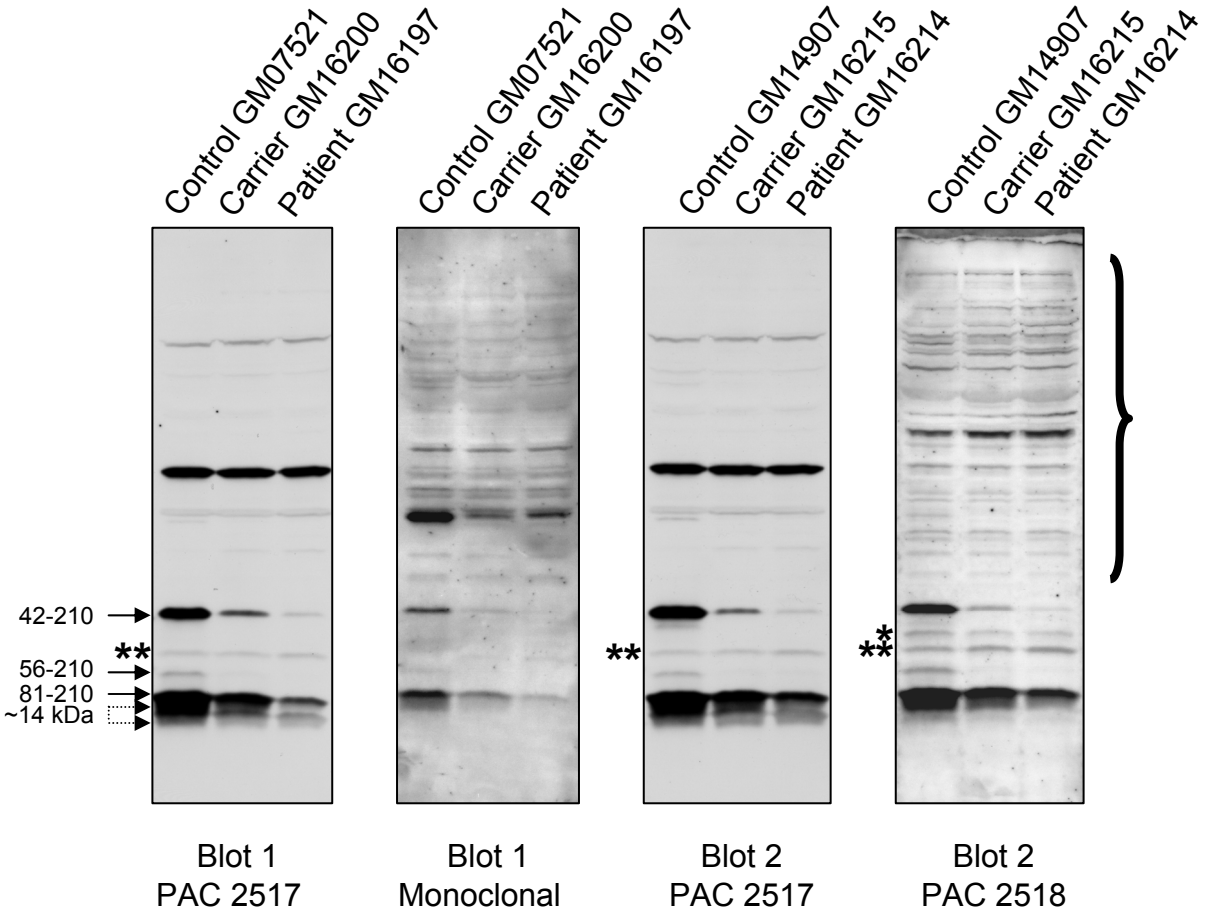
