

An *RMND1* Mutation Causes Encephalopathy Associated with Multiple Oxidative Phosphorylation Complex Deficiencies and a Mitochondrial Translation Defect

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Mutations in the genes composing the mitochondrial translation apparatus are an important cause of a heterogeneous group of oxidative phosphorylation (OXPHOS) disorders. We studied the index case in a consanguineous family in which two children presented with severe encephalopathy, lactic acidosis, and intractable seizures leading to an early fatal outcome. Blue native polyacrylamide gel electrophoretic (BN-PAGE) analysis showed assembly defects in all of the OXPHOS complexes with mtDNA-encoded structural subunits, and these defects were associated with a severe deficiency in mitochondrial translation. Immunoblot analysis showed reductions in the steady-state levels of several structural subunits of the mitochondrial ribosome. Whole-exome sequencing identified a homozygous missense mutation (c.1250G>A) in an uncharacterized gene, *RMND1* (required for meiotic nuclear division 1). *RMND1* localizes to mitochondria and behaves as an integral membrane protein. Retroviral expression of the wild-type *RMND1* cDNA rescued the biochemical phenotype in subject cells, and siRNA-mediated knockdown of the protein recapitulated the defect. BN-PAGE, gel filtration, and mass spectrometry analyses showed that *RMND1* forms a high-molecular-weight and most likely homopolymeric complex (~240 kDa) that does not assemble in subject fibroblasts but that is rescued by expression of *RMND1* cDNA. The p.Arg417Gln substitution, predicted to be in a coiled-coil domain, which is juxtaposed to a transmembrane domain at the extreme C terminus of the protein, does not alter the steady-state level of *RMND1* but might prevent protein-protein interactions in this complex. Our results demonstrate that the *RMND1* complex is necessary for mitochondrial translation, possibly by coordinating the assembly or maintenance of the mitochondrial ribosome.

Mitochondria contain their own translation machinery for the synthesis of the 13 mtDNA-encoded polypeptides that are essential structural components of the oxidative phosphorylation (OXPHOS) complexes. Whereas the mitochondrial genome (mtDNA) itself codes for the two rRNAs and 22 tRNAs necessary for translation, all of the other components of the translation apparatus are encoded in the nucleus and must be targeted to the mitochondrion. Mutations in genes affecting mitochondrial translation are common causes of OXPHOS disorders in adults and children.^{1,2} For instance, mutations in all of the mitochondrial tRNAs have been associated with disease, and although they make up less than 10% of mtDNA, they account for a large proportion of mtDNA mutations associated with human mitochondrial disease.³ Most of the diseases that have been identified in the nuclear-encoded components of the translation machinery are early-onset, fatal disorders that are inherited in an autosomal-recessive fashion.² Although not universally true, many of the translation defects cause deficiencies in more than one OXPHOS complex. There is, however, a great deal of heterogeneity (often gene specific, but not always) in phenotypic presentation, which is an enduring mystery. In addition, it is likely that many translation factors remain unknown as new factors continue to be identified by the investigation of individuals with translation defects.⁴

In this study, we investigated a subject who presented with an encephalopathy and lactic acidosis. Informed consent was obtained, and the institutional review board at the Montreal Neurological Institute approved the research studies. The subject, a girl, was the fourth child of consanguineous parents. There are three older female siblings, the first two of whom are normal. The third daughter developed intractable seizures at 2 months of age, and a computed tomography (CT) scan showed cerebral atrophy and microcephaly. She died at the age of 13 months. The proband was normal at birth but developed seizures on day 6. She was hypotonic and required tube feeding. At 4 months of age, she had unremitting seizures and her head circumference was not increasing. The liver was slightly enlarged, but there was no splenomegaly. The blood lactate concentration was 3.56 mmol/l (the normal range is 0.63–2.44 mmol/l), and the cerebrospinal-fluid lactate concentration was 3.43 mmol/l (the normal range is 0.90–2.80 mmol/l). The lactate to pyruvate ratio in the blood was 21.2 (the normal ratio is 10–20). Blood ammonia, urine amino acids, and organic acids and lysosomal-enzyme studies were all normal. Cytochrome *c* oxidase (COX) activity in cultured fibroblasts was 4 nmol/mg protein/min (the normal range is 30–90), and the COX to citrate synthase ratio was 0.11 (normal is >1). The subject died at 5 months of age. At autopsy,

