8-27-2010

Frataxin and Mitochondrial FeS Cluster Biogenesis

Timothy L. Stemmler
Wayne State University, tstemmle@med.wayne.edu

Emmanuel Lesuisse
Institut Jacques Monod

Debumar Pain
University of Medicine and Dentistry, New Jersey

Andrew Dancis
University of Pennsylvania

Frataxin and mitochondrial Fe-S cluster biogenesis

Timothy L. Stemmler[1], Emmanuel Lesuisse[2], Debumar Pain[3], Andrew Dancis[4]

1. Department of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, Detroit, Michigan 48201 2. Laboratoire Mitochondrie, Metaux et Stress Oxydant, Institut Jacques Monod, CNRS-Universite Paris Diderot, France 75205 3. Department of Pharmacology and Physiology, UMDNJ, New Jersey Medical School, Newark, New Jersey 07101 4. Department of Medicine, Division of Hematology-Oncology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract

Friedreich's ataxia is an inherited neurodegenerative disease caused by frataxin deficiency. 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 sulfur, thus placing it at the center of mitochondrial Fe-S cluster biosynthesis.

Introduction

Friedreich's ataxia is an inherited disease 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 mellitis 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). The protein is found primarily in mitochondria (3). 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, evolutionary 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 archaebacteria) (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 clusters assembly: *nif*, *suf* and *isc*. The *nif* operon is specialized for high volume biosynthesis needed to support nitrogenase synthesis during diazotropic 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 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 homolog 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 unicellar parasite, a modified and stripped-down organelle called the hydrogenosome performs Fe-S cluster assembly and contains a frataxin homolog 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 *Δyfh1* 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 the accumulated iron was in the form of ferric phosphate micelles (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, indicating a remarkable degree of conservation of the iron homeostatic

machineries (17). The presumption is that there exists a conserved regulatory Fe-S cluster protein or proteins mediating these effects. At this time however, although an unusual Fe-S cluster (liganded by glutathione and glutaredoxins) has been found to mediate control of cellular iron uptake in yeast (18,19), the controlling protein(s) for mitochondrial iron uptake or accumulation has not been identified for any species, and so the latter piece is still missing from our understanding.

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, homologs of the other Isc Fe-S cluster assembly components have been found in mitochondria (20). 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 (21). An Fe-S cluster intermediate is first 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 sulfur 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 (22,23). The sulfur 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 (24). Frataxin may play a role here and has been shown to genetically interact with the mitochondrial carrier proteins. This is shown by the exacerbated slow growth and mitochondrial iron starvation in the combined *Δmrs3Δmrs4Δyfh1* mutant (25). 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 (26). The Isu protein appears to be conserved in the context of primary sequence and function, and structural information exists for a few bacterial homologs. Key features are three critical cysteines and an aspartate that bind the cluster intermediate probably on each subunit of the Isu dimer (27).

Following formation of the Isu scaffold intermediate, several additional components are required for transfer of the intermediate to recipient apoproteins. 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) (28,29). This interaction is modulated by the ATP hydrolysis cycle in

conjunction with accessory proteins Jac1 and Mge1 (30). A monothiol glutaredoxin is also implicated here (31). The proteins destined to become Fe-S cluster proteins in mitochondria are translated on cytoplasmic ribosomes as precursor proteins with mitochondrial targeting sequences. Upon import into mitochondria, the unfolded precursors are subjected to various processing steps that remove the targeting sequences (32), and the mature apoproteins thus generated become substrates for transfer of Fe-S clusters from Isu (Fig. 1).

