

Biallelic mutations in *MRPS34* lead to instability of the small mitoribosomal subunit and Leigh syndrome

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Abstract

The synthesis of all 13 mitochondrial DNA (mtDNA)-encoded protein subunits of the human oxidative phosphorylation (OXPHOS) system is carried out by mitochondrial ribosomes (mitoribosomes). Defects in the stability of mitoribosomal proteins or mitoribosome assembly impair mitochondrial protein translation, causing combined OXPHOS enzyme deficiency and clinical disease. Here we report four autosomal recessive pathogenic mutations in the gene encoding the small mitoribosomal subunit protein, MRPS34, in six subjects from four unrelated families with Leigh syndrome and combined OXPHOS defects. Whole exome sequencing was used to independently identify all variants. Two splice-site mutations were identified, including homozygous c.321+1G>T in a subject of Italian ancestry and homozygous c.322-10G>A in affected sibling pairs from two unrelated families of Puerto Rican descent. In addition, compound heterozygous *MRPS34* mutations were identified in a proband of French ancestry; a missense (c.37G>A, p.Glu13Lys) and a nonsense variant (c.94C>T, p.Gln32*). We demonstrated that these mutations reduce MRPS34 protein levels and the synthesis of OXPHOS subunits encoded by mtDNA. Examination of the mitoribosome profile and quantitative proteomics showed that the mitochondrial translation defect was caused by destabilization of the small mitoribosomal subunit and impaired monosome assembly. Lentiviral-mediated expression of wild-type *MRPS34* rescued the defect in mitochondrial translation observed in skin fibroblasts from affected subjects, confirming the pathogenicity of *MRPS34* mutations. Our data establish that MRPS34 is required for normal function of the mitoribosome in humans, and furthermore demonstrate the power of quantitative proteomic analysis to identify signatures of defects in specific cellular pathways in fibroblasts from subjects with inherited disease.

Main Text

Introduction

The oxidative phosphorylation (OXPHOS) system generates the majority of cellular energy required by the body. Mitochondrial DNA (mtDNA) encodes 13 protein subunits of the OXPHOS system via 11 mRNAs, two of which are bicistronic (*MT-ATP8/MT-ATP6* and *MT-ND4L/MT-ND4*), as well as 22 transfer RNAs (mt-tRNA) required for translation and 2 ribosomal RNAs (mt-rRNA) necessary for

the assembly of mitochondrial ribosomes (mitoribosomes). Nuclear genes encode all of the other proteins required for mitochondrial translation, including mitoribosomal proteins, tRNA- and rRNA-modifying enzymes and additional factors that mediate mitoribosome biogenesis and translation initiation, elongation, and termination.¹

Mammalian mitoribosomes contain two subunits; a small (28S) subunit that decodes mRNA and mediates tRNA delivery of required amino acids, and a large (39S) subunit that catalyzes the formation of peptide bonds between the amino acids.^{2,3} In humans, 30 mitochondrial ribosomal small subunit proteins (MRPSs) assemble with the 12S mt-rRNA to form the small 28S subunit, while 50 mitochondrial ribosomal large subunit proteins (MRPLs) assemble with the 16S mt-rRNA and mt-tRNA^{Val} to form the large 39S subunit.⁴⁻⁶

Molecular defects that impair different components of the mitochondrial translation machinery can cause combined OXPHOS deficiency.⁷ Specifically, 7 of the 80 genes encoding mitochondrial ribosomal proteins have had pathogenic mutations reported, including autosomal recessive mutations in *MRPS7* (MIM: 611974),⁸ *MRPS16* (MIM: 609204),⁹ *MRPS22* (MIM: 605810),¹⁰ *MRPS23* (MIM: 611985),¹¹ *MRPL3* (MIM: 607118),¹² *MRPL12* (MIM: 602375),¹³ and *MRPL44* (MIM: 611849).¹⁴ Disorders caused by mutations in mitoribosomal proteins are clinically heterogeneous and multi-systemic, with common features including neurodevelopmental disabilities, brain abnormalities, liver disease, kidney disease, cardiomyopathy, and lactic acidosis.^{11,15} They generally lead to death in infancy or early childhood,¹⁵ although survival into teenage years and adulthood has been reported.^{8,14,16} The small number of mitoribosomal genes known to underlie OXPHOS diseases is surprising, since nearly two-thirds of mitoribosomal genes are essential for OXPHOS based on a high-throughput knock-out death screen in cell models.¹⁷

Here, we report four pathogenic recessive mutations in a small mitoribosome subunit gene not previously associated with human disease, *MRPS34*, that were identified in six individuals from four families with combined OXPHOS defects and Leigh syndrome or Leigh-like disease (MIM: 256000).

MRPS34, which lies within the foot of the small 28S mitoribosome subunit, is one of 15 mammalian mitochondria-specific MRPSs not found in the ancestral bacterial ribosome.⁵ We previously showed that mice with a homozygous *Mrps34* missense mutation that caused reduced MRPS34 protein stability developed cardiac hypertrophy and pronounced liver dysfunction due to impaired mitoribosome assembly.¹⁸ We now demonstrate that human *MRPS34* mutations cause Leigh or Leigh-like syndrome by destabilizing the small mitoribosomal subunit and impairing mitochondrial protein translation.