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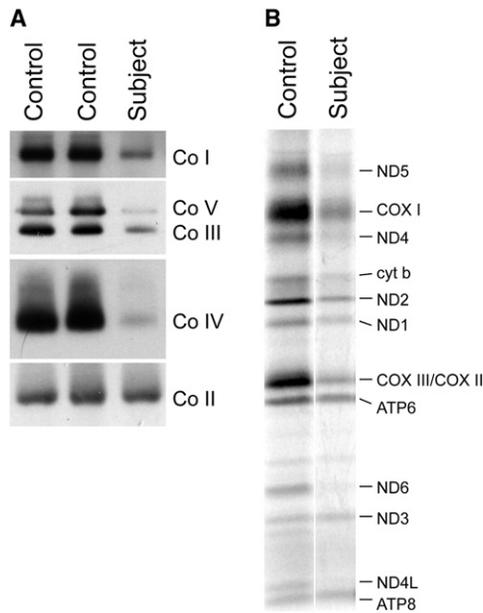


Figure 1. Characterization of Biochemical and Molecular Defects in Subject Fibroblasts

Control and subject fibroblasts were analyzed by BN-PAGE (A) and by pulse labeling of the mtDNA-encoded polypeptides (B). (A) Each of the five OXPHOS complexes (I–V) was visualized with a subunit-specific antibody that recognizes the native complex as follows: CoI (NDUFA9), CoII (SDHA), CoIII (UQCRC1), CoIV (COX4), CoV (ATP5A1). Complex II is the loading control. (B) The seven subunits of complex I (ND), one subunit of complex III (cyt b), three subunits of complex IV (COX), and two subunits of complex V (ATP) are indicated to the right of the figure.

the brain showed marked atrophy of the cortex but a relatively normal cerebellum and brain stem. The cord was also atrophic. There was symmetrical ventricular dilatation, and the corpus callosum was thin. Microscopically, there was widespread vacuolation of cortical gray matter and status spongiosus and extensive loss of myelin in the brain stem.

To further investigate the nature of the biochemical defect, we first immortalized the subject fibroblasts as described previously.⁵ The COX activity in these cells was $26 \pm 11\%$ of control levels ($n = 34$). BN-PAGE analysis⁶ revealed marked assembly defects in complexes I, III, IV, and V (Figure 1A), suggesting a mitochondrial-translation defect. Confirming this prediction, the synthesis of the mtDNA-encoded polypeptides, investigated by pulse labeling the mitochondrial translation products with a mixture of [³⁵S] methionine and [³⁵S] cysteine,⁷ was severely and uniformly decreased to $<20\%$ of control levels (Figure 1B). Although this pattern of mitochondrial-translation deficiency is similar to that found in other subjects with genetically defined mitochondrial-translation defects,^{8–10} it represents the most severe generalized mitochondrial-translation decrease that we have observed.

Southern blot analysis of subject fibroblasts showed no decrease in the level of mtDNA when normalized to the cytoplasmic 18S rRNA gene (Figure 2A), excluding a mtDNA depletion syndrome. Northern blot analysis

