Frataxin and mitochondrial Fe-S cluster biogenesis

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Frataxin is a conserved mitochondrial protein that plays a role in Fe-S cluster assembly in mitochondria. Fe-S clusters are modular cofactors that perform essential functions throughout the cell. They are synthesized by a multi-step and multi-subunit mitochondrial machinery that includes a scaffold protein Isu for assembling a protein bound Fe-S cluster intermediate. Frataxin interacts with Isu, iron, and with the cysteine desulfurase Nfs1 that supplies sulfide, thus placing it at the center of mitochondrial Fe-S cluster biosynthesis.

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Introduction: Friedreich’s ataxia is an inherited disease which is characterized by progressive symptoms of ataxia and sensory loss, often leading to gait impairment and the need for a wheelchair. A progressive and sometimes lethal cardiomyopathy is another feature, and in some cases diabetes mellitus is associated. At the pathological level, degenerative changes affect certain large sensory neurons, heart cells and islet cells, thus involving a unique target tissue distribution (1). The gene was identified by genome mapping of affected kindreds, and the encoded protein, frataxin, is decreased in affected individuals most often due to a GAA repeat in the first intron of the gene (2). Human frataxin is synthesized on cytoplasmic ribosomes with a mitochondrial targeting sequence that mediates organelle targeting and is subsequently removed by proteolytic processing steps in the mitochondrial matrix (4-7). Deficiencies of aconitase and other mitochondrial iron-sulfur (Fe-S) proteins occur as first noted in cardiac biopsies of affected individuals (8). Fe-S clusters are modular cofactors consisting of iron and sulfur, most often anchored by bonds joining cysteine sulfur atoms in the polypeptide chain of a protein and iron atoms of the cluster. They perform essential and diverse biochemical functions (electron transfer in cellular respiration, substrate interaction, biological signal transduction among other functions), and their biogenesis is catalyzed by a multisubunit machinery (9). Frataxin has been a subject of intense study with contributions from many disciplines: structural biology, cell biology, genetics of model organisms, evolution biology, and medicine. A picture is emerging of a direct role of frataxin in the complex and highly conserved machinery of Fe-S cluster biogenesis in mitochondria. Additional functions may be mediated by direct frataxin-iron effects or by other protein-protein interactions.

Frataxin Evolution: Frataxin is highly conserved throughout evolution, being present in humans, plants, flies, worms, and bacteria (proteobacteria but not archaeabacteria) (10). Some hints about frataxin function can be gleaned from the evolutionary record. Three different bacterial operons have been identified capable of mediating Fe-S cluster assembly: nif, suf and isc. The nif operon is specialized for high volume biosynthesis needed to support nitrogenase supply during diazotrophic growth of some organisms (e.g. Azotobacter vinlandii). The suf operon is specially adapted for Fe-S cluster synthesis in conditions of iron starvation and oxidative stress. Finally the isc operon handles housekeeping Fe-S cluster synthesis.

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biogenesis (9). Although not found on any of these operons, frataxin is strongly associated with the isc operon. It appeared in proteobacteria about the time that the specialized chaperones hscA and hscB appeared on the isc operon. A frataxin ortholog was subsequently lost in this phylogenetic lineage on two separate occasions, coinciding with loss of hscA and hscB. Frataxin’s appearance in eukaryotes occurred about the time of the endosymbiotic event creating mitochondria from the purple bacterial ancestor, and it was probably acquired by mitochondria together with other components of the isc operon (10). This notion of co-evolution of frataxin and Fe-S assembly components of the isc operon is supported by studies of primitive eukaryotes that lack typical mitochondria. In Trichomonas vaginalis, a unicellular parasite, a modified and stripped-down organelle called the hydrogenosome performs Fe-S cluster assembly and contains a frataxin ortholog able to complement the phenotypic defects of a yeast mutant lacking its own frataxin (11). An even more severe case of organelle simplification is presented by mitosomes. These are rudimentary organelles of obligate intracellular parasites called microsporidia, and they lack respiratory complexes or mitochondrial DNA. Virtually all that remains are a few components recognizable for their homology to Fe-S cluster assembly components (e.g. Isu, Nfs1, Hsp70 homologs) and frataxin (12). The co-retention of frataxin with these very few other components involved in Fe-S cluster assembly in the mitosome emphasizes their close functional relationship.

