Mitochondrial frataxin interacts with ISD11 of the NFS1/ISCU complex and multiple mitochondrial chaperones

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The neurodegenerative disorder Friedreich's ataxia (FRDA) is caused by mutations in frataxin, a mitochondrial protein whose function remains controversial. Using co-immunoprecipitation and mass spectrometry we identified multiple interactors of mitochondrial frataxin in mammalian cells. One interactor was mortalin/GRP75, a homolog of the yeast ssq1 chaperone that integrates iron–sulfur clusters into imported mitochondrial proteins. Another interactor was ISD11, recently identified as a component of the eukaryotic complex Nfs1/ISCU, an essential component of iron–sulfur cluster biogenesis. Interactions between frataxin and ISD11, and frataxin and GRP75 were confirmed by co-immunoprecipitation experiments in both directions. Immunofluorescence analysis demonstrated that ISD11 co-localized with both frataxin and with mitochondria. The point mutations I154F and W155R in frataxin cause FRDA and are clustered to one surface of the protein, and these mutations decrease the interaction of frataxin with ISD11. The frataxin/ISD11 interaction was also decreased by the chelator EDTA, and was increased by supplementation with nickel but not other metal ions. Nickel supplementation rescued the defective interaction of mutant frataxin I154F and W155R with ISD11. Upon ISD11 depletion by siRNA in HEK293T cells, the amount of the Nfs1/ISCU protein complex declined, as did the activity of the iron–sulfur cluster enzyme aconitase, while the cellular iron content was increased, as seen in tissues from FRDA patients. Furthermore, ISD11 mRNA levels were decreased in FRDA patient cells. These data suggest that frataxin binds the iron–sulfur biogenesis Nfs1/ISCU complex through ISD11, that the interaction is nickel-dependent, and that multiple consequences of frataxin deficiency are duplicated by ISD11 deficiency.

INTRODUCTION

Friedreich's ataxia (FRDA) is the most common autosomal recessively ataxia, with an incidence of ~1:50 000 (1). Like the autosomal dominant spinocerebellar ataxias, the major clinical sign of the disease is loss of coordination and unsteadiness of gait. However, hypertrophic cardiomyopathy and insulin resistance are also common in FRDA (1–3).

FRDA is usually caused by inheritance of two expanded alleles of (GAA)n triplet repeat in the first intron of frataxin gene (4). Expansions are thought either to partially inhibit transcription elongation (5), or to condense chromatin structure (6,7) and thus reduce frataxin protein expression (8). Both the severity of the FRDA and the age of onset are directly related to size of the smaller GAA expansion (9).

Frataxin is thought to support the biogenesis of iron–sulfur clusters, since its deficiency specifically affects iron–sulfur cluster enzymes (10) and because it interacts with ISCU, which is thought to be the main scaffold on which iron–sulfur clusters are built (11–14). Recent in vitro studies proved that frataxin interacts with the iron–sulfur cluster containing enzymes mitochondrial aconitase (15) and ferrochelatase (an ISC protein in mammals) (16–18), while in yeast and Caenorhabditis elegans it interacts with succinate dehydrogenase (19,20). Knockdown of the frataxin message causes a decrease in the maturation of iron–sulfur cluster proteins (21–23). Microarray analysis of human cells has shown that frataxin depletion affects iron–sulfur cluster-related transcripts preferentially (24).

To identify frataxin's interactors, we carried out co-immunoprecipitation, electrophoresis, band cutting and
mass spectrometric identification. We isolated GRP75, an ssq1 homolog and ISD11, a recently identified component of the Nfs1/ISCU scaffold complex (25,26), and confirmed their interaction with frataxin by multiple means, i.e. co-immunoprecipitation in both directions, GST-pulldown, and colocalization. Here we show that frataxin interacts with ISD11 in a mutation-dependent and nickel-dependent fashion, and that knockdown of ISD11 phenocopies two consequences of frataxin inhibition, namely aconitase deficiency and cellular iron overload.

RESULTS
Frataxin associates with multiple mitochondrial proteins
We searched for novel interaction partners of frataxin in two ways. One method included the preparation of protein extracts from lymphoblast mitochondria, followed by immunoprecipitation with anti-frataxin antibody, separation of proteins via electrophoresis, band isolation, digestion and mass spectrometry. However, the efficiency of the process was further enhanced by overexpressing frataxin. So, a vector (pcDNA3.1-frataxin-flag) was constructed which expressed frataxin protein with a C-terminal flag epitope tag. This construct drove high frataxin expression in COS7 cells as detected by anti-frataxin antibody, and maximal amount of mature mitochondrial frataxin was produced at post-transfection day 5 (Fig. 1A).