Subjects and Methods

Samples from probands and family members were obtained after receiving informed consent for diagnostic or research investigations, and associated studies were performed in accordance with the Declaration of Helsinki and approved by the respective human research institutional review board responsible for each research site.

Clinical Information

A summary of features in the six affected subjects from four unrelated families is shown in Table 1, with detailed descriptions provided in the Supplemental material (Case Reports and Table S1). Most subjects had normal neonatal periods, with subsequent onset of developmental delay in all patients by 6 months of age that typically evolved to neurodevelopmental regression. Subjects 1 and 4 had failure to thrive in the first few months of life, and episodic metabolic acidosis with respiratory distress, with death during an episode between 8 to 9 months of age. They had progressive clinical courses with brain MRI and/or neuropathology analysis showing lesions in the basal ganglia, brainstem and/or midbrain that were diagnostic of the progressive neurodegenerative disorder Leigh syndrome (Figure S1 for subject 4 MRI).¹⁹ Similar but milder clinical courses typical of Leigh or Leigh-like syndrome have been seen in subjects 2a, 2b, 3a and 3b, with all still alive at ages ranging from 2 to 17 years. The subject with Leigh-like syndrome had neuroradiological imaging that did not fulfil stringent diagnostic criteria for Leigh syndrome.¹⁹ The three older subjects have developed dystonic and/or choreoathetoid movements, with wheelchair dependence. Four of the subjects had OXPHOS

enzymology performed in skeletal muscle, liver and/or skin fibroblast cell lines, which showed deficiency of one or more OXPHOS complexes (Tables 1 and S2).

Enzyme assays

Spectrophotometric enzyme assays assessing mitochondrial OXPHOS enzyme and citrate synthase activities in cultured fibroblasts, skeletal muscle and liver biopsy were performed as described previously for subjects 1 and 4.^{20,21} For subject 2a, clinical skeletal muscle OXPHOS enzymology testing was performed at 19 months at All Children's Hospital (St. Petersburg, Florida, USA) and at 12 years at MNG Laboratories (Atlanta, Georgia, USA). Enzyme studies for subject 3a were performed at Baylor Medical Genetics Laboratory. Dipstick assays to measure complexes I and IV activity in lentiviral rescue studies were performed on 15 µg of fibroblast lysates as described previously.²²

Whole exome sequencing

Whole exome sequencing (WES) of subject 1 was performed at the Broad Institute using Illumina Capture Exome technology (version 1) supplemented with additional baits to ensure capture of mtDNA. Data were mapped to NCBI hg19/GRCh37 human genome reference sequence using BWA,²³ and then analyzed using GATK Best Practices recommendations,²⁴ HaplotypeCaller,^{25,26} Variant Effect Predictor,²⁷ and Seqr. Analysis of mtDNA was performed as described previously.²⁸

Clinical WES testing was performed for subjects 2a, 2b, 3a and their parents as described.¹⁹ DNA libraries were generated using the SureSelect Human All Exon V4 or Clinical Research Exome kit (Agilent Technologies, Santa Clara, California, USA). Data were mapped to the NCBI hg19/GRCh37 human genome reference sequence and analyzed using GeneDx's XomeAnalyzer. Variants identified by WES were evaluated and classified according to published guidelines.²⁰ The homozygous variant identified in subject 3a was confirmed as homozygous in the similarly affected sibling subject 3b by Sanger sequencing. Whole mitochondrial genome sequencing analysis in blood was performed for

subjects 2a and 3a; mtDNA sequence was assembled and analyzed relative to the revised Cambridge Reference Sequence (rCRS) and MITOMAP database as previously described.²⁹

Prior to clinical WES, blood DNA from subjects 2a and 2b were also processed for WES in the Mount Sinai Genomics Core Facility using SureSelect V5 libraries (Agilent). Alignment and variant calling using an in-house GATK-based pipeline, plus variant filtering using Ingenuity Variant Analysis (Qiagen), were performed as described previously.³⁰

WES for subject 4 utilized Agilent SureSelect Human All Exon V libraries. Reads were mapped to the NCBI hg19/GRCh37 human genome reference sequence and variant calling utilized GATK, SAMtools and Picard Tools. Single nucleotide variants were called with GATK Unified Genotyper, whereas indel calls were made with the GATK IndelGenotyper_v2. Variants were annotated and filtered using in-house software PolyWeb as described elsewhere.³¹

RNA and DNA Analyses

DNA was extracted from primary skin fibroblasts as described previously.²² For cDNA studies of subject 1, cultured fibroblasts were grown with and without cycloheximide treatment, as described previously.³² For cDNA studies of subjects from families 2 and 3, cultured fibroblasts or Epstein-Barr Virus transformed lymphoblasts were grown without cycloheximide treatment. Total RNA was extracted using the miRNeasy Mini kit (Qiagen) as per manufacturer's protocol and as described previously.³² Synthesis of cDNA was performed using the SuperScript IV First-Strand Synthesis System (ThermoFisher Scientific) as per manufacturer's protocol and as described previously.³² To examine the effect of the c.321+1G>T and the c.322-10G>A mutations on mRNA splicing, PCR primers were designed to amplify exons 1-3 of *MRPS34* from cDNA (using either pair 1 [forward primer 5'CGGGAGCAACTGAACAGG, reverse primer 5'TGCGTATCCTCTGCACATTC] or pair 2 [forward primer 5'AGCTCTACGCGGTGGACTAC, reverse primer 5'GATCCAGGCAGAGAGAGCAC] respectively). PCR products amplified from the *MRPS34* transcript containing the c.322-10G>A mutation were cloned into the pCRTM4-TOPO TA vector

using the TOPO TA cloning kit (ThermoFisher Scientific). The vector was transformed into TOP10 competent cells (Invitrogen), and individual colonies were examined and sequenced.

