Frataxin-bypassing Isu1: characterization of the bypass activity in cells and mitochondria

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Abstract

Frataxin is a conserved mitochondrial protein, and deficiency underlies the neurodegenerative disease Friedreich's ataxia. Frataxin interacts with the core machinery for Fe–S cluster assembly in mitochondria. Recently we reported that in frataxin-deleted yeast strains, a spontaneously occurring mutation in one of two genes encoding redundant Isu scaffold proteins, bypassed the mutant phenotypes. In the present study we created strains expressing a single scaffold protein, either Isu1 or the bypass mutant M107I Isu1. Our results show that in the frataxin-deletion strain expressing the bypass mutant Isu1, cell growth, Fe–S cluster protein activities, haem proteins and iron homoeostasis were restored to normal or close to normal. The bypass effects were not mediated by changes in Isu1 expression level. The persulfide-forming activity of the cysteine desulfurase was diminished in the frataxin deletion (Δyfh1 ISU1) and was improved by expression of the bypass Isu1 (Δyfh1 M107I ISU1). The addition of purified bypass M107I Isu1 protein to a Δyfh1 lysate conferred similar enhancement of cysteine desulfurase as did frataxin, suggesting that this effect contributed to the bypass mechanism. Fe–S cluster-forming activity in isolated mitochondria was stimulated by the bypass Isu1, albeit at a lower rate. The rescuing effects of the bypass Isu1 point to ways that the core defects in Friedreich's ataxia mitochondria can be restored.

Keywords

cysteine desulfurase; eukaryote; frataxin; iron–sulfur; Isu1 scaffold; mitochondrion

INTRODUCTION

Frataxin is a conserved mitochondrial protein implicated in causation of Friedreich's ataxia, an inherited neurodegenerative disease [1]. It is the deficiency of frataxin in certain tissues, such as the dorsal root ganglia and heart, that is responsible for the disease. The cellular disease phenotype is characterized by deficiencies of Fe–S cluster proteins and haem proteins [2]. Iron homoeostatic abnormalities also ensue, including accumulation of mitochondrial iron in the biologically unavailable form of ferric phosphate particles [3]. The frataxin protein is highly conserved, and the human protein complements the yeast deletion mutant, indicating an orthologous relationship [4]. In isolates of slow-growing yeast lacking the frataxin homologue (Yfh1), an outgrowth of clones with frataxin-bypassing activity was observed. A single point mutation in Isu1, the Fe–S scaffold protein, was identified as the mediator of the frataxin-bypassing effect [5].

This observation is interesting because of the implications for frataxin function, which is still poorly defined [6]. Fe–S clusters, essential cofactors for numerous proteins, are synthesized by a multisubunit machinery in mitochondria. Frataxin has been found to be a constituent of an Fe–S cluster assembly complex that also includes Nfs1 (NiFS-like)–Isd11 (iron–sulfur desulfurase-interacting protein) and Isu [7–9]. This assembly complex is needed to form Fe–S cluster intermediates on the Isu scaffold, which are subsequently transferred to target apoproteins by the mediation of chaperones, glutaredoxins and other components. The Nfs1–Isd11 complex provides the essential cysteine desulfurase activity for sulfur donation, binding the amino acid cysteine, generating a persulfide on Nfs1 and transferring the sulfur for synthesis of Fe–S cluster intermediates on the Isu scaffold [10]. The source of iron for the intermediate is unknown, and frataxin may play a role in the iron donation step [11]. Another function proposed for frataxin is as an allosteric activator of the cysteine desulfurase activity of the complex [12]. How exactly the mutant Isu bypasses frataxin is not known, but most probably it is able to fulfil some of frataxin's functions in Fe–S cluster assembly in the absence of frataxin.

A curious feature of the frataxin-bypassing mutation of Isu1 is that it involves substitution of an amino acid present in all eukaryotic scaffold proteins with an amino acid present in many prokaryotic proteins [5]. The methionine residue at position 107 of Isu1 is present in all eukaryotes, including metazoans, flies, worms, plants and even primitive protists. In contrast, the isoleucine residue is found in prokaryotic scaffolds such as *Escherichia coli*, *Shigella* and *Salmonella*. Isu sequences and functions are highly conserved across evolution, and so this change is likely to be significant. The metamorphic structure extrapolated from the bacterial proteins consists of α-helices superimposed over a β-sheet platform, and the methionine to isoleucine mutation occurs on an exposed surface of a large C-terminal α-helix predicted to be close to interaction sites for Nfs1 [13], frataxin and the Hsp70 (heat-shock protein 70) chaperone Ssq1 (stress-seventy subfamily Q) [14]. In prokaryotes such as *E. coli*, the lack of frataxin induces a mild phenotype without altering iron homoeostasis or sensitivity to oxidants [15], as contrasted with eukaryotes where frataxin loss is severely deleterious or lethal [4]. Thus the methionine to isoleucine mutation, which improves mitochondrial function in the absence of frataxin, can be envisaged as creating a more

prokaryotic-like Fe–S cluster machinery. The mechanistic details of how this occurs are not known.

The bypass mutation in Isu1 was identified in a strain of yeast deleted for *YFH1*. In yeast there are two functionally redundant genes encoding Isu1 and Isu2 [16]. Thus the bypass activity, conferred by the Isu1 substitution in the presence of an unaltered Isu2 implies some degree of genetic dominance. The M107I Isu1 protein might work as an alternative scaffold or might work *in trans* via the normal scaffold, and experiments up to now have not distinguished between these possibilities. In the present study we constructed strains in which a single Isu, wild-type or mutant, is expressed, either in the setting of normal frataxin (*YFH1*) or frataxin deletion (△*yfh1*). Assays of these cells and mitochondria isolated from these cells have been performed to more fully characterize the features and mechanisms of frataxin bypass.

MATERIALS AND METHODS

Yeast strains, plasmids and growth conditions

Strains were constructed in the YPH499 background of *Saccharomyces cerevisiae* (Table 1). To construct the single copy Isu strains, the *ISU2* gene from YPH499 was interrupted by insertion of pRS406-containing γ-oriented fragments of the gene. A *GAL1* promoter swap was introduced upstream of *ISU1*, creating the strain 106-61, which was dependent on galactose for growth and Isu1 expression [17]. Plasmids were introduced containing wild-type Isu1 or the bypass Isu1 carrying a single amino acid substitution (M141I in the precursor protein or M107I in the mature protein, referred to as M>I). The CEN (centromere)-based YCplac22 plasmids contained 700 bp 5′ of the ORF and 200 bp 3′ of the ORF in a polylinker (EagI-Isu1 promoter-Nde1-Isu1 ORF-XhoI-stop-3′non-coding-Sac1). The resulting strains, 115-18 or 115-21 respectively, were viable and expressed only the plasmid-borne Isu1 or M>I when grown in media with raffinose or glucose as a carbon source. *YFH1* was knocked out in these strains by transforming pRS405 with γ-oriented gene fragments and selecting for leucine prototrophy in an argon-flushed container. Three different colony sizes were noted: tiny colonies that were leucine auxotrophs on restreaking, intermediate sized colonies that were confirmed by PCR to be deleted for *YFH1* and large colonies which resulted from incorrect integration. The confirmed △*yfh1* knockout strains exhibited moderately slow growth that was mitigated by the microaerophilic conditions in the argon-flushed chamber. The genotypes were confirmed by PCR amplification of △*yfh1* or *YFH1* flanking regions. The resulting strains carried △*yfh1* and YCplac22-Isu1 (strain 115-26) or △*yfh1* and YCplac22-M107I (strain 115-28). For simplicity the four strains are referred to by a simple name or short genotype (Table 1). Strain 115-18 is called 'WT' or YFH1 ISU1. Strain 115-21 is called YFH1 M>I. Strain 115-26 is called 'deletion' or △yfh1 ISU1. Strain 115-28 is called 'bypass' or △yfh1 M>I. The 'bypass' terminology is meant to refer to the ability of the M107I mutant form of Isu1 to bypass the effects of frataxin deficiency (Table 1).

A second set of strains was constructed in which the tet07 promoter [18] replaced the native *ISU1* promoter. Isu1-His$_6$ or Isu1-M107I-His$_6$ was subcloned into the pCM184 vector. The plasmids were inserted into the Gal-Isu1 △isu2 strain 106-61, and *YFH1* was knocked out as

above. The set of strains 116-14, 116-15, 116-9 and 116-11 are referred to by their abbreviated genotypes YFH1 tet-ISU1, YFH1 tet-M>I, yfh1 tet-ISU1 and yfh1 tet-M>I respectively (Table 1). The plasmid pCM184-Isu1-His$_6$ was modified by site-directed mutagenesis, changing amino acid 100 of the mature Isu1 from proline to alanine. The P100A mutant was inserted into the parent generating strain 116-16 (Table 1), and this strain was used as a control for detecting Isu1 stabilization as described previously [19]. For most experiments, cells were cultured in complete defined medium with raffinose as the carbon source. Air exposure was minimized by maintaining the cells in argon-flushed flasks. The cultures were brought out in air for the duration of the experiments, usually ranging between 15 and 48 h. Several independent clones of the *yfh1* strains were tested to ensure that new genetic changes were not altering the phenotypes.