Mitochondria were purified from day 5-transfected COS7 cells, lysed, and frataxin-associated molecules were immunoprecipitated by anti-frataxin antibody and analyzed by SDS/PAGE followed by silver staining. Three visible bands were cut from the gel and analyzed by mass spectrometry, and the results are shown in Table 1. A few sporadic hits that occurred in non-mitochondrial proteins (A2M, COL1A2, KCTD3, CAD) were removed from the list. The remaining mitochondrial peptides were ranked first by the percent sequence coverage, and secondly by the number of hits (i.e. peptide fragments precipitated), because these both relate to the confidence in the interaction (Table 1). The chaperone GRP75/Mortalin/ HSPA9B, a mitochondrial stress-induced peptide, was at the top of the list, with 28% sequence coverage. The ISD11 homolog (CH6ORF149) was second on the list with 14% sequence coverage. ISD11 was recently identified to be a component of the Nfs1/ISCU complex of mitochondrial iron–sulfur cluster biogenesis (25,26). ATP5L, a component of the mitochondrial ATP

![Figure 1. Immunoaffinity purification of human frataxin.](https://academic.oup.com/hmg/article-abstract/16/8/929/553069/1)

**Table 1. Mitochondrial proteins associated with human frataxin**

<table>
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<tr>
<th>Sequence coverage (%)</th>
<th>Protein identified</th>
<th>Abbreviation</th>
<th>Peptides matched</th>
<th>Total MW</th>
<th>Accession #</th>
</tr>
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<td>28</td>
<td>Mortalin, GRP75, hsp70 9B</td>
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<td>11</td>
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<td>C6orf149/ISD11</td>
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<td>AFG3L1</td>
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Caption. COS7 = COS7 cells, Lymph = lymphoblasts, mul = molecular weight.
Frataxin interacts with GRP75/mortalin

The highest sequence coverage of immunoprecipitated proteins was for GRP75/mortalin, a mitochondrial chaperone and homolog of yeast chaperone ssq1, which participates in folding and insertion of iron–sulfur clusters in recently imported mitochondrial proteins (31). We confirmed an interaction between flag-tagged frataxin and myc-tagged GRP75 by a different set of co-immunoprecipitation epitopes (Fig. 2). In Figure 2A, three different bands were detected by anti-flag antibody in the HEK293T cells transfected with plasmid p3xflag-CMV-frataxin, three different bands indicated precursor frataxin, intermediate frataxin and mature form frataxin. And in Figure 2B, only one band was recognized by anti-myc antibody in the HEK293T cells transfected with pCMV-myc-GRP75. In Figure 2C, tier II, lane 2, it is demonstrated that a 75 kDa protein immunoprecipitated by anti-flag antibody is detected by anti-myc antibody. Conversely, Figure 2C tier IV demonstrates that a protein immunoprecipitated by anti-myc, which is only attached to GRP75, is detected by anti-flag antibody. Thus, it was demonstrated that frataxin interacts with GRP75 by immunoprecipitation and probing in both directions. In Figure 2C tiers I and III are controls, demonstrating that the anti-flag (tier I) and anti-myc (tier III) antibodies are effective both for immunoprecipitation and immunoblotting.

ISD11 colocalizes with frataxin and is targeted to the mitochondria

ISD11 has recently been demonstrated in yeast to interact with both Nfs1 and ISCU of the mitochondrial iron–sulfur cluster biogenesis machinery (25), and so finding a mammalian ISD11 homolog as an interactor of frataxin was of particular interest. If frataxin interacts with ISD11, the protein should colocalize with frataxin. Frataxin and flag-tagged ISD11 constructs were co-transfected into COS7 cells. The expressed proteins were then stained with antibodies to frataxin and flag, respectively, and visualized by immunofluorescence microscopy. A merge of the two stains demonstrated colocalization (Fig. 3C), in a punctate, extranuclear distribution.

In addition to its known mitochondrial location, frataxin has recently been reported to occur outside the mitochondria (32,33). To experimentally determine the cellular compartment to which ISD11 localizes, we transfected p3×flag-ISD11 into COS7 cells and stained mitochondria with MitoTracker red. The triple flag epitope was fused to the C terminus of ISD11 to allow normal processing of the putative mitochondrial leader sequence. The results clearly indicated that the ISD11–3×flag fusion protein accumulates in the mitochondria (Fig. 3F). We produced the ISD11 poly-antibody in rabbit and this antibody could recognize both transfected ISD11 and endogenous ISD11, and western blot showed that endogenous ISD11 was localized on the mitochondria (Fig. 3G).

