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# Understanding the molecular mechanisms of Friedreich's ataxia to develop therapeutic approaches

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**Friedreich's ataxia (FRDA) is a neurodegenerative disease caused by reduced expression of the mitochondrial protein frataxin. The physiopathological consequences of frataxin deficiency are a severe disruption of iron–sulfur cluster biosynthesis, mitochondrial iron overload coupled to cellular iron dysregulation and an increased sensitivity to oxidative stress. Frataxin is a highly conserved protein, which has been suggested to participate in a variety of different roles associated with cellular iron homeostasis. The present review discusses recent advances that have made crucial contributions in understanding the molecular mechanisms underlying FRDA and in advancements toward potential novel therapeutic approaches. Owing to space constraints, this review will focus on the most commonly accepted and solid molecular and biochemical studies concerning the function of frataxin and the physiopathology of the disease. We invite the reader to read the following reviews to have a more exhaustive overview of the field [Pandolfo, M. and Pastore, A. (2009) The pathogenesis of Friedreich ataxia and the structure and function of frataxin. *J. Neurol.*, 256 (Suppl. 1), 9–17; Gottesfeld, J.M. (2007) Small molecules affecting transcription in Friedreich ataxia. *Pharmacol. Ther.*, 116, 236–248; Pandolfo, M. (2008) Drug insight: antioxidant therapy in inherited ataxias. *Nat. Clin. Pract. Neurol.*, 4, 86–96; Puccio, H. (2009) Multicellular models of Friedreich ataxia. *J. Neurol.*, 256 (Suppl. 1), 18–24].**

## INTRODUCTION

Friedreich's ataxia (FRDA) is the most frequent hereditary ataxia, with an estimated prevalence of 1 of 50 000 in Caucasians (1). FRDA is an autosomal recessive neurodegenerative disease characterized by progressive gait and limb ataxia, dysarthria, lower limb areflexia, decreased vibration sense, muscular weakness of the legs and positive extensor plantar response (2,3). Most neurological symptoms are a consequence of degeneration of the large sensory neurons of dorsal root ganglia (DRG) and spinocerebellar tracts. Non-neurologic signs include hypertrophic cardiomyopathy (4) and increased incidence of diabetes mellitus. Onset of symptoms usually occurs around puberty, and typically before the age of 25 years. Life expectancy averages between 40 and 50 years. The gene associated with the disease has been mapped to chromosome 9q13-q21.1 and encodes a small mitochondrial protein called frataxin (5–7). The most common mutation is a GAA triplet-repeat expansion within the first intron of the frataxin gene. In healthy patients, the number of repeats ranges from 6 to 36 repeats, whereas in FRDA patients, the number of repeats ranges from 70 to 1700 repeats, most commonly 600 to 900 GAA. The severity of the disease correlates with the number of repeats. Most patients (96%) carry two expanded GAA alleles, which lead to strongly reduced frataxin expression in all tissues (6). A small but significant number of FRDA patients (4%) are compound heterozygous with a $(GAA)_n$ mutation on one allele and a micromutation on the other (8,9). These mutations are small deletions or point mutations (at least 15 have been described) that can disturb the normal function of frataxin, either by affecting protein folding or by affecting functional residues.

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To date, no FRDA patients homozygous for point mutation have been identified, suggesting that at least some functional frataxin protein is crucial for survival. Indeed, in multicellular eukaryotes, frataxin is essential for embryonic development and complete frataxin deletion leads to early-embryonic lethality in plants and mice (10). Alternatively, the lack of patients homozygous for point mutation alleles could be caused by the rarity of these mutations or to an atypical clinical presentation that has not yet been associated with the FRDA locus.