Lentiviral Transduction

For subject 1, lentiviral transduction of fibroblasts was performed as described previously.²² For subject 4, fibroblasts were transduced with lentiviral particles expressing *RFP* or wild-type *MRPS34* using the p.Lenti7.3 (ThermoFisher Scientific) for 12 hours and then incubated for an additional 20 days until the cells were harvested.

SDS-PAGE and Blue Native Gel Electrophoresis (BN-PAGE)

Protein was extracted from cultured fibroblasts, lymphoblasts, and liver biopsies, and 10-30 µg of each protein lysate was analyzed by SDS-PAGE as described previously.^{32,33} BN-PAGE was performed on 100 µg of fibroblast mitochondria lysate in 1% Triton X-100 as described previously,^{34,35} or on 15 µg fibroblast mitoplast extract in 1% dodecyl maltoside as described previously.³⁶

Immunoblotting

Protein lysates analyzed by SDS-PAGE were probed with primary antibodies against MRPS34 (Sigma-Aldrich), MRPL37 (Proteintech or Sigma-Aldrich), MRPL11 (Proteintech), MRPS2 (Abcam), MRPS5 (Abcam), MRPS16 (Proteintech), MRPS18B (Proteintech), MRPS35 (Proteintech), GAPDH (Cell Signalling), NDUFS3 (Abcam), complex II 70 kDa subunit (Molecular Probes), COXI (Abcam), VDAC1 (MitoSciences or Abcam), Beta-Actin (Abcam), Citrate Synthase (GeneTex,) and Total OXPHOS Human WB Antibody Cocktail (consisting of five antibodies ATP5A, UQCRC2, SDHB, COXII, and NDUFB8; Abcam). Isolated mitochondria and mitoplasts analyzed by BN-PAGE were probed with Total OXPHOS Rodent WB Antibody Cocktail (consisting of five antibodies ATP5A, UQCRC2, COXI, SDHB, and NDUFB8; Abcam) or with primary antibodies against NDUFA13, SDHA, UQCRC2, COXIV, ATP5A (Abcam) respectively. Blots were incubated with anti-mouse or anti-rabbit IgG secondary antibodies (VWR International) and developed with Clarity Western ECL

Substrate (Bio-Rad Laboratories), or with IRDye 800CW/680LT Goat Anti-Rabbit or Anti-Mouse IgG (Li-Cor) secondary antibodies and visualized using an Odyssey Infrared Imaging System (Li-Cor). Relative band intensities were quantitated using Image J software, where protein levels were normalized to VDAC1.

Sucrose Gradient Subfractionation

Fibroblast mitochondria were prepared from 8 x 15cm² dishes as described previously,³⁷ and the isolated mitochondria were lysed with 2% digitonin in 260 mM Sucrose, 10 mM Tris HCl, pH 7.5, 100mM KCl and 20 mM MgCl₂ in the presence of 1x Complete EDTA-free Protease inhibitor cocktail for 20 min as previously described.³⁸ The mitochondrial lysate was centrifuged for 45 min at 9200 g at 4°C, the clarified lysate was loaded onto a continuous 10-30% sucrose gradient (in 10 mM Tris-HCl, pH 7.4, 100 mM KCl, 20 mM MgCl₂ in the presence of protease inhibitors) and centrifuged at 71,000 g in an Optima Beckman Coulter preparative ultracentrifuge for 15 hours. Fractions were collected and precipitated with 0.02% sodium deoxycholate and 12% trichloroacetic acid (final concentration), washed twice with acetone, and the entire precipitate was resolved by SDS-PAGE. Protein markers of the mitochondrial ribosomal subunits were detected by immunoblotting, as described above.

Mitochondrial protein synthesis assay

Mitochondrial protein synthesis was analyzed in fibroblasts using previously described methods.^{37,39-41}

