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Supplemental Data

Infantile Encephaloneuromyopathy and Defective Mitochondrial Translation Are Due to a Homozygous *RMND1* Mutation

Beatriz Garcia-Diaz, Mario H. Barros, Simone Sanna-Cherchi, Valentina Emmanuele, Hasan O. Akman, Claudia C. Ferreiro-Barros, Rita Horvath, Saba Tadesse, Nader El Gharaby, Salvatore DiMauro, Darryl C. De Vivo, Aly Shokr, Michio Hirano, and Catarina M. Quinzii

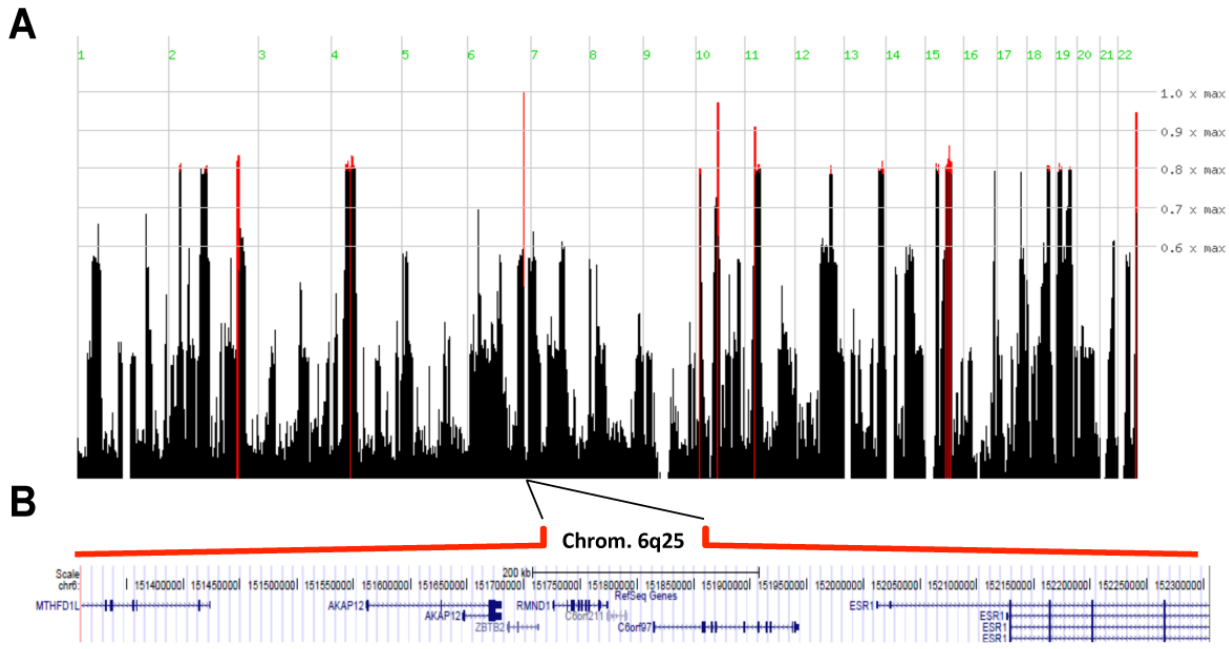

Figure S1. Genome-wide Homozygosity Mapping in the Family

Genome-wide homozygosity mapping identified a single region of homozygosity due to shared identity by descent in all affected patients (A). The region on chromosome 6q25 spanned <1Mb between the markers rs519861 and rs926777 and included only 7 coding genes (B).

