Supporting Information

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SI Materials and Methods

Plasmid Construction. To generate *mCOX2* and *RP-mCOX2* constructs, the *mCOX2* cDNA was PCR amplified from mouse mtDNA, using the primers 5′-GAGAAGATCTATGGCCTAACCCATTCCAAC-3′ or 5′-GAGAAGATCTATGTCTCCCTGAGCTTCAGGGAGGATGGCCTAACCCATTCCAAC-3′ and 5′-CCGCCGCTCGAGTTAAATTATTGAAGCAGATCAGTTTTCGA-3′, and inserted into the PQsuper expression vector. To generate *hCOX2* and *RP-hCOX2* constructs, *hCOX2* cDNA was PCR amplified from human mtDNA, using the primers 5′-CGGCCGCACCGGTATGGCACATGCAGCGC-3′ or 5′-CGGCCGCACCGGTATGTCTCCCTGAGCTTCAGGGAGGATGGCACATGCAGCGC-3′ and 5′-CGCGGATCCCTATAGGGTAAATACGGGC-3′, and inserted into the PQCXIP expression vector. To generate *Leu* and *RPLeu* constructs, $tRNA_{UUR}^{Leu}$ with 5′ and 3′ presequences was PCR amplified from human mtDNA, using the primers 5′-CGGCCGCACCGGTATGGAGAAATAAGGCCTACTTCAC-3′ or 5′-CGGCCGCACCGGTATGTCTCCCTGAGCTTCAGGGAGGGAGAAATAAGGCCTACTTCAC-3′ and 5′-CGCGGATCCCGTTCGGTAAGCATTAGG-3′, and inserted into the PQCXIP expression vector. To generate *Lys* and *RPLys* constructs, $tRNA_{AAA}^{Lys}$ with 5′ and 3′ presequences was PCR amplified from human mtDNA, using the primers 5′-CGGCCGCACCGGTATGCATGCATGCCCATCGTCCTAG-3′ or 5′-CGGCCGCACCGGTATGTCTCCCTGAGCTTCAGGGAGGCATGCCCATCGTCCTAG-3′ and 5′-CGCGGATCCGGGTGATGAGGAATAGTG-3′, and inserted into the PQCXIP expression vector. To make *LeuA* and *RPLeuA* constructs, the reverse primer for *Leu* and *RPLeu* was replaced with 5′-CCGCCGCTCGAGGGGTTTGTTAAGAAGAGGAATTGAACC-3′. To make *LysA* and *RPLysA* constructs, the reverse primer for *Lys* and *RPLys* was replaced with 5′-CCGCCGCTCGAGAGAGCCCACTGTAAAGAGGTGTTG-3′. To make *LeuM*, *RPLeuM*, *LeuAM*, *RPLeuAM*, *LysM*, *RPLysM*, *LysAM*, and *RPLysAM* constructs the 3′-UTR of *MRPS12* was PCR amplified from human gDNA with primers 5′-CGCGGATCCCATCAGAAGAAGTGACGGCTG-3′ and 5′-CCGGAATTCTAGTGGTCCTGATGGAA-3′ and inserted into *Leu*, *RPLeu*, *LeuA*, *RPLeuA*, *Lys*, *RPLys*, *LysA*, and *RPLysA* constructs.

Isolation of mtRNA and mtDNA. Mitochondria (1 mg/mL) were isolated using mannitol-sucrose buffer (0.225 M mannitol, 75 mM sucrose, 5 mM Hepes, pH 7.4). Mitochondria were treated with 25 μg/mL of micrococcal nuclease S7 in nuclease buffer (0.6 M sorbitol, 20 mM $MgCl_2$, 5 mM $CaCl_2$, 20 mM Tris, pH 8.0) supplemented with digitonin (0.1 mg/mg protein) for 30 min at 27 °C. The reaction was stopped by addition of 20 mM EGTA. Mitochondria were collected and solubilized in SDS buffer (100 mM NaCl, 1% SDS, 20 mM Tris, pH 7.4) at 65 °C for 5 min. RNA was purified using TRIzol reagent and treated with RNase-free DNase I (Roche) for 1 h at 37 °C. DNase I was inactivated by heating at 65 °C for 10 min. Phenol-chloroform extractions were used for DNA purification from the mitochondrial lysates.