Enzyme assays and iron labelling

Mitochondrial lysates were assayed for activities of Fe–S cluster enzymes, succinate dehydrogenase and aconitase as described previously [20]. Iron labelling was performed by growing cells to steady state (four doublings) in the presence of radioactive ^{55}Fe in a standard defined medium (100 nM ^{55}FeCl$_3$, 10 μM ferrous sulfate and 100 μM ascorbic acid). The cells were washed free of unincorporated iron and separated into cytoplasmic and mitochondrial fractions. The mitochondria were processed further by sonication in the presence of 0.1 % Triton X-100 in 50 mM Hepes/KOH and 150 mM NaCl. The supernatant (soluble) and pellet (insoluble) portions were separated by centrifugation (10 000 ***g*** for 15 min at 4 °C). All the fractions were analysed by scintillation counting and protein quantitation with BCA.

Purified proteins

pST39-Nfs1-His$_6$/Isd11 was expressed and purified as described previously [21]. The N1-51 mature form of Yfh1 was expressed and purified. The N1-34 truncation of Isu1 was cloned into pET21b with a His$_6$ tag, expressed in BL21(DE3) codon plus cells, and purified on Ni-NTA (Ni^{2+}-nitrilotriacetate)–agarose. Additional washing steps in the presence of 0.5 M NaCl and 6 M urea while the protein was bound to the beads allowed a high level of purity to be obtained (greater than 95 %). The M107I substitution was introduced by QuikChange mutagenesis of the plasmid pET21b-Isu1-His$_6$, and the mutant protein was similarly expressed and purified. Isd11 was expressed and purified as described previously [22]. Mitochondrial proteins were separated by SDS/PAGE (12 % gel), and immunoblotting was performed with specific rabbit antisera against various mitochondrial proteins. The secondary antibody was a goat anti-(rabbit IgG)–Alexa Fluor™ 680 (Life Technologies). Following scanning with the LiCor Odyssey infrared scanner, signal intensities were recorded.

Organelle assays

Persulfide formation on Nfs1 present in intact mitochondria was measured as described in [22]. Isolated and intact mitochondria were depleted for endogenous nucleotides by incubation for 10 min at 30 °C in buffer (20 mM Hepes/KOH, pH 7.5, and 0.6 M sorbitol). Labelling was carried out by the addition of 1 μl (10 μCi) of [^{35}S]cysteine for 20 min at 30

°C. Mitochondria were recovered, proteins were separated by SDS/PAGE (12 % gel) and the persulfide was viewed by radioautography. Fe–S cluster formation was measured as described in [21]. Briefly, isolated mitochondria were labelled in the presence of added nucleotides (4 mM ATP, 1 mM GTP and 5 mM NADH) and iron (10 μM ferrous ascorbate). Mitochondria were recovered, and soluble proteins were released by freeze–thaw sonication and separated on a native gel. The signal from newly formed [Fe–^{35}S]aconitase was visualized by radioautography. Quantification of radiolabelled protein bands was performed by densitometric analysis using NIH ImageJ software.

Statistics

All data were analysed using Student's t test for unpaired data in a two-tailed analysis (except for the data in Figure 6C, which used a one-tailed analysis). $P < 0.05$ was considered statistically significant.

RESULTS

Single-copy Isu strains

S. cerevisiae has two redundant genes encoding the Fe–S scaffold proteins, *ISU1* and *ISU2*, and a single gene encoding the yeast frataxin homologue *YFH1*. In several independent isolates of yfh1 yeast, an altered *ISU1* was found to confer improved growth and recovery of many of the yfh1-related phenotypes. The bypass effect was traced to a point mutation in *ISU1*, changing amino acid 107 in the mature protein from methionine to isoleucine (M>I) [5]. We considered different scenarios for explaining the function of the bypass Isu1 protein. The bypass Isu1 mutant might work by interacting with the wild-type and unmutated Isu, encoded by *ISU2*, activating it during the course of Fe–S cluster intermediate formation or transfer. Alternatively, the bypass Isu1 might function as a scaffold on its own, being used in preference to the other unmutated Isu2. To distinguish between these possibilities we constructed congenic strains expressing a single Isu. The frataxin deletion strain (yfh1 ISU1) grew more slowly than the others under all conditions (Figure 1). The slow growth was exacerbated by use of non-fermentable substrates that require respiratory activity for their metabolism (Figure 1, right-hand panel). Individual clones of the frataxin deletion (yfh1 ISU1) appeared heterogeneous with larger and smaller colonies, posing a problem for phenotypic characterization. However, if the strains were maintained in an argon-flushed jar under low oxygen tension, growth was uniform, albeit slower than the wild-type, and new mutations did not appear. Therefore for construction and maintenance of these yeast strains, they were kept in the argon-flushed jar as much as possible, taking them out only for use in experiments. In terms of growth characteristics in standard medium, the bypass strain (yfh1 M>I) was indistinguishable from the wt (YFH1 ISU1) strains. The strain with the mutated Isu1 in the YFH1 context (YFH1 M>I) also grew normally (Figure 1).

Enzyme activities

Activity of succinate dehydrogenase, a mitochondrial enzyme complex (complex II of the respiratory chain with haem, Fe_2S_2, Fe_3S_4 and Fe_4S_4 cofactors) [23] was measured. In the frataxin deletion (yfh1 ISU1), succinate dehydrogenase activity was approximately 24 %,

significantly lower ($P = 0.001$) than in the wt (YFH1 ISU1) (Figure 2A). In the bypass mutant (yfh1 M>I), activity was restored and was no longer different from the wt (Figure 2A). For aconitase, an abundant Fe_4S_4 cluster protein of the mitochondrial tricarboxylic acid cycle, the activity was very low or undetectable in the frataxin-deletion strain (yfh1 ISU1), which was significantly lower ($P = 0.012$) than in the wt (YFH1 ISU1). By contrast, in the bypass mutant (yfh1 M>I), the activity was almost completely restored and was no longer different from the wt (Figure 2B). The activity gel assay for aconitase corroborated these findings, showing absent aconitase in the deletion and restoration in the bypass (Figure 2C).

Isu1 and other protein levels in mitochondria

Aconitase protein was decreased in mitochondria from the frataxin-deletion strain (yfh1 ISU1), but recovered in the bypass (yfh1 M>I) (Figure 3). Equivalent levels were present in the frataxin-plus strains (YFH1 ISU1 and YFH1 M>I) (Figure 3). As the frataxin-deletion (yfh1 ISU1) strain had no detectable aconitase activity, the results are consistent with partial destabilization and increased turnover of the apoprotein. The levels of the individual components of the Fe–S cluster assembly complex consisting of Nfs1, Isd11, Isu1 and frataxin [7–9] were measured. Nfs1 levels were equivalent in all cases, showing no signs of regulatory variation. Isd11 also was unchanged. Yfh1 protein expression was consistent with the genotype, being absent in the deleted strains and present in the YFH1 strains as expected. Only Isu levels showed regulatory changes as has been reported previously [19]. Isu1 abundance was increased in the yfh1 ISU1 strain by 3–4-fold, but returned to normal in the bypass yfh1 M>I (Figure 3).

The amounts of several key proteins in mitochondria were measured by quantitative immunoblotting of wt (YFH1 ISU1) mitochondria. The quantities of Nfs1 and Isd11 present in mitochondria were similar (0.9 and 0.8 pmol per 100 μg of mitochondrial proteins respectively), consistent with the idea that they are tightly associated with each other [24] and form a cysteine desulfurase enzyme complex. Nfs1 and Isd11 in turn are associated with Isu and Yfh1 as part of an Fe–S cluster assembly complex. Isu1 was present in similar amounts as Nfs1–Isd11 (0.8 pmol per 100 μg of mitochondrial proteins) (Supplementary Figure S1 at http://www.biochemj.org/bj/459/bj4590071add.htm). Interestingly, Yfh1 was less abundant than the other proteins, being present at 0.5 pmol per 100 μg of mitochondrial proteins, significantly less than the amounts of Nfs1, Isd11 or Isu1 in wt strain mitochondria. The results suggest that Yfh1 may serve a stimulatory or catalytic role, coming on and off the Fe–S cluster assembly complexes as required. In the frataxin-deletion strain (yfh1 ISU1), Isu1 abundance was increased compared with the wt strain (Figure 3), suggesting that, in these mitochondria, it is present at molar excess over Nfs1 and Isd11. As such, the excess Isu might perform functions independently of the Fe–S cluster assembly complex. For example surplus Isu might store iron or Fe–S cluster intermediates in mitochondria.