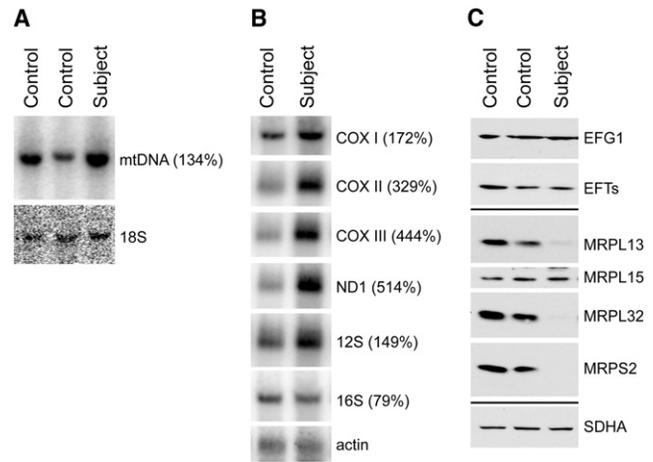


Figure 2. Steady-State Levels of Mitochondrial DNA, mRNAs, rRNAs, and Mitochondrial-Translation Proteins

(A) Southern blot analysis of genomic DNA extracted from control and subject fibroblasts. Hybridization was performed with probes directed against a 16 kb fragment of the mitochondrial genome, and the nuclear 18S rRNA gene was used as a loading control. (B) Northern blot analysis carried out with total RNA extracted from control and subject fibroblasts. Hybridization was performed with probes specific to mitochondrial mRNAs encoding the three COX subunits, one of the complex I subunits (ND1), and the 12S and 16S mitochondrial ribosomal RNAs. Beta-actin was used as the loading control. (C) Immunoblot analysis of control and subject fibroblasts with antibodies against the mitochondrial-translation elongation factors (EFG1 and EFTs) and the mitochondrial ribosomal proteins MRPL32 (a kind gift of T. Langer, Cologne), MRPL13, MRPL15, and MRPS2 (kind gifts of L. Spremulli, UNC Chapel Hill). The 70 kDa subunit of complex II (SDHA) was used as a loading control.

of several mitochondrial mRNAs (normalized to actin mRNA) showed an increase of 2–5 fold in all of those examined (Figure 2B). The 12S mitochondrial rRNA level was also $1.5\times$ higher in subject fibroblasts than in controls; however, the 16S rRNA was relatively decreased ($0.8\times$ the control) (Figure 2B). Immunoblot analysis, carried out on fibroblasts solubilized in 1.5% lauryl maltoside, showed near control levels of the mitochondrial-translation elongation factors but severely decreased levels of several mitochondrial ribosomal subunits (MRPL13, MRPL32, and MRPS2) (Figure 2C), suggesting that the mitochondrial-translation defect could be due to impaired assembly or maintenance of the mitochondrial ribosome.

Microcell-mediated chromosome-transfer studies¹¹ and sequencing of several candidate genes failed to reveal a causative mutation, so we used whole-exome sequencing to search for a homozygous mutation. DNA from exonic regions was captured with the Agilent SureSelect Human All Exon Kit v.1 and sequenced on one lane of an Illumina GAIIX with single-end sequencing of 76 bp reads. Read alignment and variant identification were performed as previously described.¹² The average coverage in the National Center for Biotechnology Information Consensus Coding Sequence was $23.5\times$, resulting in approximately 65,000 variants passing the SAMtools varFilter¹³ (default parameters, as well as a minimum SNP