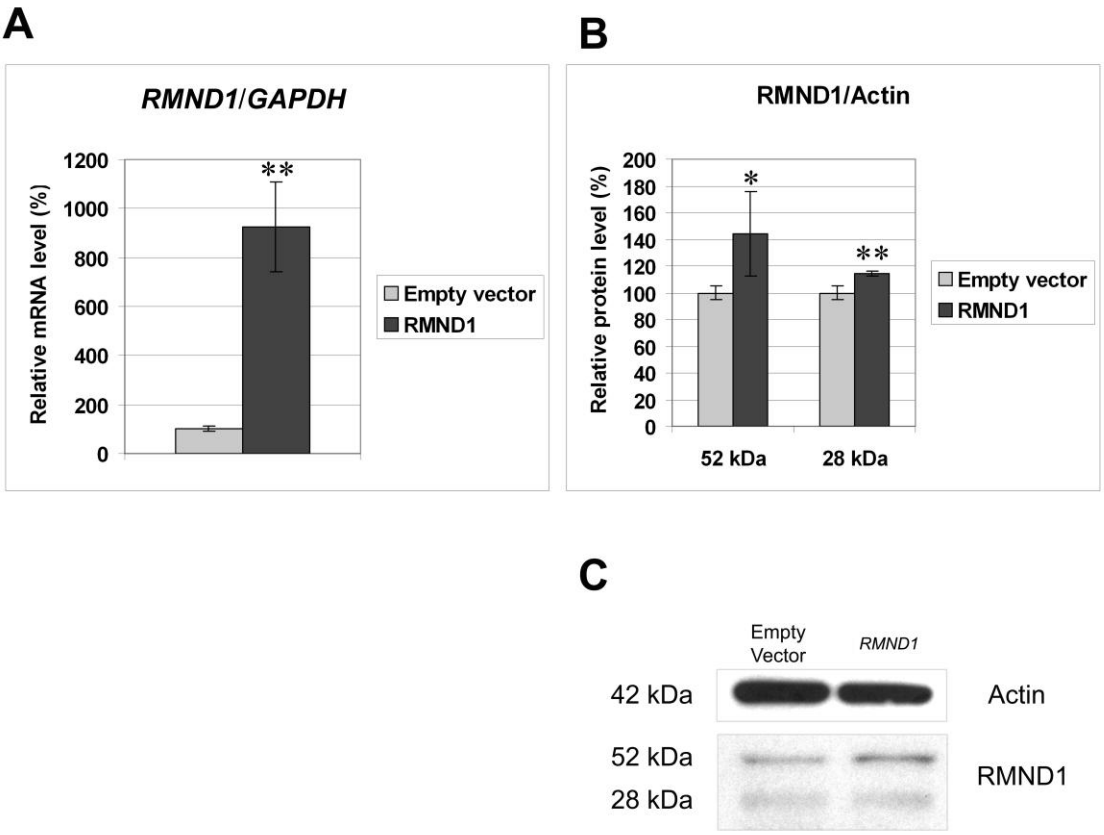

Figure S2. Assessment of *RMND1* Overexpression

Quantitative PCR to assess transcript levels (A) and immunoblot to determine protein levels as determined by ratios of RMND1/β-actin bands (B) and immunoblots showing 52 and 28 kDa bands detected by anti-RMND1 antibody (C) to assess transfection efficiency of a construct encoding RMND1 in VI-3 fibroblasts. The full-length human *RMND1* cDNA cloned in the pCMV-SPORT6 vector was generated with the Open Bio system (Thermo Fisher Scientific), and fidelity of the final product was confirmed by sequencing. Cells were cultured in high glucose DMEM medium supplemented with 10% FBS (Invitrogen). When 70% confluent, cells were transiently transfected with 5 µg of wild-type *RMND1* or empty CMV-SPORT6 vector plus Lipofectamine 2000 (Invitrogen) in FBS-free medium. After 5 hours, cells were cultured in high-glucose medium supplemented with 2% FBS for 24 hours. Values are expressed as percentages of control and are represented as mean ± standard deviation. The asterisk (*) indicates Student's t test $p < 0.05$; (**) indicates Student's t test $p < 0.01$.