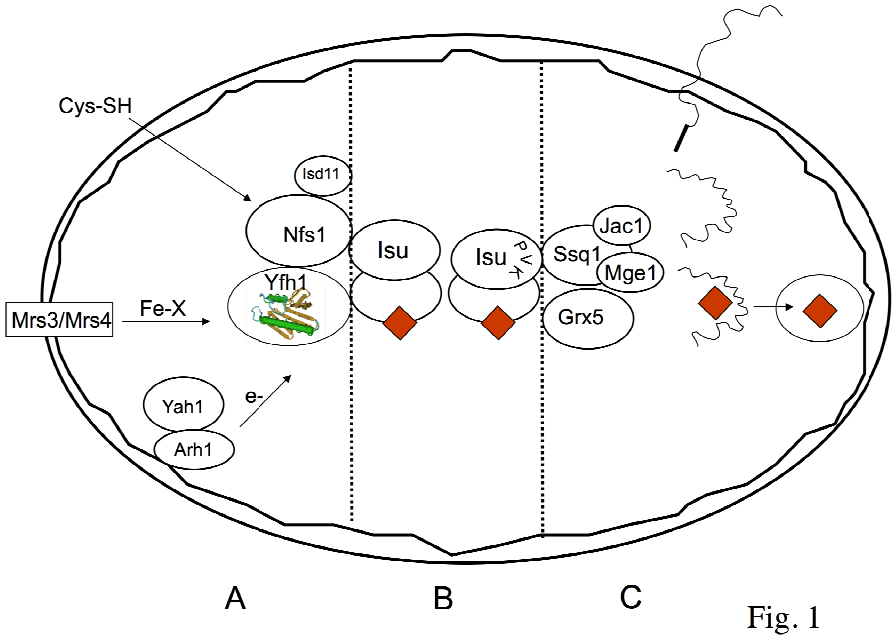

Fig. 1

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.

Epistasis and placement of Yfh1 within the pathway of Fe-S cluster assembly

The central component in Fe-S cluster assembly in mitochondria is the Isu scaffold. In order to begin to address the function of frataxin, it was important to determine if frataxin acts before Isu, in formation of the Fe-S cluster intermediates, or after Isu, in the transfer of the Isu intermediates to apoproteins. 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 mismetallation. Specifically, these mutants, such as *ssq1* and *grx5*, belonged to Isu downstream events in transfer of Fe-S cluster intermediates to apoproteins (35). By contrast, although frataxin mutants *(Δyfh1)* dramatically accumulated iron in mitochondria, the iron was not available for (mis)incorporation into MnSod (36). Similarly Isu mutants (depletion or dominant negatives) were also not associated with MnSod inactivation (35). These data suggest 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.

Effects of frataxin in promoting Fe-S cluster assembly in mitochondria

Two experiments are presented to illustrate the role of frataxin in promoting Fe-S cluster assembly in mitochondria. In the first experiment, mitochondria were isolated from wild-type or *Δyfh1* strains. New synthesis of Fe-S clusters in the isolated organelles was examined by providing ^{35}S-cysteine as a source of sulfur. In the wild-type, radioactive sulfur 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 *Δyfh1* mutant mitochondria and the mitochondria were recovered by centrifugation. When a similar ^{35}S-cysteine labeling experiment was performed, the mutant mitochondria demonstrated restored ability to make Fe-S clusters on both aconitase 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 *Δyfh1* 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 activates or mediates the process (25).

In the second experiment, a more reductionist approach was taken with purified proteins. Isu was expressed and purified from bacteria in the apoprotein form, without any cluster. If Isu was incubated with elemental iron and sulfur, a small amount of Fe-S cluster intermediate could be detected as shown by appearance of the characteristic absorption spectrum (Fig. 3, tracing B). In a parallel incubation, Isu was added to elemental sulfur and iron loaded frataxin monomer instead of elemental iron alone. The absorbance tracing (Fig. 3, tracing A) followed over time showed tremendous facilitation in the presence of frataxin. In this experiment, Drosophila frataxin was used because of its greater stability, and a D37A mutant of yeast Isu was used to trap and stabilize the intermediate on Isu (37). A similar assay showed activity of human frataxin in facilitating Fe-S cluster formation on the human Isu scaffold protein (38). These experiments with purified components demonstrate that frataxin possesses the ability to facilitate Fe-S cluster formation on Isu.