RT-PCR. RNA was extracted using TRIzol and first-strand cDNA synthesized, using the AccessQuick RT-PCR kit (Promega) and a specific reverse primer. Avian myeloblastosis virus reverse transcriptase was denatured at 95 °C for 5 min. Specific forward primers were added and PCR amplifications were carried out in the same tubes.

Western Blot. Mitochondrial lysates (50 μg) were resolved by SDS/PAGE, transferred to nitrocellulose membranes, and incubated for 1 h in 5% milk TBS-T and for 1 h with primary antibodies in 5% milk TBS-T. Antibodies included α-PNPASE (1:5,000) (1, 2), α-COX2 (1:1,000) (Santa Cruz Biotechnology), α-ND6 (1:1,000) (Santa Cruz Biotechnology), and α-TOMM40 (1:1,000). ECL Reagent (GE Healthcare) was used for chemiluminescent detection.

In Vitro Transcription. RNAs were synthesized using the megascript kit (Ambion). For radiolabeled RNA synthesis, [^{32}P]CTP (MP Biomedical) was incorporated. The RNAs were purified using TRIzol reagent.

RNA Import Assay. In vitro RNA import assays were performed as previously described (3) in a 200-μL volume containing 0.5 μg RNA, 100 μg mitochondria, 0.225 M mannitol, 0.075 M sucrose, 2 mM KH_2PO_4, 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 5 mM L-methionine, 1 mg/mL BSA, 5 mM ATP, 2 mM DTT, 20 mM succinate, and 50 mM Hepes, pH 7.1, at room temperature (RT) for 10 min. Mitochondria were pelleted at 11,000 × *g* for 5 min and washed once with wash buffer (0.225 M mannitol, 0.075 M sucrose, 20 mM Tris, pH 8.0). Mitochondria were spun again and resuspended in 200 μL nuclease buffer containing 25 μg/mL of micrococcal nuclease S7 and incubated for 30 min at 27 °C. Mitochondria were collected and solubilized in SDS buffer at 65 °C for 5 min. RNA was purified using TRIzol reagent and analyzed by SDS/PAGE and autoradiography.

In Organello Translation. Following a 2-min incubation at RT with the in vitro synthesized tRNAs in the in vitro RNA import buffer, 250 μg/mL of rNTP was added and the samples were incubated at RT for an extra 5 min. RNase A was added to digest the non-imported RNA at 27 °C for 20 min. The mitochondria were then pelleted at 11,000 × *g* for 5 min and resuspended in translation buffer containing 0.225 M mannitol, 0.075 M sucrose, 100 mM KCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 10 mM Tris, 10 mM K_2HPO_4, pH 7.4, 10 mM glutamate, 2.5 mM malate, 1 mM ADP, 1 mg/mL fatty acid free BSA, 100 μg/mL emetine, 10 μM of each amino acid, and 100 μCi of PRO-MIX L-[^{35}S]methionine and cysteine (MP Biomedical) and incubated at 37 °C for 30 min. Translation products were analyzed by 14% SDS/PAGE and autoradiography.

In Vivo Mitochondrial Translation. In vivo mitochondrial translation assays were performed as previously described (4). Semi-confluent cells (0.5×10^6) were incubated in DMEM with 10% dialyzed FCS lacking methionine and cysteine and supplemented with 0.2 mg/mL emetine for 5 min at 37 °C. A total of 200 μCi/mL of PRO-MIX L-[^{35}S]methionine and cysteine (MP Biomedical) was added, followed by a 30-min incubation at 37 °C. Cells were PBS washed, lysed, and analyzed by 14% SDS/PAGE and autoradiography.

Oxygen Consumption. Cells were seeded at 50,000 cells per well in an XF24 Extracellular Flux Analyzer cell culture plate (Seahorse Bioscience) and incubated in the 37 °C incubator with 5% CO_2 for 24 h. The oxygen consumption rate was measured using the XF24 Extracellular Flux Analyzer, using protocols supplied by the manufacturer.