Frataxin interacts with ISD11 in vitro

To confirm the interaction between frataxin and ISD11, we performed pull-down experiments with recombinant
GST-tagged frataxin. As shown in Figure 4A, ISD11 bound to glutathione-Sepharose 4B beads containing GST-D55frataxin (lane 3) but not to control glutathione-Sepharose 4B beads containing GST alone (lane 2).

Endogenous, unamplified frataxin protein interacts with ISD11 in vivo

We also confirmed an interaction between ISD11 and frataxin by a different set of co-immunoprecipitations (Fig. 4B–D). In Figure 4B (tier II, lane 2), it is demonstrated that a protein immunoprecipitated by anti-frataxin antibody is detected by anti-flag antibody, of which there is only one expressed, i.e. flag-ISD11. Conversely, Figure 4B tier IV demonstrates that a 15kDa protein immunoprecipitated by anti-flag in lane 1 is detected by anti-frataxin antibody. Since the cells were not transfected with frataxin vector, we conclude that the 15 kD protein identified by the anti-frataxin antibody is endogenous frataxin. A 17 kDa protein was immunoprecipitated by anti-flag in lane 2 and detected by anti-frataxin antibody exactly corresponds to the size we expect for frataxin plus the HA tag. Thus, it was demonstrated that frataxin interacts with ISD11 by immunoprecipitation and probing, in both directions. In Figure 4B tiers I and III are controls, demonstrating that the flag (tier I) and frataxin (tier III) antibodies are effective both for immunoprecipitation and immunoprobing.

We designed multiple experiments to test and therefore demonstrate that the interaction between frataxin and ISD11 was not the result of overexpression of frataxin.

After showing that ISD11 immunoprecipitates endogenous frataxin (Fig. 4B, Tier IV lane 1), we tested whether endogenous frataxin could immunoprecipitate ISD11; so we transfected...
The interaction between frataxin and ISD11 is reversed by a chelator

As frataxin has been shown to bind metal ions, and specifically iron (34–37), we tested the influence of known chelators upon the frataxin-ISD11 interaction. EDTA specifically decreased the interaction of ISD11 with frataxin, in a concentration-dependent fashion, i.e. the amount of ISD11 associated with the immunobeads is significantly lower in lysate with a buffer containing 1 and 10 mM EDTA (Fig. 5). Unexpectedly, the interaction of frataxin with ISD11 was inhibited by a buffer containing physiological concentrations (50 μM) of ferrous iron, and this interaction could be rescued by iron chelator deferoxamine. The interaction was also inhibited by Ca²⁺, Mg²⁺, Mn²⁺, Cu²⁺, Co²⁺, Zn²⁺, Fe³⁺, but strikingly nickel, Ni²⁺, increased the interaction between frataxin and ISD11 (Fig. 5).

The frataxin point mutation I154F inhibits the interaction with ISD11, and is rescued by nickel

Most disease-causing alleles of frataxin are expansions of a GAA repeat in intron 1; however, multiple substitution mutations of frataxin also exist, which decrease protein function and cause FRDA. Using the pcDNA-frataxin-HA vector as a template, five of the known frataxin mutant constructs were generated: G130V, I154F, W155R, W173G and L156P. These constructs...
were transfected into HEK293T cells, blotted and probed with anti-frataxin antibody. The mature form of frataxin was not expressed in the G130V, W173G and L156P mutations (Fig. 6A), suggesting that these mutations decrease mature protein expression. In contrast, the I154F and W155R construct produced a reasonable amount of the mature mitochondrial form of frataxin. The frataxin I154F mutant and ISD11 constructs were transfected into HEK293T cells, and their interaction was analyzed by co-immunoprecipitation. The results showed that no ISD11 was co-immunoprecipitated by frataxin antibody under these conditions (Fig. 6B, left lane). But when nickel was added into the Heps buffer, a band was co-immunoprecipitated. (Fig. 6B, middle lane 2). To determine whether this band was immunoprecipitated by frataxin I154F contained on the plasmid, or the much lower level of endogenous wild-type cellular frataxin, we transfected pcDNA empty vector and ISD11 into HEK293 cells, and lysed the cells with Heps buffer treated with and without nickel. If there were no interaction between I154F and ISD11, then we should observe the same bands in middle lane and right lane of Figure 6C. But we observed no band in right lane of Figure 6C, suggesting that nickel rescued the interaction between frataxin I154F and ISD11. For the W155R mutant, only weak interaction was observed between ISD11 and W155R mutant (Fig. 6D, middle lane). Nickel could partially rescue the interaction between ISD11 and W155R mutant.