Quantitative Proteomics

Mass-spectrometry on primary fibroblast material was performed label-free, using sample preparation methodology previously described with modifications.⁴² Fibroblasts from three separate controls and two subcultures of subject 1 cultured as above were solubilized in 1% w/v sodium deoxycholate, 100 mM Tris-HCl (pH 8.1) prior to incubation at 99°C for 10 minutes with vortexing. Samples were further incubated for 10 minutes at 60°C in a sonicator waterbath, followed by the addition of 5 mM Tris(2-carboxyethyl)phosphine (TCEP), 20 mM chloroacetamide and incubation for 5 min at 99°C

with vortexing. Denatured and alkylated proteins were digested with trypsin overnight at 37°C. Detergent was removed by extraction into ethyl acetate in the presence of 2 % formic acid (FA), followed by concentration of the aqueous phase through vacuum centrifugation. Peptides reconstituted in 0.5 % FA were loaded onto small cation exchange (Empore Cation Exchange-SR, Supelco Analytical) stage-tips made in-house.⁴³ Tips were washed with 20 % acetonitrile (ACN), 0.5 % FA and eluted over five fractions of increasing amounts (45-300 mM) freshly prepared ammonium acetate, 20 % ACN, 0.5 % FA, followed by a final elution of 5 % ammonium hydroxide, 80 % ACN. Fractions were concentrated by vacuum centrifugation and desalted on SDB-XC poly(styrene-divinylbenzene; Supelco Analytical) stage-tips as previously described.^{40,43} Peptides were reconstituted in 0.1% trifluoroacetic acid (TFA), 2% ACN and analyzed by online nano-HPLC/electrospray ionization-MS/MS on a Q Exactive Plus connected to an Ultimate 3000 HPLC (Thermo-Fisher Scientific). Peptides were loaded onto a trap column (Acclaim C₁₈ PepMap nano Trap x 2 cm, 100 µm I.D, 5 µm particle size and 300 Å pore size; ThermoFisher Scientific) at 15 µL/min for 3 min before switching the pre-column in line with the analytical column (Acclaim RSLC C₁₈ PepMap Acclaim RSLC nanocolumn 75 µm x 50 cm, PepMap100 C₁₈, 3 µm particle size 100 Å pore size; ThermoFisher Scientific). The separation of peptides was performed at 250 nL/min using a non-linear ACN gradient of buffer A (0.1% FA, 2% ACN) and buffer B (0.1% FA, 80% ACN), starting at 2.5% buffer B to 35.4% followed by ramp to 99% over 120 minutes. Data were collected in positive mode using Data Dependent Acquisition using m/z 375 - 1800 as MS scan range, HCD for MS/MS of the 12 most intense ions with $z \geq 2$. Other instrument parameters were: MS1 scan at 70,000 resolution (at 200 m/z), MS maximum injection time 50 ms, AGC target 3E6, Normalized collision energy was at 27% energy, Isolation window of 1.8 Da, MS/MS resolution 17,500, MS/MS AGC target of 1E5, MS/MS maximum injection time 100 ms, minimum intensity was set at 1E3 and dynamic exclusion was set to 15 sec.

Raw files were analyzed using the MaxQuant platform⁴⁴ version 1.5.5.1 searching against the Uniprot human database containing reviewed, canonical and isoform variants in FASTA format (June 2016) and a database containing common contaminants. Default search parameters for a label-free (LFQ)

experiment were used. Briefly, multiplicity was set to 1 (unlabeled), “LFQ”, “Re-quantify” and “Match between runs” were enabled with default settings. Unique and razor peptides were used for quantification, using a minimum ratio count of 2. Using the Perseus platform version 1.5.5.3,⁴⁵ proteins identified using < 2 unique peptides were excluded, as were identifications marked “Only identified by site”, “Reverse”, and “Potential Contaminant”. Mitochondrial proteins were defined through matching of gene names and Ensembl gene IDs to the Mitocarta2.0 dataset.⁴⁶ LFQ Intensity values were Log₂ transformed, and mean and standard deviations were calculated for each experimental group, consisting of either 3 control fibroblast biological replicates or 2 subject fibroblast technical replicates. Values from a group with a standard deviation > 0.3 were invalidated by conversion to “NaN” and rows were filtered to contain at least 2 valid values in both experimental groups. A two-tailed ratio paired t-test was performed on the linearised Log₂ LFQ Intensity Mean values for controls and subject 1. The D'Agostino and Pearson normality test was used to confirm that the log of the ratios followed a Gaussian distribution. The Bonferroni correction was used to determine the p-value threshold for significance.

Mapping of subunit levels to complex I (PDB: 5LDW) was performed as previously described.^{42,47} For mitoribosome subunit mapping the human mitoribosome structure (PDB: 3J9M) was used.⁴⁸ For complexes III and IV homologous human subunits were mapped to the bovine structures PDB: 1BGY and PDB: 5B1A respectively,^{49,50} whereas for complex II homologous human subunits were mapped to those found in the porcine structure (PDB: 1ZOY).⁵¹

Results

Whole exome sequencing identified *MRPS34* autosomal recessive mutations in individuals with Leigh (-like) syndrome