Recovery of haem proteins, iron homoeostasis and iron solubility

Ccp1 (cytochrome *c* peroxidase) and Cyc1 (cytochrome *c*) are haem proteins. These proteins reside in the intermembrane space and co-ordinate *b* and *c* type haems respectively. In the single-copy Isu strains, Ccp1 protein was decreased to approximately 50 % in the absence of Yfh1 (yfh1 ISU1), but completely recovered in the bypass strain (yfh1 M>I) (Figure 4).

Cyc1, which strongly depends on haem for both its transcription and protein stability [25], was not detected at all in the frataxin-deletion strain (yfh1 ISU1), but was nearly normal in the bypass strain (yfh1 M>I) (Figure 4). The results suggest that haem was deficient in the frataxin-deletion yeast as reported in [26], whereas it was recovered in the bypass, similar to frataxin-plus yeast.

Iron homoeostasis was evaluated by growing cells to steady state in the presence of ^{55}Fe radionuclide. Cells were fractionated into cytoplasm and mitochondria, and iron in the fractions was measured by scintillation counting. The expected iron homoeostatic phenotype [26,27] was observed for the frataxin-deletion strain (yfh1 ISU1), as reflected by increased total cellular iron ($P = 0.010$) (Figure 5A) and mitochondrial iron ($P = 0.013$) (Figure 5C) compared with the wt strain. In the bypass strain (yfh1 M>I), the abnormal iron uptake and distribution were reverted, and these values were not significantly different from the wt strain (YFH1 ISU1) (Figures 5A–5C). A feature of the iron homoeostatic abnormality associated with lack of frataxin is the formation of ferric phosphate nanoparticles in mitochondria [26,28]. Thus the accumulated mitochondrial iron in the frataxin mutant strain (yfh1 ISU1) was predominantly insoluble, as determined by a biochemical test involving detergent solubilization and centrifugation of isolated mitochondria. Significantly more iron was present in the insoluble fraction in the frataxin mutant ($P = 0.002$) compared with the wt strain (Figure 5D). By contrast, the bypass mutant behaved like the wt strain; iron did not accumulate in mitochondria (Figure 5C) and iron in the insoluble fraction was not increased significantly compared with wt strain (Figure 5D). The M>I substitution in the YFH1 context did not lead to any discernible changes in cellular iron content, distribution or solubility (Figure 5).

Forced overexpression of Isu1

Isu abundance was increased in the frataxin-deletion strain (Figure 3) and in other settings with deficient Fe–S cluster synthesis [13]. We wondered if this increase in Isu could perform some function, perhaps acting to improve viability or Fe–S cluster synthesis. To test this notion, a set of single-copy Isu strains was constructed in which Isu1 was placed under control of the tet07 promoter. Driven by the tet07 promoter, expression of Isu1 was increased by more than 5-fold compared with the native promoter (Figure 6B, Isu1 in lanes 1–4 compared with lane 6). The phenotypes of the Isu-overexpressing strains were examined further. The frataxin-deleted strain (yfh1 tet-ISU1) was slower growing than the others. As before, the overexpressed bypass Isu1 strain (yfh1 tet-M>I) completely restored normal growth and was indistinguishable from the frataxin-plus strains (YFH1 tet-ISU1 and YFH1 tet-M>I) in terms of growth and colony size on YPAD agar plates (Figure 6A). There was no discernible effect of the strong overexpression of Isu1 in enhancing (or inhibiting) growth under standard conditions, compared with the strains with much lower Isu1 expression from the native promoter (Figure 6A). However, Cyc1 protein, which was undetectable in the frataxin-deletion strain (yfh1 ISU1), was increased to 47 % of the wt strain levels as a consequence of overexpression of Isu1 in the yfh1 tet-ISU1 strain (Figure 6B, lane 1 compared with lane 6). Succinate dehydrogenase was increased from 16 % to 31 % by overexpression of Isu1 in the *yfh1* context (Figure 6C, left-hand panel), although this difference did not reach statistical significance ($P = 0.072$). Aconitase activity, which was

barely detected, was significantly higher in the Isu1-overexpressing strain ($P = 0.018$), increasing to 14 % of the wt strain level (Figure 6C, right-hand panel). In conclusion, overexpression of Isu1 resulted in uneven phenotypic effects, with no enhancement of growth, but with significant improvement in some biochemical traits of *yfh1* yeast. An effect of increased Isu level in enhancing fitness and responses to some forms of environmental stress has been described previously [19]. Conversely, decreases in Isu levels, as occured in *isu1* strains, are synthetically lethal when combined with *yfh1* [29]. By contrast, the M>I substituted Isu1 produced a more complete correction of the *yfh1* mutant phenotypes, and its bypass activity was independent of the expression level (Figure 6).

Mechanism of Isu regulation

The Isu level was increased in the *yfh1*-deletion strain (Δyfh1 ISU1) and it returned to normal in the bypass strain (Δyfh1 M>I). To assess Isu1 protein stability, pulse–chase experiments were performed. The strains with overexpressed Isu1 under the tet07 promoter were grown to log-phase, pulsed with [^{35}S]methionine and chased with unlabelled methionine. The Isu1 protein was recovered with a C-terminal His$_6$ tag, and protein turnover was evaluated. The results showed that Isu1 was rapidly turned over, with the signal declining to less than 20 % of the starting signal within 30 min of chase (Supplementary Figure S2B at http://www.biochemj.org/bj/459/bj4590071add.htm). The rapid turnover was observed for Isu1 or M>I in the YFH1 strains, and similarly rapid turnover was observed in the *yfh1* context (Supplementary Figure S2A). Numerous other labelled proteins were recovered on the Ni-NTA–agarose and visualized on the gel (Supplementary Figure S2), probably due to non-specific binding, and most of these proteins were stable during the time frame of the experiment. A single amino acid substitution was introduced into the plasmid pCM184-Isu1-His$_6$ (P100A), and protein stability of this mutant protein was tested. As reported previously [19], the P100A Isu1 protein was completely stabilized, showing almost no decline in the labelled cohort after 180 min. Steady-state levels of Isu1 in the tet07 strains were almost equal (Figure 6B) except in the case of the stabilized P100A mutant for which the level was increased (results not shown). The *ISU1* promoter has binding sites for Aft1/2 (activator of ferrous transport 1/2) [13,16], the transcriptional regulators for iron. Thus the increased Isu1 expression in the *yfh1* setting could be due, at least in part, to perturbed iron homoeostasis and increased Aft1/2-dependent transcription. The *ISU1* transcripts were directly evaluated by real-time PCR. The deletion had a C_T value of 4.2, which was significantly higher ($P = 0.020$) than the wt strain ($C_T = 3.3$), using *TAF1* (TATA-binding protein-associated factor 1) as an endogenous standard. The difference corresponded to a 1.8-fold difference in the RNA levels. There was no difference between the C_T of the bypass strain and the wt strain (Supplementary Figure S3 at http://www.biochemj.org/bj/459/bj4590071add.htm). Thus changes in Isu1 protein level in the deletion (Δyfh1 ISU1) were attributable principally to mRNA level regulation and were corrected in the bypass mutant (Δyfh1 M>I).

Nfs1 persulfide formation

An initial step in formation of the Fe–S cluster assembly intermediate on the Isu scaffold is sulfur donation. This requires the activity of the Nfs1 cysteine desulfurase in mitochondria [30]. As part of its reaction cycle, Nfs1 binds the amino acid cysteine at its substrate-binding

site. Then, in a step that depends on a conformational change induced by the accessory protein Isd11, the Nfs1 active-site cysteine forms a persulfide with the -SH group of the substrate cysteine [22]. The persulfide can be labelled with [^{35}S]cysteine substrate, allowing detection by gel electrophoresis and providing a quantitative measure of a single round of cysteine desulfurase activity in mitochondria [21].