Silencing ISD11 decreases aconitase and NFS1/ISCU and increases iron concentration

Frataxin knockdown in cells and animals produces both defects in iron–sulfur cluster enzyme activity, and cellular iron over-
load (10,23). If frataxin causes its effects on iron–sulfur cluster status through its interaction with ISD11, then the knockdown of ISD11 should produce the same consequences (i.e. phenocopy) as frataxin knockdown. siRNA directed against ISD11 or a scrambled control was transfected into the HEK293T cells. The anti-ISD11 siRNA resulted in a 50% reduction of the ISD11 mRNA relative to transfection with the control siRNA by quantitative RT–PCR (Fig. 7A). At the protein level, co-transfection of ISD11 siRNA and p3/C2/flag-ISD11 knocked down ISD11 protein expression in HEK293T cells (Fig. 7B). ISD11 siRNA also efficiently knocked down endogenous ISD11 protein in HEK293T cells (Fig. 7E).

We measured total cell iron content in siRNA transfected cells. Compared to the scrambled control siRNA transfected, the ISD11 siRNA transfected cells showed a significant increase in total iron content (Fig. 7C), as do Δyfh yeast cells (38). Aconitase activity also was examined in HEK293T cells depleted of ISD11. The results showed that ISD11 siRNA reduces both cytosolic and mitochondrial aconitase activities, even while the protein level of mitochondrial aconitase was similar, and IRP1 slightly decreased (Fig. 7D). Nfs1/ISCU protein expression was examined as well. The results showed that ISD11 siRNA reduces both cytosolic and mitochondrial Nfs1/ISCU level (Fig. 7E).

**ISD11 mRNA levels decrease in FRDA patients**

We have demonstrated that frataxin interacts with ISD11 and that ISD11 knockdown duplicated multiple consequences of frataxin deficiency. Previously, we have demonstrated that ISCU and Nfs1 (ISCS) transcript levels are decreased in FRDA patients’ cells (24). If frataxin and ISD11 interact at the protein level, we reasoned that their mRNAs might be co-regulated and we investigated this possibility by quantitative RT–PCR. The results showed ~75% reduction of the frataxin transcript and ~60% reduction of the ISD11 mRNA in FRDA patients (Fig. 8). Thus, frataxin-deficiency causes a transcriptional co-repression of multiple transcripts involved in iron–sulfur cluster biosynthesis, including ISD11, ISCU and Nfs1 (ISCS) in FRDA patients. This is expected if frataxin’s primary function is in iron–sulfur cluster biosynthesis, and less expected if frataxin’s primary function is in iron metabolism or transport.

**DISCUSSION**

**Frataxin associates with multiple mitochondrial proteins**

Deficiency of frataxin protein causes FRDA. Substantial debate about frataxin’s primary physiological role still exists, and prime candidates include iron–sulfur cluster biogenesis and repair, iron transport and metabolism, and anti-oxidative action. We immunoprecipitated frataxin from mitochondrial extracts to isolate its partners, to help determine frataxin’s most likely physiological role. Multiple mitochondrial proteins were isolated, which were consistent between lymphoblasts and COS7 cells, and some previous findings in

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**Figure 7.** Silencing ISD11 decreases aconitase and Nfs1/ISCU and increases iron concentration. (A) HEK293T cells were transfected with ISD11 siRNA or control siRNA, mRNA prepared and analyzed by QPCR, using primers specific to the ISD11 gene, error bars represent standard deviation of four experiments. ***P < 0.001). (B) Western blot detection of ISD11-flag fusion protein, using anti-flag antibody, 48 h post-transfection with control siRNA or ISD11 siRNA. (C) Total cellular iron concentrations of HEK293T cells transfected with ISD11 siRNA or control siRNA. The experiment was repeated three times and **P < 0.005. (D) Aconitase activity analysis of HEK cells transfected with Control siRNA or ISD11 siRNA. (E) Down-regulation of ISD11 affects Nfs1 and ISCU protein level.
Frataxin is a homolog of the well-known HSP10 forms a mitochondrial chaperonin complex (44). HSP60 is a known interactor of GRP75, and together we observed several peptide interaction hits of frataxin with the mitochondrial chaperone HSP60. Frataxin associates with the mitochondrial chaperone GRP75/mortalin/HSPA9B (hereafter GRP75) suggesting that frataxin is well associated with a known mitochondrial chaperone. GRP75 is a known interactor of the mitochondrial chaperone HSP60/GroEL/SPG13/HSPD1 (28), the best represented interactor on the basis of peptides isolated (Table 1). Amino acids comparison analysis showed that there is 49% identity and 70% similarity between GRP75 and Ssq1. Ssq1 is a mitochondrial matrix protein which was shown to be essential for the import and maturation of the yeast frataxin homolog Yhf1 (39) and for iron–sulfur (Fe/S) clusters assembly in mitochondria (40); mutants in Ssq1 were reported to have low levels of Fe/S cluster-containing enzymes and accumulate iron within mitochondria (41). Also, Ssq1 interacts with Isu (the yeast ortholog of human ISCU) and Jac1 (31,42); and it is involved in iron–sulfur cluster biogenesis (31,43).