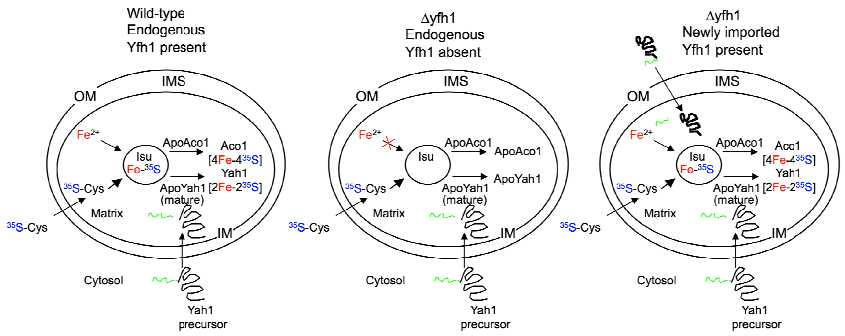

Fig. 2A

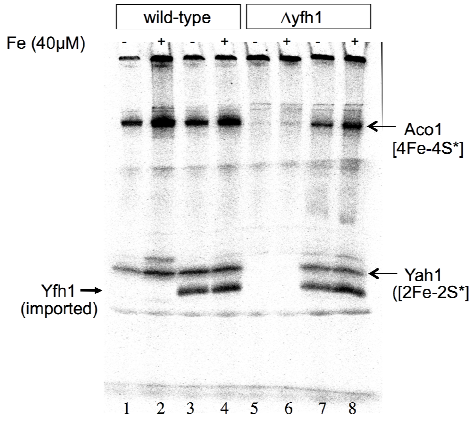

Fig. 2B

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* (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, ^{35}S-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) (25).

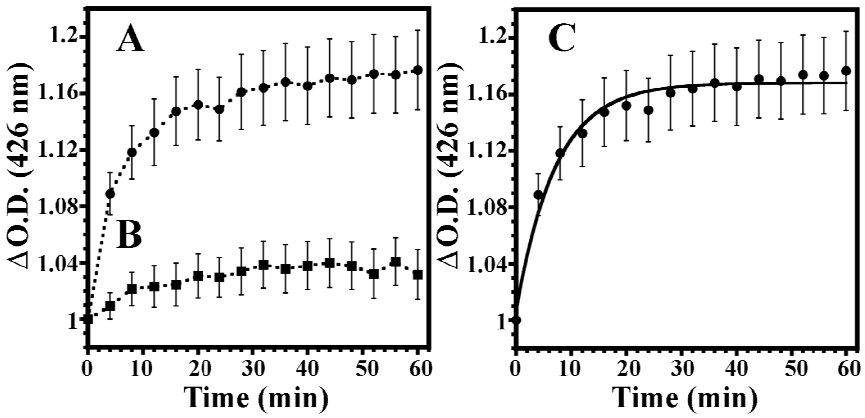

Fig. 3

Fig. 3: Frataxin facilitates the rate and efficiency of Fe-S cluster formation on Isu scaffold. Yeast Isu was expressed and purified from bacteria in the apoprotein form (D37A mutated form of Isu1). Mature drosophila frataxin Dfh was likewise expressed and purified from bacteria. The reactions contained 100 μM Isu scaffold. To this was added sulfur (2.4 mM Na_2S) and iron (100 μM ferrous ammonium sulfate) (tracing B) or iron-frataxin (100 μM of Dfh-iron monomer) (tracing A). The formation of cluster on Isu was followed by absorbance at 426 nm. Time dependence data from tracing A was fit with a rate equation for a first order process (panel C) (37).

Frataxin structures

Structures have been obtained for yeast (39), human (40) and bacterial (41) 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 sheets; a sixth and possibly seventh strand intersect the planes depending on the species (42). 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 (43). A negatively charged surface is created by clustering of contiguous acidic residues contributed by portions of helix 1 and beta sheet 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, with more hydrophobic towards

the center and more hydrophilic towards the periphery. This region contains a number of perfectly conserved residues that appears to be well suited to mediate interaction with a protein partner.

Isu interaction

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) (44). Physical interactions between frataxin and Isu were subsequently demonstrated in pull-down experiments from mitochondrial lysate. Interestingly the pull-down of Yfh1 by Isu, or reciprocally of Isu by Yfh1, was dependent on iron addition to the buffer (50 μM ferrous ammonium sulfate), and then inhibited by the presence of the metal chelator EDTA (45). The clearest demonstration of the importance of frataxin-Isu interaction in Fe-S cluster assembly was provided by careful analyses of mutant alleles of frataxin that failed to interact. A frataxin mutant (N122A/K123T/Q124A) was constructed by alteration of conserved surface exposed residues of the strand 3 in an area predicted to mediate protein interactions (46)(Table 1, column 1). These mutations did not interfere with protein expression or stability, and *in vitro* studies showed no effect on iron binding. However, interaction with Isu was severely impaired as assessed by pull-down from mitochondrial lysates onto immobilized Yfh1-His_6. In these experiments, the affinity of frataxin binding to Isu was markedly decreased from about 1 μM for the wild-type to a number greater than 12 μM for the mutant. Experiments with purified Yfh1 and Isu1, as distinguished from lysate containing these and other proteins, also showed the interaction and the reduced affinity of the mutant, confirming that the mutational effect was direct. Importantly, *in vivo* experiments performed with the same *yfh1* mutant demonstrated reduced Fe-S cluster protein activities and increased iron accumulation in mitochondria. The N122K mutant similarly was associated with decreased Isu interaction and decreased Fe-S cluster enzyme activities. The N122K mutation corresponds to N146K in the human ortholog and has been associated with Friedreich's ataxia. The wild-type and mutant proteins placed under control of a regulated promoter and expressed over a wide range of concentrations confirmed mutant phenotypes occurred over a wide range of expression levels (46). Other mutations were constructed that altered amino acids in exposed areas of the beta sheet (Q129A in strand 4, W131A in strand 4 and R141A in strand 5). These mutations were examined *in vitro* and *in vivo*, and they too showed decreased frataxin-Isu interaction and deficiency of Fe-S cluster proteins (47) (Table 1, column 2, Fig. 4). The studies with mutant alleles were especially informative because they avoided many of the secondary phenotypes