## MOLECULAR MECHANISMS OF THE GAA EXPANSION

The mechanism by which the GAA expansion induces the partial transcriptional inhibition has been investigated both *in vitro* and *in vivo*, and two non-exclusive models have emerged: non-B DNA conformation and/or a heterochromatin-mediated gene silencing. *In vitro* and *in vivo*, bacterial plasmids containing expanded GAA repeats can adopt a triple helical structure that directly interferes with transcriptional elongation (11). The first evidence of higher-order structures came from observations that plasmids containing long GAA expansions present a retarded profile in agarose gel. As GAA repeats are mirror repeats that contain only purines (R) on one strand and pyrimidines (Y) on the other strand, they can form intramolecular triple-helix structures (12). Such triple-helix structures have been demonstrated by nuclear magnetic resonance experiments (13). Furthermore, RRY triplex can adopt higher-order conformations called "sticky DNA" that have been shown to inhibit transcription *in vitro* and in mammalian cells (14,15). Experiments using DNA triplex-stabilizing conjugates, based on benzoquinoquinoxaline, provide direct chemical evidence for the triplex formation and stabilization into higher-order structures at expanded GAA sequences (16). Recently, a slightly different model has been proposed with a transcription-dependent RNA–DNA hybrid leading to transcriptional arrest (17). Whether the sticky DNA structures are directly involved in transcriptional inhibition *in vivo* where the DNA is tightly packaged in chromatin remains to be proven, but the transcription-dependent RNA–DNA hybrid structure might cause repeat instability in the cell. Indeed, the GAA expansion is not only unstable when transmitted from parent to child, but also shows somatic instability, including in post-mitotic cells such as DRG sensory neurons (18–20).

The role of an epigenetic mechanism in GAA-mediated gene silencing was pointed out by the discovery that long GAA repeats suppress the expression of a nearby heterochromatin-sensitive cell surface reporter gene in transgenic mice by position effect variegation (PEV) (21). PEV is a phenomenon occurring when a gene is located abnormally near a heterochromatin region. The transcriptional silencing of the heterochromatin-sensitive transgene was correlated with decreased promoter accessibility and was enhanced by overexpression of HP-1, a well-known heterochromatin modifier. Silent heterochromatin is characterized by specific post-translational modifications of histones, including changes in acetylation and methylation, as well as DNA methylation on CpG islands (22,23). In agreement with the GAA-mediated epigenetic hypothesis, the heterochromatin status of the frataxin gene has been highly documented in the past 3 years. Studies of the region upstream of the GAA repeat in primary FRDA lymphoblastoid cells have shown increased trimethylation of histone H3 lysine 9 (H3K9) and a global hypoacetylation of the histone H3 and H4 tails (H3K14, H4K5, H4K8 and H4K12), all markers of heterochromatin (24). No obvious changes were observed in the promoter region. An increase in H3K9 dimethylation was also observed in lymphoblast cell lines from patients (25). Patient fibroblasts were also reported to have H3K9 hypermethylation in the promoter region of the gene with concomitant recruitment of the heterochromatin protein HP1 (26). Furthermore, global histone deacetylation within the locus was observed in human brain biopsies from FRDA patients; however, this was more pronounced in the downstream region of the GAA (27). Despite these differences, which are probably linked to the dynamic state of heterochromatin, the common hallmarks in patient material are H3K9 di- and trimethylation and a significant decrease in H3K9 acetylation in the GAA region. Interestingly, these hallmarks are also found in the YG8 and YG22 humanized mouse lines, yeast artificial chromosome transgenic mice carrying the full human locus with GAA expansions of 190 + 90 and 190 GAA repeats, respectively, as well as in a GAA knock-in mouse model (27,28). In addition to the histone modifications, CpG residues were found hypermethylated in the upstream region of the GAA with a tendency to be hypomethylated after the expansion in patient biopsies and lymphoblasts and in humanized mice heart and brain (25,27). The consequences of DNA methylation could be directly linked to promoter activity and transcription initiation, as one of the sites of methylation is located within an E-box (25), the binding site for members of the basic-helix–loop–helix family of transcription factors. Interestingly, a recent report demonstrated a specific depletion of CTCF at the frataxin promoter (26). CTCF is a multi-zinc-finger protein considered as a chromatin insulator that might prevent spreading of heterochromatin by creating barrier between inactive and active chromatin (29). In addition, the expression of an antisense transcript (FAST-1) of the frataxin gene was shown to be specifically increased in FRDA patient cells (26). Although the authors suggest that the CTCF depletion and the antisense transcript might be involved in the heterochromatin formation, this potential molecular link needs further experimental evidence.

