Supporting Information

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SI Materials and Methods

Confocal Microscopy. Anti-FLAG (Sigma) and anti-Mito (ImmunoVision) antibodies were used for immunofluorescence assay. Cells were visualized by using Nikon confocal instruments.

Construction of Mfrn Chimeras. Mfrn chimeras Ch1 and Ch2 were constructed by using overlapping PCR. Chimera 1 consists of the first 50 amino acid residues of Mfrn1 and amino acid residues 51–364 of Mfrn2. Chimera 2 consists of the first 25 amino acid residues of Mfrn1 and amino acid residues 26–364 of Mfrn2.

cRNA Microinjections and Genotyping. We subcloned BT-Mfrn1 and Mfrn chimera cDNA clones into the pCS2+ vector and prepared 5′-capped cRNA by using the SP6 mMessage mMachine kit (Ambion) for expression in zebrafish. Fertilized eggs from *frascati* (frs^{tq223}) heterozygous pairs were injected at the one- to two-cell stage with roughly 100 pg of cRNA and phenol red dye as a tracer. Genotyping of all zebrafish embryos participating in the microinjection studies was performed with allele-specific oligonucleotides (1) as described previously (2).

1. Farr CJ, et al. (1988) Analysis of RAS gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes. *Proc Natl Acad Sci USA* 85:1629–1633.
2. Shaw GC, et al. (2006) Mitoferrin is essential for erythroid iron assimilation. *Nature* 440:96–100.