Mitochondria were isolated from the four single-copy Isu strains, and these were briefly (10 min) incubated at 30 °C to deplete endogenous nucleotides including ATP, GTP and NAD(P)H. The nucleotide-depleted intact mitochondria, although able to form persulfide on Nfs1, cannot transfer the persulfide because of a block in the nucleotide-dependent steps [21]. These intact mitochondria were labelled with [^{35}S]cysteine, recovered and analysed by non-reducing SDS/PAGE. The Nfs1 persulfide signal in the frataxin-deletion mitochondria (yfh1 ISU1) was found to be diminished to 28 % of the wt strain level (Figure 7A, lane1). In the bypass mitochondria (yfh1 M>I) the persulfide signal was recovered to 91 % of the wt strain level (Figure 7A, lane 2). The persulfide was detected at a level comparable with the wt stain in the mutant Isu1 in the YFH1 context (YFH1 M>I) (Figure 7A, lane 4). MA14, carrying a hypomorphic allele of *NFS1*, showed no detectable persulfide in mitochondria, and the purified Nfs1–Isd11 showed a strong persulfide signal, migrating slightly slower than the mitochondrial signal due to the His$_6$ tag on the purified Nfs1 [21]. The changes in mitochondrial persulfide were not due to changes in Nfs1 protein levels, as the Nfs1 protein levels were equivalent in these mitochondria (Figure 3).

Persulfide on Nfs1, reflecting cysteine desulfurase activity, can also be assayed in mitochondrial lysates. A lysate was generated from frataxin-deletion mitochondria (yfh1 ISU1), and this exhibited low, but detectable, Nfs1 persulfide (Figure 7B, lane 1). Mature frataxin, expressed and purified from bacteria, was added back to the *yfh1* mitochondrial lysate. A stimulatory effect was observed, and the stimulation increased with increasing amounts of added frataxin (Figure 7B, lanes 2–4). Similarly, mature M>I Isu1 protein was expressed and purified from bacteria. Addition of this substituted bypass Isu1 also produced a stimulatory effect on Nfs1 persulfide-forming activity in the lysate (Figure 7B, lanes 5–7). The degree of stimulation was similar to the frataxin effect, with the maximum occurring at approximately 2 μg of added protein. Thus either frataxin or the M>I bypass Isu was able to stimulate cysteine desulfurase activity in the mitochondrial lysate from the frataxin-deletion (yfh1 ISU1) strain. In summary, the mitochondrial Nfs1 persulfide level, reflecting a single round of cysteine desulfurase activity, was decreased in mitochondria lacking Yfh1 (yfh1 ISU1 compared with YFH1 ISU1), consistent with a role of Yfh1 as a positive regulator of cysteine desulfurase [12]. The M>I substituted Isu1 (yfh1 M>I) restored persulfide-forming activity when expressed as the sole Isu1 in mitochondria lacking frataxin (Figure 7A), or when added to mitochondrial lysate lacking frataxin, but already carrying endogenous Isu1 (Figure 7B). Frataxin or M>I bypass Isu1 exhibited similar stimulatory effects on the cysteine desulfurase in a *yfh1* mitochondrial lysate, suggesting that they exert their effects by a similar mechanism.

Fe–S cluster assembly in isolated mitochondria

Isolated and intact mitochondria possess a complete machinery for synthesis of new Fe–S clusters. When supplied with [^{35}S]cysteine, iron and nucleotides, the machinery is activated, and newly formed [Fe–^{35}S] clusters are made and inserted into apoaconitase, providing a measure of the efficiency of the entire biosynthetic pathway [21]. In wt (YFH1 ISU1) mitochondria, new aconitase Fe–S cluster synthesis was apparent even at 15 min, increasing further at the 30 and 60 min time points (Figure 8A, lanes 1–3). In mitochondria with frataxin present and the substituted Isu1 (YFH1 M>I), the synthesis of new clusters was still quite active, although slightly less than that in the wt strain (Figure 8A, lanes 4–6). By contrast, in the frataxin-deletion strain (yfh1 ISU1), Fe–S cluster synthesis activity was severely impaired (Figure 8A, lanes 7–9). In the bypass mutant mitochondria (yfh1 M>I), biosynthetic activity was restored to some extent, but the amount was small and the rate of formation remained low (Figure 8A, lanes 10–12), reaching only 15 % of the wild-type amount after 60 min. These same bypass (yfh1 M>I) mitochondria have normal levels of aconitase protein (Figure 3) and almost normal aconitase activity (Figure 2). The data suggest that turnover of these slowly formed Fe–S clusters in living cells is slow enough to allow for eventual recovery of normal amounts of aconitase holoprotein.

Iron dependence of Fe–S cluster synthesis was evaluated by adding different amounts of ferrous ascorbate to the isolated mitochondria during the labelling assay (Figure 8B). Iron dependence of new Fe–S cluster formation was observed for the wt strain (Figure 8B, lanes 1–3). Little new Fe–S clusters were seen in the deletion mitochondria (yfh1 ISU1) (Figure 8B, lanes 7–9). The addition of up to 50 μM iron enhanced Fe–S cluster assembly in the bypass mutant mitochondria (yfh1 M>I), but the improvement was modest and the amount of synthesis after 60 min was still quite low compared with the wild-type (Figure 8B, lanes 10–12). Thus the kinetic defect in Fe–S cluster assembly in the M>I mitochondria was not overcome by the addition of iron *in vitro*, suggesting that the function of frataxin could not be replaced by exogenously supplied iron.

Peroxide sensitivity

Frataxin deficiency has been associated with increased sensitivity to oxidative stress [31]. With this in mind, the strains were subjected to oxidative stress by spotting on to agar plates supplemented with H_2O_2. The frataxin-deletion strain (yfh1 ISU1) was sensitive as expected, exhibiting no colony formation at higher H_2O_2 concentrations (Figure 9, row 1 at 4 mM H_2O_2). The wt frataxin-plus strain was resistant (Figure 9, row 3). However, examination of the bypass strain (yfh1 M>I) revealed a unique phenotype (Figure 9, row 2). Growth was identical to the wt at low peroxide concentrations (Figure 9, 0.25 mM and 2 mM H_2O_2), but at higher H_2O_2 concentrations it exhibited decreased growth indicative of sensitivity to H_2O_2 (Figure 9, row 2 at 4 mM H_2O_2). Overexpression of wild-type or mutant Isu1 did little to alter the H_2O_2 sensitivity phenotypes (Figure 9, rows 5–8). The increased H_2O_2 sensitivity of the bypass mutant (yfh1 M>I) compared with the wt strain (YFH1 ISU1) is not likely to be explained by iron accumulation and the resultant free radical damage, because iron homoeostasis was corrected in that strain. Instead, a possible explanation is that H_2O_2 exposure induced a more rapid turnover of Fe–S clusters or their

precursors. The low rate of synthesis of Fe–S clusters in the bypass strain then made it unable to keep up with the increased demand created by the oxidative stress conditions.

DISCUSSION

Mitochondria synthesize Fe–S clusters in a multi-step process, involving formation of Fe–S cluster intermediates on a scaffold and transfer to substrate apoproteins [10]. Frataxin is part of an Fe–S cluster assembly protein complex in mitochondria, interacting with the cysteine desulfurase, Nfs1–Isd11, and the scaffold, Isu [7–9]. Frataxin is required for efficient formation of the Fe–S cluster intermediate on the scaffold and for Fe–S cluster synthesis in cells [7]. An amino acid substitution in one of two redundant genes encoding Isu, the M>I change of amino acid 107 of Isu1, was found to promote recovery of almost all of the mutant phenotypes, including growth, Fe–S cluster protein activities, haem and iron homoeostasis [5]. In the present study we found that the recovery could be effected by single-copy Isu1 with the M>I substitution. The implication is that the bypass Isu1 was able to function as the sole Isu in the cell, mediating the scaffold function and also conferring the frataxin-bypass effect. An interaction with a wild-type copy of Isu1 or Isu2 was not required.

The process of Fe–S cluster assembly in mitochondria requires iron and sulfur. For the sulfur component, the assembly process involves the release of sulfur from the amino acid cysteine, formation of a persulfide on the cysteine desulfurase Nfs1 and donation of the sulfur to the Isu scaffold [32]. In eukaryotic cells, the Nfs1 cysteine desulfurase activity is apparently strongly regulated, with the enzyme remaining 'off' until the crucial activating steps occur [12,22]. In experiments with intact mitochondria and mitochondrial lysates, the persulfide signal generated by a single cycle of cysteine desulfurase was found to be decreased in frataxin-deletion mitochondria (yfh1 ISU1) compared with the wt strain (YFH1 ISU1). The activity was restored *in vitro* by the addition of frataxin to the mitochondrial lysate. Interestingly, the mutant form of Isu1, M>I, performed very much in the same way as frataxin. The bypass mitochondria expressing the single-copy Isu1 M>I mutant in the absence of frataxin were more active for cysteine desulfurase, and the addition of purified M>I mutant Isu1 protein stimulated persulfide formation in the deletion mitochondrial lysate. These data strongly suggest that the frataxin-bypassing effect of the M>I Isu1 is mediated, at least in part, by a stimulatory effect on cysteine desulfurase. In recent work with purified proteins, the stimulation was traced back to an effect on enhancing substrate binding to Nfs1 [33]. Either frataxin or M>I Isu1 increased the binding of the amino acid cysteine to Nfs1 in an early step of the reaction cycle, and the persulfide formation was subsequently stimulated by Isd11 interaction with Nfs1 in a later step of the reaction cycle [33]. How this might occur in terms of structural changes in the proteins is unknown, as no crystal structures of eukaryotic cysteine desulfurases or scaffolds have been solved. One can imagine that the M107I substitution might alter the interaction of Isu1 with Nfs1 in such a way to expose Nfs1 substrate-binding sites and enhance substrate binding. Thus frataxin or M>I could act in similar fashion as an allosteric effector of the cysteine desulfurase [12,33].