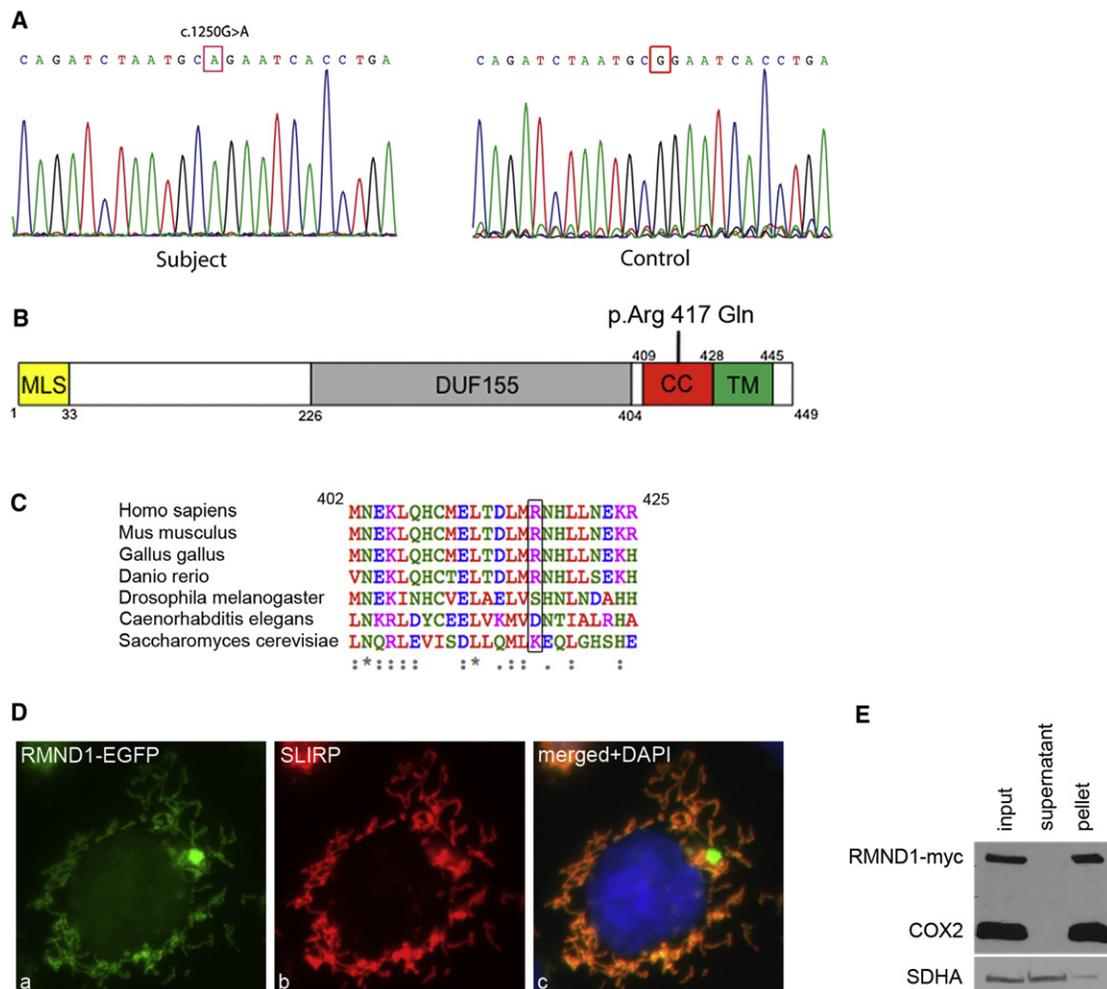


Figure 3. Mutational Analysis of *RMND1* and Mitochondrial Localization of the Protein

(A) DNA sequence analysis of *RMND1* cDNA indicates the position of the homozygous c.1250G>A mutation in the subject compared to the control.

(B) A schematic representation of *RMND1* (not to scale) shows the predicted domains and the position of the p.Arg417Gln substitution. The following abbreviations are used: MLS, mitochondrial localization signal; DUF155, domain of unknown function 155; CC, coiled-coil domain; and TM, transmembrane domain.

(C) The alignment of the amino acid sequences of *RMND1* homologs in different species shows that the mutated arginine (black rectangle) is conserved only in the vertebrates.

(D) Control fibroblasts transiently expressing *RMND1-EGFP* (left panel, green) were incubated with an antibody against the mitochondrial protein SLIRP (middle panel, red). The far right panel showing the overlay is counterstained with DAPI for visualization of the nucleus.