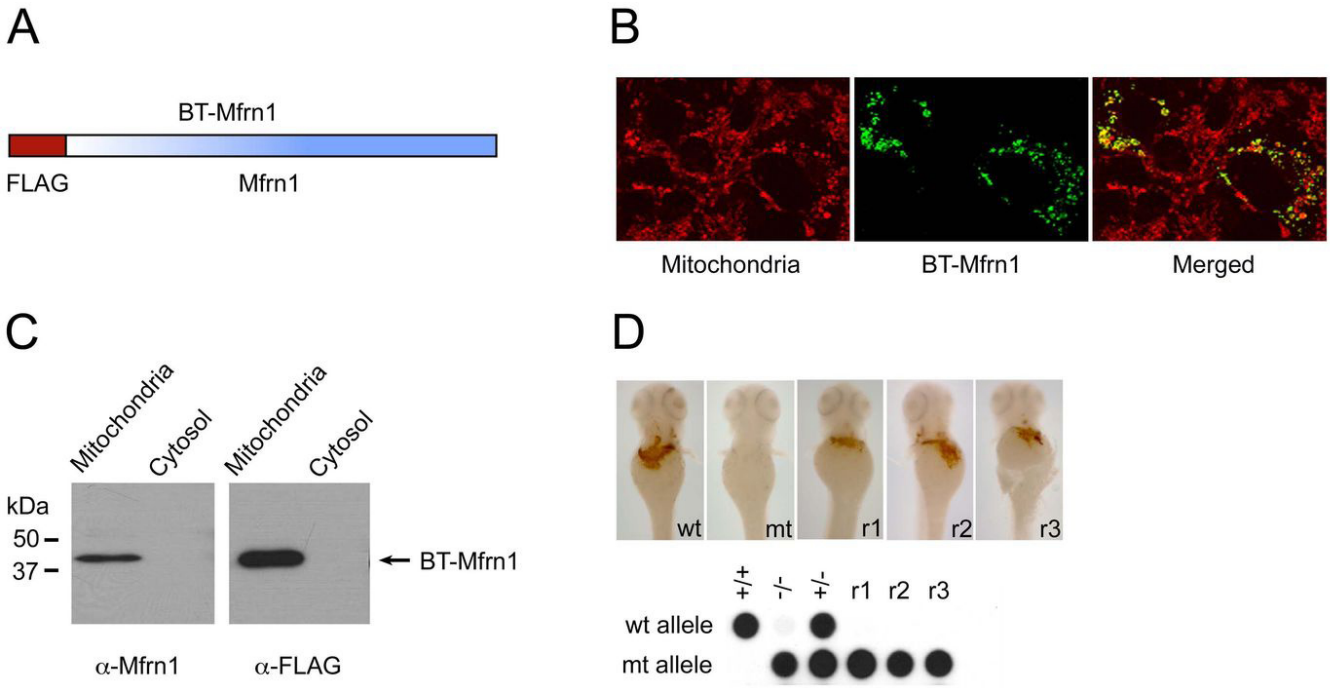

Fig. S1. BT-Mfrn1 properly targets to the mitochondrial compartment and complements anemia of *frs* mutants, and so it is validated as a functional bait for affinity purification. (*A*) Mouse Mfrn1 was FLAG-tagged at its N terminus as bait for affinity purification. The FLAG-tagged construct was referred as BT-Mfrn1. (*B*) Immunolocalization of BT-Mfrn1 protein to mitochondria of transfected COS7 cells. Fluorescence confocal images were obtained from immunostained resident mitochondrial proteins (red) and BT-Mfrn1 (green). Colocalized expression of BT-Mfrn1 in the mitochondria is indicated by the yellow signal. (*C*) BT-Mfrn1 proteins properly targeted to the mitochondrial fraction. Mitochondrial and cytosolic fractions were isolated from BT-Mfrn1-transfected COS7 cells and immunoblotted by anti-Mfrn1 and anti-FLAG antibodies, respectively. (*D*) Expression of BT-Mfrn1 cRNA rescued the anemia of *frascati* (*frs*^{tq223}) embryos. Control wild-type (wt), *frs* mutant (mt), and rescued *frs* embryos (r1–3) were stained with *o*-dianisidine to detect hemoglobinized cells. Control wild-type, mutant, heterozygote, and rescued *frs* (r1–3) embryos were genotyped. Genotyping results in the *Right Lower* confirmed that these three putative rescued embryos (r1–3) are mutants.

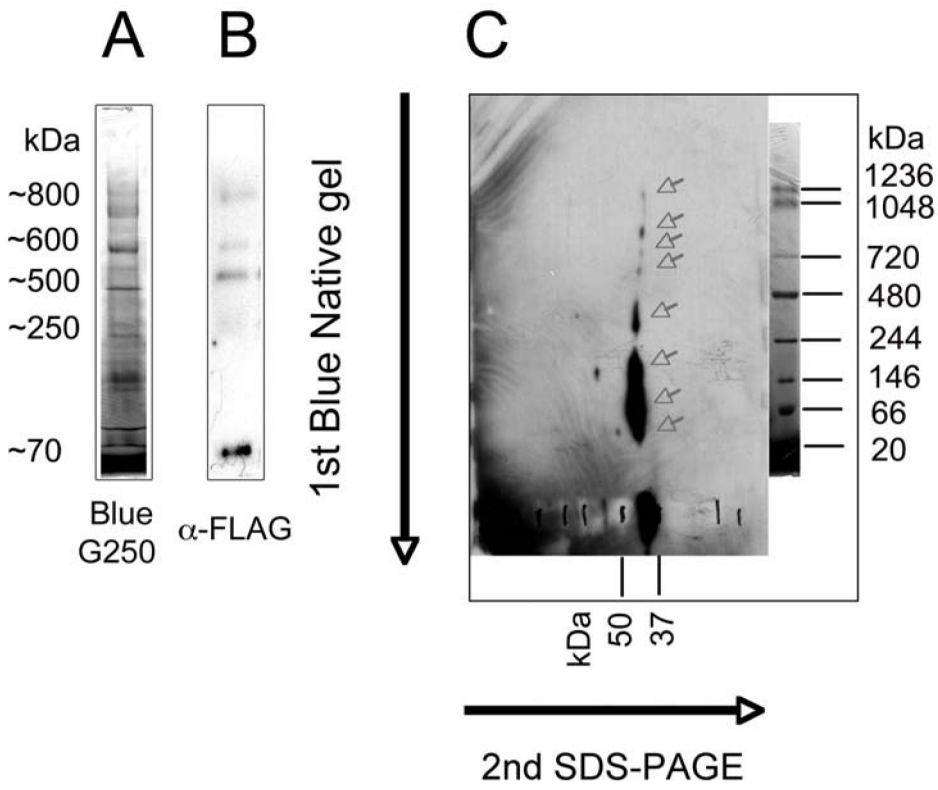

Fig. S2. BT-Mfrn1 forms higher-order oligomeric complexes with other mitochondrial proteins. (*A*) Mitochondria were solubilized with 1% Nonidet P-40 and subjected to separation on a neutral-pH, nondenaturing Blue Native gel with Coomassie G250 dye. (*B*) Western blot analysis with anti-FLAG antisera shows at least four higher-order complexes. (*C*) When the entire lane from Blue Native gel (lane A) was subjected to a second-dimensional separation in an SDS/PAGE, followed by Western blot analysis, additional protein complexes containing BT-Mfrn1 were uncovered (arrows). Monomeric and homodimeric BT-Mfrn1 are the most abundant (lowest 2 arrows).

