at least in part, for the oxidative stress in mitochondria of FA patients [3,7,107]. In fact, studies have demonstrated this outcome in both yeast and animal models of FA, which further illustrate the significance of frataxin in maintaining iron homeostasis for cellular function and vitality [107,151]. Frataxin deficiency in FA leads to the down-regulation of ISC expression that subsequently causes a dysfunctional respiratory chain [123,124]. This dys-regulation, together with the defective utilization of mitochondrial iron, and presence of the redox active iron aggregates in the mitochondria, could increase the generation of ROS [106,107,150]. This is supported by studies examining tissues from FA patients, as well as from animal models of FA, in which these alterations could potentiate the oxidative stress in FA [114,139,147,152,153].

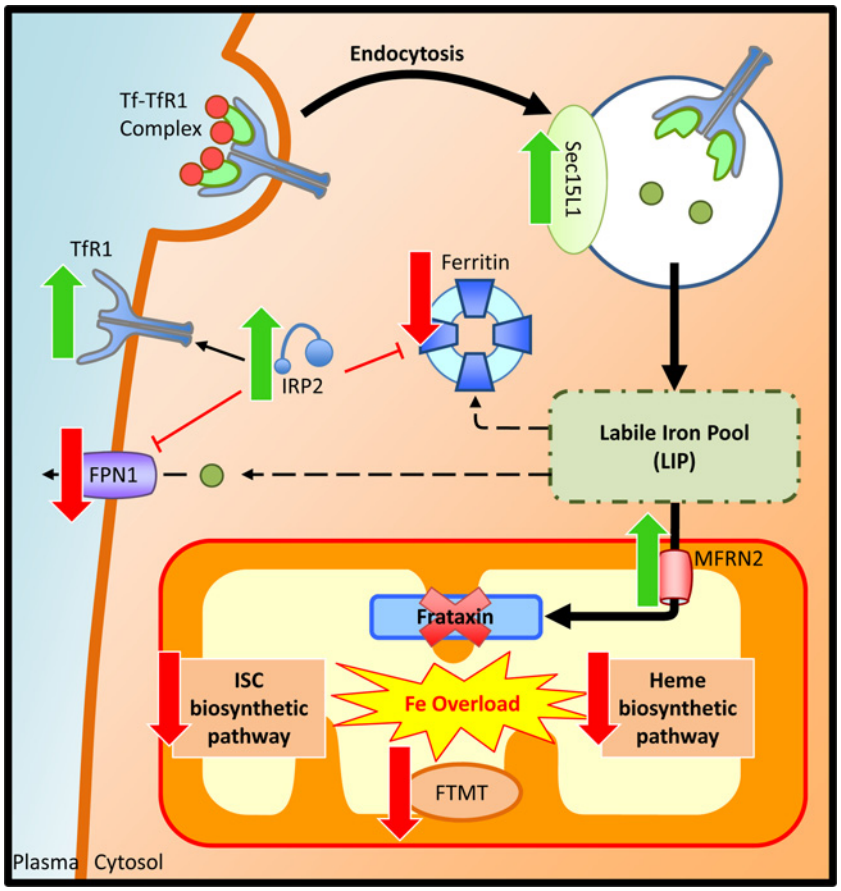

Figure 7 **Schematic illustrating the alterations in cellular and mitochondrial iron metabolism due to the deficiency of frataxin in the heart of the frataxin KO mouse**

Frataxin deficiency leads to severe alterations in cellular and mitochondrial iron metabolism. TfR1 is up-regulated, whereas ferritin and FPN1 are down-regulated. These effects are probably mediated by increased mRNA-binding activity of the IRP2. These changes occur in conjunction with the up-regulation of the exocyst complex protein, Sec15l1, that could aid cellular iron uptake. There is also mitochondrial-targeted iron trafficking evident by an increase in the mitochondrial transporter, MFRN2. However, major iron utilization processes in the mitochondria are down-regulated, namely ISC and heme biosynthesis, and mitochondrial iron storage via FTMT. Therefore, the suppression of these latter metabolic pathways, combined with the increase in iron uptake and its targeting to the mitochondrion, ultimately leads to mitochondrial iron-loading in the absence of frataxin.

It is of interest to note that Myers et al. [154] have indicated that the levels of F_2-isoprostanes in FA patients were not different from controls and were not significantly associated with age, GAA repeat length, disability levels or antioxidant. However, these authors indicate that the lack of significant alteration in F_2-isoprostanes could be due to the fact that ROS production in FA does not target membrane lipids. Furthermore, it is known that increased F_2-isoprostane levels are also not observed in other neuro-degenerative conditions such as Parkinson's disease where there is documented redox stress [154]. In contrast, previous studies that examined the DNA oxidation marker, 8-hydroxy-2′-deoxyguanosine, have observed a significant increase in FA patients relative to controls [148]. Moreover, treatment with the antioxidant idebenone was able to significantly reduce 8-hydroxy-2′-deoxyguanosine levels in FA patients [148]. This finding was contrary to a later investigation that demonstrated no significant difference in this oxidative metabolite between idebenone-treated and -untreated FA patients [155]. In all these later studies, measurements of oxidative markers correlated poorly with disease duration or the number of GAA repeats. Therefore, although most studies identified a role for oxidative stress in models of FA and FA patients, there is still a need for identifying an appropriate biomarker for oxidative stress and understanding the mechanism of its generation.