(E) Alkaline carbonate extraction of mitochondria from HEK cells stably expressing a C-terminal Myc-tagged *RMND1*. Immunoblot analysis with a Myc antibody shows that *RMND1* is an integral membrane protein. SDHA (soluble, membrane-associated protein) and COX subunit 2 (integral inner membrane protein) were used as controls.

quality of 20 and a minimum indel quality of 50, were used). This number was significantly reduced to approximately 1,000 candidates after we filtered any previously identified variants in dbSNP (v.131), the 1000 Genomes Project (July 2010 release), and ten local exomes. In addition, because of the subject's consanguinity, homozygous nonsynonymous alleles were prioritized, leading to only 22 candidates. Finally, we searched for known and predicted mitochondrial proteins (by using the Mitocarta database¹⁴), and *RMND1* (required for meiotic nuclear division 1), an uncharacterized gene, emerged as the only candidate. Sanger sequencing of *RMND1* in the subject

cDNA and genomic DNA confirmed the homozygous missense mutation (c.1250G>A) identified by whole-exome sequencing (Figure 3A); this mutation was predicted to cause amino acid substitution p.Arg417Gln (RefSeq accession number NM_017909.2). DNA was not available for analysis from any of the other family members.

Bioinformatic analysis of the protein sequence revealed the presence of a DUF155 domain (Figure 3B) that is shared by members of the RMD1/Sif2 family comprising *RMD1* (the yeast homolog of *RMND1*) and four other yeast genes, viz., *RMD8*, *SIF2*, *SIF3*, and *YDR282C*. *RMND1* is also predicted to contain a mitochondrial localization signal at

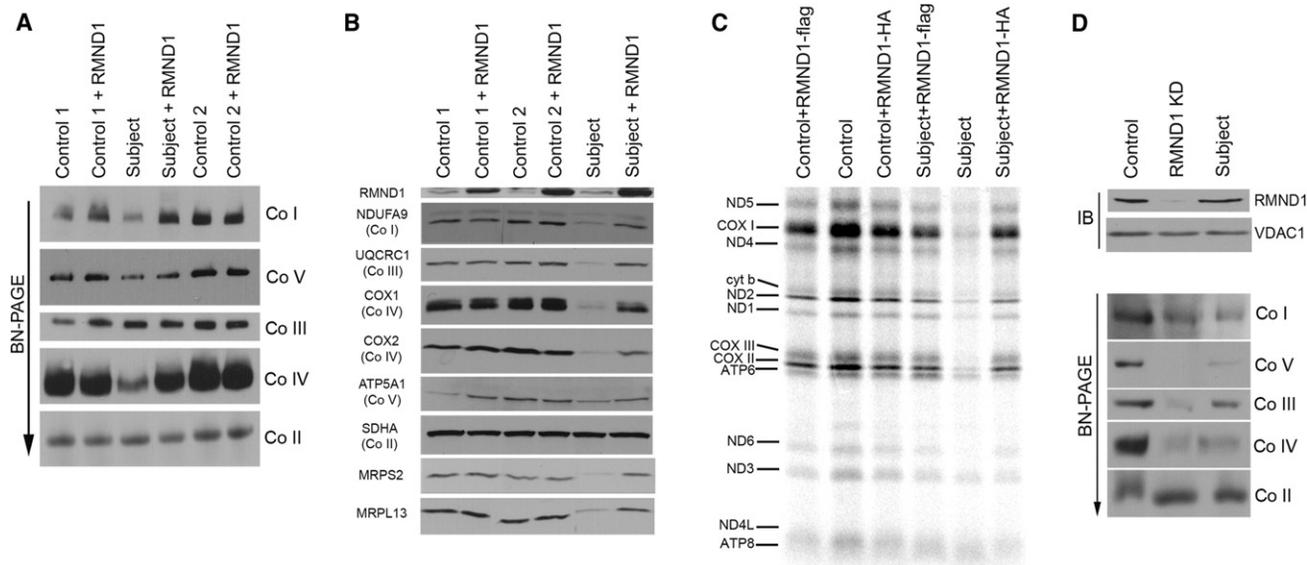


Figure 4. Rescue of the Biochemical Phenotype by *RMND1* Expression and Recapitulation of the Defect by siRNA-Mediated Knockdown of the Protein

(A) BN-PAGE analysis of controls and subject fibroblasts expressing *RMND1* from a retroviral vector (pBABE). Each of the five OXPHOS complexes (I–V) was visualized with a subunit-specific antibody.