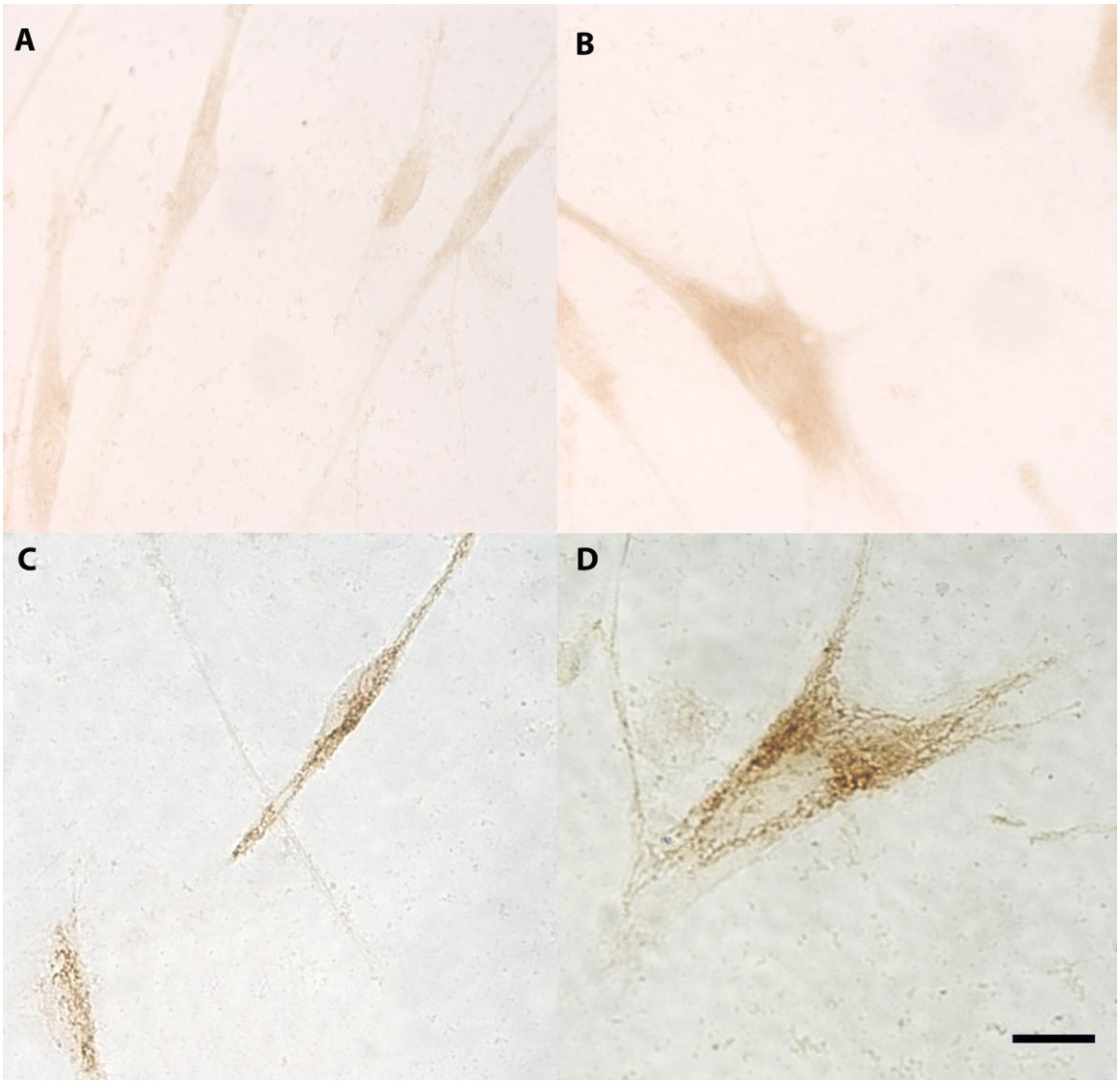

Figure S3. Transient Expression of *RMND1* cDNA Increases Cytochrome *c* Oxidase (COX) Activity

Cytochemical assessment of COX activity in VI-3 fibroblasts before (A and B), and after transient transfection with a *RMND1* expression vector (C and D). Three COX-positive cells are evident in the field. Scale bar represents 20 μm.