Sodium Carbonate Extraction. A total of 50 μg of mitochondria was pelleted and then resuspended in 200 μL of 0.1 M Na_2CO_3 and incubated on ice for 15 min. The membrane was pelleted at 100,000 × *g* for 15 min at 4 °C. The proteins in the pellet and the supernatant were precipitated with 10% trichloroacetic acid, separated on 12% SDS/PAGE, and analyzed with α-poly-

nucleotide phosphorylase (α-PNPASE) (1:5,000), α-cytochrome oxidase 2 (α-COX2) (1:1,000), and α-TOMM40 (1:1,000).

Complex I Assembly and Activity Assay. A total of 50 μg of mitochondria was separated on a 6–16% continuous gradient blue-native gel. Gels were incubated with 2 mM Tris-HCl, pH 7.4, 0.1 mg/mL NADH, and 2.5 mg/mL nitrotetrazolium blue overnight at RT. Gels were then washed with distilled water and fixed with 50% methanol and 10% acetic acid for 1 h (5).

1. Chen HW, et al. (2006) Mammalian polynucleotide phosphorylase is an intermembrane space RNase that maintains mitochondrial homeostasis. *Mol Cell Biol* 26:8475–8487.
2. Rainey RN, et al. (2006) A new function in translocation for the mitochondrial i-AAA protease Yme1: Import of polynucleotide phosphorylase into the intermembrane space. *Mol Cell Biol* 26:8488–8497.
3. Wang G, et al. (2010) PNPASE regulates RNA import into mitochondria. *Cell* 142:456–467.
4. Hao H, Moraes CT (1996) Functional and molecular mitochondrial abnormalities associated with a C —> T transition at position 3256 of the human mitochondrial genome. The effects of a pathogenic mitochondrial tRNA point mutation in organelle translation and RNA processing. *J Biol Chem* 271:2347–2352.
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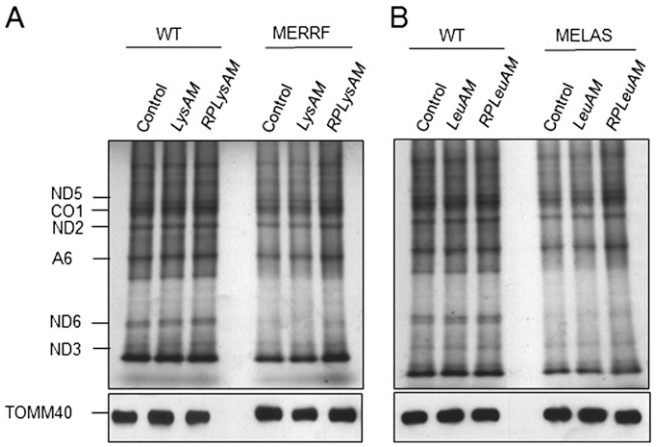

Fig. S1. Analysis of mitochondrial translation in control experiments for Fig. 2. (*A*) Mitochondrial (mt)-tRNA precursors with or without *RP* were imported into isolated WT or myoclonic epilepsy with ragged red fibers (MERRF) mitochondria from cybrid lines for 2 min at RT, followed by an additional 5 min with rNTP supplementation. Following RNase A digestion of the nonimported mt-tRNA, mitochondria were pelleted and resuspended in an in organello translation buffer with radiolabeled methionine and cysteine for 30 min at 37 °C. Mitochondrial translated proteins were separated by SDS/PAGE and visualized by autoradiography. (*B*) As in *A*, with mitochondria from the mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) cell line.