We analyzed six subjects from four families with Leigh or Leigh-like syndrome and OXPHOS defects (Figure 1A and Table 1). WES studies identified homozygous or compound heterozygous pathogenic variants in *MRPS34* (GenBank: NM_023936.1) in all subjects, which were confirmed by Sanger sequencing (Figure 1B). A homozygous essential splice site mutation, c.321+1G>T, was identified in

subject 1, an Australian child with consanguineous parents of Italian ancestry. This variant is predicted to cause abnormal splicing by abolishing the donor splice site of exon 1. The *MRPS34* c.321+1G>T variant is absent from dbSNP and the Genome Aggregation Database (gnomAD) Browser.^{52,53} A homozygous extended splice site mutation, c.322-10G>A, was identified in subjects 2a, 2b, 3a and 3b of Puerto Rican ethnicity; these subjects were ascertained with assistance from the GeneMatcher tool.⁵⁴ This variant is predicted to abolish the acceptor splice site and create a new cryptic splice acceptor site within intron 1, causing abnormal gene splicing. The *MRPS34* c.322-10G>A variant is reported in dbSNP (dbSNP: rs563189672), and 2 heterozygous individuals (both of Latino ethnicity) were reported in the gnomAD Browser (2 of 236,804 alleles examined, no homozygotes observed). Principal component analysis of available WES data revealed that individuals from families 2 and 3 all fall in the Admixed American (AMR) group, and their close clustering indicated they come from a very similar population (Table S3 and Figure S2). Kinship analysis showed that there is no consanguinity at the level of 2nd cousins or closer between the parents either within or across families 2 and 3 (Table S4). Analysis of the variants in the *MRPS34* genomic region using WES data from families 2 and 3 showed a shared haplotype of ~590kb in size between chr16:1,306,986-1,894,912 (Table S5), implying that the c.322-10G>A mutation is a founder mutation.

Compound heterozygous *MRPS34* mutations were identified in subject 4, a c.37G>A (p.Glu13Lys) missense variant and a c.94C>T (p.Gln32*) nonsense variant. The c.37G>A (p.Glu13Lys) variant is absent from dbSNP and the gnomAD Browser. Protein sequence alignment of human MRPS34 with its homologs in nine other vertebrate species indicated that the p.Glu13 residue is highly conserved (Figure 1C). The *MRPS34* c.37G>A (p.Glu13Lys) missense variant is predicted as ‘Damaging’ by SIFT (score 0.02), ‘Disease Causing’ by MutationTaster (score 1.0), ‘Probably Damaging’ by PolyPhen-2 (score 0.97) and likely to interfere with function by AlignGVGD (Class C55, second highest class of 7). The *MRPS34* c.94C>T (p.Gln32*) variant is reported in dbSNP (dbSNP: rs763672163), and 39 heterozygous individuals were reported in the gnomAD Browser (39 of 82,878 alleles examined, no homozygotes observed). Investigation of family members with DNA available

confirmed that all variants segregated with disease (Figures 1A and 1B). The *MRPS34* variants not reported in dbSNP have been submitted to ClinVar (see Accession Numbers).

The *MRPS34* c.321+1G>T and c.322-10G>A mutations cause abnormal mRNA splicing

The c.321+1G>T variant (subject 1) lies within the highly conserved donor splice site of exon 1, while the c.322-10G>A variant (families 2 and 3) is situated within the extended acceptor site of exon 2. To examine the effect of these variants on *MRPS34* splicing, PCR was performed on cDNA synthesized from fibroblast or lymphoblast RNA extracted from controls and subjects 1 and 2a. For the c.321+1G>T variant, gel electrophoresis of PCR products containing exons 1-3 of *MRPS34* revealed an amplicon in subject 1 that was smaller than that in control (Figure 2A). Sequencing determined that this amplicon lacked the last 24 bases of exon 1, indicating the use of an upstream donor splice site within exon 1 (Figure 2B). The c.321+1G>T variant therefore produces a shortened, stable transcript that results in an in-frame deletion of 8 amino acids, p.Val100_Gln107del (Figure 2C). Protein sequence alignment of human *MRPS34* with its homologs in nine other vertebrate species indicated that the missing 8 amino acids are highly conserved (Figure 2D).

To determine the effect of the c.322-10G>A variant on splicing, *MRPS34* PCR products amplified from fibroblast and lymphoblast cDNA were cloned and sequenced. Two abnormally spliced *MRPS34* transcripts resulting in frameshifts and premature truncations were detected in subject 2a; an amplicon with 8 nucleotides inserted prior to exon 2 due to the utilization of a cryptic intronic acceptor site (p.Asn108Leufs*12), which represented 80% or 68% of total PCR products analyzed in fibroblasts and lymphoblasts respectively, and an amplicon which skipped exon 2 (p.Asn108Glyfs*50) observed in 10% or 18% of total PCR products analyzed in fibroblasts or lymphoblasts respectively. The remaining analyzed PCR products were wild-type (10% or 15% in fibroblasts and lymphoblasts respectively) (Figure 2C). Quantitative RT-PCR analysis of fibroblast cDNA revealed a ~75% reduction in *MRPS34* transcript level in cells from subject 3a relative to control (Figure S3). Collectively, these results confirm that the c.321+1G>T and c.322-10G>A variants cause impaired

splicing of the *MRPS34* transcript. The latter variant allows some wild-type mRNA to be made, implying it is a hypomorphic allele.

MRPS34 protein levels are reduced in individuals with *MRPS34* recessive mutations

To investigate the effect of the mutations on MRPS34 protein levels, immunoblotting was performed on available tissue and cells from controls and subjects 1, 2a, 2b and 4. Immunoblotting of fibroblasts and liver tissue confirmed the absence of wild-type MRPS34 protein in subject 1 (Figure 2E), and longer exposure of the immunoblots revealed two bands in subject 1 that were faint but detectable, one of which may represent the mutant MRPS34 protein (p.Val100_Gln107del). These results suggest that the mutant MRPS34 protein is likely degraded in subject 1. In cells from subjects 2a, 2b and 4, a substantial decrease in MRPS34 protein levels was identified relative to controls (Figures 2F and 2G). The residual protein in subjects 2a and 2b with the homozygous c.322-10G>A mutation likely reflects the presence of residual wild-type transcript. These findings establish that all the *MRPS34* mutations identified in the affected subjects result in decreased MRPS34 protein levels.