In summary, although it is clear that the GAA expansion mutation leads to a decrease in frataxin transcripts, the specific molecular mechanisms involved need further refinement. It is important to note that the two models presented are not mutually exclusive as it is possible that the sticky DNA structure adopted by the GAA expansion is the signaling mechanism leading to the recruitment or displacement of factors involved in chromatin modification. Given that the mutation leaves the coding region intact, gene-based strategies designed to increase the frataxin level are very promising therapeutic approaches (Fig. 1). The first proof of the concept that targeting the alternative DNA structures found in FRDA might be a viable therapeutic option came from experiments demonstrating that oligodeoxyribonucleotides specifically designed to block triplex structures are capable of increasing transcription through the GAA repeats (30). Since

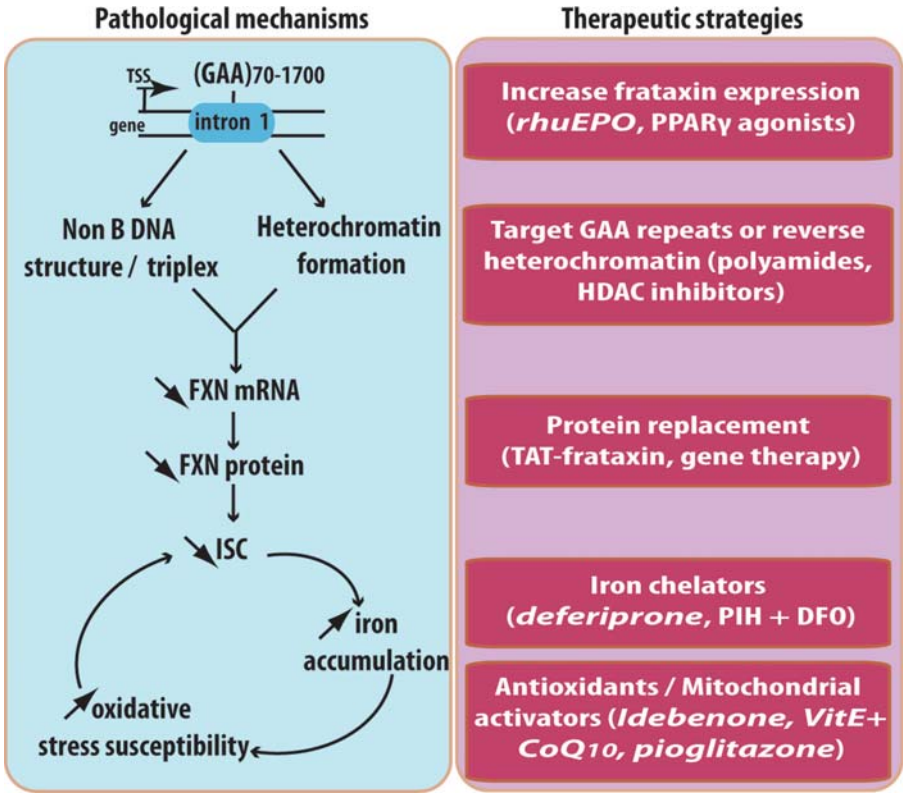


**Figure 1.** Pathological mechanisms involved in FRDA and associated therapeutic strategy. FRDA is caused by a GAA expansion mutation within the first intron of frataxin gene. The mutation, through either a triplex-helix or a heterochromatin formation, impairs transcription and causes a severe decrease of frataxin mRNA expression and protein level. Physiological consequences of frataxin deficiency are specific ISC enzyme deficits, an intramitochondrial iron accumulation and a higher sensitivity to oxidative stress. In the right panel, the different therapeutic strategies currently under investigation to counteract defects observed in patients and to develop future treatments are presented. Therapeutics in bold and italic are currently under (pre)clinical trial. rhuEPO, recombinant human erythropoietin; PPAR, peroxisome proliferator-activated receptor; HDAC, histone deacetylase; PIH, pyridoxal isonicotinoyl hydrazone; DFO, deferroxiamine; VitE, vitamin E; $CoQ_{10}$, coenzyme $Q_{10}$.