Mitochondrial iron accumulation: In Saccharomyces cerevisiae, Yfh1 is the yeast frataxin homolog, and the initial studies of the Aft1Δ deletion strain described a pleiomorphic phenotype. The mutant exhibited constitutive upregulation of the cellular iron uptake system and tremendous accumulation of iron in mitochondria (13). The accumulated iron appeared as dense aggregates in unstained electron micrographs. Chemical analyses revealed that the accumulated iron was in the form of ferric phosphate nanoparticles (14). Many additional studies have established a close association of the mitochondrial iron accumulation phenotype and deficiency of Fe-S clusters. Cellular depletion studies, in which Yfh1 was put under control of a regulated promoter and turned off, recapitulated the phenotypes of deficient Fe-S cluster proteins and mitochondrial iron accumulation. If the promoter was turned on again, Fe-S clusters were restored and the accumulated iron returned to a normal distribution (15). Other mutants of the mitochondrial Fe-S cluster assembly pathway were similarly associated with this iron homeostatic phenotype (ssq1, jac1, nfs1, others). In human cells, mitochondrial iron accumulation has also been observed, both in tissues from Friedreich’s patients (e.g. heart, dorsal root ganglia) and in cultured cells engineered to exhibit stable frataxin deficiency (16). Thus low level frataxin is associated with mitochondrial iron accumulation and Fe-S cluster deficiency in both human and yeast cells. However, an Fe-S cluster protein capable of mediating these effects and conserved between yeast and humans has not been identified. The best candidate protein for this function, human IRP1, is not conserved with yeast, and the yeast iron regulatory machinery, including Aft1/2, is not found in humans (17).

Fe-S cluster assembly in mitochondria: Frataxin has been found in mitochondria of virtually all eukaryotes using biochemical and microscopic tools to ascertain its subcellular location. Similarly, orthologs of many of the other Isc Fe-S cluster assembly components have been found in mitochondria (18). The steps and components involved in the biogenesis of Fe-S cluster proteins in mitochondria are quite similar to those in bacteria. The process can be understood in terms of the central role of scaffolds as first shown by Dennis Dean and now well established (19). An Fe-S cluster intermediate is formed on the scaffold protein Isu and then transferred to apoproteins (Fig. 1). Many steps must occur in a coordinated fashion for proper Fe-S cluster synthesis on Isu. The sulfide for Fe-S clusters originates from cysteine via the action of Nfs1, a pyridoxal phosphate containing cysteine desulfurase. In eukaryotes, Nfs1 must be assembled with Isd11, a small accessory subunit of unknown function (20,21). The sulfide is probably transferred to Isu from the Nfs1 active site as a persulfide. The source of the iron for the Isu intermediate is unclear, although a role for mitochondrial carrier proteins Mrs3 and Mrs4 in yeast (mitoferrin in
humans) has been proposed (22). Frataxin may play a role here and has been shown to genetically interact with the mitochondrial carrier proteins. This was shown by the exacerbated slow growth and mitochondrial iron starvation in the combined ∆mrs3∆mrs4∆yfh1 mutant (23). Other components implicated at this stage are Arh1 and Yah1 (adrenodoxin reductase and adrenodoxin in humans), which are needed to provide reducing equivalents in an electron transfer chain, although the substrate requiring reduction has not been defined (24). The Isu protein appears to be conserved in the context of primary sequence and function, and structural information exists for a few bacterial orthologs. Key features are three critical cysteines and an aspartate that bind the cluster intermediate probably on each subunit of the Isu dimer (25).

Most mitochondrial proteins, including Fe-S cluster proteins, are translated on cytoplasmic ribosomes as precursors with mitochondrial targeting sequences. Upon import into mitochondria, the unfolded precursors are subjected to various processing steps that remove the targeting sequences (26). At this point, the processed apoproteins become substrates for a machinery dedicated to transferring the Isu cluster intermediate to recipient proteins. The scaffold Isu displays an interaction site consisting of the tripeptide sequence, PVK, and this mediates binding to the Hsp70 chaperone (Ssq1 in yeast and Ssc1 in humans) (27,28). This interaction is modulated by the ATP hydrolysis cycle in conjunction with accessory proteins Jac1 and Mge1 (29). A monothiol glutaredoxin is also implicated here (30). Through concerted action of the chaperones, glutaredoxin and accessory proteins, the Isu Fe-S cluster intermediate is transferred to apoproteins.

A compartment problem exists for eukaryotic cells in that many Fe-S cluster proteins reside in the cytoplasm or in the nucleus. The biogenesis of these Fe-S clusters requires both mitochondrial and extramitochondrial machineries (31,32). In this regard, it is notable that small amounts of frataxin have been found outside mitochondria, and a role in synthesis of extramitochondrial Fe-S clusters has been proposed (7).

**Placement of Yfh1 in the pathway of mitochondrial Fe-S cluster assembly:** An experiment is described here that illustrates the role of frataxin in promoting Fe-S cluster assembly in mitochondria. Mitochondria were isolated from wild-type or ∆yfh1 yeast strains. New synthesis of Fe-S clusters in the isolated organelles was examined by providing ^35^S-cysteine as a source of sulfide. In the wild-type, radioactive sulfide was incorporated into a pool of apoaconitase by the mitochondrial Fe-S cluster machinery. Synthesis of new clusters was also detected by labeling of imported ferredoxin, and these could be distinguished by signals on a native gel (Fig. 2A, left panel; Fig. 2B, lanes 1 and 2). In the mutant, however, no such labeling occurred, indicating failure to make Fe-S clusters (Fig. 2A, middle panel; Fig. 2B, lanes 5 and 6). Subsequently, frataxin was imported into the ∆yfh1 mutant mitochondria, and the mitochondria were recovered by centrifugation. Now when a similar ^35^S-cysteine labeling experiment was performed, the mutant mitochondria demonstrated restored ability to make Fe-S clusters on both aconitate and imported ferredoxin (Fig. 2A, right panel; Fig. 2B, lanes 7 and 8). Thus a very small amount of frataxin (estimated less than one picomole) imported into isolated ∆yfh1 mitochondria even without iron addition was able to rapidly (in less than 10 minutes) restore Fe-S cluster synthesizing activity. This effect in isolated mitochondria shows that everything is present in mitochondria for Fe-S cluster formation and that a small quantity of frataxin is capable of activating or mediating the process (23).