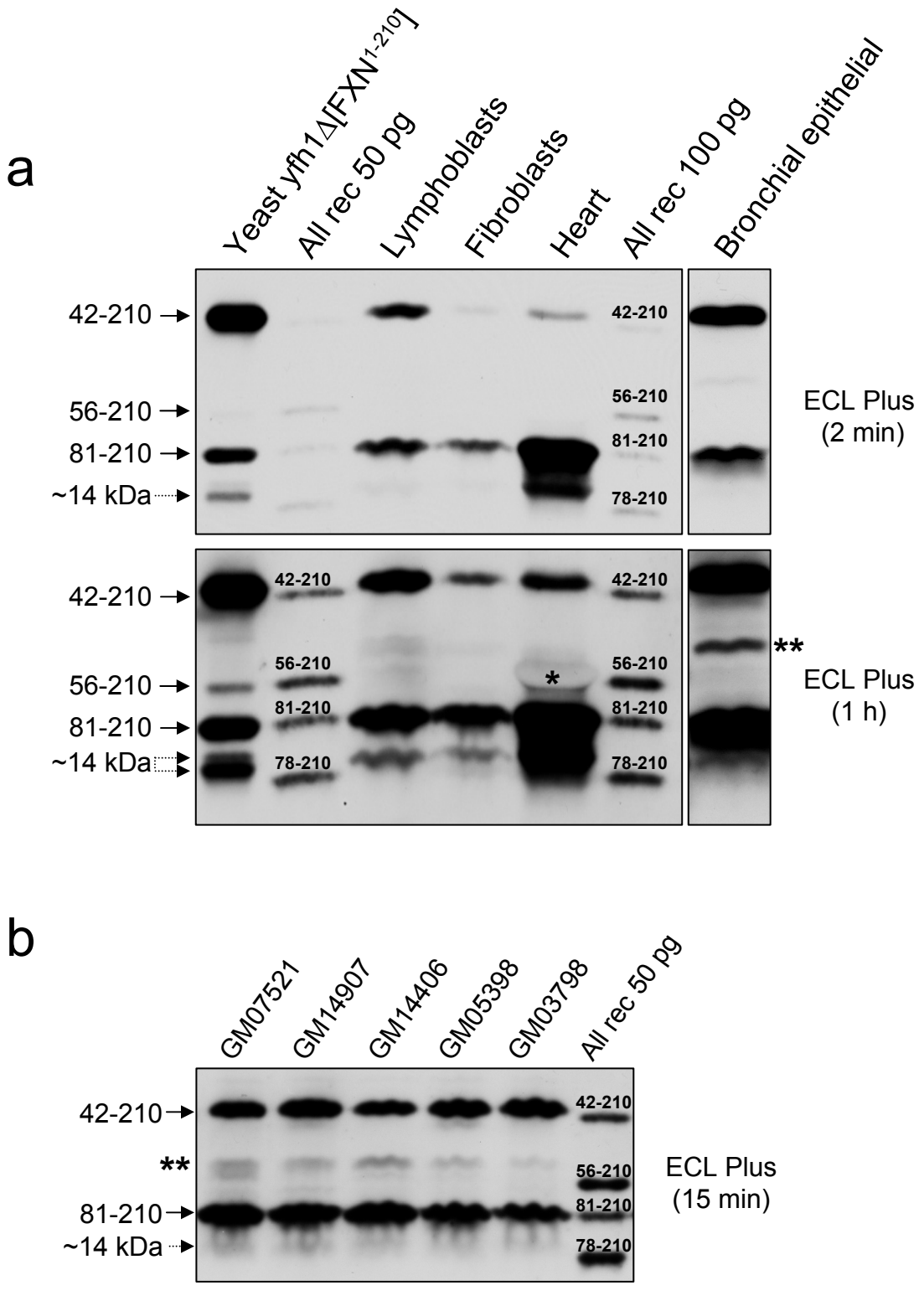