then, many studies have demonstrated that DNA-binding compounds, such as pyrrole-imidazole polyamides or pentamidine, are effective at increasing frataxin transcription in primary FRDA lymphocytes (31,32), although the exact mechanism involved awaits further characterization. A promising therapeutic strategy that is currently the subject of intense research is to directly target the heterochromatin state of the GAA repeat expansion with histone deacetylase inhibitors (HDACi) to restore frataxin levels (reviewed in 33). HDACi prevent the deacetylation of histones, making heterochromatin revert to an open active conformation that is conducive for gene expression (34). Human cells from FRDA patients treated with the highly active HDACi compound 4b [N1-(2-aminophenyl)-N7-phenylheptanediamide] resulted in a substantial increase in frataxin, accompanied by increased histone H3 acetylation near the GAA repeat (24). Similarly, GAA knock-in mice treated with a novel HDACi compound 106 [N1-(2-aminophenyl)-N7-p-tolylheptanediamide] show increased histone H3 and H4 acetylation in chromatin near the GAA repeat and restored the wild-type frataxin levels in the nervous system and heart (28). The development of new more potent HDACi specific to the frataxin locus, as well more complete animal studies to determine bioavailability and efficacy, is currently underway. In addition to such molecules that directly target the GAA repeat, a number of additional molecules have also been shown to increase frataxin mRNA or protein levels. In particular, recombinant human erythropoietin (rhuEPO) has been reported to increase frataxin protein in lymphocytes from FRDA patients (35), and clinical trials with rhuEPO are currently in progress. Although the molecular basis of the increase in frataxin protein remains to be determined, it is hypothesized that it is related to increase translation or stabilization of the frataxin protein.

## PHYSIOPATHOLOGICAL MECHANISMS OF FRATAXIN DEFICIENCY

The physiological consequences of frataxin deficiency are a severe disruption of iron–sulfur cluster (ISC) enzymes (36–39), mitochondrial iron overload coupled to cellular iron dysregulation (38,40,41) and an increased sensitivity to oxidative stress (40,42,43). Owing to the tight link between these important cellular pathways, it is difficult to identify the primary cause of the disease. The first evidence linking frataxin to iron metabolism was the identification of increased iron content in FRDA patient hearts (44) and in mitochondria of yeast strains with a deletion of the frataxin homologue (Yfh1) (40,41). These studies suggest that frataxin plays a

major role in regulating mitochondrial iron transport (40). Furthermore, early biochemical studies in the endomyocardial biopsies of FRDA patients demonstrated deficiencies of the ISC-containing subunits of the mitochondrial electron transport complexes I, II and III and of iron–sulfur proteins aconitases (39). Moreover, disruption of the *Yfh1* gene resulted in multiple ISC-dependent enzyme deficiencies in yeast (45). Although these ISC enzyme deficits were initially thought to be a consequence of increased oxidative stress generated through the Fenton reaction by mitochondrial iron accumulation, studies using conditional mouse models with tissue-specific frataxin deletions demonstrate that the primary deficit in the disease is the ISC protein deficiency followed by secondary mitochondrial iron accumulation, with no overt sign of oxidative stress damage (38,46). It is now commonly accepted that frataxin deficiency leads to primary mitochondrial and extramitochondrial ISC deficits (36,38,39). Whether iron dysregulation has a causal role in the disease pathogenesis remains to be demonstrated and further cellular and animal studies will be crucial to determine whether a subtle increase in redox-active mitochondrial iron plays a role in the disease pathogenesis. The role of oxidative stress in the pathogenesis of FRDA was discovered very early with the demonstration that frataxin-deficient yeast and cultured cells from FRDA patients exhibit increased sensitivity to oxidative stress reagents (42,47,48). Numerous studies have demonstrated an impaired response of antioxidant enzymes in cell lines and model organisms (42,43,46,49,50). This disabled response was recently proposed to be the consequence of a disorganization of the Nrf2-dependent phase II antioxidant-signaling pathway (51). Finally, reports of increased levels of oxidative stress markers are not consistent, with initial studies showing low but significant increases in oxidative stress markers in blood and urine samples from FRDA patients (52–54), whereas more recent studies have failed to replicate these data (55–58). Interestingly, a recent study presented data suggesting increases in nuclear and mitochondrial DNA damage in peripheral blood samples from FRDA patients (59). Whether this increase in DNA damage is a consequence of an impaired antioxidant defense or directly linked to an ISC deficit remains to be determined, as several damage recognition and DNA repair proteins are ISC proteins, (36,60). Finally, to date, there is no clear evidence that increased oxidative stress induces pathogenic cellular damage in frataxin-deficient cells or tissues.