In order to begin to address where frataxin might act to promote Fe-S cluster assembly, it was important to determine if frataxin acts before or after Isu. In one approach to this problem, a form of metabolic labeling was devised in which radioactive iron was added to growing yeast cells, and the radiolabeled Fe-S cluster Isu intermediate was recovered by immunoprecipitation (33). This approach was combined with promoter swaps to produce cellular depletion of one or another of the assembly component(s) prior to iron labeling and Isu recovery. A reduction in iron labeling of Isu would indicate decreased formation of Fe-S cluster intermediates. In that case, the depleted component was placed upstream of Isu, because it
was necessary for intermediate formation. In the case of increased iron labeling of Isu, the component placed downstream of Isu, because it was needed for transfer of the Isu clusters to recipient apoproteins. Using this assay design, Nfs1, Arh1, Yah1 were placed upstream, and Ssq1, Jac1, and Grx5 were placed downstream of Isu in the Fe-S cluster assembly process. Frataxin was placed upstream, because frataxin depleted cells failed to efficiently form the radiolabeled Isu intermediate (34).

A completely independent set of observations led to a similar conclusion. The manganese superoxide dismutase (MnSod), the dismutase of the mitochondrial matrix, was found to incorporate iron instead of manganese in some settings, with concomitant inactivation of the enzyme. For example, some of the Fe-S cluster assembly mutants with a mitochondrial iron accumulation phenotype were associated with MnSod inactivation secondary to iron incorporation. Specifically, these mutants, such as ssh1 and grx5, belonged to Isu downstream events in transfer of Fe-S cluster intermediates to apoproteins (35). By contrast, although frataxin mutants (∆yfh1) dramatically accumulated iron in mitochondria, MnSod was active because the iron was not available for insertion (36). Similarly Isu mutants (depletion or dominant negatives) were also not associated with MnSod inactivation (35). These data suggest that frataxin should be grouped with Isu in the upstream part of the Fe-S cluster biogenesis pathway and distinguished from Ssq1 and Grx5. Although mismetallation is an aberrant situation, these observations may point to a physiological role of frataxin in modulating bioavailable iron pools in mitochondria.

Frataxin structure, Isu interaction and iron interaction: Structures have been obtained for yeast (37), human (38) and bacterial (39) frataxins. They are strikingly alike, characterized by an alpha-beta sandwich motif creating the two protein planes. The α-helical plane consists of two parallel (N- and C-terminal) alpha helices, and the β-sheet plane consists of five anti-parallel beta strands; a sixth and possibly seventh strand intersect the planes depending on the species (40). The structure and length of the respective N-termini vary among orthologs, while the unstructured C-termini, also of variable length, help control protein stability (41). A negatively charged surface is created by clustering of contiguous acidic residues contributed by portions of helix 1 and beta strand 1. Although the individual residues are not always conserved, all known frataxins are characterized by the presence of such an acidic surface. A second conserved structural feature is a neutral flat area on the beta sheet surface, which is more hydrophobic towards the center and more hydrophilic towards the periphery. This region contains a number of perfectly conserved residues and appears to be well suited to mediate an interaction with a protein partner.

Several lines of evidence point to Isu as a frataxin protein partner. A functional relationship of frataxin and Isu was initially suggested by genetic experiments showing synthetic lethality of Δyfh1 (YFH1 gene deleted) and Δisu1 (deletion of one of two homologous ISU genes causing lowered levels of Isu) (42). Physical interactions between frataxin and Isu were subsequently demonstrated in pull-down experiments from mitochondrial lysates. Interestingly the pull-downs of Yfh1 by Isu, or reciprocally of Isu by Yfh1, were dependent on iron addition to the buffer and were inhibited by the presence of metal chelators (43). Iron dependence of an Isu-frataxin interaction was also shown for the purified human proteins (44). The clearest demonstration of the importance of frataxin-Isu interaction in Fe-S cluster assembly was provided by careful analyses of frataxin proteins with substitutions of surface exposed amino acids in the interaction interface (Fig. 3A).

Alteration of conserved surface exposed residues of β-strand 3 (N122A/K123T/Q124A) was associated with severely impaired frataxin-Isu interaction (pull down of Isu from a lysate on immobilized Yfh1-His6). The same mutant frataxin expressed in cells was associated with decreased Fe-S cluster protein enzyme activities. Other substituted forms of frataxin with alterations in exposed areas of the beta sheets (Q129A in strand 4, W131A in strand 4 and R141A in strand 5) were studied in vitro and in vivo. These various amino acid changes were each associated with decreased frataxin-Isu interaction and deficient activities of Fe-S cluster proteins (45) (Fig. 3A). These studies with minimally altered frataxins
were especially informative, because they avoided many of the secondary phenotypes associated with yfh1 deletion. The conclusion from these studies is the interaction between frataxin and Isu in mitochondria is required for Fe-S cluster assembly.