(B) Immunoblot analysis of the same samples as in (A) for expression of *RMND1*, individual structural subunits of the five OXPHOS complexes, and two mitochondrial ribosomal subunits. The 70 kDa subunit of complex II (SDHA) was used as a loading control.

(C) Analysis of mitochondrial translation products in control and subject fibroblasts transduced with retroviral vectors expressing *RMND1-HA* or *RMND1-flag*.

(D) Stealth RNAi-mediated knockdown of *RMND1*. The upper panel shows the level of knockdown of *RMND1* on an immunoblot with VDAC1 (porin) as a loading control. The bottom panel shows the BN-PAGE analysis in the control, *RMND1* knockdown, and subject cells.

the N terminus and a coiled-coil domain (where the mutation is localized) juxtaposed to a transmembrane domain at the extreme C terminus (Figure 3B).

RMND1 is evolutionarily conserved down to yeast (from which the name is derived), but the *S. cerevisiae* gene product is reported to be cytosolic in a high-throughput study of the cellular localization of the gene products expressed from genes chromosomally tagged with green fluorescent protein.¹⁵ All the other members of the *SIF2* family are thought to have either nuclear or cytosolic localizations. Alignment of the amino acid sequences of *RMND1* homologs (Figure 3C) shows that the mutated residue is only conserved in vertebrates (40 of 40 vertebrates for which the complete *RMND1* sequence is available in Ensembl). For confirming the mitochondrial localization of *RMND1*, control fibroblasts transiently expressing *RMND1-EGFP* were grown on coverslips, fixed with 4% paraformaldehyde, and analyzed by immunofluorescence. This analysis showed a nearly perfect colocalization of the EGFP signal with mitochondria detected with a SLIRP antibody (Figure 3D). Alkaline carbonate extraction of isolated mitochondria⁵ from a stable human embryonic kidney (HEK) cell line expressing *RMND1-Myc* demonstrated that it behaves as an integral membrane protein (Figure 3E). Similar results were obtained for the native protein in both control and subject fibroblasts (data not shown).

To confirm the pathogenicity of the *RMND1* mutation, we used a retroviral vector to express the wild-type

RMND1 cDNA in subject fibroblasts. The *RMND1* cDNA was amplified by OneStep RT-PCR (QIAGEN) with specific primers modified for Gateway-compatible vectors and was cloned into pBABE, modified to be Gateway-compatible.⁴ The Phoenix packaging cell line was transiently transfected with the retroviral construct according to the HBS/Ca₃(PO₄)₂ method (see Web Resources). Fibroblasts were infected 48 hr later by exposure to virus-containing medium in the presence of 4 μg/ml of polybrene.⁴ Expression of the wild-type *RMND1* cDNA rescued the assembly defects in the OXPHOS complexes (Figure 4A). (*RMND1* C-terminally tagged with either a flag or Myc epitope also rescued the assembly defects; data not shown). Immunoblot analysis with a polyclonal antibody (Sigma) raised against a peptide antigen (amino acids 336–412) of *RMND1* showed a specific 35 kDa band that increased in intensity when the cDNA was expressed from the retroviral vector (Figure 4B), suggesting that *RMND1* is processed beyond the mitochondrial localization sequence (amino acid residue 32, predicted by MITOPROT) because the molecular weight of that species would be 48 kDa. Consistent with this interpretation, analysis of immunoprecipitated *RMND1* by mass spectrometry (n = 7) failed to identify a peptide N terminal of amino acid 127, whereas the coverage of the protein C terminal to this residue was 73%. The steady-state level of *RMND1* in subject fibroblasts was similar to that in controls (Figure 4B), indicating that the mutation does not destabilize the protein and that