associated with the *yfh1* deletion. The conclusion from these studies is the interaction between frataxin and Isu is required for Fe-S cluster assembly in the mitochondria.

Table 1: Amino acids with effects on Yfh1-Isu interaction and Fe-S cluster proteins

structure	amino acid no.	Ref 1	Ref 2	Ref 3
beta 1	D101			x
beta 1	E103			x
beta 3	N122	x		
beta 3	K123	x		
beta 3	Q124	x		
beta 4	Q129		#1	
beta 4	W131		#2	
beta 5	R141		#3	

Ref 1 (46): Triple Yfh1 mutant N122A/K123T/Q124A, diminished Isu interaction, low aconitase and succinate dehydrogenase, iron accumulation

Ref 2 (47): Single mutants #1 Q129A #2 W131A #3 R141A, diminished Isu interaction, low aconitase

Ref 3 (55): Double mutant D101A/E103A, no interaction with Isu, aconitase decreased

Iron binding

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 (48). Analysis of iron interaction with yeast frataxin monomer showed two ferrous atoms binding with affinity of 2.5 to 5 μM as high spin iron. Chemical shifts and line broadening induced by exposure of ^{15}N-labeled yeast frataxin to iron (ferrous iron under anaerobic conditions) further defined iron binding residues as primarily carboxylate-containing amino acids in the helix 1-strand 1 region (39,49). Studies with other frataxin orthologs gave similar results although with some variation in iron binding stoichiometry and affinity (human bound 6 atoms with K_d 12-55 μM (38), *E. coli* bound 2 atoms with K_d 4 μM (50), Drosophila bound 1 atom with K_d 6 μM (37)). 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. In addition, various effects of iron binding to frataxin have been observed, some of which may be physiologically important and some of which may not be relevant.

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 (51). Mutation of three carboxylates D86, E90, and E93 to alanine completely abrogated iron dependent oligomerization of the yeast protein (Table 2, column 1, Fig. 4). 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 (52). Thus frataxin oligomerization is probably not required for Fe-S cluster synthesis. Other mutations were tested in both *in vitro* and *in vivo* assays within yeast. A D79A/D82A mutation abrogated ferroxidase activity. The D79A/D82A or E93A/D97A/E103A mutants showed decreased frataxin-iron interactions *in vitro*, and both of these mutant alleles were associated with increased oxidant sensitivity and decreased life span *in vivo*. However, these mutations had no effect on aconitase activity and no effect on mitochondrial iron accumulation (53) (Table 2, column 2), and thus they are probably not important for Fe-S cluster biogenesis.