Frataxins interact with iron in vitro, although not in the manner of typical iron binding proteins. Binding is relatively low affinity, occurs on the protein surface rather than in a cavity, and is mediated primarily by carboxylic amino acids rather than cysteines and histidines (46). Analysis of iron interactions with yeast frataxin monomers showed two ferrous iron atoms binding with affinity of 2.5 to 5 µM as high spin ferrous iron. Chemical shifts and line broadening induced by exposure of 15N-labeled yeast frataxin to paramagnetic iron (ferrous iron under anaerobic conditions) further defined iron binding residues as primarily carboxylate-containing amino acids in the helix 1-strand 1 region (37,47). Studies with other frataxin orthologs gave similar results although with some variation in iron binding stoichiometry and affinity (human bound 6 atoms with Kₐ 12-55 µM (44), E. coli bound 2 atoms with Kₐ 4 µM (48), drosophila bound 1 atom with Kₐ 6 µM (49)).

Mutagenesis studies performed with the objective of correlating in vitro iron binding and in vivo function have been problematic in part because of the redundant nature of the iron binding site(s) distributed over a surface and mediated by multiple iron binding amino acids. Alterations of single acidic residues showed minimal effects. However, frataxins with multiple acidic residues changed to alanine were compromised in terms of iron binding and in terms of supporting Fe-S cluster formation (Fig. 3B). For example, an altered frataxin with residues D86/E90/E93/D101/E103 all changed to alanine was associated with decreased iron binding by tryptophan fluorescence titration and decreased Fe-S cluster enzyme activities (50). Similarly, D86/E89/D101/E103 changed to alanine was associated with decreased aconitase, activity, iron accumulation in mitochondria, and abrogation of iron-dependent Isu interaction in a mitochondrial lysate (51) (Fig. 3B). Some frataxins oligomerize when exposed to iron. Exposure of yeast or E. coli frataxins to 20-fold molar excess of elemental iron in the absence of competing cations induced oligomerization, but this did not occur with the human protein (52). Mutation of three carboxylates D86, E90, and E93 to alanine completely abrogated iron dependent oligomerization of the yeast protein (Fig. 3B). This triple mutant, when tested in vivo, showed no deleterious effects on Fe-S cluster protein activities and iron homeostasis, even when expressed at low levels (53), and thus frataxin oligomerization is probably not required for Fe-S cluster synthesis.

How frataxin might work: In summary, frataxin interacts with the Fe-S cluster assembly scaffold protein Isu using frataxin’s beta sheet surface. It also interacts with iron via surface carboxylates of the helix 1-beta 1 area. Furthermore the frataxin-Isu interaction is iron dependent. Various in vitro assays have demonstrated these interactions, and in vivo experiments with mutant alleles have shown the importance of these interactions for Fe-S cluster formation. Biochemical data show that frataxin facilitates iron use for Fe-S cluster formation on Isu (44,49). Frataxin might work as a metallochaperone, similar to copper metallochaperones. Copper chaperones function by high affinity interactions with copper and with recipient proteins. Copper is bound to the chaperone, then liganded between chaperone and target protein, and finally handed off to the target protein. The copper thus is never free in solution and is targeted to its correct destination in the cell by the protein-protein interactions between chaperone and target (54). Frataxin might work in a similar fashion by transiently binding iron in mitochondria, interacting with Isu in an iron dependent fashion, transferring protein bound iron to Isu, and finally disengaging from Isu after metal delivery. However, the affinity of frataxin for iron is relatively low and coordination incomplete, with the carboxylate binding sites being complete by water molecules. The driving force and mechanism of metal transfer to recipients also might be different than for the copper chaperones. A prediction of this model is that the iron liganding sites should overlap or be contiguous with the Isu binding sites. This may be the case especially for the iron binding site on the strand 1 sheet consisting of the conserved D101/E103
residues (Fig. 3A and Fig. 3B). Other functions in mitochondrial iron trafficking are also possible. In line with the iron chaperone idea, frataxin might bind iron and bind to other proteins, thereby targeting the metal for delivery to aconitase (55), ferrochelatase (14,37) and succinate dehydrogenase (56). Iron specific activities of frataxin have also been suggested, and some bypass of frataxin mutant phenotypes including Fe-S cluster deficiencies has been observed as a result of forced expression of mitochondrial ferritin (57).