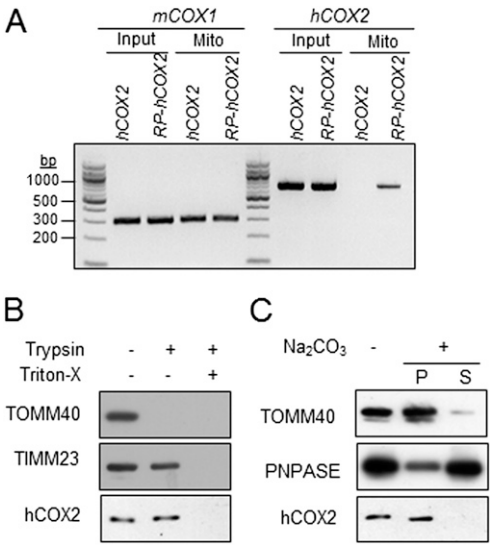

Fig. S2. (*A*) The *RP-hCOX2* transcript but not the *hCOX2* transcript is imported into mitochondria when stably expressed in mouse embryonic fibroblasts (MEFs). Mitochondria were isolated from MEFs stably expressing *hCOX2* or *RP-hCOX2*. Mitoplasts were generated by digitonin treatment, followed by nuclease addition to degrade RNA localized outside of the matrix. RNA was then isolated from total cell lysates (Input, without nuclease treatment) or from nuclease-treated mitoplasts (Mito) and analyzed by primer-specific RT-PCR. *mCOX1* is a primer set for the mouse *COX2* transcript and is included as a control for total and mitochondria-isolated RNAs. (*B*) Mitochondria isolated from MEF cells expressing *RP-hCOX2* were treated with trypsin and Triton-X 100, as indicated. The proteins were separated on SDS/PAGE and analyzed with antibodies for TOMM40 (OM), TIMM23 (IM), and hCOX2. (*C*) Mitochondria isolated from MEF cells expressing *RP-hCOX2* were treated with Na_2CO_3, followed by centrifugation to separate soluble proteins (supernatant, S) and integral membrane proteins in the pellet (P). The proteins were separated on SDS/PAGE and analyzed with antibodies for TOMM40, PNPASE, and hCOX2.

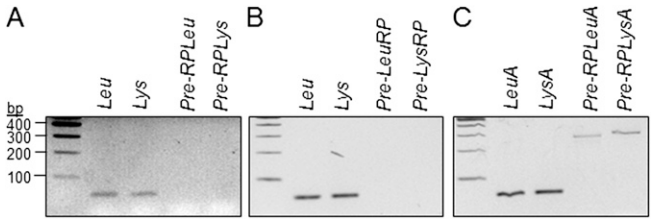

Fig. S3. Analysis of tRNA expression in the cytosol in control experiments for Fig. 4. tRNAs were stably expressed in wild-type cells to analyze the export requirements to shuttle the tRNA from the nucleus to the cytosol. RNA was isolated from the cytosol fraction of the wild-type cells stably expressing tRNAs for (*A*) *Pre-RPLeu*, *Pre-RPLys* and (*B*) *Pre-LeuRP*, *Pre-LysRP*. Export was analyzed by RT-PCR, using primers for processed tRNAs and their precursors or for precursors only. Controls include tRNAs for *Leu* and *Lys*, which were exported to the cytosol. (*C*) tRNAs were modified with the addition of several ribonucleotides adjacent to the aminoacyl stem (schematic in Fig. 4*A*), designated *LeuA*, *LysA*, *Pre-RPLeuA*, and *Pre-RPLysA*. Export was analyzed as in *A* and *B*.

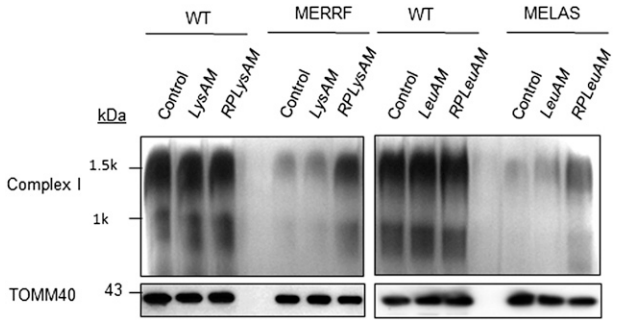

Fig. S4. Mitochondria isolated from the wild-type, MERRF, and MELAS mutant cell lines expressing *Pre-LysAM*, *Pre-RPLysAM* or *Pre-LeuAM*, *Pre-RPLeuAM* were separated on a 6–16% blue-native gel and assayed for complex I activity by staining with nitrotetrazolium blue. TOMM40 immunoblot serves as a loading control.