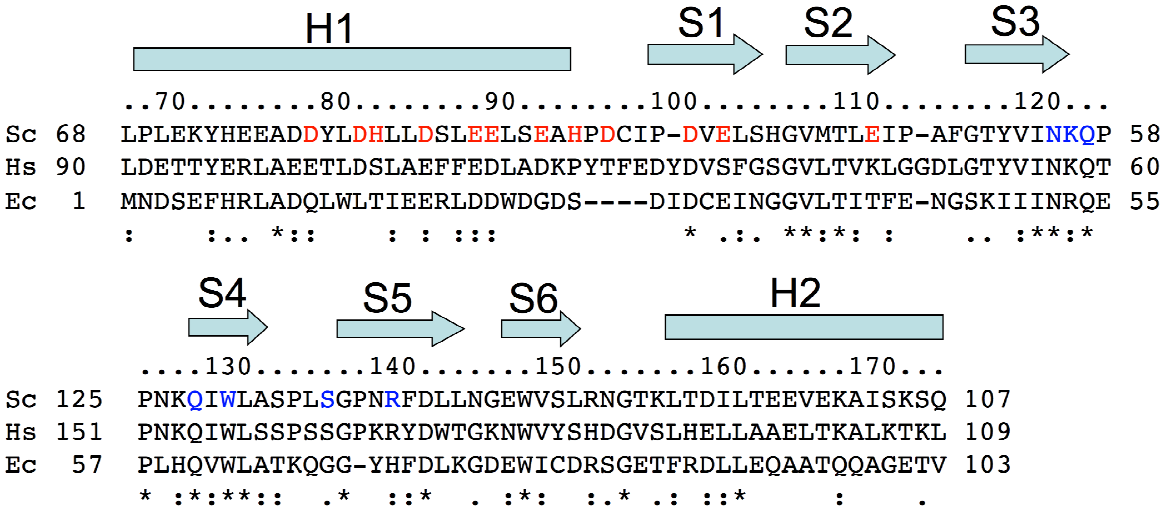

Fig. 4A

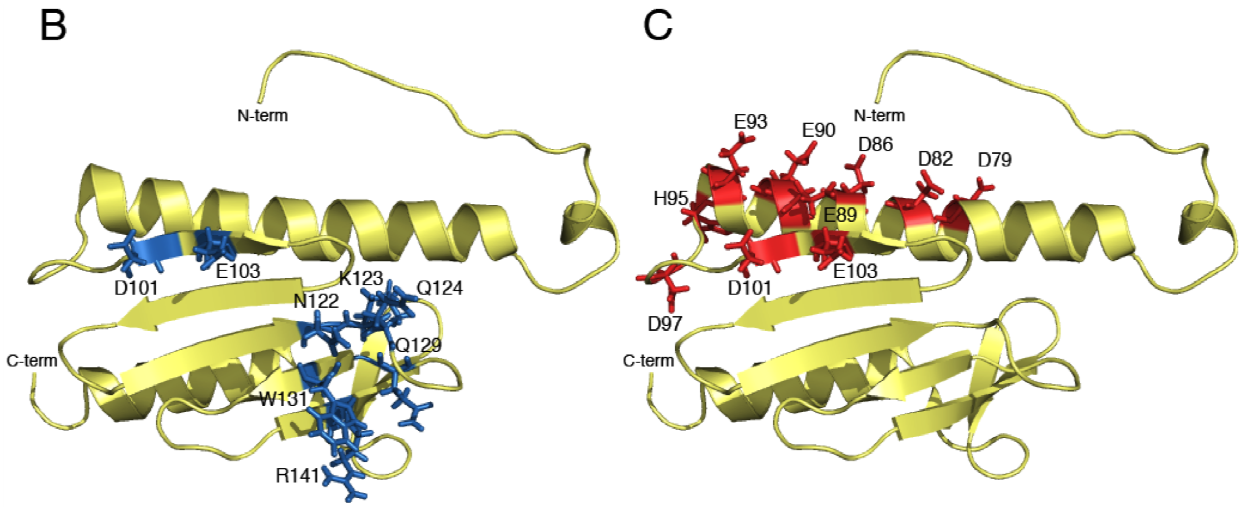

Fig 4: Frataxin ortholog sequence alignment and Yfh1 structure showing locations of mutations with effects on iron binding and/or Isu binding. A. Sequence alignment of yeast, human and *E. coli* frataxin using ClustalW with structural features on top. The colored amino acids have been studied by mutagenesis and are implicated in iron interaction (red), or Isu interaction (blue). B. Solution structure of monomeric apo-Yfh1 (PDB ID# 2GA5) with residues involved in Isu binding identified in blue. C. Solution structure of monomeric apo-Yfh1 with residues involved in Fe binding identified in red (39).

By contrast, mutation of four acidic residues D86/E89/D101/E103 to alanine was more deleterious for Fe-S clusters: aconitase was decreased to 30% of wild-type levels and mitochondrial iron accumulation occurred. Iron binding to this mutant frataxin was not measured directly, although iron dependent Isu interaction in mitochondrial lysate was markedly decreased (54) (Table 2, column 3). Another set of experiments illustrates the problem of redundancy in the acidic iron binding sites. A yeast frataxin mutant D86/E90/E93 gave no *in vivo* phenotype (compared with wild-type), regardless of the frataxin expression level. The D101/E103 mutant displayed a modest phenotype with moderately lower aconitase. However, the combined mutant exhibited a severe phenotype with growth inhibition and 40% residual aconitase activity. Iron binding affinity of this combined mutant protein was reduced by tryptophan fluorescence titration but was not abolished