Several therapeutic strategies for FRDA have been developed to intervene in the pathogenetic cascade downstream of frataxin (Fig. 1). Some pharmacological compounds such as antioxidants or iron chelators have shown promise in improving some of the symptoms of the disease and are already in clinical testing. The most advanced experimental therapeutic is in the use of parabenzoquinone derivatives, such as idebenone or coenzyme $Q_{10}$ (ubiquinone) (61–64). The rationale was initially based on the antioxidant activity of these molecules, but since these molecules can also stimulate oxidative phosphorylation and ATP production by transferring electrons from complexes I and II to complex III in the electron transport chain (65), they may also contribute to counteract the electron transport impairment due to ISC deficits observed in FRDA patients. FRDA patients who were given ubiquinone in addition to vitamin E, an antioxidant compound, exhibited cardiac and skeletal muscle improvements after several months of treatment (66). Idebenone was efficient in decreasing cardiac hypertrophy in several open trials in humans (reviewed in 67) and was shown to be cardioprotective in the conditional cardiac mouse model with a clear effect on both cardiac hypertrophy and function (64). A large 12-month phase III clinical trial to test the efficacy of idebenone on neurological symptoms is currently underway (sponsored by Santhera Pharmaceuticals, Switzerland). Iron chelation designed to specifically target the mitochondrial iron accumulation is another major potential strategy for the treatment of FRDA that is being intensively investigated. Deferiprone, an orally active, blood–brain barrier permeable iron chelator, can potentially redistribute iron from the mitochondria to other cellular compartments and to blood transferrin (68). Therefore, deferiprone has been tested in a preliminary study on a small cohort of patients and showed some signs of neurological improvements, although the conclusions should be taken with caution (69). A phase II clinical trial (sponsored by Apopharma, Canada) is currently underway.

## FRATAXIN FUNCTION

Frataxin is a highly conserved protein that is ubiquitously expressed. It is synthetized as a precursor of 210 amino acids that is imported and undergoes maturation within mitochondria by a two-step process leading to the successive generation of an intermediate form of 19 kDa and a mature form of 14 kDa (70–72). An important feature of the frataxin structure is the presence of an acidic ridge at the surface of the protein, proposed to be involved in iron binding (73–76). Structurally, frataxin displays a platform of β-sheets and two α-helices in a separate plane, a hydrophobic core and a charged groove that may provide a surface for the interaction with protein partners (77,78). Although much effort has led to significant advances in understanding the role of frataxin in mitochondrial iron homeostasis, the precise role of frataxin remains unclear. Frataxin has been proposed as an iron donor for both Fe–S and heme biosynthesis, as an iron chaperone during Fe–S re-assembly, or as an iron-storage protein (Fig. 2A) (reviewed in 79).

In agreement with the multiple roles proposed for frataxin, many protein partners have been reported *in vivo* and/or *in vitro*, including components of the ISC machinery (ISCU, IscS/NFS1 and ISD11), mitochondrial aconitase, ferrochelatase, subunits of complex II of the mitochondrial respiratory chain, frataxin itself and several chaperones (GRP75, Ssc1) (75,80–84). Nevertheless, only the interaction with components of the ISC machinery is widely accepted and largely experimentally supported in the literature (80,82,85–89). ISCs are versatile cofactors of proteins involved in electron transport, enzyme catalysis and regulation of gene expression. The synthesis and insertion of ISC into apoproteins require three multicomponent systems in eukaryotes, with the participation of over 20 components (Fig. 2B) (90). The ISC machinery, located within the mitochondria, performs the central role of *de novo* ISC synthesis on a scaffold protein (Isu1/2 in yeast, ISCU in humans), with sulfur being delivered by a cysteine desulfurase (yeast and human NFS1/ISD11 complex). Both synthesis of the transiently