the phenotype is most likely due to a loss of function of the mutant *RMND1*. The steady-state levels of all analyzed OXPHOS-complex components, as well as mitochondrial ribosomal proteins in subject fibroblasts expressing the wild-type *RMND1* cDNA, were also comparable to levels in controls (Figure 4B). Finally, we tested whether expression of the wild-type *RMND1* rescued the mitochondrial-translation defect in subject fibroblasts. Mitochondrial protein synthesis was completely restored by retroviral expression of either *RMND1-Myc* or *RMND1-flag* in subject fibroblasts (Figure 4C).

To further investigate the function of *RMND1*, we used RNAi-mediated knockdown of the protein. Three different Stealth RNAi duplexes were designed with Block-iT RNAi Designer (Invitrogen). The different RNAi constructs were transiently transfected into control fibroblasts with Lipofectamine RNAimax (Invitrogen) at a final concentration of 12 nM. The transfections were repeated on days 3 and 6, and the cells were harvested and analyzed on day 9. The Stealth KD2 duplex (5'–CAAACCAAUCUGUU GGGUUCUAAA-3') produced the most robust *RMND1* knockdown (Figure 4D, top panel), so we continued the BN-PAGE analysis with this construct. The OXPHOS-assembly defects produced by RNAi-mediated knockdown of *RMND1* were very similar to those found in the *RMND1* subject (Figure 4D), confirming that *RMND1* is required for normal assembly of the OXPHOS complexes.

We next asked whether *RMND1* was part of a protein complex. We isolated mitochondria from HEK cells stably expressing *RMND1-Myc*, solubilized them, and analyzed them by gel filtration as previously described.¹⁶ *RMND1-Myc* and the endogenous *RMND1* eluted in the fractions between the peak fractions from COX (230 kDa, Figure 5A) and LRPPRC¹⁰ (250 kDa, Figure 5A). To confirm that *RMND1* formed a high-molecular-weight complex, we carried out a similar analysis by using BN-PAGE. The complex containing *RMND1* ran slightly slower than that of COX on the first-dimension BN-PAGE, confirming that it is approximately 240 kDa in size. Strikingly, this complex almost completely failed to assemble in the subject cells but was restored by retroviral expression of wild-type *RMND1-Myc* (Figure 5B). These data indicate that the mutation in the coiled-coil domain of *RMND1* prevents the formation of a higher-order complex and that the complex is essential for *RMND1* function. To establish whether the complex contained other proteins, we immunoprecipitated *RMND1-Myc* with a Myc antibody and ran the immunoprecipitate on a BN-PAGE gel. Mass-spectrometry analysis of the purified complex identified only *RMND1* (data not shown). Although we cannot rule out the presence of other proteins at the moment, this analysis suggests that *RMND1* very likely assembles into a homopolymeric complex.

Although we do not know the exact molecular function of *RMND1*, our data show that it is part of a large mitochondrial-membrane complex that is required for the translation of the mitochondrially encoded polypeptides

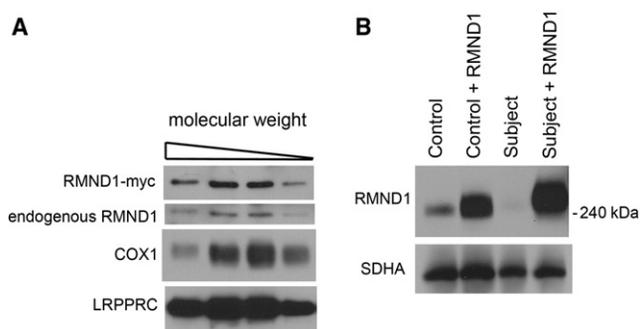


Figure 5. *RMND1* Is Part of a High-Molecular-Weight Protein Complex that Does Not Assemble in the Subject

(A) Size-exclusion-chromatography analysis was carried out with mitochondria from HEK cells expressing *RMND1-Myc*. Immunoblotting with antibodies against either the native protein or the Myc epitope demonstrated that both the endogenous and overexpressed proteins are part of a complex of about 240 kDa. The COX subunit 1 of COX (230 kDa) and LRPPRC (250 kDa) were used as molecular-weight references.