**Frataxin interacts with the mitochondrial chaperone GRP75/mortalin**

First, there were multiple hits in the mitochondrial chaperone protein GRP75/mortalin/HSPA9B suggesting that frataxin is well associated with a known mitochondrial chaperone. In vitro studies have shown that in E.Coli GroEL interacts with rhodanese (46), a mitochondrial enzyme involved in the repair of Fe/S clusters (47) transcript levels of which we have previously shown to be decreased in FRDA lymphoblasts (24). GroEL also inserts iron–sulfur cluster into adrenodoxin (48), a protein involved in electron transfer from NADPH+ (via a reductase) to cytochrome P-450 in the adrenal gland. And we also observe that adrenodoxin activity is deficient in frataxin-deficient cells (Napoli et al., in preparation). In yeast, decreases of hsp60 cause iron increase and negatively affect the activity of Fe/S enzymes upon oxidative stress (49).

So, by inference from the combined yeast and mammalian studies, the interaction of frataxin with GRP75 and HSP60 is consistent with the idea that frataxin is part of a chaperone complex that inserts iron–sulfur clusters into recently imported apoproteins (50). This association of frataxin with mitochondrial chaperones may help explain the interesting results of Bulteau et al. (15), who observed a stress-dependent association of frataxin with mitochondrial aconitase. If frataxin is already associated with mitochondrial chaperones, and upon stress these chaperones preferentially bind unfolded proteins, then the stress-dependent association of frataxin with aconitase may occur through linkage of each to mitochondrial chaperones.

**Frataxin associates with the mitochondrial chaperone HSP60**

We observed several peptide interaction hits of frataxin with HSP60. HSP60 is a known interactor of GRP75, and together with HSP10 forms a mitochondrial chaperonin complex (44). HSP60 is a homolog of the well-known E.Coli GroEL (45). In vitro studies have shown that in E.Coli GroEL interacts with rhodanese (46), a mitochondrial enzyme involved in the repair of Fe/S clusters (47) transcript levels of which we have previously shown to be decreased in FRDA lymphoblasts (24). GroEL also inserts iron–sulfur cluster into adrenodoxin (48), a protein involved in electron transfer from NADPH+ (via a reductase) to cytochrome P-450 in the adrenal gland. And we also observe that adrenodoxin activity is deficient in frataxin-deficient cells (Napoli et al., in preparation). In yeast, decreases of hsp60 cause iron increase and negatively affect the activity of Fe/S enzymes upon oxidative stress (49).

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**Confirmation of the interaction of frataxin with succinate dehydrogenase**

Our co-immunoprecipitation results from mitochondria of lymphoblasts and COS cells confirm the study of frataxin’s partners in yeast and C. elegans (19,20), in which frataxin was observed to interact with succinate dehydrogenase. Succinate dehydrogenase is an enzyme complex embedded in the mitochondrial inner membrane, interacting with the matrix but not the intermembrane space. These results further localize frataxin’s interactors to the mitochondrial matrix and matrix side of the mitochondrial inner membrane.

**Frataxin interacts with an ISD11 ortholog**

The second greatest sequence coverage of frataxin’s interactors occurred with C6ORF149, an ortholog of yeast ISD11, which has recently been demonstrated to be a component of the eukaryotic Nfs1/ISCU iron–sulfur biogenesis complex (25,26). Frataxin physically interacts with ISD11 by several criteria, including immunoprecipitation in both directions, GST-pulldowns and colocalization within the mitochondria.