(55) (Table 2, column 4). Interestingly, the frataxin-Isu iron dependent interaction tested with purified proteins was abrogated by the D101/E103 or the combined D886/E90/E93/D101/E103 mutations (55). We determined mutational effects of amino acids that showed strong interactions with Fe^{2+} in an NMR study with ^{15}N–labeled Yfh1. Two possible iron binding sites were identified, one site consisting of D68/E93/H95 (site 1) and the other consisting of D101/E103 (site 2). Mutation of site 1 residues to alanine had no discernable effect on *in vivo* phenotypes, mutations of site 2 had a very mild effect (aconitase 90%), although iron binding stoichiometry for these mutants was decreased such that only one iron bound per molecule. Combined mutations of site 1 and site 2 showed no iron binding by ITC or NMR titrations, and the *in vivo* phenotypes were quite severe, with aconitase of 37%, slow growth, and mitochondrial iron accumulation. These data show a correlation of frataxin iron binding and Fe-S cluster assembly activity i.e. the mutated iron binding sites were correlated with severely compromised Fe-S cluster assembly activity (Table 2, column 5, manuscript in preparation).

TABLE 2: Yfh1 iron binding amino acids and their effects on Fe-S cluster assembly.

structure	amino acid no.	Ref 1	Ref 2	Ref 3	Ref 4	Ref 5
alpha 1	D79		#1			
alpha 1	D82		#1			
alpha 1	D86	x		x	#1	#1
alpha 1	E89			x		
alpha 1	E90	x			#1	
alpha 1	E93	x	#2		#1	#1
alpha 1	H95					#1
alpha 1–beta 1	D97		#2			
beta 1	D101			x	#2	#2
beta 1	E103		#2	x	#2	#2

Ref 1 (52): Triple Yfh1 mutant D86A/E90A/E93A , no oligomerization in response to iron exposure in vitro, normal Isu interaction, no Fe-S deficit, no iron accumulation

Ref 2 (53): Double Yfh1 mutant #1 D79A/D82A, low ferroxidase, oxidant sensitivity, normal aconitase, no iron accumulation. Triple Yfh1 mutant #2 E93A/D97A/E103A, oxidant sensitivity, normal aconitase, no iron accumulation .

Ref 3 (54): Quadruple mutant D86A/89A/E101A/E103A , decreased iron dependent Isu interaction, low aconitase, iron accumulation .

Ref 4 (55): Triple Yfh1 mutant #1 D86/A/E90A/E93A, decreased iron binding affinity, normal aconitase, no iron accumulation. Double mutant #2 D101A/E103, decreased iron binding affinity, mild low aconitase, no

growth defect. Combined mutant #1 and #2: D86A/E90A/E93A/D101A/E103A , decreased iron binding affinity, lower aconitase, slow growth.

Ref 5 (unpublished): Triple Yfh1 mutant #1 D86A/E90A/E93A, decreased iron binding stoichiometry, normal aconitase, no iron accumulation. Double mutant #2 D101A/E103A, decreased iron binding stoichiometry, slight decrease aconitase, no growth defect. Combined mutant #1 and #2 D86A/E90A/E93A/E101A/E103A, no iron binding in vitro, decreased aconitase, iron accumulation, slow growth.

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-strand 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. Frataxin might work as a metal chaperone similar to copper chaperones involved in copper trafficking and copper metallation of proteins. 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 (56). Frataxin might work in a similar fashion by transiently binding iron in mitochondria, interacting with Isu in an iron dependent fashion, and finally transferring protein bound iron to Isu. However, the affinity of frataxin for iron is relatively low and coordination incomplete, with the carboxylate binding sites being completed 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 consisting of the conserved D101/E103 residues. Biochemical data suggest frataxin facilitates iron use for Fe-S cluster formation on Isu (38) (Fig. 3). 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 (57), ferrochelatase (14,39) and succinate dehydrogenase (58). 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 (59).

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 (45) and Isd11 (46), the functional cysteine desulfurase complex responsible for providing sulfur 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 sulfur 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 sulfur 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 sulfur transfer has been proposed in bacteria (60,61). For eukaryotes, the interaction with Nfs1/Isd11 is not well defined and regulatory functions have not been defined for sulfur transfer. Structural details of the frataxin-Nfs1/Isd11 interaction also remain to be clarified. The details of the frataxin-Isu interaction from the Isu side have not been solved, and these interactions might be competing in some manner with Isu-chaperone interactions that mediate transfer of the cluster intermediate to apoproteins.