Alternatively, the function of frataxin in Fe-S cluster biogenesis might be primarily a regulatory one. The iron dependent frataxin-Isu interaction might signal formation of a protein complex for creation of the Fe-S intermediate on Isu. Of note, pull down experiments from mitochondria with yeast frataxin as bait yielded not only Isu, but also Nfs1 (43) and Isd11 (58), the functional cysteine desulfurase complex responsible for providing sulfide for iron-sulfur. Perhaps the data are pointing to the existence of a multi-subunit complex consisting of frataxin, Isd11-Nfs1, and Isu. Such a complex would be well situated to mediate formation of the Isu bound Fe-S cluster intermediate. Iron and sulfide are toxic intermediates, and their insertion into Fe-S clusters must be controlled to avoid toxicity. Frataxin might regulate the flux of these key intermediates, permitting delivery of enough for physiologic Fe-S cluster formation and not so much as to create iron and/or sulfide toxicity. The source of iron and its biological form are not well understood. The sulfide generated by the action of Nfs1/Isd11 on cysteine may be provided to Isu as persulfide by direct protein interaction as observed for some bacterial components. A role for frataxin in regulating sulfide transfer has been proposed in bacteria (59,60). For eukaryotes, regulation of sulfide delivery for FeS cluster formation and not so much as to create iron and/or sulfide toxicity.