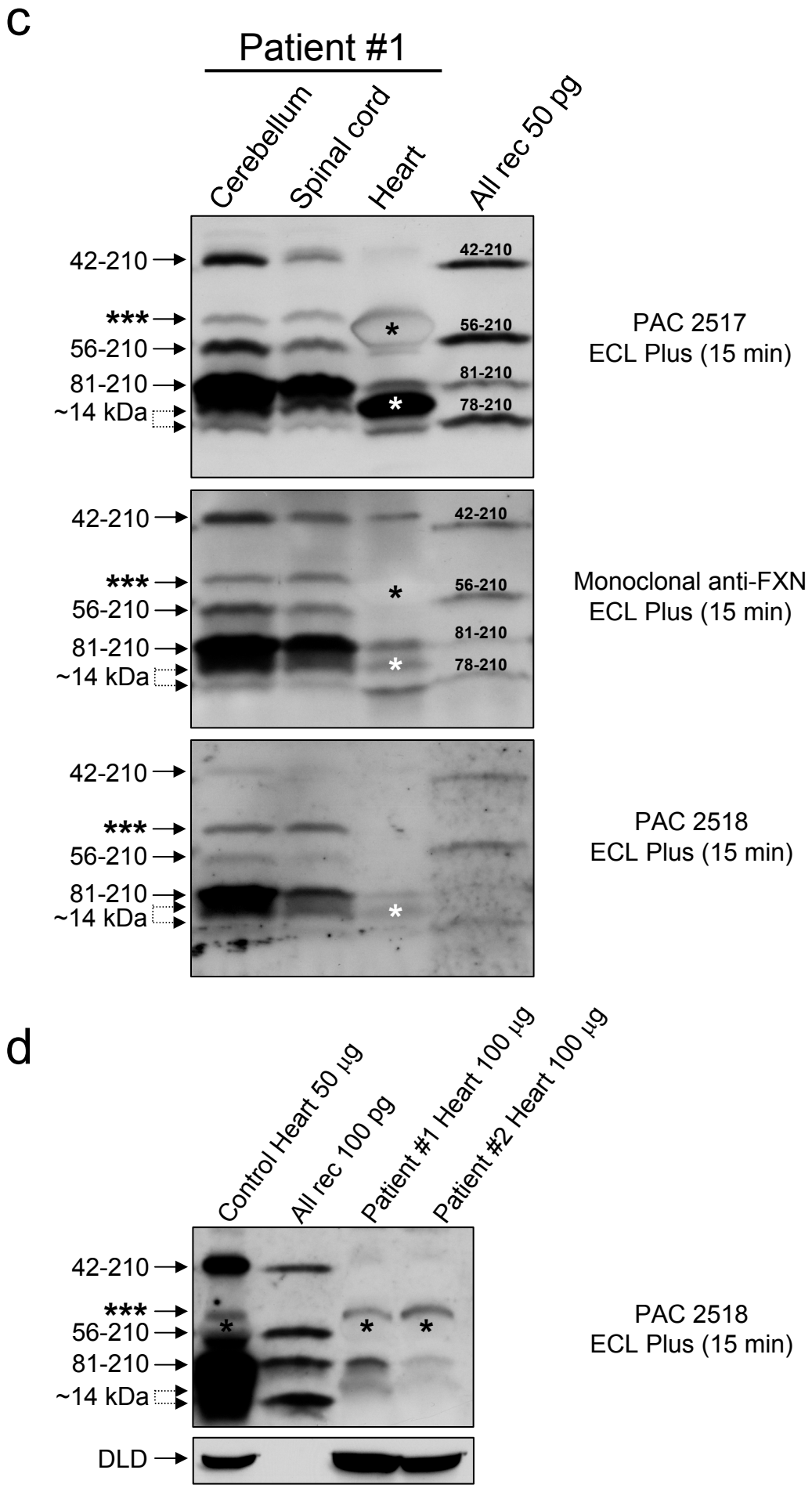
Supplementary Fig. S1

Supplementary Fig. S1 - Characterization of anti-FXN polyclonal antibodies and recombinant FXN proteins used in this study

Rec-FXN$^{56-210}$ was used to produce two polyclonal antisera in rabbits (PAC 2517 and PAC 2518). The two antibodies were validated via western blotting analysis of total extracts from lymphoblastoid cells from two FRDA carriers and their affected children, and from normal controls. The indicated samples were analyzed by western blotting with PAC 2517 (approximately 35 μg of total IgG in 50 ml) as described in the legend of Fig. 1B. Afterwards, the membrane was stripped per manufacturer's protocol (acetonitrile for 10 min, followed by incubation for 30 min at 50 °C in 62.5 mM Tris-HCl buffer, pH 6.7, in the presence of 2% SDS and 100 mM β-mercaptoethanol) and cut in two halves (designated Blot 1 and Blot 2), which were probed with anti-FXN monoclonal antibody (MitoSciences, Inc.) and PAC 2518, respectively (approximately 140 μg of total IgG in 40 ml and 35 μg in 50 ml, respectively). All blots shown in this figure were developed by incubation with ECL Plus Western Blotting Detection Reagents (GE Healthcare) for 15 min. All three antibodies recognized FNX isoforms although with decreasing sensitivities (Monoclonal<PAC 2518<PAC 2517). Each antibody detected a different set of cross-reacting bands in the high-molecular-weight region of the blot (denoted by the bracket). Both PAC 2517 and PAC 2518 detected non-specific bands in the region of the membrane between FXN$^{42-210}$ and FXN$^{81-210}$ (denoted by asterisks), which unlike FXN isoforms were present in equivalent levels in controls, carriers and patients.