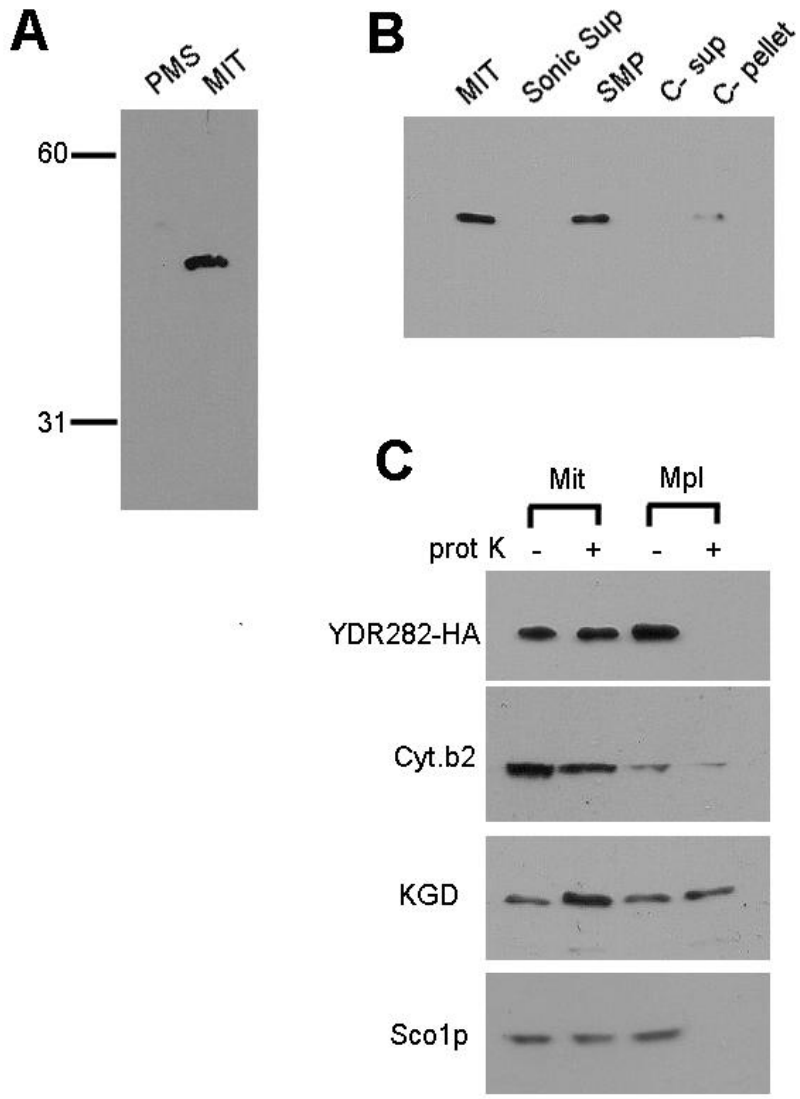

Figure S4. Subcellular Localization of the Yeast Ortholog (Ydr282p) of Human *RMND1* in the Mitochondrial Inner Membrane

After PCR amplification of the *Ydr282c* ORF with the oligonucleotides 5'-ggcctgcagtcaagcgtagtctgggacgtcgtatgggtactttgtagcatctagatc and 5'-cctttaaagatctgtgcggcg, the PCR product was digested with *Bgl*II and *Pst*I and ligated into YEp351 previously digested with the same enzymes. The recombinant plasmid was used to transform *W303ΔYDR282c* and generated YDR282p with a C-terminal HA tag. Immunoblot with the anti–hemaglutinin (HA) antibody detected a ~50 kDa protein (A). HA-tagged Ydr282p is present in the mitochondrial (MIT), but not post-mitochondrial supernatant (PMS) (A). Post-sonic disruption of mitochondria, the protein was recovered predominantly in the membrane fraction (sub-mitochondrial particles [SMP]) and was not extracted with carbonate (C-sup) with a partial recovery in post-carbonate pellet (C-pellet) indicating strong association of Ydr282p with membranes (B). The HA-tagged protein was susceptible to proteinase K in mitoplasts (Mpl) but not in mitochondria (Mit) indicating that the C-terminus protrudes into the intermembrane space (C). Cyt b_2 is a mitochondrial intermembrane space protein; α-ketoglutarate dehydrogenase (KGD) is a matrix protein; and Sco1p is an inner membrane protein.