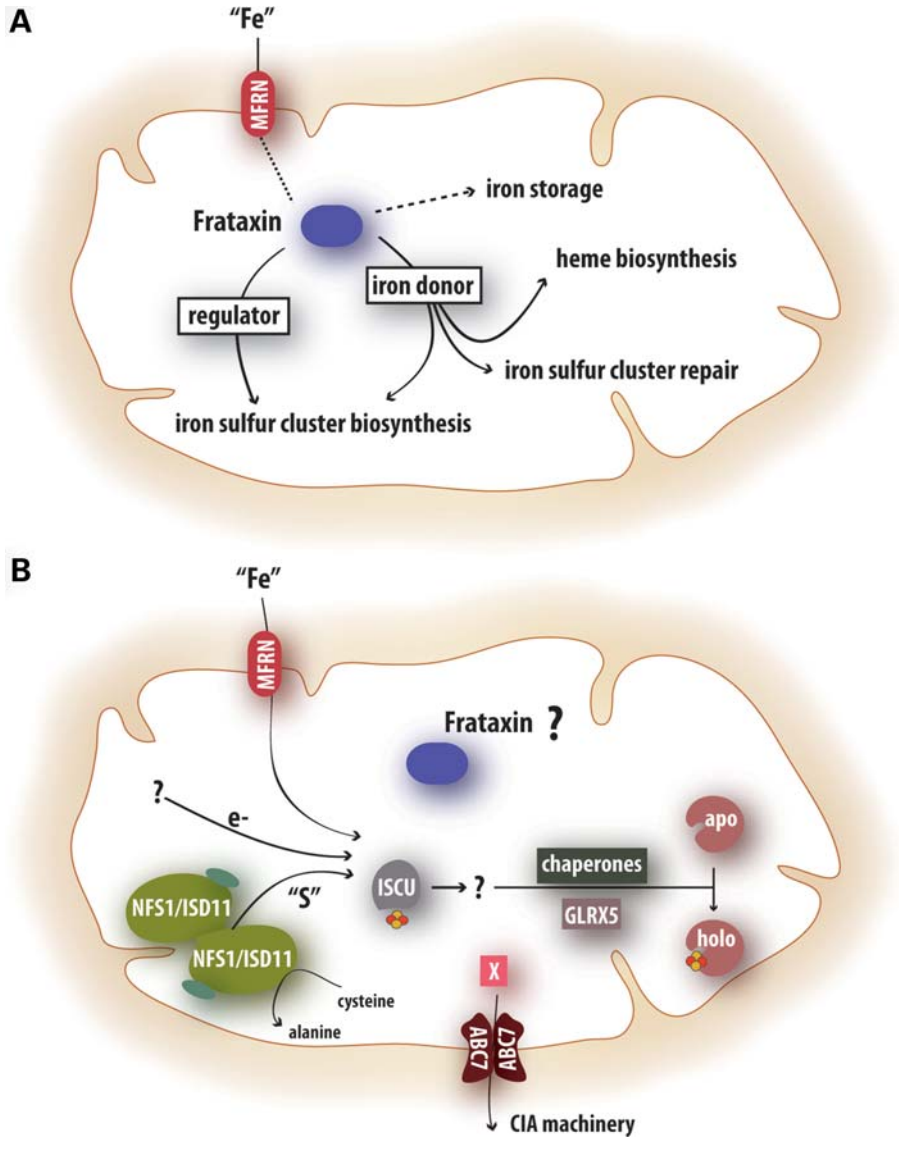


**Figure 2.** Proposed functions for frataxin. (**A**) Schematic representation of the different hypotheses of the frataxin function within mitochondria. Frataxin is a small protein targeted to mitochondria that was shown to bind iron *in vitro*. Frataxin was proposed to be an iron-storage protein or an iron chaperone that could provide iron for heme biosynthesis, ISC biosynthesis/repair. Recently, frataxin was proposed to be a regulator of ISC biosynthesis. (**B**) Simplified schematic representation of mammalian ISC biosynthesis machinery. Briefly, ISC *de novo* biosynthesis occurs within the mitochondria where sulfur is provided by NFS1, a cysteine desulfurase. NFS1 interacts with ISD11, a small protein whose function is unclear. Iron and sulfur atoms are assembled on the scaffold protein ISCU to give an ISC. ISCs are then transferred to apoproteins possibly with the help of chaperones and glutaredoxin 5 (GLRX5). In parallel, a still uncharacterized compound made by the mitochondrial machinery was proposed to be exported to the cytosol by ABC7, an ABC transporter localized in the mitochondrial inner membrane. The compound is used by the cytosolic ISC assembly machinery to generate ISC for cytosolic and nuclear proteins. The reaction needs electrons and iron. The electron donor is not known in mammals. Frataxin was proposed to be the iron donor (90). Recently, three human diseases associated with abnormal ISC biosynthesis were described in addition to FRDA. Mutations in ISC scaffold ISCU were associated with a myopathy with exercise intolerance; a patient with a mutation in GLRX5 developed a microcytic anemia. Finally, ABC7 is mutated in X-linked sideroblastic anemia with ataxia. These four pathologies share similarities like an ISC defect and iron accumulation, suggesting common mechanisms and demonstrating the crucial role of the ISC biosynthesis machinery (97). MFRN, mitoferrin.