(B) BN-PAGE analysis of control and subject fibroblasts expressing *RMND1-Myc* from a retroviral vector. An antibody directed against the native protein shows that *RMND1* forms a 250 kDa complex that does not assemble in the subject. The complex is restored in subject cells expressing *RMND1-Myc*. *SDHA* was used as a loading control.

and is thus essential for the biogenesis of the OXPHOS complexes. The gene products of all the other members of the *RMD/SIF2* family localize to the cytoplasm or nucleus and have a function in some aspect of meiosis or sporulation in yeast species. *Sif2p*, for example, is part of the Set3 histone deacetylase complex that represses a set of meiotic genes.¹⁷ Although the function of *Rmd1p*, the *S. cerevisiae* homolog of *RMND1*, remains unknown, there is no evidence that it has a role in mitochondrial translation or OXPHOS function. *RMND1* has apparently evolved a new function related to mitochondrial translation in mammalian cells. The substantial decreases in the steady-state levels in some of the mitochondrial ribosomal subunits and the relative decrease in the 16S rRNA in the *RMND1* subject cells both point to a role for *RMND1* in some aspect of the assembly or maintenance of the mitochondrial ribosome.

Ribosome assembly is a complex, highly coordinated process in which protein binding and posttranscriptional nucleotide modifications drive and stabilize rRNA structures until the native state is reached. How this is regulated in mammalian mitochondria is largely unknown, although a few ribosome-biogenesis factors have been identified. *ERAL1*^{18,19} and the PPR protein *PTCD3*²⁰ bind and stabilize the 12S rRNA, and the mitochondrial transcription factor *TFB1M* is responsible for dimethylating two highly conserved adenines in a stem loop near the 3' end of the 12S rRNA.^{21,22} A GTPase, *C4orf14* (*NOA1*), associates with the mt-SSU, but its precise molecular role has not been defined.²³ Knockdown of any of these factors compromises mt-SSU assembly and mitochondrial translation.

The only known protein that binds the 16S rRNA is mTERF4, which forms a complex with the RNA dimethyl transferase NSUN4 presumably to methylate a cytosine residue (not yet identified) on the 16S rRNA.²⁴ Interestingly, a tissue-specific knockout of *Mterf4* in the heart results in a large increase in both mt-SSU and mt-LSU without a corresponding increase in monosome formation, suggesting that it is not required for the assembly or stability of the individual subunits per se. The GTPase MTG1 is thought to play a role in mt-LSU assembly on the basis of its ability to partially rescue a deletion of the yeast ortholog, but its precise role has not been defined.²⁵ Assembly of the mature ribosome requires proteolytic processing of MRPL32, a task performed by the m-AAA protease.²⁶ C7orf30, a protein of the widely expressed DUF143 family, was shown to associate with the mt-LSU,^{27,28} specifically with MRPL14,²⁹ but its role in the assembly of mt-LSU and the downstream effects on translation remain controversial. Aside from the m-AAA protease in the list above, RMND1 stands out because of all the proteins involved in ribosome biogenesis, it is the only one that forms a membrane-anchored complex. We speculate that this could act to stabilize or position the mitochondrial ribosome perhaps by linking it through to the outer membrane. Further detailed studies will be necessary for testing this hypothesis.

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Web Resources

The URLs for data presented herein are as follows:

BLOCK-iT RNAi Designer, <http://rnaidesigner.invitrogen.com/rnaiexpress>

Transfection protocol for Phoenix retroviral packaging cell line, http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html

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