Supplementary Fig. S2a,b



Supplementary Fig. S2c-d

Supplementary Fig. S2 - Human frataxin protein profiles in different control cells and tissues

(a) Total cell extracts were prepared from the indicated cell lines and tissues from non-FRDA controls as well as a yeast frataxin knock-out strain expressing the wild type human frataxin precursor (yfh1Δ[$FRDA^{1-210}$]) as described in Experimental Procedures. Fifty μg total protein was loaded per lane (5 μg for yeast) and analyzed by western blotting with PAC 2517 anti-FXN antibody as described in Supplementary Fig. S1. The asterisk denotes an abundant protein present in heart, probably myoglobin, which co-migrates with FXN^{56-210}. The double asterisk denotes a non-specific cross-reacting band as described in Supplementary Fig. S1. **(b)** The FXN protein profile was analyzed by western blotting with PAC 2517 in lymphoblastoid cell lines (Coriell Cell Repository) from 5 healthy individuals: GM07521 (19 years old female); GM14907 (28 years old male); GM14406 (41 years old female); GM05398 (44 years old male); GM03798 (10 years old male). The double asterisk denotes a non-specific cross-reacting band as described above. **(c)** Duplicates of the samples from Patient #1 analyzed in Fig. 2A were analyzed by western blotting with anti-FXN monoclonal antibody. Afterwards, this blot was stripped and reprobed with PAC 2518. The initial blot with PAC 2517 (the same blot shown in Fig. 2A) is shown for reference. The triple asterisk denotes a band in cerebellum and spinal cord, which does not co-migrate with any of the known FXN isoforms; this band was detected with both PAC 2517 and PAC 2518 as well as the monoclonal antibody and its significance is undetermined. The single asterisk denotes an abundant protein present in heart, probably myoglobin, which migrates close to FXN^{56-210}. The white asterisk denotes a band that was detected with PAC 2517 but not with PAC 2518 or the monoclonal antibody. Immunodetection with monoclonal antibody required ~4 fold higher amounts of total purified IgG compared to polyclonal PAC

2517 or PAC 2518 (~140 μg vs. ~35 μg). **(d)** The FXN protein profile was analyzed once again by western blotting with PAC 2518 in a non-FRDA heart sample (the same sample analyzed in Supplementary Fig. S2a) side by side with heart samples from FRDA Patients #1 and #2. The single and triple asterisks are as described in (c) above.

Native FXN42-210

1 **42**

MWTLGRRAVAGLLASPSPAQAQTLTRVPRPAELAPLCGRRGLRTDIDATCTPRR

81

ASSNQRGLNQIWNVKKQSVYLMNLRKSGTLGHPGSLDETTYER/LAEETLDSLAE
FFEDLADKPYTFEDYDVSFGSGVLTVK/LGGDLGTYVINKQTPNKQIWLSSPSSGP
KRYDWTGKNWVYSHDGVSLHELLAAELTKALKTKLDLSSLAYSGKDA

Rec-FXN42-210

42

ARTDIDATCTPRR

81

ASSNQRGLNQIWNVKKQSVYLMNLRKSGTLGHPGSLDETTYER/LAEETLDSLAE
FFEDLADKPYTFEDYDVSFGSGVLTVK/LGGDLGTYVINK/QTPNKQIWLSSPSSG
PKRYDWTGKNWVYSHDGVSLHELLAAELTKALKTKLDLSSLAYSGKDA

Native FXN81-210

1 **42**

MWTLGRRAVAGLLASPSPAQAQTLTRVPRPAELAPLCGRRGLRTDIDATCTPRR

81

ASSNQRGLNQIWNVKKQSVYLMNLRKSGTLGHPGSLDETTYER/LAEETLDSLAE
FFEDLADKPYTFEDYDVSFGSGVLTVK/LGGDLGTYVINKQTPNKQIWLSSPSSGP
KRYDWTGKNWVYSHDGVSLHELLAAELTKALKTKLDLSSLAYSGKDA