Is Friedreich's ataxia a disease of Fe-S cluster assembly?

If Friedreich's ataxia is caused by frataxin deficiency and frataxin mediates Fe-S cluster assembly, the question arises whether the disease is caused by Fe-S cluster deficiency. 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 produces the peculiar tissue specific effects characteristic of the disease. 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 these decreased levels of frataxin, some Fe-S cluster proteins are deficient and iron accumulates in mitochondria as in the human disease (16). In humans, effects of frataxin deficiency are likely to be buffered to different degrees in

different tissues depending on redundancy with other proteins and tissue specific needs. The recently demonstrated role of mitochondrial ferritin and its ability to mitigate the effects of frataxin deficiency might be important in this regard (59), and thus red cell precursors which have the ability to express mitochondrial ferritin in some settings perhaps would not be as affected by frataxin deficiency as other tissues e.g. large bore sensory neurons. An inherited disease ascribed to Isu deficiency has been described, and this is characterized by some degree of Fe-S cluster deficiency and iron accumulation in affected tissues (62,63). Given the close functional association of frataxin and Isu, one might expect that the human deficiencies would phenocopy each other. However in Friedreich's ataxia, a unique tissue distribution targets dorsal root ganglia, cerebellum, and heart muscle. In the Isu deficiency disease, by contrast, the features are skeletal muscle swelling and exercise intolerance; neurodegeneration is not a feature. Thus Isu deficiency and frataxin deficiency in humans bear some resemblance but the tissue specificities are distinct with the differences still unexplained.

References

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., Adamec, J., Kalousek, F., Taroni, F., and Isaya, G. (1999) *J Biol Chem* **274**, 22763-22769
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. (1996) *Trends Neurosci* **19**, 465-468
11. Dolezal, P., Dancis, A., Lesuisse, E., Sutak, R., Hrdy, I., Embley, T. M., and Tachezy, J. (2007) *Eukaryot Cell* **6**, 1431-1438

