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online data

SUPPLEMENTARY ONLINE DATA

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

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MATERIALS AND METHODS

Levels of mitochondrial proteins

Serial dilutions of purified Isu1–His_6 [1] analysed by SDS/PAGE alongside serial dilutions of pure lysozyme as a standard. The gel was stained with Coomassie Blue and Isu1–His_6 amounts were determined by scanning at 700 nm using a LiCor Odyssey and comparing the integrated intensity to the lysozyme standard curve. Mitochondria (10–100 μg of proteins) from three independent mitochondrial preparations of the wt strain (YFH1 ISU1) were analysed by SDS/PAGE using purified Isu1 as a standard. The gel was blotted and probed with anti-Isu1 antibodies, and the signal was developed with secondary antibody [goat anti-(rabbit IgG)–Alexa Fluor™ 680; Life Technologies] followed by scanning with the LiCor Odyssey infrared scanner. The protein amounts were calculated using the accompanying software and Microsoft Excel. A similar procedure was followed for quantitation of Yfh1, Nfs1 and Isd11 proteins in mitochondria.

Pulse–chase labelling of Isu1

Cells were grown in defined medium lacking methionine, restarted into logarithmic phase and allowed to reach a D_{600} of 1.5. For labelling of each time point 40 D_{600} equivalents of cell culture were concentrated to 1 ml and incubated for 10 min at 30 °C in the presence of 10 μl EasyTagEXPRESS^{35}S Protein Labeling mix (11 μCi; PerkinElmer) and 20 μM of a mixture of all amino acids except cysteine and methionine [2]. The labelled cells were recovered and washed once followed by dilution into 20 ml of fresh medium with excess unlabelled methionine and cysteine (0.4 mg/ml). Time points were obtained after chase of 30, 60, 90 and 180 min. Cell pellets were frozen, thawed and subjected to glass bead lysis in buffer containing 50 mM Hepes/KOH (pH 7.5), 150 mM NaCl and protease inhibitors. After brief centrifugation (20 000 ***g*** for 3 min) the supernatants were recovered. The lysate thus obtained was adjusted to 0.5 % Triton X-100 and 10 mM imidazole, and the samples were loaded on to 20 μl of Ni-NTA–agarose beads by tumbling for 1 h at 4 °C. After washing the beads with binding buffer containing 30 mM imidazole, samples were eluted with SDS sample buffer, and eluates were analysed on a 13 % SDS reducing gel run at 200 V for 140 min. Proteins were transferred on to nitrocellulose membranes and radioactivity was visualized using the STORM phosphorimager. The specific signal attributed to radiolabelled Isu1–His_6 (14 kDa) was present only in the pCM184-transformed cells.

Real-time PCR for *ISU1* transcript quantitation

Three separate cultures were prepared for each strain. RNA was extracted by hot phenol buffered at pH 5 [3]. The isolated total RNA was treated with DNase I and cleaned further using an RNAeasy kit (Qiagen). The quality of RNA was assessed by electrophoresis on a formaldehyde gel, and the RNA was quantified using a NanoDrop spectrophotometer. First strand cDNA was synthesized using Superscript II reverse transcriptase (Invitogen) with random p$(dN)_6$ primers. Quantitative PCR was performed using an ABI 7500 System with Taqman methodology. Primers and probes were obtained from Life Technologies. The primer parameters were: *ISU1* (YPL135W), amplicon size 101 bp and ABI Assay ID Sc04171412-s1; and *TAF1* (YGR274C), amplicon size 69 bp and ABI Assay ID Sc04128090-s1. Following UNG activation (2 min at 50 °C) and Taq polymerase activation (10 min at 95 °C), the two-step amplification parameters were 40 cycles of denaturation (15 s at 95 °C) and annealing/elongation (1 min at 50 °C). C_T values were calculated using ABI 7500 System SDS 1.2.3 software with baselines set manually and thresholds set automatically. C_T values were exported to Excel for comparative C_T and statistical analysis [4].

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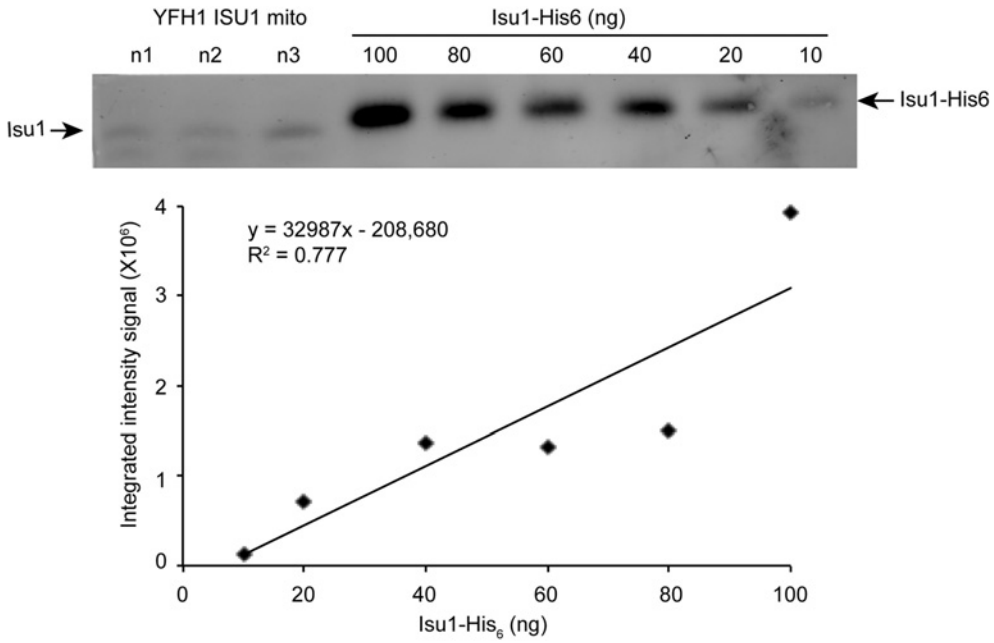