scaffold-bound ISC and its final transfer to apoproteins requires the help of chaperones. The maturation of extramitochondrial ISC proteins necessitates the export machinery, which includes the ATP-binding cassette transporter (atm1 in yeast, ABC7 in humans). ISCs are then assembled and transferred to cytosolic and nuclear apoproteins by a cytosolic machinery (90). The first evidence of a direct interaction between frataxin and the ISC biosynthesis machinery comes from glutathione *S*-transferase (GST) pull-down and co-immunoprecipitation experiments showing a clear interaction between Yfh1 (yeast frataxin homolog) and the Isu1/Nfs1 complex (80). As this interaction was increased under high ferrous iron, it was hypothesized that frataxin might be the iron-loading protein for Isu1, suggesting that frataxin is the iron donor for the system. Furthermore, as the interaction increased with increase concentration of Isu1, it was suggested that the interaction with the complex

was through a direct interaction between Isu1 and Yfh1. More recently, *in vivo* mutagenesis experiments followed by co-immunoprecipitation demonstrated that the mutations localized in the third β-strand of yeast Yfh1 (N122K, N122A/K123T/Q124A and W131A) strongly weakens the interaction with Isu1 (87,89). Similar experiments using the human frataxin have shown that the W130 residue is critical for maintaining frataxin interaction with the complex (S.S. and H.P., unpublished data). However, a GST pull-down experiment with the bacterial homolog, CyaY, provided evidence for a direct interaction with the cysteine desulfurase, IscS (85,86), whereas co-immunoprecipitation experiments with the human frataxin proposed a direct interaction with ISD11 (82), a protein proposed to stabilize NFS1 in eukaryotes. Therefore, although it is clear that frataxin interacts with the *de novo* ISC assembly machinery, the precise molecular interactions need further refinement. Recently, the iron donor function of frataxin was challenged as frataxin was proposed as an uncompetitive inhibitor of ISC biosynthesis (85). In *in vitro* reconstitution experiments, the addition of CyaY was demonstrated to slow the incorporation of a 2Fe–2S on IscU through a direct interaction with IscS. Interestingly, frataxin interaction did not affect the cysteine desulfurase activity of IscS, leaving the question of the inhibition mechanism unanswered. Finally, it is worth noting that *in vivo* data from yeast and mouse models suggest that frataxin cannot be the sole iron donor for ISC biosynthesis, as in the total absence of frataxin, there is a residual amount of ISC being synthesized. Therefore, a regulatory role of frataxin in ISC biosynthesis seems more probable.

Besides its role as an iron chaperone in ISC biosynthesis, frataxin has also been proposed to act as an iron-storage protein within mitochondria (91–95). Yeast frataxin monomers can self-assemble *in vitro* in an iron-dependent manner into larger oligomeric subunits capable of detoxifying iron by catalyzing the oxidation of Fe(II) to Fe(III) and storing the latter form as ferrihydrite mineral within the large oligomer (91,92). Although the iron-storage property of yeast frataxin is well-established *in vitro*, the relevance for *in vivo* function remains unclear. Indeed, Yfh1 mutants of the acidic residues D86N/D90Q/DE93Q, which are defective in iron-induced oligomerization, fully rescue Δ*Yfh1* yeast strain, even at low level and under conditions where cellular iron is high, suggesting that the oligomerization properties are dispensable *in vivo* (96). Therefore, if the iron-storage function of frataxin is relevant *in vivo*, it is clearly not the major function of frataxin.

## CONCLUSION

Recent advances in understanding of the molecular mechanisms involved in FRDA allow the development of a set of new potential therapies. Research on GAA expansion behavior and consequences has opened the road to molecules interfering with the DNA triplex or new promising HDAC inhibitors. Similarly, the refinement of pathophysiological consequences of frataxin deficiency in different models, such as the determination of the exact impact of iron accumulation on the disease progression, will certainly bring new interesting therapeutic strategies. Although the role of frataxin in the ISC biosynthesis is now strongly demonstrated, the exact function of frataxin during the process remains to be elucidated. Identification of the direct partner(s) of frataxin (ISCU, NFS1 or ISD11) would provide useful evidence in this way. A better collaboration between *in vitro* and *in vivo* approaches will also provide an improved view of frataxin function. Much additional effort is required to investigate the frataxin role in pathways such as heme biosynthesis or iron storage. Moreover, the identification of new ISC proteins will lead to a better understanding of the pathways affected in FRDA patients lead to a better understanding of the mechanisms involved in the neurodegenerative process.

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