Frataxin deficiency has also been associated with a decrease in the antioxidant response upon exposure to oxidative stress stimuli [156]. In frataxin-depleted cells of FA patients and in a yeast model of FA, the antioxidant, glutathione (GSH), was markedly decreased [157,158]. Notably, frataxin deficiency has also been linked to the impaired activity of the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), which is crucial for the regulation of genes that are involved in anti-oxidant defense [7,159]. This has been demonstrated in dorsal root ganglia of mouse models of FA, as well as in numerous other cell-types with frataxin depletion [160]. However, the specific molecular mechanism of how frataxin regulates the Nrf2

antioxidative signaling pathway is elusive and remains to be investigated.

In general, frataxin deficiency leads to dys-regulated cellular and mitochondrial iron metabolism [106]. The marked accumulation of toxic iron in the mitochondria along with a defective antioxidant response exacerbates oxidative damage that could play a role in the pathogenesis of FA [13,160].

Neuro-degeneration is a major phenotypic feature of FA [4]. The dorsal root ganglia is the primary site of neuro-degeneration that progressively affects both the central and peripheral nervous system, causing lesions in the dentate nuclei of the cerebellum and corticospinal tracts, and fibre loss in dorsal columns and the spinocerebellar tracts [3,161]. It is thought that oxidative stress contributes to the development of neuro-degeneration in which cells of FA patients show increased sensitivity to oxidative stress and decreased expression of cellular antioxidants [148,149,160,162]. This could be accounted for by the dys-regulation of enzymatic systems that would normally respond to oxidative stress and provide antioxidant protection [158,160].

POTENTIAL TREATMENT OF FA

Antioxidants, Nrf2 and GSH inducers

It has been shown *in vivo* that GSH levels can be supplemented by *N*-acetylcysteine (NAC), which acts on Nrf2 and elevates the biosynthesis of GSH, thus increasing antioxidant protection [163]. Notably, NAC has shown potential therapeutic effects with an increase in glutathione reductase activity in mouse models of Alzheimer's disease [164]. These studies have led to clinical trials investigating the effects of NAC as a GSH precursor to induce GSH synthesis in patients with Alzheimer's disease [165].

Nrf2 is a transcription factor responsible for the expression of genes important for antioxidant defense and maintaining homeostasis of GSH and ROS [166,167]. The overall effect of Nrf2 expression is an increase in reducing potential, making cells more resistant to oxidative insult [168]. Therefore, agents that promote cellular Nrf2 expression may provide a potential therapeutic avenue for the prevention of neuro-degeneration in FA and other diseases such as Parkinson's disease and Alzheimer's disease [168].

There are a variety of antioxidants designed for the treatment of FA that are under development [169]. For instance, the ubiquinone analogue, idebenone, functions to scavenge free radicals in order to potentially restore mitochondrial ATP production [170]. Studies have found that idebenone can improve cardiac and neurological functions of FA patients [170], although clinical trials have produced contradictory results [171,172]. Another antioxidant, Coenzyme Q10, is a lipophilic molecule structurally similar to idebenone that functions to facilitate mitochondrial electron transport [173]. In addition, α-tocopheryl quinone is a potent antioxidant that was found to cause a dose-dependent improvement in neurological functions among adult FA patients [174]. The use of antioxidants for the treatment of FA has been ongoing and further assessments are required to verify the effectiveness of these agents.

Treatment with iron chelators and antioxidants

The potential treatment of FA with iron chelators has been assessed in previous studies [170,175–178]. Since iron accumulates in the mitochondria of FA patients [179,180], it has been suggested that the chelation of iron would lower its toxicity [175,181]. Moreover, it has been shown that chelating and removing the mitochondrial iron-load reduced cardiac hypertrophy *in vivo* [138]. Nevertheless, chelation did not entirely reverse the phenotype, as it did not rescue the function of frataxin in mitochondrial iron metabolism [138]. Similarly, treatment strategies utilizing antioxidants alone, including idebenone and CoQ10/vitamin E have demonstrated mixed results [148,155,182]. Subsequent studies in FA patients demonstrated that therapies which combine the iron chelator, deferiprone and the antioxidant, idebenone, significantly improved the heart hypertrophy and reduced the iron deposits in the dentate nucleus [183]. In addition, this combination treatment induced a stabilizing effect in the neurological dys-function, as shown by improved kinetic functions in the patients [170,183]. In the absence of an appropriate therapy to replace frataxin, these studies demonstrate the potential of the combination of using both an iron chelator and antioxidant for the treatment of FA. However, it is essential to further assess the effectiveness of this combined therapy in comprehensive animal and clinical studies, as it could result in new therapeutic avenues for FA patients.

SUMMARY

FA is a neuro- and cardio-degenerative disease caused by the deficient expression of the mitochondrial protein, frataxin. It is known that frataxin-deficiency leads to alterations in cellular and mitochondrial iron metabolism, resulting in excessive iron loading to the mitochondria from the cytosol. This accumulation of iron is a major pathological feature of FA in the heart and may be at least partially responsible for instigating oxidative stress. Concurrently, cellular antioxidant defense is also dys-regulated when frataxin is deficient, which exacerbates oxidative damage in FA. Despite our current limitations in restoring frataxin expression in FA patients, studies have investigated potential therapeutics using antioxidants to restore antioxidant defense in this disease. Similarly, iron chelators have been of great interest in the treatment of FA, as these agents are able to remove toxic iron from the mitochondria to relieve the oxidative stress. The prospect of combining these two approaches is a critical therapeutic strategy and requires further investigation, as it could lead to more effective treatments for FA patients.

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