In yeast, frataxin interacts with Nfs1/ISCU, and this interaction is increased by ferrous iron (12). Unexpectedly, we observed that iron inhibited the interaction between ISD11 and frataxin, and that the iron-specific chelator desferoxamine could rescue the interaction. The chelator EDTA clearly inhibited the interaction between frataxin and ISD11 in a concentration-dependent manner, and so a survey of other metal ions was undertaken. We found that nickel at a 50 μM concentration increased the interaction between frataxin and ISD11. Nickel is an essential nutrient for eukaryotes.
Some data have demonstrated that deficiency of nickel causes poor absorption of ferric iron (51), and in A549 cells, nickel treatment decreases total cellular iron level (52). Nickel forms Ni-Fe-S clusters in bacteria (53), and nickel ‘bridges’ 4-iron, 4-sulfur clusters on some peptides that act as scaffolds (54). So, the data suggest that nickel supports the interaction of frataxin and ISD11, and thus iron–sulfur cluster biogenesis.

The I154F and W155R mutations decrease the interaction between frataxin and ISD11

Approximately 4% of FRDA patients are compound heterozygotes. Three point mutations, G130V, I154F and W173G, were found in 40% of families with identified point mutations (55). We generated these three mutant frataxin plasmids and transfected them into HEK293T cells. Both G130V and W173G were poorly expressed in the mature form, suggesting that these two mutations affect expression, maturation, or degradation of frataxin. In contrast, the I154F mutant was well expressed as mature frataxin. However, the frataxin I154F mutation, even expressed at high levels, was not immunoprecipitable by ISD11, whereas normal frataxin was. Intriguingly, 50-micromolar nickel could rescue the interaction between the mutant frataxin and ISD11. This result suggests that the I154F mutation causes FRDA through defective interaction with ISD11. This suggests ISD11 is the functional binding partner of both frataxin and the Nfs1/ISCU complex.

Crystal structure analysis showed that frataxin protein is a compact αβ sandwich (56) and none of FRDA point mutations overlap the proposed iron-binding region (17,57). The I154 residue is located on the central β sheet, β4, which we propose contains the interaction surface with ISD11. We generated other point mutations occurring on the B4 sheet and causing FRDA, W155R and L156P (55,58); we found that only the W155R mutant was well expressed as mature frataxin, and the W155R mutant only weakly interacts with ISD11. Fifty-micromolar nickel could increase the interaction between the mutant frataxin and ISD11. Nickel could cause increased interaction between frataxin and ISD11 either through bridging their interaction or by stabilizing mutant frataxin.

siRNA against ISD11 phenocopies multiple aspects of frataxin deficiency

If frataxin’s upstream interaction with ISD11 is necessary for NFS1/ISCU function, then conversely silencing ISD11 should phenocopy the same consequences of frataxin depletion, which include iron–sulfur cluster deficiency and iron accumulation. We silenced the ISD11 by siRNA in HEK293T cells. Our results showed that when ISD11 was depleted by siRNA, both mitochondrial and cytosolic aconitase activity decreased, and that this was not a consequence of decreased protein levels. ISD11 silencing also decreased both the mitochondrial and cytosolic forms of the Nfs1/ISCU protein, suggesting that ISD11 functions in both compartments. Although either deficiency of frataxin or ISD11 affects the Nfs1/ISCU complex, knockdown of ISD11 did not cause a deficiency of frataxin mRNA or protein. We suggest that frataxin deficiency causes mitochondrial and cytosolic aconitase deficiency through the defective interaction with ISD11.

A function for frataxin in iron–sulfur cluster biogenesis

In Escherichia coli, the CyaY protein (the bacterial ortholog of frataxin) is thought to specifically interact with IscS (Nfs1) to donate iron to build [2Fe-2S] on the scaffold IscU (59). But up to now no interaction has been observed between frataxin and Nfs1 in human cells (14). Isd11 exists only in eukaryotes (60). Our data demonstrate that frataxin interacts with ISD11, which is essential for the proper function of the Nfs1/ISCU complex (25,26). The data presented here support the view that ISD11 serves as an adaptor between frataxin and Nfs1/ISCU. This is consistent with our previous work in cells from FRDA patients showing that the mRNA and protein level of Nfs1 and ISCU were decreased in frataxin-deficient cells (24). There has been substantial debate over the role of frataxin, including iron transport, iron bioavailability, antioxidant status, mitochondrial stimulator and iron–sulfur cluster function. Data presented here suggest that in human cells, frataxin is directly associated with ISD11 of the iron–sulfur cluster biogenesis complex, and that two mutations that cause FRDA decrease this specific interaction, and that nickel increases it. Furthermore, there is interaction of frataxin with both GRP75 (an ssq1 homolog) and HSP60, and it has previously been shown in yeast that the chaperone system is essential for insertion of iron–sulfur clusters into newly imported mitochondrial proteins. These data strongly support a direct role of frataxin function in iron–sulfur cluster biogenesis and insertion into apoproteins as part of a mitochondrial chaperone complex (Fig. 9).