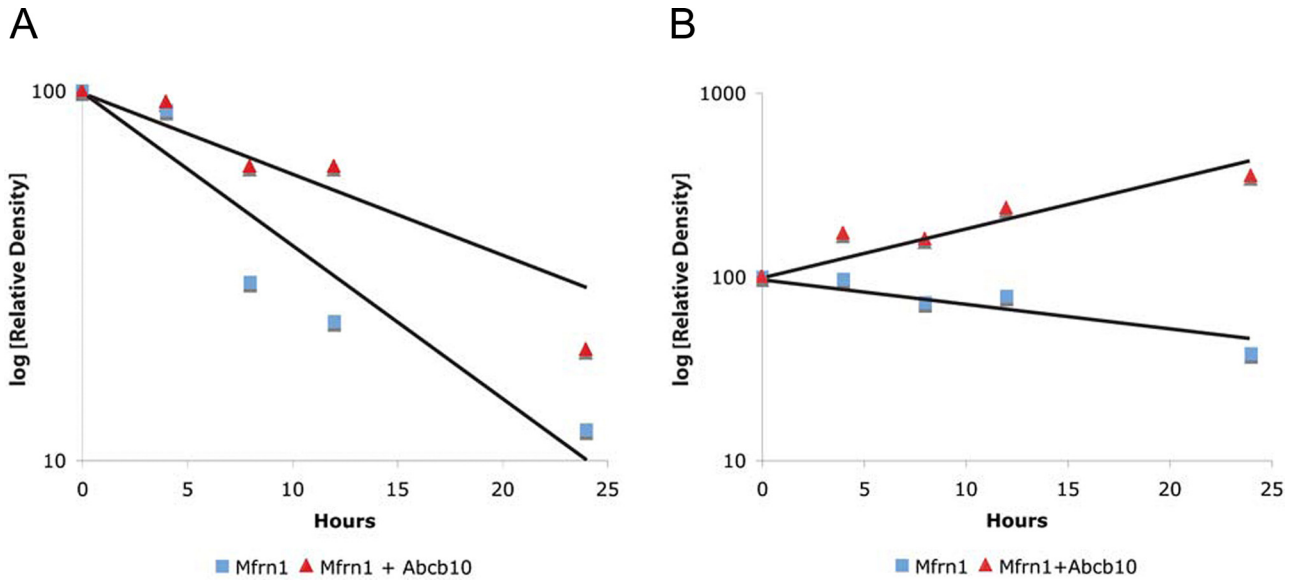

Fig. S3. Quantitation of pulse–chase assay and steady-state Western blot analysis of Mfrn1 stability. (*A*) Quantitation figure for Fig. 2A pulse–chase assay of Mfrn1. (*B*) Quantitation figure for Fig. 2B Western blot analysis of steady-state Mfrn1 proteins after normalization to the signal of the control protein Hsp60. The quantifications of the autoradiographic bands using Quantity One software (Bio-Rad) are plotted as the relative density compared with that at time 0 h. Both figures show Abcb10 stabilizes Mfrn1 protein half-life.

Table S1. Abcb10 was identified as an interacting protein of Mfrn1 by affinity purification and MS

	No. of peptides identified			
Protein name	Experiment 1	Experiment 2	Experiment 3	Total
Mfrn1	24	24	19	67
Abcb10	7	8	9	24
Aralar1	11	10	7	28
OGCP	6	2	2	10

Mfrn1 affinity-purified samples were analyzed by MS in triplicate analyses. Abcb10 protein was consistently identified as a Mfrn1-interacting protein with the coverage of 24 total peptides. No peptide of Abcb10 protein was recovered in three control samples.