Rec-FXN81-210

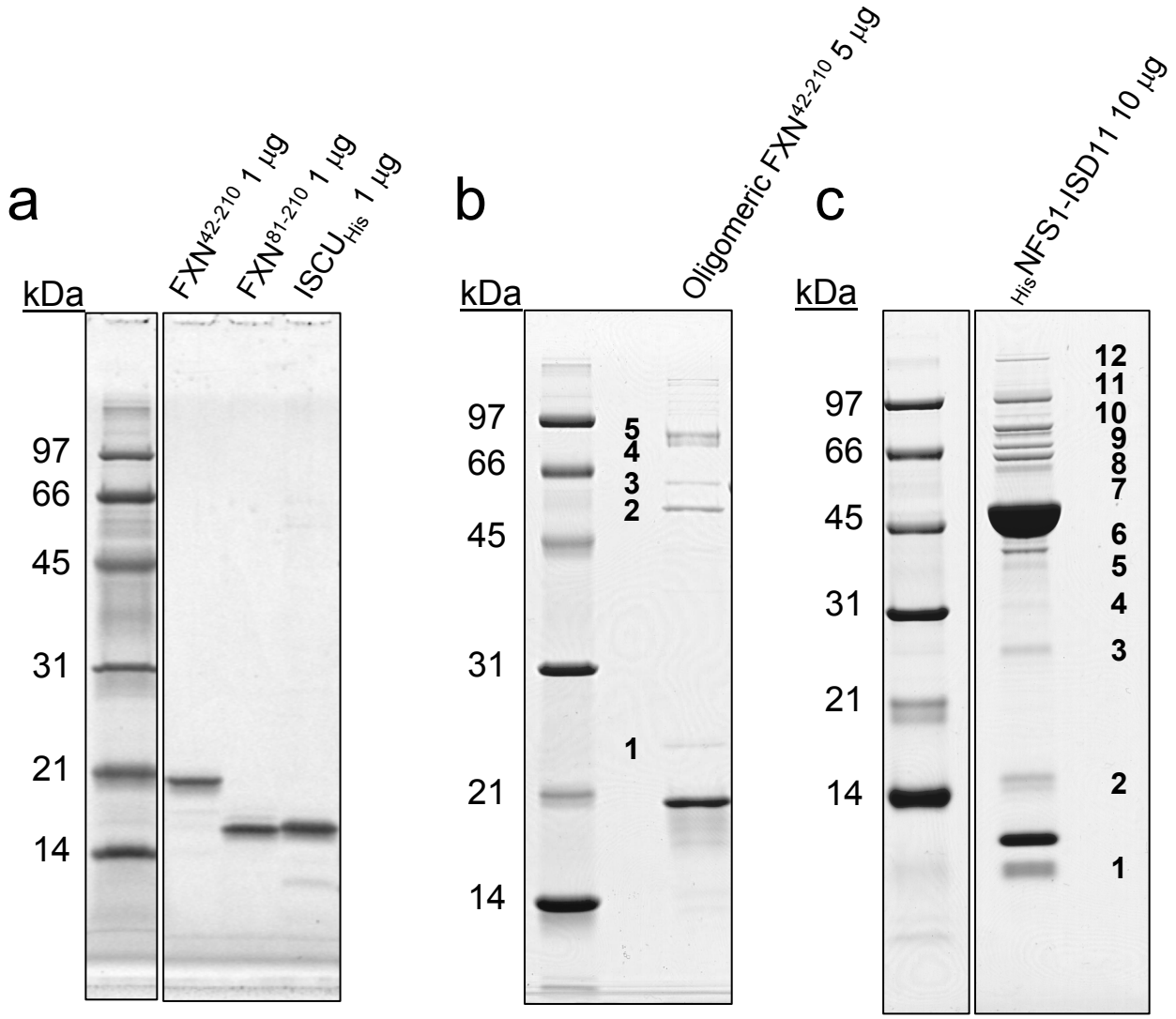
81

SGTLGHPGSLDETTYER/LAEETLDSLAE
FFEDLADKPYTFEDYDVSFGSGVLTVK/LGGDLGTYVINK/QTPNKQIWLSSPSSG
PKRYDWTGKNWVYSHDGVSLHELLAAELTKALKTKLDLSSLAYSGKDA

Supplementary Fig. S3

Supplementary Fig. S3 - Peptide mass finger-printing analysis of native FXN^{42-210} and FXN^{81-210}