There is an evolutionary story here too. The Isu protein is highly conserved between prokaryotes and eukaryotes, consistent with the evolutionary origin of mitochondria from purple bacteria [14]. The M>I change occurs in a conserved amino acid. At residue 107 the methionine is present in all eukaryotes including plants, animals and protists, whereas the isoleucine is present in many prokaryotes including *E. coli*. Other branched chain amino acids such as leucine and valine are present in a similar position in Isu homologues from other prokaryotes [5]. The M>I change then can be considered to confer a prokaryotic feature to Isu and to the Fe–S cluster assembly apparatus. In this regard it is interesting that the *E. coli* Fe–S cluster assembly apparatus does not depend on frataxin as much as the eukaryotic apparatus [34]. The *E. coli* knockout has very mild phenotypes and does not accumulate iron or show severe Fe–S cluster deficiencies [15]. Also the cysteine desulfurase does not depend on CyaY, the prokaryotic frataxin, for activation in the way that the eukaryotic Nfs1 depends on Yfh1, the yeast frataxin homologue [35]. Rendering the mitochondrial Fe–S cluster assembly apparatus more prokaryote-like might also make it more frataxin independent.

Isolated and metabolically active mitochondria were examined in an assay for new Fe–S cluster synthesis on apo-aconitase [36]. There was strong Fe–S cluster biosynthetic activity in the wt strain (YFH1 ISU1) and negligible activity in the frataxin-deletion strain (yfh1 ISU1), consistent with the *in vivo* phenotypes. Although mitochondria with the bypass (yfh1 M>I) recovered activity, Fe–S clusters were made slowly such that the newly synthesized [Fe–^{35}S] aconitase clusters were only approximately 15 % of the wild-type level after 60 min (Figure 8A). With this information, i.e. that the bypass mitochondria possess normal aconitase activity, but a very slow rate of new aconitase cluster synthesis, we can envision what occurs in living cells. The slow synthesis of Fe–S clusters in the absence of frataxin does not restore function instantaneously, but, over time, the mutant 'catches up' eventually accumulating enough Fe–S clusters to reconstitute holoenzymes in normal amounts. Cellular Fe–S clusters are slowly restored, and these include the regulatory Fe–S clusters that control iron homoeostasis [37,38]. The implications of this scenario are first, that the normally functioning Fe–S cluster assembly machinery has tremendous spare capacity, and secondly, that the turnover rate of assembled Fe–S clusters within target enzymes is low. As far as we know neither of these parameters, spare Fe–S cluster biosynthetic capacity or Fe–S cluster turnover, has been measured directly. The exposure of cells to H_2O_2, a strong oxidant, by damaging Fe–S clusters and Fe–S cluster intermediates might decrease the spare capacity and increase Fe–S cluster turnover. Thus, because the bypass mutant (yfh1 M>I) lacked spare biosynthetic capacity, it was able to grow normally under non-stress conditions, but only very slowly under H_2O_2 stress conditions. The deficit of spare biosynthetic capacity in the bypass mutant M>I might be accounted for by other frataxin functions that were not corrected as well as the cysteine desulfurase. The bypass mutant might effectively replace frataxin in terms of stimulating cysteine desulfurase, but it might fail to replace some other key function of frataxin, for example iron donation for Fe–S cluster assembly [11].

In summary, a detailed analysis has been presented of the frataxin-bypass phenotype conferred by the M>I mutant Isu1, expressed as the sole cellular Fe–S scaffold protein. Most

yfh1 phenotypes were corrected in the bypass strain (yfh1 M>I). Stimulation of the cysteine desulfurase contributed to the bypass effect, although a kinetic defect in Fe–S cluster assembly in mitochondria remained. The results point to the multifaceted roles of Yfh1 in controlling mitochondrial Fe–S cluster assembly, and also suggest therapeutic targets for bypassing frataxin in Friedreich's ataxia and for improving Fe–S cluster assembly generally.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

Aft1/2	activator of ferrous transport 1/2
Ccp1	cytochrome *c* peroxidase
Cyc1	cytochrome *c*
Isd11	iron–sulfur desulfurase-interacting protein
Nfs1	NiFS-like 1
Ni-NTA	Ni^{2+}-nitrilotriacetate

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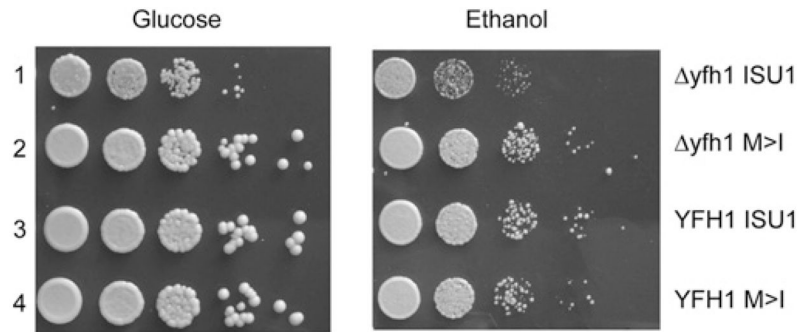
Figure 1. Growth of single-copy Isu strains

The four single-copy Isu strains (Δyfh1 ISU1, Δyfh1 M>I, YFH1 ISU1 and YFH1 M>I) were grown overnight in rich medium (YPAD), and 10^6 cells were subjected to serial dilutions and spotted on to agar plates containing 2 % glucose or 3 % ethanol as a carbon source. The plates were incubated at 30 °C for 3 days and photographed.
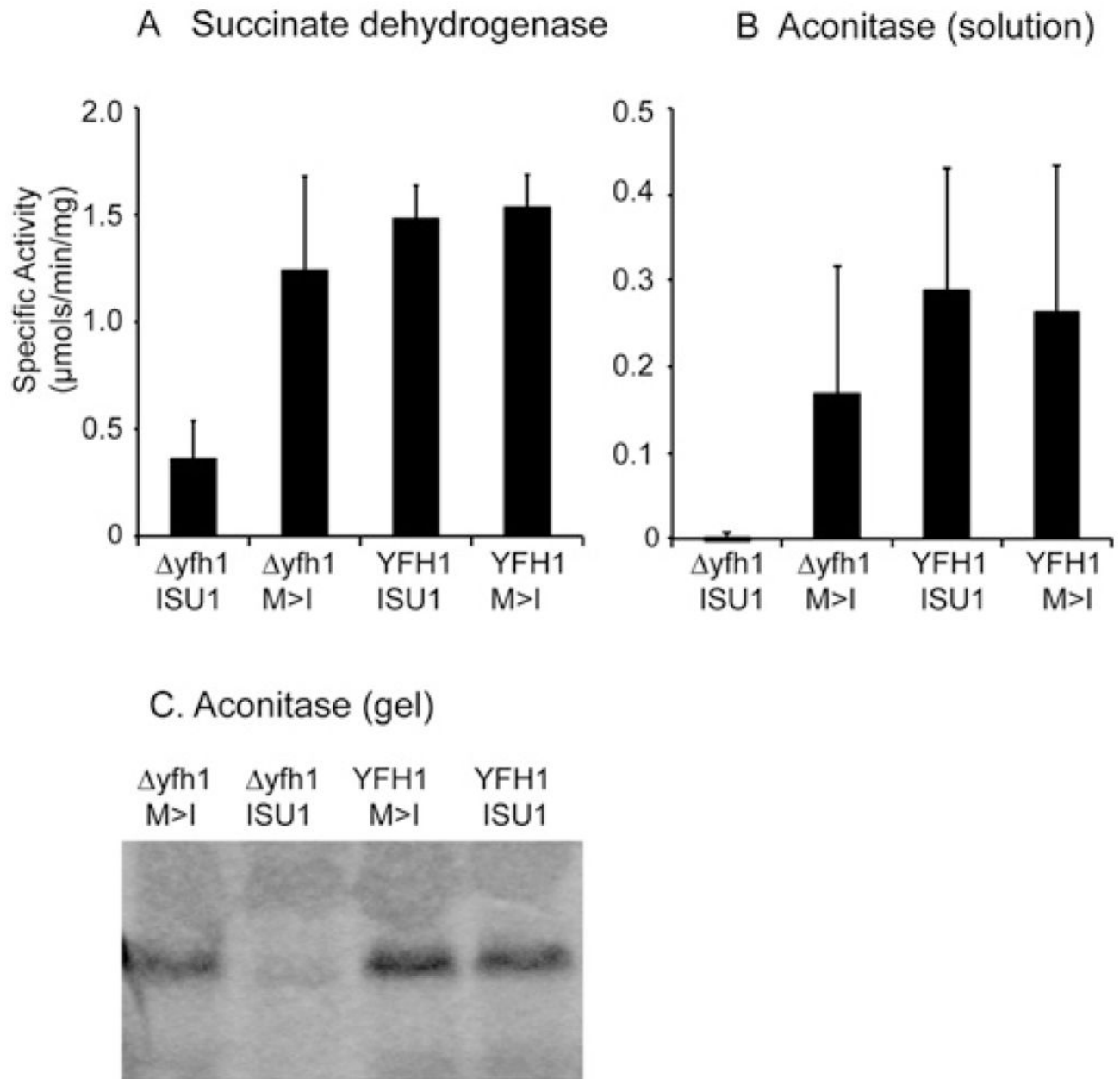
Figure 2. Enzyme activities