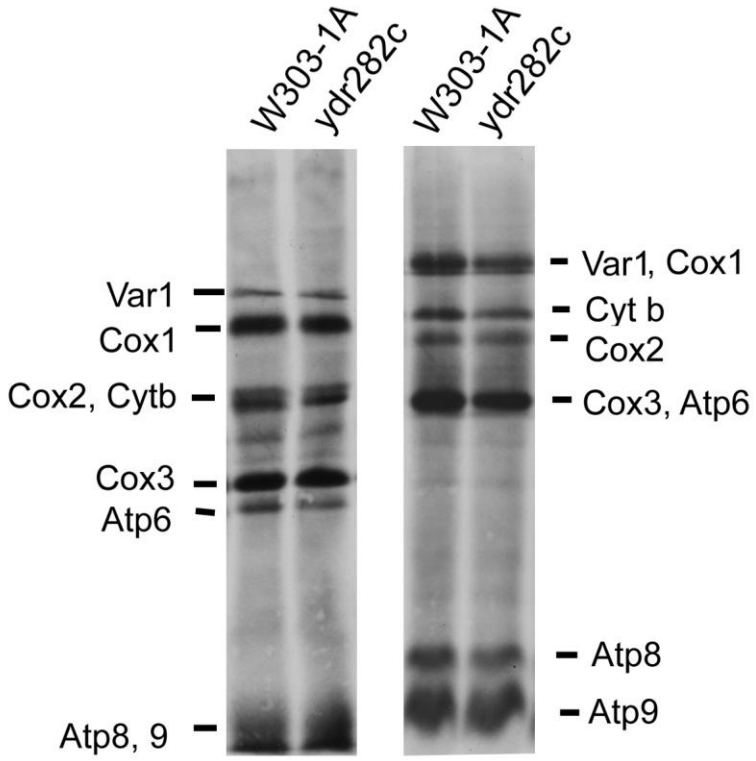

Figure S5. Mitochondrial Protein Synthesis in Wild-Type (W303-1A) and *rmnd1*-Null (Δydr282c) *S. cerevisiae* Strain

To generate a disrupted version of *Saccharomyces cerevisiae rmnd1* (*RMND1* ortholog), ORF YDR282c was PCR-amplified from total yeast nuclear DNA with primers 5'-cctttaaagatctgtgcggcg and 5'-cccctgcagtttagatccgagattcc. The 1756 bp fragment containing the gene flanked by 305 nucleotides of 5'-untranslated and 224 nucleotides of 3'-untranslated sequences was digested with *Bgl*II and *Pst*I, and then transferred to *YEp352* and *YIp352*[28]. The recombinant plasmid (pDR282/ST1) was digested with *Bcl*I, then the 725bp *Bcl*I internal fragment was replaced by a 1.1 kb fragment containing the yeast *HIS3* gene. The *YDR282:HIS3* allele was isolated from this plasmid (pDR282/ST8) as a linear 2.1 kb *Sac*I-*Pst*I fragment, which was used to replace the wild-type gene in the respiratory competent haploid strain W303-1A by homologous recombination. Histidine-independent transformants were shown to have the null allele by PCR and restriction analysis, confirming the successful construction of W303ΔYDR282.