Is Friedreich’s ataxia a disease of Fe-S cluster assembly? The molecular basis of Friedreich’s ataxia is deficiency of frataxin protein. The question then arises of how frataxin deficiency causes the disease? As far as is known, neither the cellular phenotype nor the human disease phenotype can be explained by deficiency of a single Fe-S cluster protein. Perhaps it is the combined effects from decreased activities of many Fe-S cluster proteins that is pathogenic. Also the degree of frataxin deficiency may be important for producing the unique cellular disease phenotypes. Complete lack of frataxin is lethal for growing mammalian cells, and somewhere between 20-30% is needed for normal growth. In cells with decreased levels of frataxin, some Fe-S cluster proteins are deficient, iron accumulates in mitochondria, and oxidant sensitivity is observed as in the human disease (16). The causal links among these effects are not well defined, and an Fe-S cluster protein mediating the mitochondrial iron accumulation or oxidant sensitivity has not been identified. Conversely, several lines of evidence indicate that mitochondrial iron accumulation (61,62) and oxidative stress (63) worsen the cellular phenotypes, perhaps mediated by detrimental effects on Fe-S clusters or perhaps mediated independently. Interventions that limit cellular iron have improved growth of yeast mutants in some cases and have had some efficacy in treatment of the human disease (64). However opposite effects have also been observed (i.e. worsening of phenotypes associated with iron starvation) (65,66). One final issue merits mention, that is the tissue specificity of the disease. Iron sulfur cluster biogenesis is required for every cell in every tissue, as many essential processes require Fe-S cluster proteins. Frataxin is ubiquitously expressed in all cell types. Therefore it is unclear why some tissues are compromised by frataxin deficiency and some are spared. The target tissue distribution for the disease includes dorsal root ganglia, cerebellum and heart muscle and does not involve skeletal muscle or blood for example. By contrast an inherited disease ascribed to Isu deficiency, also characterized by deficient Fe-S cluster proteins, affects primarily skeletal muscle and spares nervous tissues (67,68). Thus Isu deficiency and frataxin deficiency in humans bear some resemblance but the tissue specificities are distinct with the differences still unexplained.
| References                                                                 | Details |
|---------------------------------------------------------------------------|---------|
| 1. Pandolfo, M. (2009)                                                   | J Neurol 256 Suppl 1, 3-8 |
| 2. Campuzano, V., Montermini, L., Molto, M. D., Pianese, L., Cossee, M.,  |         |
| Cavalcanti, F., Monros, E., Rodius, F., Duclos, F., Monticelli, A., Zara, |         |
| F., Canizares, J., Koutnikova, H., Bidichandani, S. I., Gellera, C.,     |         |
| Brice, A., Trouillas, P., De Michele, G., Filla, A., De Frutos, R.,      |         |
| Palau, F., Patel, P. I., Di Donato, S., Mandel, J. L., Cocozza, S.,      |         |
| Koenig, M., and Pandolfo, M. (1996)                                       | Science 271, 1423-1427 |
| 3. Wilson, R. B., and Roof, D. M. (1997)                                  | Nat Genet 16, 352-357 |
| 4. Campuzano, V., Montermini, L., Lutz, Y., Cova, L., Hindelang, C.,    |         |
| Jiralerspong, S., Trottier, Y., Kish, S. J., Faucheux, B., Trouillas, P.,|         |
| Authier, F. J., Durr, A., Mandel, J. L., Vescovi, A., Pandolfo, M., and   |         |
| Koenig, M. (1997)                                                        | Hum Mol Genet 6, 1771-1780 |
| 5. Gordon, D. M., Shi, Q., Dancis, A., and Pain, D. (1999)                | Hum Mol Genet 8, 2255-2262 |
| 6. Branda, S. S., Cavadini, P., Adamiec, J., Kalousek, F., Taroni, F.,   | J Biol Chem 274, 22763-22769 |
| and Isaya, G. (1999)                                                     |         |
| 7. Condo, I., Malisan, F., Guccini, I., Serio, D., Rufini, A., and Testi,|         |
| R. Hum Mol Genet                                                         |         |
| 8. Rotig, A., de Lonlay, P., Chretien, D., Foury, F., Koenig, M., Sidi, |         |
| D., Munnich, A., and Rustin, P. (1997) Nat Genet 17, 215-217             |         |
| 9. Johnson, D. C., Dean, D. R., Smith, A. D., and Johnson, M. K. (2005)  | Annu Rev Biochem 74, 247-281 |
| 10. Gibson, T. J., Koonin, E. V., Musco, G., Pastore, A., and Bork, P.   | Trends Neurosci 19, 465-468 |
| (1996)                                                                   |         |
| 11. Dolezal, P., Dancis, A., Lesuisse, E., Sutak, R., Hrdy, I., Embley,  | Eukaryot Cell 6, 1431-1438 |
| T. M., and Tachezy, J. (2007)                                             |         |
| 12. Goldberg, A. V., Molik, S., Tsaousis, A. D., Neumann, K., Kuhnke, G.| Nature 452, 624-628 |
| , Delbac, F., Vivares, C. P., Hirt, R. P., Lill, R., and Embley, T. M.   |         |
| (2008)                                                                   |         |
| 13. Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, |         |
| S., Montermini, L., Pandolfo, M., and Kaplan, J. (1997) Science 276,     |         |
| 1709-1712                                                                |         |
| 14. Lesuisse, E., Santos, R., Matzanke, B. F., Knight, S. A., Camadro, J.| Hum Mol Genet 12, 879-889 |
| M. J., and Dancis, A. (2003)                                              |         |
| 15. Chen, O. S., Hemenway, S., and Kaplan, J. (2002) Proc Natl Acad Sci U|         |
| S A 99, 12321-12326                                                       |         |
| 16. Calmels, N., Schnucker, S., Wattenhofer-Donze, M., Martelli, A.,     |         |
| Vauclamps, N., Reutenauer, L., Messaddeq, N., Bouton, C., Koenig, M.,    |         |
| and Puccio, H. (2009) PLoS One 4, e6379                                   |         |
| 17. Rouault, T. A., and Tong, W. H. (2005) Nat Rev Mol Cell Biol 6,      |         |
| 345-351                                                                  |         |
| 18. Lill, R., Dutkiewicz, R., Elsasser, H. P., Hausmann, A., Netz, D. J.,|         |
| Pierik, A. J., Stehling, O., Urzica, E., and Muhlenhoff, U. (2006)       |         |
| Biochim Biophys Acta 1763, 652-667                                         |         |
| 19. Frazzon, J., Fick, J. R., and Dean, D. R. (2002) Biochem Soc Trans 3 |         |
| 600-685                                                                  |         |
| 20. Wiedemann, N., Urzica, E., Guiard, B., Muller, H., Lohaus, C., Meyer |         |
| H. E., Ryan, M. T., Meisinger, C., Muhlenhoff, U., Lill, R., and Pfanner,| Embo J 25, 184-195 |
| N. (2006) Embo J 25, 174-183                                              |         |
| 21. Adam, A. C., Bornhovd, C., Prokisch, H., Neupert, W., and Hell, K.   |         |
| (2006) Embo J 25, 174-183                                                 |         |
| 22. Muhlenhoff, U., Stadler, J. A., Richhardt, N., Seubert, A., Eickhorst,|         |
| T., Schweyen, R. J., Lill, R., and Wiesenberger, G. (2003) J Biol Chem 278|         |
| 40612-40620                                                              |         |
23. Zhang, Y., Lyver, E. R., Knight, S. A., Pain, D., Lesuisse, E., and Dancis, A. (2006) *J Biol Chem* **281**, 22493-22502
24. Lange, H., Kaut, A., Kispal, G., and Lill, R. (2000) *Proc Natl Acad Sci U S A* **97**, 1050-1055
25. Bandyopadhyay, S., Chandramouli, K., and Johnson, M. K. (2008) *Biochem Soc Trans* **36**, 1112-1119
26. Neupert, W., and Herrmann, J. M. (2007) *Annu Rev Biochem* **76**, 723-749
27. Dutkiewicz, R., Schilke, B., Cheng, S., Knieszner, H., Craig, E. A., and Marszalek, J. (2004) *J Biol Chem* **279**, 29167-29174
28. Schilke, B., Williams, B., Knieszner, H., Puksztza, S., D'Silva, P., Craig, E. A., and Marszalek, J. (2006) *Curr Biol* **16**, 1660-1665
29. Dutkiewicz, R., Schilke, B., Knieszner, H., Walter, W., Craig, E. A., and Marszalek, J. (2003) *J Biol Chem* **278**, 29719-29727
30. Rodriguez-Manzaneque, M. T., Tamarit, J., Belli, G., Ros, J., and Herrero, E. (2002) *Mol Biol Cell* **13**, 1109-1121
31. Rouault, T. A., and Tong, W. H. (2008) *Trends Genet* **24**, 398-407
32. Lill, R., and Muhlenhoff, U. (2008) *Annu Rev Biochem* **77**, 669-700
33. Pierik, A. J., Netz, D. J., and Lill, R. (2009) *Nat Protoc* **4**, 753-766
34. Muhlenhoff, U., Gerber, J., Richhardt, N., and Lill, R. (2003) *Embo J* **22**, 4815-4825
35. Naranuntarat, A., Jensen, L. T., Pazicni, S., Penner-Hahn, J. E., and Culotta, V. C. (2009) *J Biol Chem* **284**, 22633-22640
36. Yang, M., Cobine, P. A., Molik, S., Naranuntarat, A., Lill, R., Winge, D. R., and Culotta, V. C. (2006) *Embo J* **25**, 1775-1783
37. He, Y., Alam, S. L., Proteasa, S. V., Zhang, Y., Lesuisse, E., Dancis, A., and Stemmler, T. L. (2004) *Biochemistry* **43**, 16251-16262
38. Dhe-Paganon, S., Shigeta, R., Chi, Y. I., Ristow, M., and Shoelson, S. E. (2000) *J Biol Chem* **275**, 30753-30756
39. Nair, M., Adinolfi, S., Pastore, C., Kelly, G., Temussi, P., and Pastore, A. (2004) *Structure* **12**, 2037-2048
40. Bencze, K. Z., Kondapalli, K. C., Cook, J. D., McMahon, S., Millan-Pacheco, C., Pastor, N., and Stemmler, T. L. (2006) *Crit Rev Biochem Mol Biol* **41**, 269-291
41. Adinolfi, S., Nair, M., Politou, A., Bayer, E., Martin, S., Temussi, P., and Pastore, A. (2004) *Biochemistry* **43**, 6511-6518
42. Ramazzotti, A., Vanmansart, V., and Fourny, F. (2004) *FEBS Lett* **557**, 215-220
43. Gerber, J., Muhlenhoff, U., and Lill, R. (2003) *EMBO Rep* **4**, 906-911
44. Yoon, T., and Cowan, J. A. (2003) *J Am Chem Soc* **125**, 6078-6084
45. Leidgens, S., De Smet, S., and Fourny, F. *Hum Mol Genet* **19**, 276-286
46. Pastore, C., Franzese, M., Sica, F., Temussi, P., and Pastore, A. (2007) *Febs J* **274**, 4199-4210
47. Cook, J. D., Bencze, K. Z., Jankovic, A. D., Crater, A. K., Busch, C. N., Bradley, P. B., Stemmler, A. J., Spaller, M. R., and Stemmler, T. L. (2006) *Biochemistry* **45**, 7767-7777
48. Bou-Abdallah, F., Adinolfi, S., Pastore, A., Laue, T. M., and Dennis Chasteen, N. (2004) *J Mol Biol* **341**, 605-615
49. Kondapalli, K. C., Kok, N. M., Dancis, A., and Stemmler, T. L. (2008) *Biochemistry* **47**, 6917-6927
50. Correia, A. R., Wang, T., Craig, E. A., and Gomes, C. M. *Biochem J* **426**, 197-203