Individuals with *MRPS34* mutations have combined OXPHOS deficiency associated with reduced mitochondrial translation

Spectrophotometric enzyme assays performed on liver, skeletal muscle and/or fibroblasts identified a combined OXPHOS deficiency in subjects 1, 2a, and 3a, and isolated complex IV deficiency in subject 4 (Table S2). Immunoblotting of cell lysates from controls and subjects 1, 2a, 2b, and 4 revealed decreased levels of OXPHOS complex I and IV subunits in affected subjects relative to controls (Figures 3A - 3C). This was associated with a decrease in the steady-state levels of assembled complexes I and IV in fibroblasts from subject 1, as demonstrated by BN-PAGE (Figure 3D). BN-PAGE also showed a decrease in assembled complex I in subject 4, but complex IV assembly was not clearly decreased below controls (Figure 3E). Given that MRPS34 is a component of the mitoribosome, we sought to determine whether the combined OXPHOS deficiency was due to reduced mitochondrial translation. Examination of mitochondrial *de novo* protein synthesis in fibroblasts from subjects 1 and 4 by ³⁵S-methionine radiolabeling revealed an overall decrease in

protein synthesis of mtDNA-encoded OXPHOS subunits in the affected subjects relative to controls (Figures 3F, 3G and S4). BN-PAGE analysis of pulse-chase ³⁵S-methionine labeled mitochondrial lysates suggested that subject 1 cells had a reduced rate of complex IV formation relative to control (Figure 3H). These findings demonstrate that MRPS34 is required for efficient mitochondrial translation in humans.

***MRPS34* mutation reduces mitochondrial translation by destabilizing the small mitoribosomal subunit**

We have previously established that decreased protein levels of MRPS34 in mice with a *Mrps34* missense mutation destabilized the small mitoribosomal subunit.¹⁸ To determine the effect of *MRPS34* mutation on the stability of the small mitoribosomal subunit in affected human subjects, we investigated the steady state levels of other small mitoribosomal subunit proteins in fibroblast lysates from controls and subjects 1 and 4 by immunoblotting. The steady state abundance of various small mitoribosomal subunit proteins was reduced in subjects 1 and 4 relative to controls (Figures 4A and 4B). Interestingly, the abundance of large mitoribosomal subunit proteins was not affected by the *MRPS34* mutation (Figures 4A and 4B). To investigate how the decrease in small mitoribosomal subunit proteins affects the mitoribosome, we analyzed the profile of the large and small mitoribosomal subunits, as well as the monosome and polysome, in fibroblast mitochondrial lysates using sucrose gradients. Immunoblotting of the sucrose gradient fractions showed a decrease in actively translating mitochondrial ribosomes in subject 1 relative to control (Figure 4C). In mitochondria isolated from subject 1 cells, the mitoribosomal proteins of the small and large subunits are both redistributed towards the top of the gradient compared to those in mitochondria from control cells, indicating a subunit assembly defect occurred in subject 1 (Figure 4C). Reduced levels of the monosome in the cells from subject 1 further indicates that the mitoribosome is destabilized by having reduced MRPS34 protein levels, which precludes correct assembly of the small ribosomal subunit and consequent association with the large subunit. Overall, these results are consistent with those observed in the *Mrps34* mutant mouse,¹⁸ and confirm that mutation of *MRPS34* destabilizes the small ribosomal subunit, resulting in impaired mitochondrial translation.

Quantitative proteomic analysis of fibroblasts from subject 1 relative to three independent control samples was also performed to permit unbiased detection of global cellular protein changes that result from the *MRPS34* mutation (Table S6). Approximately 4,500 total proteins were quantified across all samples by this approach, of which 753 were mitochondrial proteins, including 27 proteins of the small mitoribosome subunit and 47 large subunit proteins. The abundance of all detected small mitoribosome subunit proteins was significantly decreased in the subject's cell line compared to controls, while none of the proteins from the large mitoribosome subunit were substantially altered (Figures 5A and 5B). Figure 5C and the Supplemental Video show the changes in protein abundance between subject 1 and controls mapped to the human mitoribosome structure, as we have done previously for complex I.⁴² These representations clearly illustrate the marked decrease in small mitoribosomal subunit proteins in subject 1 compared to controls, as well as the relative sparing of the large subunit, consistent with destabilization of the small mitoribosomal subunit. Mapping of protein levels to the structures of OXPHOS complexes I-IV showed general turnover of the subunits of complexes I and IV, and to a lesser extent complex III (Figure 5D), with a trend to increase seen for complex II, consistent with reduced translation of mtDNA-encoded mRNAs. As a group, complex I and complex IV protein subunits were significantly decreased in subject 1 relative to controls (Figure 5B and Table S7). Interestingly, the mean subunit level of each OXPHOS complex closely matched the residual enzyme activity in the subject's fibroblasts (Tables S2 and S7). The quantitative proteomic data therefore validate destabilization of the small mitoribosomal subunit and the consequent impact on mitochondrial translation in subject 1.