To assess mitochondrial protein synthesis, the parental *Saccharomyces cerevisiae* strain W303-1A and the *rmnd1*-null mutant (Δydr282c) were grown at 28 °C and incorporation of ^{35}S-methionine into the mitochondrial translation products was assayed *in vivo* after inactivation of cytosolic translation with cycloheximide. The cells were labeled at room temperature for 10 min. Total cellular proteins were separated by SDS-PAGE on a 17% polyacrylamide gel with a 30:0.8 ratio of acylamide to bis-acrylamide, and on a 12% acylamide gel with 6M urea to separate Cox3p and Atp6p. Proteins were transferred to nitrocellulose and exposed to X-ray film. The respective positions of the yeast mitochondrial translational products: Var1, Cox1, Cyt b, Cox2, Cox3, Atp6, Atp8, and Atp9 are indicated.

Table S1. Clinical Presentation of the Five Affected Subjects Studied

#	Sex	GA (Weeks) / Birth Weight	Pregnancy / Delivery	Assisted Ventilation at Birth	Clinical Data	Blood Lactate (Normal 0.5–2.2 mM)	Age at Death
VI-1	Male	40 / 3.20 kg	C-section	Yes (mechanic ventilation)	Little spontaneous limb movement, prominent tongue fasciculations, bilateral equinus deformities of the feet, profound hypotonia of arms and legs, absent tendon reflexes myoclonic jerks at day 3.	3.2 mM	18 months
VI-3	Male	34 / 1.99 kg	Antepartum hemorrhage due to placenta previa / C-section	Yes (mechanic ventilation)	Lethargic, floppy, hyporeflexic, generalized hypotonia, bilateral equines feet deformity.	3.5 mM	12 days
VI-7	Female	38 / 2.86 kg	Mild polyhydramnios / C-section due to CPD	Yes (mechanical ventilation)	Floppy, absent Moro reflex, normal pupillary light reflex, hypotonia (proximal>distal) tongue fasciculations, muscular skeletal deformities (bilateral hyperextended knees, bilateral talipes equinovarus)	2.5-8.4 mM	8 months
VI-8	Male	38 / 2.88 kg	Uneventful / Vaginal delivery	Yes (mechanic ventilation)	Lethargic, floppy, hyporeflexic, generalized hypotonia, bilateral equines feet deformity.	5.7mM	4 months
VI-9	Female	34 / 2.01 kg	Polyhydramnios / NA	CPR attempted	No skeletal deformities	NA	Stillborn

GA, gestational age; C-section, cesarean section; CPD, Cephalopelvic Disproportion; NA, not available; and CPR, cardiopulmonary resuscitation.

Table S2. PCR Primers Used for Amplifying Human *RMND1* (Annealing Temperature: 59 °C)

Exon	**Forward (5' → 3')**	**Reverse (5' → 3')**
2	GGCAAAGTGGCAGAAACACT	CTCTTACGTTGGCGGTAAGG
3	GAATGGAGTCCAGTAGGGTG	CCACCTCAAGTAATAATGATCC
4	AGATGGTGATTGTGTTACAGAG	CACAATTACTTGACCCAAACC
5	ACCCTAAAGAATGTATTCCATC	AATTTCCCTTCTAATGTTTCAG
6	CCTTTTCCTTAAAAACATGCAA	TGCACACCTGTACTCCCACT
7	GTCAAAATGACCTCCAGAAAG	TCCCTCAGTGAAATGACAAC
8	GCGATTCAGTTTCCTCTATCC	AGATTCTGGTATCCAGTGGG
9	GCCCTGTTGACCTAATGC	ACTATGGGAATGTTTTCTTGAG
10	GGGTAACATAGCAAGACCCTC	GTACAGAGGACAAGAAGGGG
11	CTTTGGGGAGAGGACTGAG	GCCCCTGTATTTGTGAAAAG
12	CAATTCATAGGTTGCCACG	TCCTGATCTCATCTCAAGCC
cDNA	GCCGAAGAATCGGTCATCTA	AGTGATGCTTCCCAAATTGC