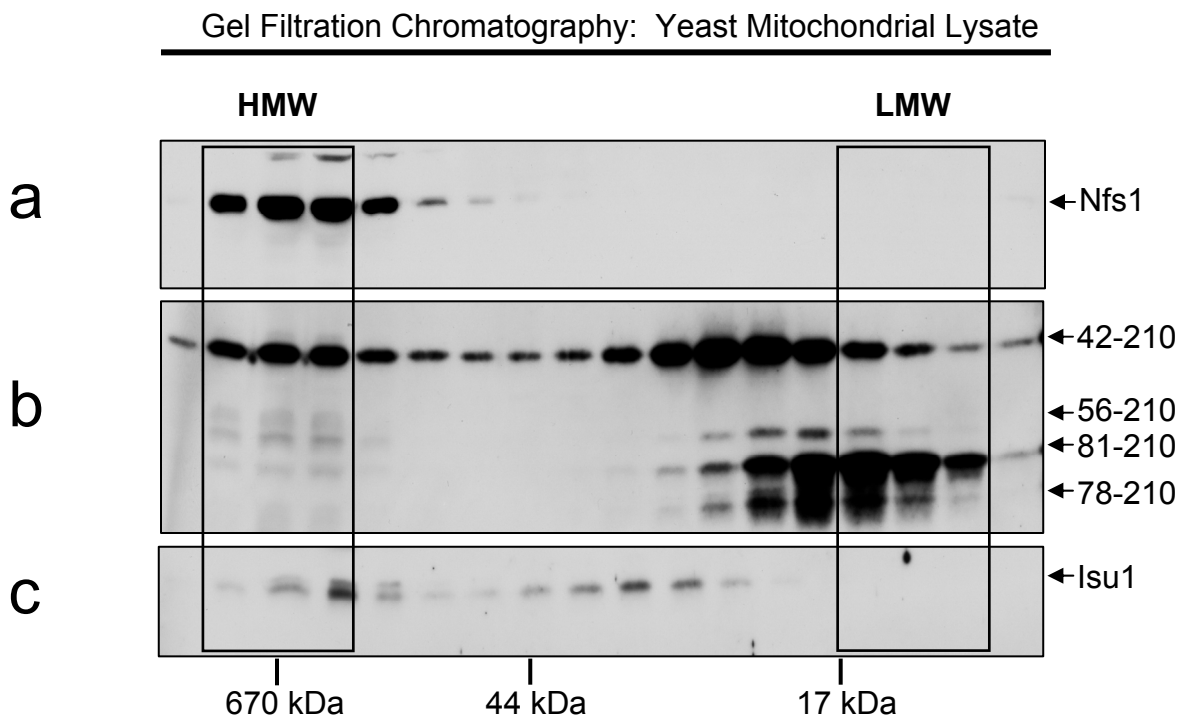
Native FXN proteins were isolated by immunoprecipitation and analyzed by in-gel trypsin digestion followed by nanoLC-MS/MS with hybrid orbitrap/linear ion trap mass spectrometry and peptide mass finger-printing, as described in detail in Supplementary Methods. The corresponding recombinant proteins were analyzed in parallel. The tryptic peptides identified for each protein are highlighted in yellow; contiguous peptides are separated by slashes. Note how the corresponding native (unknown N-terminus) and recombinant (known N-terminus) proteins yielded overlapping peptide profiles except for greater coverage in the C-terminal regions of the recombinant proteins. Three N-terminal peptides that conclusively distinguish FXN^{42-210} from FXN^{81-210} are underlined. In the case of native FXN^{42-210}, the close proximity of arginine 40 to arginine 43 does not allow to exclude that an additional very short peptide (GLR) was present upstream of peptide TDIDATCTPR. Together, these data allow mapping the N-terminus of native FXN^{42-210} between residues 41-43.