8
51. Foury, F., Pastore, A., and Trincal, M. (2007) *EMBO Rep* **8**, 194-199
52. Adinolfi, S., Trifuoggi, M., Politou, A. S., Martin, S., and Pastore, A. (2002) *Hum Mol Genet* **11**, 1865-1877
53. Aloria, K., Schilke, B., Andrew, A., and Craig, E. A. (2004) *EMBO Rep* **5**, 194-199
54. O'Halloran, T. V., and Culotta, V. C. (2000) *J Biol Chem* **275**, 25057-25060
55. Bulteau, A. L., O'Neill, H. A., Kennedy, M. C., Ikeda-Saito, M., Isaya, G., and Szweda, L. I. (2004) *Science* **305**, 242-245
56. Gonzalez-Cabo, P., Vazquez-Manrique, R. P., Garcia-Gimeno, M. A., Sanz, P., and Palau, F. (2005) *Hum Mol Genet* **14**, 2279-2288
57. Layer, G., Ollagnier-de Choudens, S., Sanakis, Y., and Fontecave, M. (2006) *J Biol Chem* **281**, 16256-16263
58. Adinolfi, S., Iannuzzi, C., Prischi, F., Pastore, C., Iametti, S., Martin, S. R., Bonomi, F., and Pastore, A. (2009) *Nat Struct Mol Biol* **16**, 390-396
59. Foury, F. (1999) *FEBS Lett* **456**, 281-284
60. Radisky, D. C., Babcock, M. C., and Kaplan, J. (1999) *J Biol Chem* **274**, 4497-4499
61. Bulteau, A. L., Dancis, A., Gareil, M., Montagne, J. J., Camadro, J. M., and Lesuisse, E. (2007) *Free Radic Biol Med* **42**, 1561-1570
62. Kakhlon, O., Manning, H., Breuer, W., Melamed-Book, N., Lu, C., Cortopassi, G., Munich, A., and Cabantchik, Z. I. (2008) *Biol Blood* **112**, 5219-5227
63. Goncalves, S., Paupe, V., Dassa, E. P., and Rustin, P. (2008) *BMC Neurol* **8**, 20
64. Li, K., Besse, E. K., Ha, D., Kovtunovych, G., and Rouault, T. A. (2008) *Hum Mol Genet* **17**, 2265-2273
65. Mochel, F., Knight, M. A., Tong, W. H., Hernandez, D., Ayyad, K., Taivassalo, T., Andersen, P. M., Singleton, A., Rouault, T. A., Fischbeck, K. H., and Haller, R. G. (2008) *Am J Hum Genet* **82**, 652-660
66. Olsson, A., Lind, L., Thornell, L. E., and Holmberg, M. (2008) *Hum Mol Genet* **17**, 1666-1672
67. Gakh, O., Park, S., Liu, G., Macomber, L., Imlay, J. A., Ferreira, G. C., and Isaya, G. (2006) *Hum Mol Genet* **15**, 467-479