MATERIALS AND METHODS

Biochemical reagents were purchased from Sigma (St Louis, MO, USA), Bio-Rad (Hercules, CA, USA) or mentioned in the text.

Cell culture

All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Lymphocytes were grown as described (61). COS7 and HEK293T cells (from ATCC) were grown in DMEM medium (Gibco-BRL) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate and penicillin/streptomycin.

Plasmid constructs and site-directed mutagenesis

Open reading frame sequence with C-terminal flag sequence (gactacaaggacgagcagtcagta) of Human frataxin cDNA (GenBank Accession No. NM_000144) was created by PCR and inserted into pcDNA3.1; mature frataxin sequence (nucleotide 521–988) was inserted into pGEX-4T-1 (Amersham Pharmacia Biotech) to produce GST-ΔN55-frataxin fusion protein. Frataxin cDNAs also was inserted in-frame into p3 × flag-CMV-14 (Sigma). The pcDNA-frataxin-HA construct

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was described previously (62). G130V, I154F, W155R, L156P and W173G mutants of frataxin were obtained from pcDNA-frataxin-HA by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) and all mutants were sequenced to confirm their sequences. GRP75 cDNAs (GenBank Accession No. NM_004134) was inserted in-frame into pCMV-myc (Clontech), ISD11 cDNAs (GenBank Accession No. NM_020408) was inserted in-frame into p3flag-CMV-14.

Frataxin pull-down and mass spectrometry

Pull-down and mass spectrometry experiments were carried out in control lymphoblasts (about 1 × 10⁶ cells) and COS7 cells. Eighteen T75 flasks of COS7 (90% confluence) were transfected with 430 μg of pcDNA-frataxin-flag plasmid constructs using Lipofectamine 2000 (Invitrogen) in the serum-free medium. After 4 h of incubation, medium was replaced with fresh complete medium, and cells were cultured for an additional 96 h before collection.

Mitochondria from COS7 and lymphoblasts were isolated as before (24), treated with a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM PMSF, 1% IGEPA, CA-630 (Sigma), at 4°C for 30 min and insoluble material removed by centrifugation. Protein concentration was estimated using the Bradford assay (Bio-Rad). Frataxin protein complexes were immunoprecipitated by rabbit polyclonal anti-frataxin antibody and rProtein G Agarose (Invitrogen). The immunoprecipitated samples were analyzed by Coomassie blue (lymphoblasts) and silver stain kit (invitrogen) (COS7) and mass spectrometry analysis (Molecular Structure Facility, McDavis, CA).

Preparation of recombinant proteins

The recombinant glutathione-S-transferase (GST) tagged proteins were expressed in E. coli strain BL21-Star (DE3)(Invitrogen) with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 22°C overnight. Cells were collected and lysed in PBS buffer (pH 7.4), supplemented with 1% TritonX-100, 10 mM DTT, 0.5 mM PMSF and 1 mg/ml lysozyme. After incubation on ice for 30 min, the samples were centrifuged and the supernatants were purified using glutathione-S-Sepharose beads (Amersham Biosciences).

Antibody production

The rabbit polyclonal antibody 5034 was raised against synthetic multiple antigenic peptide corresponding 55–72 amino acid residues (LVNKAKRDLGVIRRQVHI) of human ISD11. Antiserum was generated in New Zealand white rabbits by Bio-synthesis Inc. (Lewisville, TX).

Western blot analysis

Forty microgram whole cell lysate, or 40 μg cytoplasmic protein or 15–20 μg mitochondrial protein was analyzed on 15% SDS-PAGE. Electrophoresis was carried out at 130 V for about 1 h following a low voltage of 80 V for 10 min. After electrophoresis, the proteins were transferred to nitrocellulose membranes using a Mini Trans-Blot cell (Bio-rad). The membranes were blocked with 4% non-fat milk in Tris-buffered saline with 0.1% Tween 20 for 1 h. subsequently, the membranes were incubated overnight with the following
primary antibodies: rabbit anti-frataxin; rabbit anti-ISCU and rabbit anti-Nfs1 (kind gifts from Drs Tracey Rouault and Wing Hang Tong); rabbit anti-IRP1 (Santa Cruz Biotechnology) and rabbit anti-mitochondrial aconitase (a kind gift from Dr Luke Szweda); rabbit anti-ISD11; mouse anti-β-actin; mouse anti-cytochrome c (MitoScience), mouse anti-flag (Sigma), mouse anti-myc (Roche). Afterwards, the membranes were developed with AP-conjugated secondary antibodies using a chemiluminescent substrate (Bio-rad).