Mitochondria were prepared from three independent biological replicates from the four single-copy Isu strains. (**A**) Succinate dehydrogenase activity (means ± S.D.). yfh1 ISU1 was significantly different ($P = 0.001$) from YFH1 ISU1. YFH1 M>I was not significantly different ($P = 0.422$) from YFH1 ISU1. (**B**) Aconitase activity (means ± S.D.). yfh1 ISU1 was significantly different ($P = 0.012$) from YFH1 ISU1. YFH1 M>I was not significantly different ($P = 0.368$) from YFH1 ISU1. (**C**) The in-gel activity assay for aconitase activity on these mitochondria [39,40].
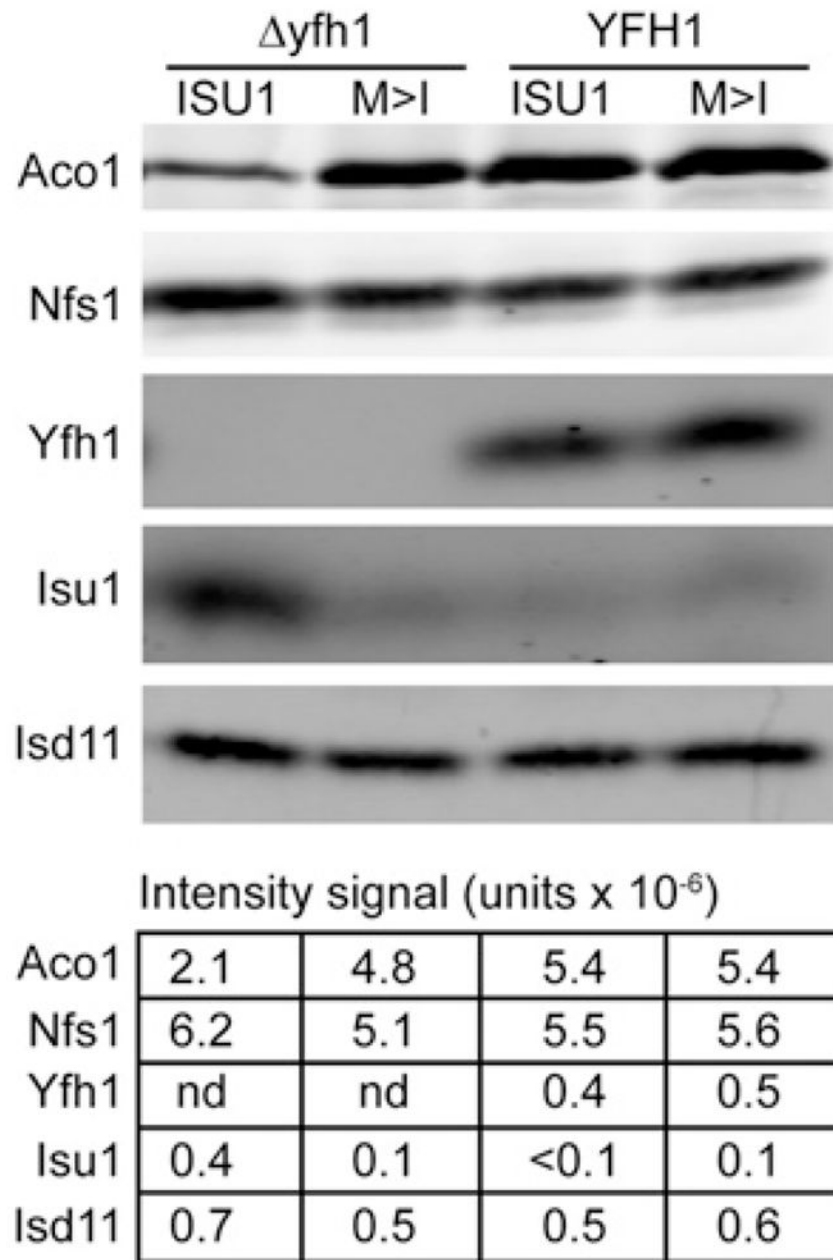
	Δyfh1		YFH1	
	ISU1	M>I	ISU1	M>I
Aco1				
Nfs1				
Yfh1				
Isu1				
Isd11				

Intensity signal (units x 10^{-6})

Aco1	2.1	4.8	5.4	5.4
Nfs1	6.2	5.1	5.5	5.6
Yfh1	nd	nd	0.4	0.5
Isu1	0.4	0.1	<0.1	0.1
Isd11	0.7	0.5	0.5	0.6

Figure 3. Protein levels in mitochondria
Mitochondria isolated from single-copy Isu strains were analysed by SDS/PAGE. After immunoblotting with specific rabbit antibodies for aconitase (Aco1), Nfs1, yeast frataxin homologue (Yfh1), Isu1 and Isd11, the images were developed using the LiCor system and infrared detector and the intensity signal was determined (shown in lower panel). Three independent preparations of mitochondria were evaluated, and a representative immunoblot is shown. nd, not detected.
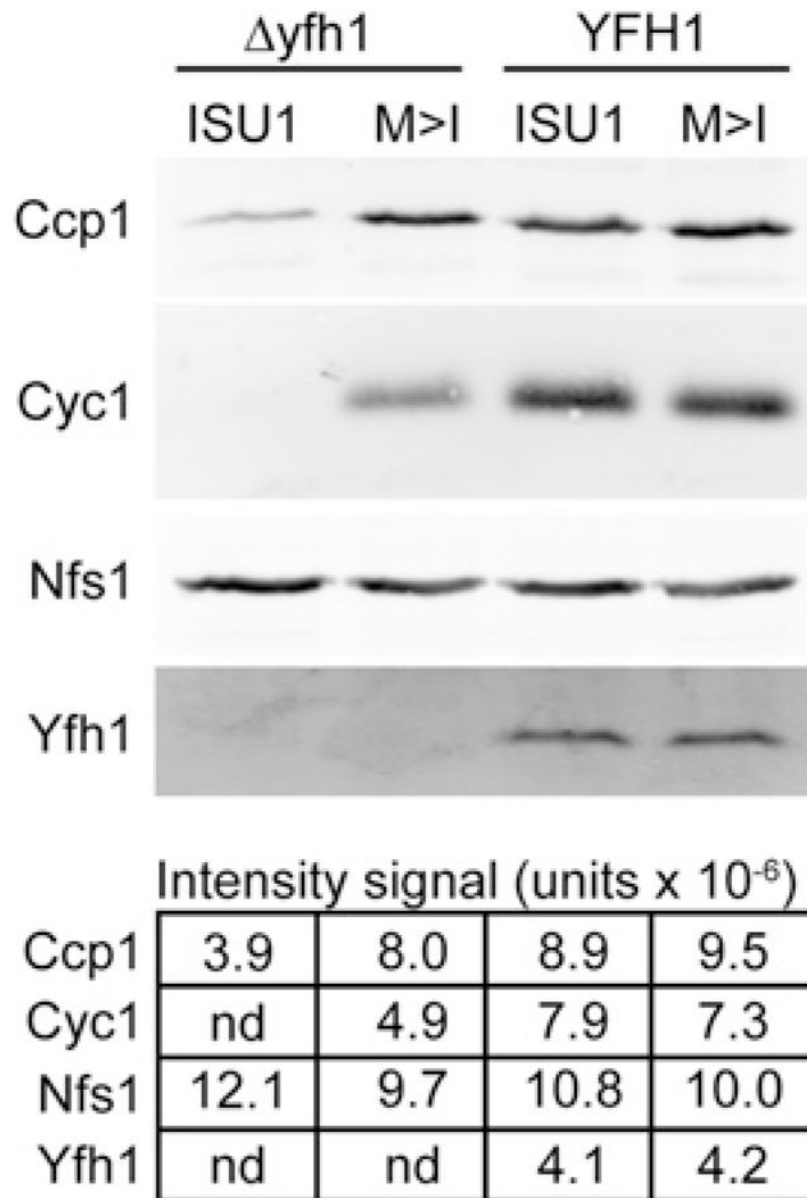
	Δyfh1		YFH1	
	ISU1	M>I	ISU1	M>I