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FIGURE LEGENDS

Fig. 1: Scheme showing role of yeast frataxin (Yfh1) in mitochondrial Fe-S cluster assembly. The mitochondrion is shown as an oval bounded by a double membrane. Yfh1 is shown as a helix-sheet sandwich (green and tan). A. Mitochondrial carriers Mrs3 and Mrs4 (box) play a role in transfer of iron in some form (Fe-X) across the mitochondrial inner membrane. Cysteine (Cys-SH) enters mitochondria and is acted on by the enzyme Nfs1 and its accessory protein Isd11 to provide sulfur for Fe-S clusters. Electrons or reducing equivalents are provided by the reductase Arh1 in the membrane and the associated
ferredoxin Yah1. Yfh1 physically interacts with Nfs1, Isd11, and Isu. B. The Fe-S cluster intermediate (red diamond) is assembled on the scaffold Isu, which interacts with Nfs1, Isd11 and Yfh1. Another complex is formed by Isu and Ssq1, Jac1, Mge1 with the binding site being provided by the PVK tripeptide on Isu1. Grx5 acts at this step. C. The precursor proteins (squiggly black lines) are nuclear encoded, translated on cytoplasmic ribosomes, and targeted to mitochondria and imported in unfolded state. After proteolytic processing they are folded and acquire the Fe-S cluster cofactor (red diamond) by action of the chaperones and glutaredoxin.

Fig. 2: Imported frataxin (Yfh1) restores Fe-S cluster assembly in isolated yeast mitochondria. A. Wild-type mitochondria (left panel) synthesize Fe-S clusters on endogenous apoaconitase and imported apoYah1. Mutant Δyfh1 mitochondria (middle panel) fail to synthesize clusters. Following Yfh1 precursor import, mutant Δyfh1 mitochondria (right panel) now synthesize clusters. B. Mitochondria were isolated from wild-type or Δyfh1 strains. Full length yeast frataxin (produced and radiolabeled on methionine and cysteine in the polypeptide) was imported (18 degrees, 10 min, lanes 3, 4, 7, 8) and mitochondria were recovered by centrifugation. In the second stage, 35S-cysteine and ferredoxin precursor Yah1 were imported 10 min, 30 degrees). The mitochondria were recovered and the matrix fraction analyzed by a native gel and autoradiography (lanes 1-8). The imported Yfh1 labeled on the polypeptide is visible on the gel. The imported ferredoxin labeled on the sulfur of the Fe-S is visible in Δyfh1 mitochondria only following Yfh1 import (lanes 5, 6). Similarly, endogenous aconitase labeled on its Fe-S cluster is visible in Δyfh1 only following Yfh1 import (lanes 5, 6) (23).

Fig 3: A. Solution structure of monomeric apo-Yfh1 (PDB ID#2GA5) with residues involved in Isu binding identified in blue. Yfh1 amino acid substitutions with effects on Isu interactions are as follows: N122A/K123T/Q124A, diminished Isu interaction, low aconitase and succinate dehydrogenase, iron accumulation (58); single amino acid changes Q129A, W131A, R141A, diminished Isu interaction and low aconitase (45); D101A/E103A, no interaction with Isu and decreased aconitase (50).

B. Solution structure of monomeric apo-Yfh1 with residues involved in Fe binding identified in red (37). Published data on frataxins with substitutions of acidic amino acids are as follows: D86A/E90A/E93A/D101A/E103A, decreased iron binding affinity, low aconitase, slow growth (50); D86A/89A/E101A/E103A, decreased iron dependent Isu interaction, low aconitase, iron accumulation (51); D86A/E90A/E93A, no oligomerization in response to iron exposure in vitro, normal Isu interaction, no Fe-S deficit, no iron accumulation (53); D79A/D82A, low ferroxidase, oxidant sensitivity, normal aconitase, no iron accumulation (69); E93A/D97A/E103A, oxidant sensitivity, normal aconitase, no iron accumulation (69).
Fig. 1
Wild-type
Endogenous
Yfh1 present

Δyfh1
Endogenous
Yfh1 absent

Δyfh1
Newly imported
Yfh1 present

Fig. 2A
Fe (40 µM) -- + - + +
Yfh1
wild-type
Δyfh1
Aco1
[4Fe-4S*]
[2Fe-2S*] (imported)
Yah1
Fig. 2B