12. Goldberg, A. V., Molik, S., Tsaousis, A. D., Neumann, K., Kuhnke, G., Delbac, F., Vivares, C. P., Hirt, R. P., Lill, R., and Embley, T. M. (2008) *Nature*
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. M., and Dancis, A. (2003) *Hum Mol Genet* **12**, 879-889
15. Chen, O. S., Hemenway, S., and Kaplan, J. (2002) *Proc Natl Acad Sci U S A* **99**, 12321-12326
16. Calmels, N., Schmucker, S., Wattenhofer-Donze, M., Martelli, A., Vaucamps, 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. Li, H., Mapolelo, D. T., Dingra, N. N., Naik, S. G., Lees, N. S., Hoffman, B. M., Riggs-Gelasco, P. J., Huynh, B. H., Johnson, M. K., and Outten, C. E. (2009) *Biochemistry* **48**, 9569-9581
19. Kumanovics, A., Chen, O. S., Li, L., Bagley, D., Adkins, E. M., Lin, H., Dingra, N. N., Outten, C. E., Keller, G., Winge, D., Ward, D. M., and Kaplan, J. (2008) *J Biol Chem* **283**, 10276-10286
20. 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
21. Frazzon, J., Fick, J. R., and Dean, D. R. (2002) *Biochem Soc Trans* **30**, 680-685
22. Wiedemann, N., Urzica, E., Guiard, B., Muller, H., Lohaus, C., Meyer, H. E., Ryan, M. T., Meisinger, C., Muhlenhoff, U., Lill, R., and Pfanner, N. (2006) *Embo J* **25**, 184-195
23. Adam, A. C., Bornhovd, C., Prokisch, H., Neupert, W., and Hell, K. (2006) *Embo J* **25**, 174-183
24. 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
25. Zhang, Y., Lyver, E. R., Knight, S. A., Pain, D., Lesuisse, E., and Dancis, A. (2006) *J Biol Chem* **281**, 22493-22502
26. Lange, H., Kaut, A., Kispal, G., and Lill, R. (2000) *Proc Natl Acad Sci U S A* **97**, 1050-1055
27. Bandyopadhyay, S., Chandramouli, K., and Johnson, M. K. (2008) *Biochem Soc Trans* **36**, 1112-1119
28. Dutkiewicz, R., Schilke, B., Cheng, S., Knieszner, H., Craig, E. A., and Marszalek, J. (2004) *J Biol Chem* **279**, 29167-29174
29. Schilke, B., Williams, B., Knieszner, H., Pukszta, S., D'Silva, P., Craig, E. A., and Marszalek, J. (2006) *Curr Biol* **16**, 1660-1665
30. Dutkiewicz, R., Schilke, B., Knieszner, H., Walter, W., Craig, E. A., and Marszalek, J. (2003) *J Biol Chem* **278**, 29719-29727
31. Rodriguez-Manzaneque, M. T., Tamarit, J., Belli, G., Ros, J., and Herrero, E. (2002) *Mol Biol Cell* **13**, 1109-1121
32. Neupert, W., and Herrmann, J. M. (2007) *Annu Rev Biochem* **76**, 723-749
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. Kondapalli, K. C., Kok, N. M., Dancis, A., and Stemmler, T. L. (2008) *Biochemistry* **47**, 6917-6927
38. Yoon, T., and Cowan, J. A. (2003) *J Am Chem Soc* **125**, 6078-6084
39. He, Y., Alam, S. L., Proteasa, S. V., Zhang, Y., Lesuisse, E., Dancis, A., and Stemmler, T. L. (2004) *Biochemistry* **43**, 16254-16262
40. Dhe-Paganon, S., Shigeta, R., Chi, Y. I., Ristow, M., and Shoelson, S. E. (2000) *J Biol Chem* **275**, 30753-30756
41. Nair, M., Adinolfi, S., Pastore, C., Kelly, G., Temussi, P., and Pastore, A. (2004) *Structure* **12**, 2037-2048
42. 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
43. Adinolfi, S., Nair, M., Politou, A., Bayer, E., Martin, S., Temussi, P., and Pastore, A. (2004) *Biochemistry* **43**, 6511-6518
44. Ramazzotti, A., Vanmansart, V., and Foury, F. (2004) *FEBS Lett* **557**, 215-220
45. Gerber, J., Muhlenhoff, U., and Lill, R. (2003) *EMBO Rep* **4**, 906-911
46. Wang, T., and Craig, E. A. (2008) *J Biol Chem* **283**, 12674-12679
47. Leidgens, S., De Smet, S., and Foury, F. *Hum Mol Genet* **19**, 276-286
48. Pastore, C., Franzese, M., Sica, F., Temussi, P., and Pastore, A. (2007) *Febs J* **274**, 4199-4210
49. 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
50. Bou-Abdallah, F., Adinolfi, S., Pastore, A., Laue, T. M., and Dennis Chasteen, N. (2004) *J Mol Biol* **341**, 605-615
51. Adinolfi, S., Trifuoggi, M., Politou, A. S., Martin, S., and Pastore, A. (2002) *Hum Mol Genet* **11**, 1865-1877
52. Aloria, K., Schilke, B., Andrew, A., and Craig, E. A. (2004) *EMBO Rep* **5**, 1096-1101
53. Gakh, O., Park, S., Liu, G., Macomber, L., Imlay, J. A., Ferreira, G. C., and Isaya, G. (2006) *Hum Mol Genet* **15**, 467-479
54. Foury, F., Pastore, A., and Trincal, M. (2007) *EMBO Rep* **8**, 194-199
55. Correia, A. R., Wang, T., Craig, E. A., and Gomes, C. M. *Biochem J* **426**, 197-203
56. O'Halloran, T. V., and Culotta, V. C. (2000) *J Biol Chem* **275**, 25057-25060
57. Bulteau, A. L., O'Neill, H. A., Kennedy, M. C., Ikeda-Saito, M., Isaya, G., and Szweda, L. I. (2004) *Science* **305**, 242-245
58. Gonzalez-Cabo, P., Vazquez-Manrique, R. P., Garcia-Gimeno, M. A., Sanz, P., and Palau, F. (2005) *Hum Mol Genet* **14**, 2091-2098
59. Campanella, A., Isaya, G., O'Neill, H. A., Santambrogio, P., Cozzi, A., Arosio, P., and Levi, S. (2004) *Hum*

Mol Genet **13**, 2279-2288

60. Layer, G., Ollagnier-de Choudens, S., Sanakis, Y., and Fontecave, M. (2006) *J Biol Chem* **281**, 16256-16263
61. 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
62. 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
63. Olsson, A., Lind, L., Thornell, L. E., and Holmberg, M. (2008) *Hum Mol Genet* **17**, 1666-1672