Isu1 (signal)	Isu1 (ng/100 µg mito protein)	mean +/- SD	mean +/- SD (pmols)
n1 166,000	11.4		
n2 103,000	9.5	11.1 ng +/- 1.5	0.8 pmol +/- 0.1
n3 203,000	12.5		

Figure S1 Measurement of Isu1 levels in mitochondria

Mitochondria (100 μg of proteins, three independent preparations) from the wt strain (YFH ISU1) were analysed by SDS/PAGE alongside purified Isu1–His_6. Following transfer on to nitrocellulose membranes, the proteins were immunoblotted with anti-Isu1 polyclonal antibodies followed by a secondary anti-(rabbit IgG)–Alexa Fluor™ 680 antibody. Reactive bands were quantified using a LiCor Odyssey. The standard curve was constructed in Microsoft Excel. The amount of Isu1 present in mitochondria was 11.1 ng or 0.8 pmol per 100 μg of mitochondrial protein.

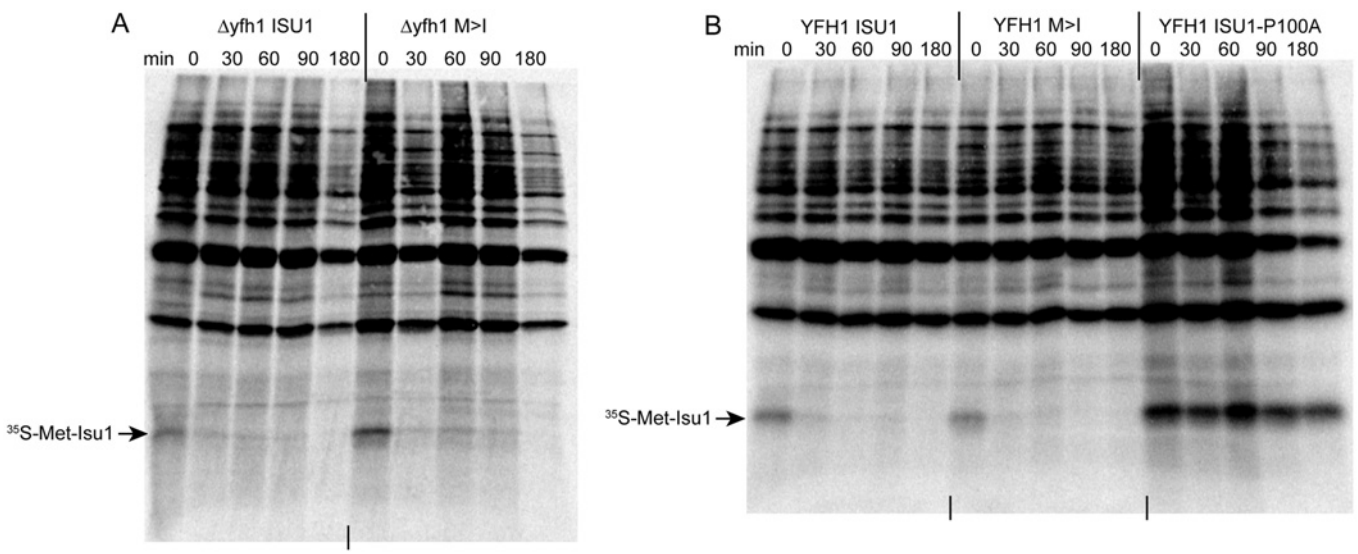

Figure S2 Pulse–chase labelling of Isu1

Cells of the indicated genotypes (Δyfh1 ISU1, Δyfh1 M>I, YFH1 ISU1, YFH1 M>I or YFH1 P100A) were grown in medium lacking methionine and incubated with [^{35}S]methionine for 10 min (pulse). Cells were diluted into fresh medium with unlabelled methionine and cysteine (0.4 mg/ml) for 0, 30, 60, 90 or 180 min (chase). After cell lysis, the His_6-tagged forms of Isu1 were recovered on Ni-NTA–agarose beads and visualized by SDS/PAGE and phosphorimager. The P100A substitution of Isu1 exhibited stabilization as described in [1]. Many background bands were observed, but the radiolabelled form of Isu1 was detected in the bottom quarter of the gel indicated by the arrow (^{35}S-Met-Isu1).

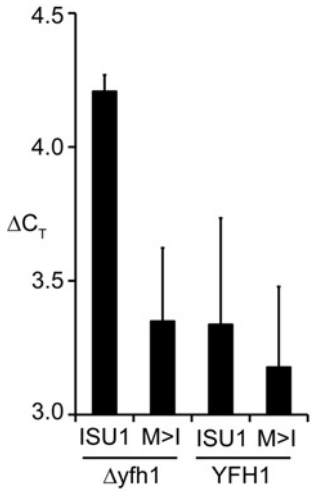

Figure S3 Real-time PCR for *ISU1* transcript levels

RNA was prepared from three independent cultures of each strain (Δyfh1 ISU1, Δyfh1 M>I, YFH1 ISU1 and YFH1 M>I). Quantitative PCR was performed using an ABI 7500 System with Taqman methodology and reagents, and *ISU1* ΔCT was determined for each RNA preparation using *TAF1* (TATA-binding protein-associated factor 1) as an endogenous standard. Results are means ± S.D. ($n = 3$).

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