Lentiviral-mediated expression of wild-type *MRPS34* in fibroblast cells from two affected subjects rescues dysfunctional mitochondrial translation and OXPHOS capacity

To validate the pathogenicity of *MRPS34* mutations, we performed complementation studies to determine if expression of wild-type *MRPS34* in subject cells rescued the mitochondrial translation defect. Fibroblasts from control, subject 1, and a subject with a homozygous pathogenic mutation in *MRPS7*⁸ were transduced with a lentiviral vector expressing wild-type *MRPS34*. The levels of

complex IV mtDNA-encoded subunit COXII and complex I nuclear-encoded subunit NDUF8, a marker of complex I stability, were examined by immunoblotting. Densitometry analysis confirmed a decrease in COXII and NDUF8 protein levels in subject 1 and in the subject with *MRPS7* mutations relative to control (Figures 6A - 6C). Lentiviral-mediated expression of wild-type *MRPS34* caused a significant increase in COXII and NDUF8 in subject 1, but not in the cell line from the subject with *MRPS7* mutations (Figures 6A - 6C), confirming that the disorder of mitochondrial translation in subject 1 is due specifically to loss of function mutations in *MRPS34*. Measurement of complex I and complex IV activities using enzyme dipstick assays confirmed that lentiviral-mediated expression of wild-type *MRPS34* significantly increased complex I and complex IV activity in cells from subject 1, but not in cells from the subject with *MRPS7* mutations (Figures 6D and 6E). Lentiviral-mediated expression of *MRPS34* in control cells caused a modest decrease in complex IV activity, suggesting a potential negative effect of *MRPS34* overexpression in cells with a stable small mitoribosomal subunit, though not in contradiction with the rescue observed in cells from subject 1. To further support *MRPS34* mutation pathogenicity, cells from subject 4 with compound heterozygous *MRPS34* mutations that had been transduced with lentiviral particles expressing wild-type *MRPS34* showed increased levels of small mitoribosomal subunit proteins MRPS5 and MRPS18B relative to cells transduced with red fluorescent protein (Figure 6F). These results provide further evidence that wild-type *MRPS34* rescue stabilized the small mitoribosomal subunit. Collectively, these data establish that recessive mutations in *MRPS34* cause Leigh syndrome.

Discussion

We describe six patients from four unrelated families in whom autosomal recessive missense, nonsense, or splice site mutations in *MRPS34* caused instability of the small mitoribosomal subunit, impaired mitochondrial translation, and defective OXPHOS capacity. Subjects with *MRPS34* mutations developed Leigh or Leigh-like syndrome, involving metabolic strokes associated with early developmental delay and/or regression. Additional clinical features including microcephaly and dysmorphic facies were also observed in several subjects. Our study expands the known genetic causes of Leigh (-like) syndrome to now include mutations in a gene that encodes a small

mitoribosomal protein.¹⁹ While Leigh-like lesions were observed in an adult subject with mutations in *MRPL44* and a clinical presentation characterized by hypertrophic cardiomyopathy, hemiplegic migraine and exercise-induced muscle pain,¹⁶ our study broadens the clinical heterogeneity of human disorders of the mitoribosome to also include infantile-onset Leigh (-like) syndrome. To date, pathogenic mutations in ~10% of all genes encoding mitoribosomal proteins have been described to cause disease. This observation is particularly striking in the context that mutations in nearly all of the genes encoding mitochondrial aminoacyl-tRNA synthetases, which are also required for mitochondrial translation, have been described to cause clinical disease.^{55,56} This contrast, alongside data indicating that a similar proportion of proteins within each group were required for OXPHOS in cell models,¹⁷ suggests an intriguing discrepancy between these two gene groups in how pathogenic mutations have been accumulated and tolerated at a population level.

Although all affected subjects experienced disease onset in early infancy, there was variability in disease severity across the cohort. Early death of subjects 1 and 4 occurred within the first year of life, while survival into childhood and late adolescence is seen in subjects from families 2 and 3. The longer survival in these subjects with the homozygous *MRPS34* c.322-10G>A variant could be related to this mutation acting as a hypomorphic allele, as we showed that small amounts of wild-type transcript are generated in these subjects' fibroblasts and lymphoblasts. To our knowledge, the *MRPS34* c.322-10G>A variant has only been identified in individuals of Latino ethnicity, including the eight individuals from families 2 and 3 of Puerto Rican descent, and the two heterozygous Latino individuals reported in gnomAD, suggesting that this variant may represent a founder mutation. Analysis of the available WES data from families 2 and 3 revealed that the *MRPS34* c.322-10G>A variant lies within a shared haplotype, providing further evidence of a founder effect. The *MRPS34* c.322-10G>A variant should therefore be considered in subjects of Puerto Rican descent with Leigh (-like) syndrome and OXPHOS defects.