Glutathione-S-transferase pull-down assay
A total of 2.5 × 10⁶ HEK293T cells were seeded in 60 mm dishes. After overnight growth, cells were 80% confluent and transfected with 8 μg p3 × flag-ISD11 plasmid using Lipofectamine 2000 (Invitrogen) in serum-free medium. After 4 h of incubation, medium was replaced with fresh complete medium, and cells were cultured for an additional 48 h before collection. The cells were lysed in TBS buffer [50 mM Tris HCl, 150 mM NaCl, 1% Triton X-100, 1 mM PMSE and EDTA-free complete protease inhibitor cocktail (Roche)]. Binding assays were precleared by incubating cell extracts in TBS buffer with 25 μg GST and 40 μl glutathione-S-Sepharose beads for 1 h at 4°C, and the supernant was incubated with 10 μg GST-ΔN55frataxin or GST protein bound to 40 μl glutathione-S-Sepharose beads for 3 h at 4°C. After three washes with TBS buffer, the bound proteins were analyzed by western blot.

Co-immunoprecipitation assays
HEK293T cells were transfected with correspond plasmids and culture for 72 h. The cells were lysed with Heps buffer (pH 7.5, containing 20 mM Heps, 150 mM KCl, 0.5% TritonX-100, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride and EDTA-free complete protease inhibitor cocktail (Roche)) with/without 50 μM ferrous chloride or other metal ion. The whole cell lysates were incubated with ANTI-FLAG-M2 resin (Sigma), Anti-c-Myc Agarose (Sigma) or rabbit polyclonal anti-frataxin antibody and rProtein G Agarose at 4°C, rotated overnight and washed four times with lysis buffer. Precipitates were analyzed by Western blotting using anti-flag mAb, anti-myc mAb or rabbit polyclonal anti-frataxin antibody. Antibodies were detected as above.

Immunofluorescence analysis
COS7 cells were plated on coverslips in six well plates. For mitochondria localization, p3 × flag-ISD11 was transfected into COS7 cells cultured for 24 h, exposed to 100 nM MitoTracker Red CMX (Molecular probe) for 1 h at 37°C and then fixed in 3.7% paraformaldehyde for 30 min at 4°C and prechilled methanol 10 min at −20°C. After washing once with PBS, the cells were permeabilized with 0.25% Triton X-100/PBS for 5 min, and then washed three times in PBS followed by blocking in fresh 1% BSA/PBS for 30 min at 37°C. Then the cells were incubated with the primary antibody at 4°C overnight. The primary antibody consisted of the rabbit polyclonal anti-frataxin antibody and anti-flag mAb. Secondary antibodies were goat anti-rabbit fluorescein conjugate (Jackson ImmunoResearch) and goat anti-mouse rhodamine (Jackson ImmunoResearch). All samples were imaged using DeltaVision microscope (APLLC).

Quantitative RT–PCR analysis
Total RNA was prepared using Qiagen Mini Kit (Qiagen, CA, USA), and RNA concentration was determined by UV spectrophotometry. Reverse transcription of 5 μg RNA was performed using an RT–PCR kit (Invitrogen, Gaithersburg, MD, USA), and reactions were performed in a 20 μl volume. Two microlitres cDNA from 20× dilution of RT reaction were used for PCR. Quantitative PCR standard curves were set up for ISD11, frataxin and beta-actin, which method was previously described (63). The primers of PCR are as follows: β-actin forward, 5'-GCC AAC ACA GTG CTT TCT GG-3'; reverse, 5'-CTG CTT GCT GAT CCA CAT CTG C-3'; frataxin forward, 5'-AAA TCT GGA ACT TGT GGC CAC, reverse, 5'-ACC TCA GCT GCA TAA TGA AGC-3'; ISD11 forward, 5'-AGA AGA GCA AGC GTT TCA GC-3'; reverse, 5'-ACT TGG CCT GGG CAT GTC TC-3'. The PCR reaction and normalization were previously described in detail (62).

RNA interference (RNAi), total cellular iron measurement and aconitase activities
ISD11 short interfering RNAi oligonucleotides and Negative control oligonucleotides pools were purchased from Dharmacon (Denver, CO). HEK293T cells at a confluency of 30–50% were transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen) every 72 h for 3–9 days (64), total cellular iron was determined under reducing conditions with Ferrozine as chelator, reactions were carried on in disposable 2.0 ml polypropylene tubes with screw caps from Sarstedt (65). Aconitase activity was analyzed in aconitase activity gels (66).

Statistics
Statistical analysis of the data was performed by Student’s t-test.

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Conflict of Interest statement. None declared.
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