Intensity signal (units x 10^{-6})

	ISU1	M>I	ISU1	M>I
Ccp1	3.9	8.0	8.9	9.5
Cyc1	nd	4.9	7.9	7.3
Nfs1	12.1	9.7	10.8	10.0
Yfh1	nd	nd	4.1	4.2

Figure 4. Haem protein levels

Mitochondria isolated from single-copy Isu strains were analysed by SDS/PAGE. The proteins were transferred on to nitrocellulose membranes and probed with rabbit polyclonal antibodies against Ccp1 and Cyc1. The blots were also probed for Yfh1 and Nfs1 to confirm the genotype and to serve as controls The images were developed using the LiCor system and infrared detector and the intensity signal was determined (shown in lower panel). Three independent preparations of mitochondria were evaluated and a representative immunoblot is shown. nd, not detected.
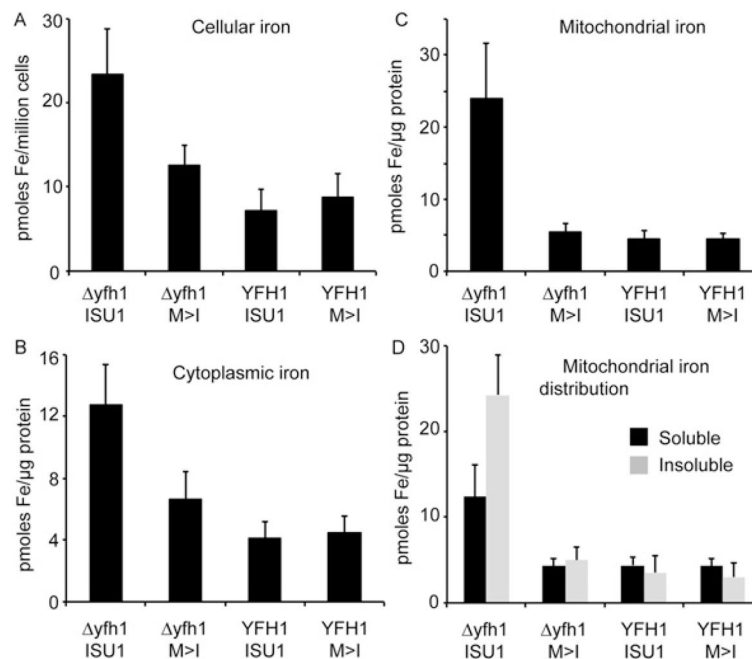
Figure 5. Iron homoeostasis

The single-copy Isu strains were grown in the presence of ^{55}Fe radionuclide tracer in defined medium supplemented with 10 μM ferrous ascorbate. Three independent labelling and fractionation experiments were performed. Results are means ± S.D. Whole cells, cytoplasmic and mitochondrial fractions were subjected to scintillation counting for ^{55}Fe. (**A**) Iron per cell was determined by cell counting, and the amount in yfh1 ISU1 was significantly greater than the amount in YFH1 ISU1 (P = 0.010), whereas the amount in YFH1 M>I was not significantly different from the amount in YFH1 ISU1. (**B**) Cytoplasmic iron was determined, and the amount in yfh1 ISU1 was significantly greater than the amount in YFH1 ISU1 (P = 0.005), whereas the amount in YFH1 M>I was not significantly different from the amount in YFH1 ISU1. (**C**) Mitochondrial iron was determined and the amount in yfh1 was significantly greater than the amount in YFH1 ISU1 (P = 0.013), whereas the amount in YFH1 M>I was not significantly different from the amount in YFH1 ISU1. (**D**) For solubility determinations, mitochondria were sonicated in buffer containing 0.1 % Triton X-100. The supernatant and pellet fractions were separated by centrifugation and subjected to scintillation counting to ascertain the iron content of each fraction. The insoluble fraction was significantly greater in the yfh1 ISU1 than in the wt YFH1 ISU1 (P = 0.002), whereas the insoluble fraction in the YFH1 M>I was not significantly different from the amount in YFH1 ISU1.
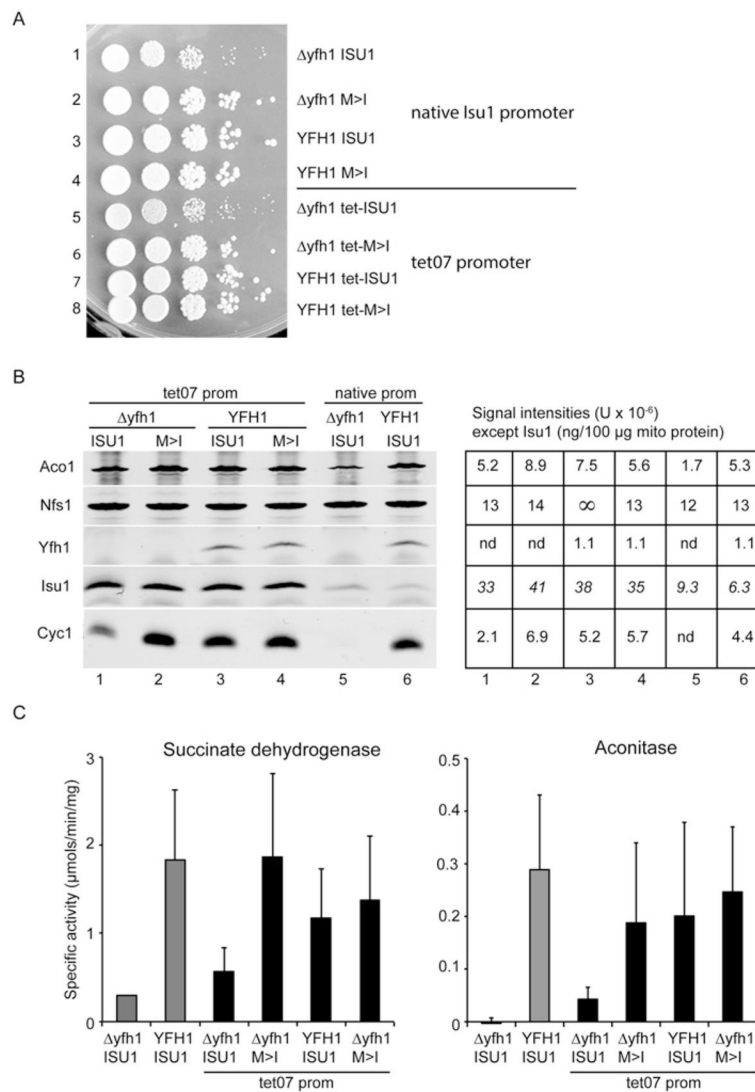
Figure 6. Overexpression of Isu1 and related phenotypes

(**A**) Plate phenotypes. Strains with the indicated genotypes (native Isu1 promoter or tet07 promoter) were serially diluted and spotted on to YPAD agar plates. After 3 days of growth at 30 °C the plates were photographed. (**B**) Mitochondria were isolated from deletion (Δyfh1 ISU1) and wt (YFH1 ISU1) strains, and from the tet promoter set including Δyfh1 tet-ISU1, Δyfh1 tet-M>I, YFH1 ISU1 and YFH1 tet-M>I. Proteins were evaluated by immunoblotting using various antibodies. The images were developed using the LiCor system and infrared detector and the intensity signal was determined (shown in right-hand panel). Three independent mitochondrial preparations were analysed, and a representative immunoblot is shown. ∞, pixel saturation. Isu1 values are reported as ng/100 μg of mitochondrial protein. (**C**) Enzyme activities for succinate dehydrogenase (left-hand panel) and aconitase (right-hand panel) were measured in mitochondria. Three independent mitochondrial preparations were assayed. Results are means ± S.D. The mean succinate dehydrogenase activity was higher in the tet07 strain Δyfh1 tet-ISU1 compared with the native promoter strain Δyfh1 ISU1 although the difference did not reach statistical significance ($P = 0.072$). The mean