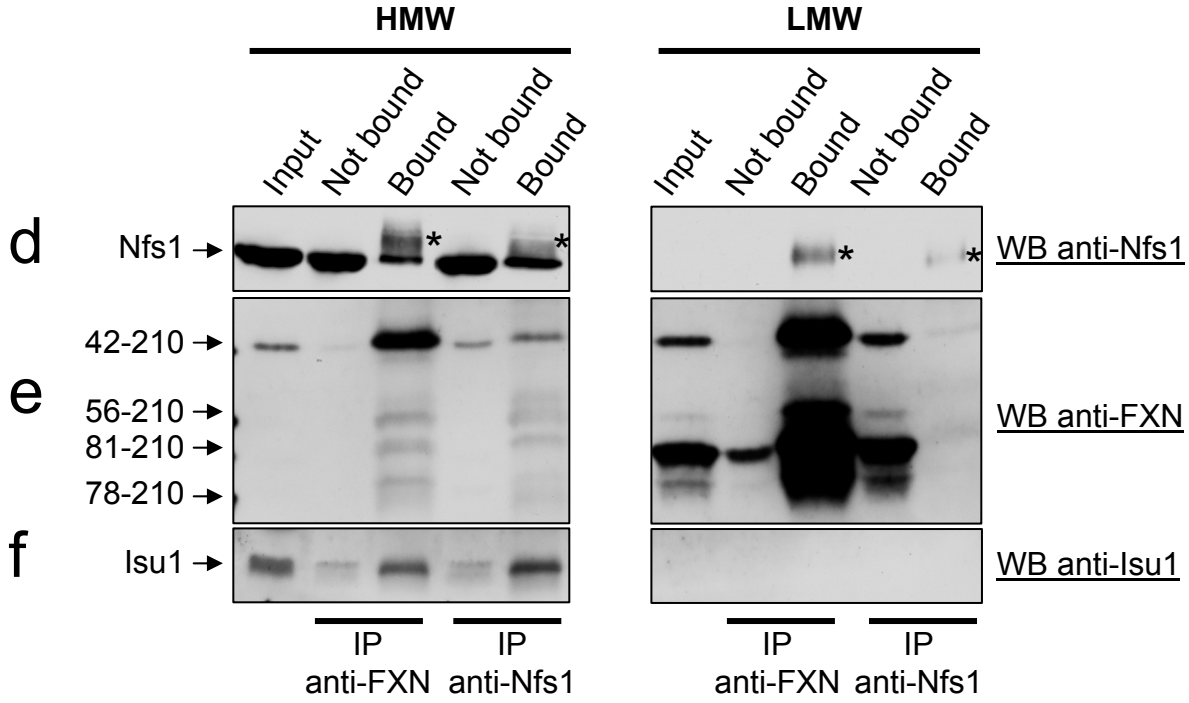


Supplementary Fig. S4

Supplementary Fig. S4 - Recombinant proteins used in this study

Proteins were expressed and purified as described in Experimental Procedures and Supplementary Methods. **(a)** One μg each of the indicated proteins was analyzed by 15% SDS-PAGE (Tris-HCl, Criterion precast gel, Bio-Rad) and protein staining with SYPRO Orange. **(b)** Five μg of purified oligomeric rec-FXN$^{42-210}$ were analyzed by 15% SDS-PAGE (Tris-HCl, Criterion precast gel, Bio-Rad) and protein staining with Coomassie blue. Several weak bands were present in addition to the predominant FXN$^{42-210}$ band. Indicated bands were excised from the gel, mixed together, digested with trypsin, and analyzed by nano-liquid chromatography/tandem mass spectrometry at the Mayo Proteomics Research Center. Peptide mass fingerprinting was used to match MS/MS spectra against the proteins in the Swiss-Prot database. This analysis revealed that the oligomeric rec-FXN$^{42-210}$ preparation contained *E. coli* proteins not implicated in iron metabolism or iron-sulfur cluster synthesis. **(c)** Ten μg of purified human $_{His}$NFS1-ISD11 complex were analyzed by 18% SDS-PAGE (Tris-HCl, Criterion precast gel, Bio-Rad) and protein staining with Coomassie blue. Several weak bands were present in addition to the predominant NFS1 and ISD11 bands. Indicated bands were excised from the gel, and subjected to MS/MS analysis as described above. This analysis revealed the presence of the *E. coli* NifU-like protein (P0ACD6; 19 peptides) and other *E. coli* proteins not implicated in iron metabolism or iron-sulfur cluster synthesis.

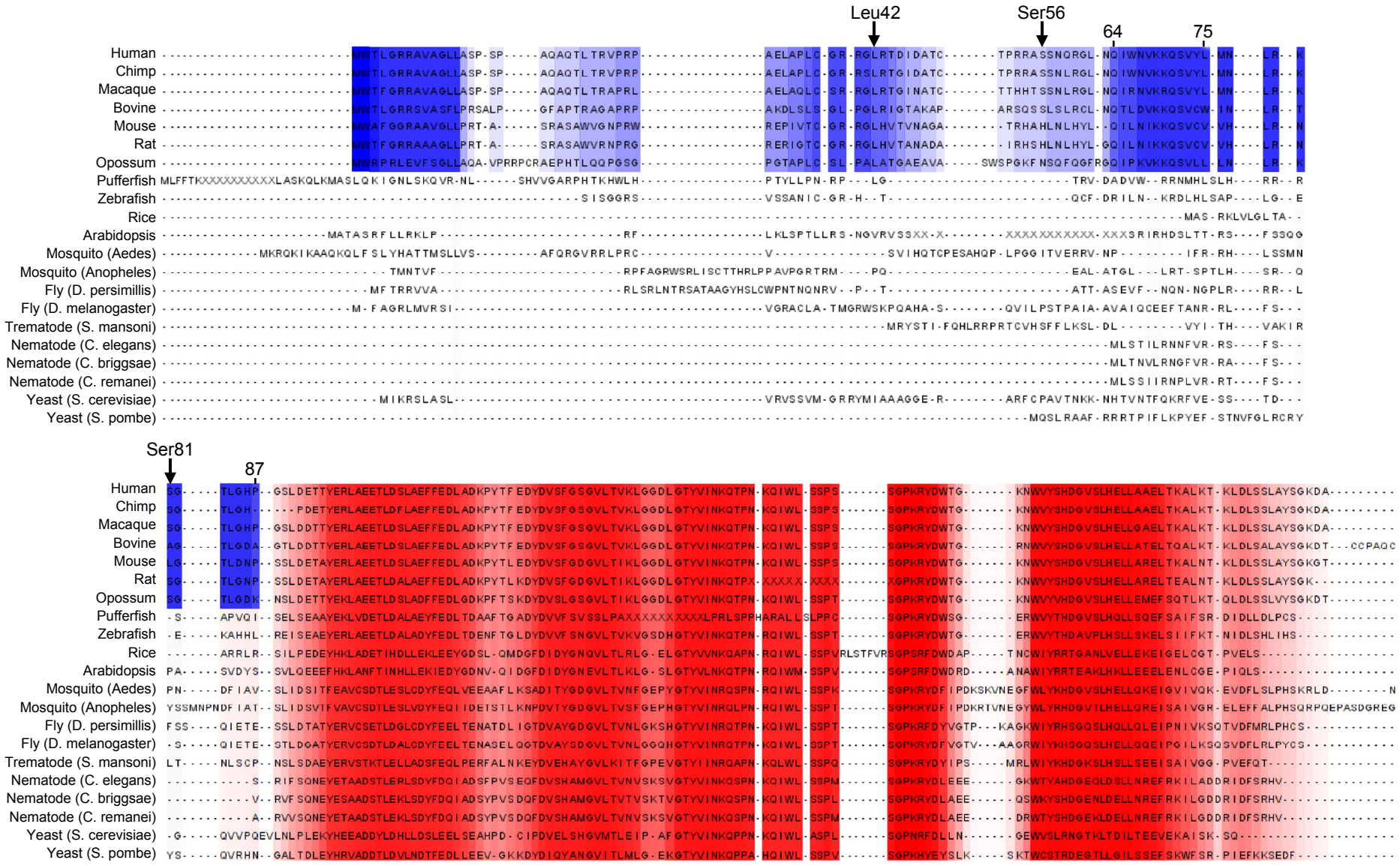




Supplementary Fig. S5

Supplementary Fig. S5 - Interactions of native FXN isoforms in yeast mitochondrial lysate

Yeast cells lacking endogenous Yfh1 and expressing FXN^{1-210} (yfh1Δ[$FRDA^{1-210}$]) were grown for ~20 h at 30 °C in rich medium with galactose as the carbon source, after which mitochondria were isolated as described in Supplementary Methods. The soluble mitochondrial fraction (~5 mg total protein) was analyzed on a Superdex 75 column. Fractions comprising the entire molecular mass fractionation range of the column were analyzed by western blotting with **(a)** anti-Nfs1, **(b)** anti-FXN or **(c)** anti-Isu1 antibody [(1) and this study]. The high- and low-molecular-weight fractions (HMW and LMW boxes) were pooled; one half of each sample was incubated overnight with anti-FXN antibodies, and the second half with anti-Nfs1 antibodies (previously immobilized on Protein A Magnetic beads from New England BioLabs by crosslinking with dimethyl pimelidate dihydrochloride) in 10 mM HEPES-KOH, pH 7.3, 100 mM NaCl, 0.01% Triton X-100, with protease inhibitors. The beads were washed 3 times with 1 ml of buffer, and bound proteins were eluted with 80 µl of 100 mM Glycine, pH 2.5. Co-eluted proteins were analyzed by western blotting with **(d)** anti-Nfs1, **(e)** anti-FXN or **(f)** anti-Isu1 antibody. The band denoted by an asterisk is the heavy IgG chain. No cross reactivity was detected between the anti-Nfs1 antibody and FXN isoforms in the low molecular weight fractions (LMW). Higher levels of FXN are detected in individual HMW fractions (panel b) relative to the HMW fraction pool (panel e; *Input* and *Not bound*) because ~4 times less total protein was loaded in the latter analysis.


Leu42
Ser56
64
75
Ser81
87
Human
Chimp
Macaque
Bovine
Mouse
Rat
Opossum
Pufferfish
Zebrafish
Rice
Arabidopsis
Mosquito (Aedes)
Mosquito (Anopheles)
Fly (D. persimillis)
Fly (D. melanogaster)
Trematode (S. mansoni)
Nematode (C. elegans)
Nematode (C. briggsae)
Nematode (C. remanei)
Yeast (S. cerevisiae)
Yeast (S. pombe)

Supplementary Fig. S6 - N-terminal sequence conservation among mammalian frataxin proteins

The sequence alignment of frataxin homologues was created with ProbAlign (2), and sequences were taken from a TreeFam phylogenetic tree of genes with fully sequenced genomes (3). The alignment reveals conservation of residues 81-210 across a broad range of eukaryotes (shown in red) and conservation of preceding residues only among mammals (shown in blue). Color density indicates the level of conservation of a residue. *Leu42*, *Ser56*, and *Ser81* indicate the N-termini of FXN^{42-210}, FXN^{56-210}, and FXN^{81-210}, respectively; *64-87* highlights a putative oligomerization domain that may enable mammalian frataxins to achieve iron-independent oligomerization.

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