aconitase activity was significantly higher in the tet07 strain compared with the native promoter strain ($P = 0.018$) as determined using a one-tailed Student's *t* test.
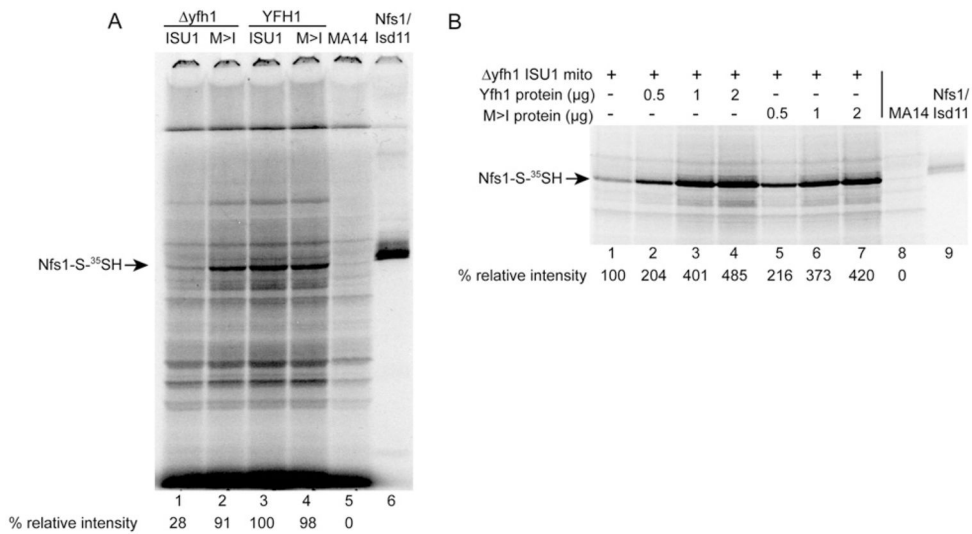
Figure 7. Persulfide-forming activity in mitochondria

Mitochondria were isolated from the single-copy Isu strains (native Isu1 promoter strains). (**A**) Nfs1 persulfide in intact mitochondria. Intact mitochondria were depleted of endogenous nucleotides by incubation for 10 min at 30 °C. After labelling with [^{35}S]cysteine, mitochondria were recovered, proteins were separated by non-reducing SDS/PAGE and Nfs1 persulfide (Nfs1-S-^{35}SH) was visualized by radioautography. Lane 1, yfh1 ISU1; lane 2, yfh1 M>I; lane 3, YFH1 ISU1; lane 4, YFH1 M>I; lane 5, MA14 mitochondria; lane 6, purified Nfs1–Isd11 complex. The Nfs1 persulfide is indicated by an arrow. Four independent experiments were performed and a representative autoradiogram is shown. (**B**) Nfs1 persulfide in mitochondrial lysate. Lysate from the deletion (yfh1 ISU1) mitochondria was supplemented with different amounts of purified Yfh1 protein (lanes 2–4) or purified mutant M>I Isu1 protein (lanes 5–7). The lysates were incubated with [^{35}S]cysteine, and radiolabelled persulfide was detected by separation on non-reducing SDS gel and radioautography. Lysate from MA14, a hypomorphic Nfs1 mutant, was included as a negative control (lane 8), and purified Nfs1–Isd11 protein complex was included as a positive control (lane 9). The experiment was repeated three times and a representative autoradiogram is shown.
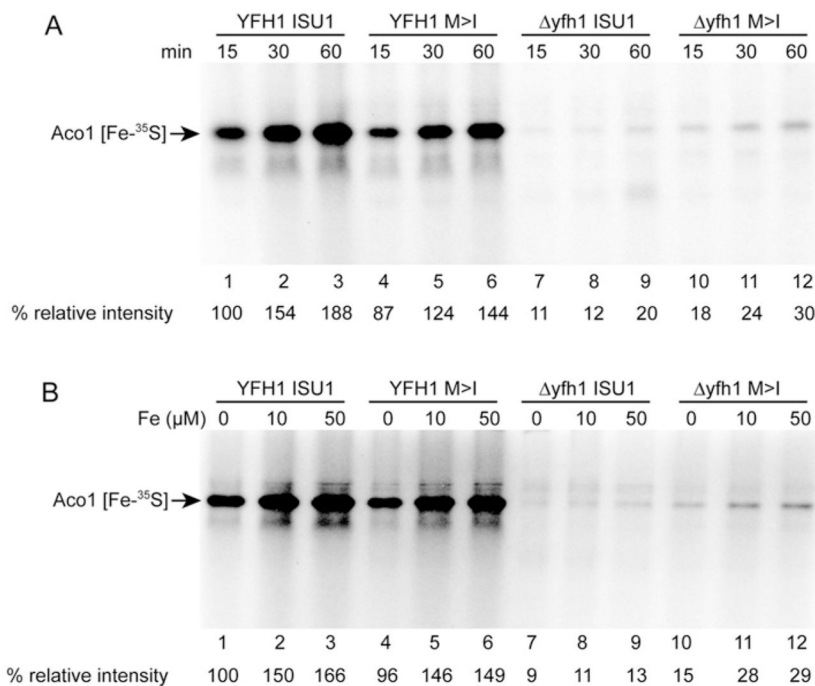
Figure 8. Fe–S cluster forming activity in intact mitochondria

(**A**) Isolated mitochondria from strains with the indicated genotypes were incubated with [^{35}S]cysteine in the presence of nucleotides (4 mM ATP, 1 mM GTP and 5 mM NADH) and ferrous ascorbate (10 μM) at 30 °C for the indicated times. Mitochondria were recovered, lysed and soluble proteins were analysed by native gel electrophoresis. Radiolabelled aconitase (Aco1 [Fe-^{35}S]) was visualized by radioautography. The time course experiment was repeated three times and a representative autoradiogram is shown. (**B**) Isolated mitochondria were labelled at 30 °C for 30 min in the presence of [^{35}S]cysteine, nucleotides and different concentrations of ferrous ascorbate (0, 10 and 50 μM). Radiolabelled aconitase was visualized as in (**A**). The iron titration experiment was repeated three times and a representative autoradiogram is shown.
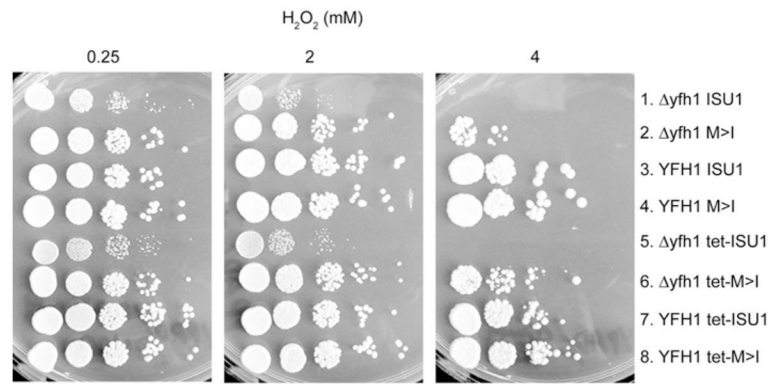
Figure 9. H$_2$O$_2$ sensitivity

Yeast strains (Isu1 driven by the native promoter or tet07 promoter) were serially diluted and spotted on to YPAD agar plates containing H$_2$O$_2$ at different concentrations (0.25 mM, 2 mM or 4 mM). The growth of the bypass strain (Δyfh1 M>I) was indistinguishable from the wt strain (YFH1 ISU1) at low H$_2$O$_2$, but severely impaired at high H$_2$O$_2$ concentration.

Table 1

List of yeast strains

Strain number	Simple name	Short genotype	Genotype
106-61	Parent	–	*MATa lys2-801(amber) ade2-101(ochre) trp1- 63 leu2- 1 pRS406-gamma isu2 (URA3) His3MX6-PGAL1-ISU1*
115-18	wt	YFH1 ISU1	Parent with *[YCplac22-Isu1] [pRS415]*
115-21	–	YFH1 M>I	Parent with *[YCplac22-M107I] [pRS415]*
115-26	deletion	yfh1 ISU1	Parent with *[YCplac22-ISU1] [pRS405-Yfh1 gamma]*
115-28	bypass	yfh1 M>I	Parent with *[YCplac22-ISU1 M107I] [pRS405-Yfh1 gamma]*
116-14	–	YFH1 tet-ISU1	Parent with *[pCM184-Isu1-His$_6$][YEp351]*
116-15	–	YFH1 tet-M>I	Parent with *[pCM184-M107I-His$_6$][YEp351]*
116-9	–	yfh1 tet-ISU1	Parent with *[pCM184-Isu1-His$_6$] yfh1::LEU2*
116-11	–	yfh1 tet-M>I	Parent with *[pCM184-M107I-His$_6$] yfh1::LEU2*
116-16	–	P100A	Parent with *[pCM184-Isu1-P100A-His$_6$]*