Demonstration of impairments in OXPHOS enzyme activities, mitochondrial translation activity, and mitoribosome assembly in affected subjects provided several lines of evidence to support the

pathogenicity of biallelic *MRPS34* mutations. The rescue of cellular defects associated with impaired mitochondrial translation by lentiviral-mediated expression of wild-type *MRPS34* transcript in fibroblasts from affected subjects further establishes that recessive pathogenic mutations in *MRPS34* cause disease. Quantitative proteomics further confirmed the cellular effects of *MRPS34* mutation, identifying a general decrease in the level of proteins from the small mitoribosomal subunit and OXPHOS complexes I and IV, which were strikingly consistent with enzyme activity results. We recently demonstrated the utility of this profiling technique for identifying proteomic signatures of expression changes in response to knockout of individual subunits of OXPHOS complex I in gene-edited cell lines relative to isogenic controls.⁴² Here, we further demonstrate that it can also serve as an effective tool for detecting specific proteomic signatures in fibroblasts from subjects relative to fibroblast controls from varied genetic backgrounds. Indeed, in the absence of any prior knowledge of disease causation, quantitative proteomic analysis of fibroblasts from subject 1 would have identified *MRPS34* as the most reduced mitochondrial protein. The decreased amounts of all other small ribosomal subunit proteins and subunits from OXPHOS complexes I and IV provide further direct support for a defect in stability of the small mitoribosome subunit. Quantitative proteomics therefore represents a powerful approach to elucidate the pathogenicity of novel gene mutations, with broad utility likely beyond investigation of primary mitochondrial disorders.

The mitoribosomal subunit assembly process in humans is poorly understood.² Our data from cells of a subject with *MRPS34* mutations, together with our previous studies on the *Mrps34* mutant mouse,¹⁸ show that *MRPS34* defects lead to a significant decrease in all small mitoribosomal subunit proteins and significantly reduced 12S rRNA levels. These data suggest that *MRPS34* plays an important role in stabilizing the 12S rRNA and is required for the stability of the small mitoribosomal subunit. The relative sparing of the large mitoribosomal subunit in *MRPS34* deficient cells is consistent with studies examining cell lines from subjects with pathogenic mutations in other small mitoribosomal subunit proteins *MRPS16* and *MRPS22*,⁵⁷ suggesting that the maintenance of large and small mitoribosomal subunit levels are not inextricably linked in humans. While the identification of affected subjects with pathogenic mutations in mitoribosomal subunit proteins enables key insight

into mitoribosome stability, more comprehensive studies are required to enable an intimate understanding of mitochondrial ribosomes and protein synthesis.

Although complexes I, III, IV and V all contain mtDNA-encoded subunits, our proteomic data suggest that complexes I and IV are more vulnerable than complexes III and V to destabilization when there is a defect in mitochondrial translation. We have previously shown that defective incorporation of mtDNA-encoded subunits into complexes I and IV can preclude assembly of these complexes, resulting in a general destabilization and turnover of unincorporated subunits.^{40,42} Most of the mtDNA-encoded subunits of complexes I and IV that we detected in proteomic analyses of MRPS34 deficient fibroblasts appeared to be decreased. This was not the case for the MT-CYB and MT-ATP8 subunits of complexes III and IV, suggesting the lesser impact on complexes III and V may relate to longer half-lives of these proteins or their stabilization by association with other polypeptides within the assembled complex. Further studies are required to determine why different OXPHOS complexes are differentially impacted by a defect in mitochondrial translation.

The *Mrps34* mutant mouse showed similar defects in mitoribosome assembly and protein synthesis to the human disorder,¹⁸ but the liver dysfunction that characterized that model was not a clinical feature of the six *MRPS34* disease subjects reported here. Further analyses of the *Mrps34* mutant mice have identified kidney dysfunction and smaller brains compared to controls (A.F., *unpublished data*), features seen in common with some of the subjects reported here. However, it remains to be determined why liver dysfunction was more prominent in *Mrps34* mutant mice. Mutations affecting mitochondrial translation machinery have often been associated with liver dysfunction.^{8,11,58} The observed differences in presentation between individual patients and the mouse model may reflect the importance of both the location and severity of the mutation (including hypomorphic versus loss of function alleles) as well as the genetic background. This consideration is also relevant to the observed differences in both onset and progression of clinical disease between the different *MRPS34* human subjects.

In conclusion, our study demonstrates that MRPS34 is required for normal function of the mitoribosome and energy-generating mitochondrial OXPHOS system in humans. Autosomal recessive splice site, missense, or nonsense mutations that destabilize MRPS34 are causal of clinical presentations of Leigh syndrome or a Leigh-like disease.

Accession Numbers

The accession numbers for the *MRPS34* variants c.321+1G>T and c.37G>A reported in this paper are ClinVar: SCV000581390 and ClinVar: SCV000583446 respectively.

Description of Supplemental Data

Supplemental Data includes detailed case reports, seven tables, four figures and a video.

Conflicts of Interest

The authors have no relevant financial conflicts of interests to disclose related to this work.

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Web Resources / Online Tools:

dbSNP: www.ncbi.nlm.nih.gov/SNP/

Exome Variant Server: <http://evs.gs.washington.edu/EVS/>

Genome Aggregation Database: <http://gnomad.broadinstitute.org/>

Genematcher: <https://www.genematcher.org/>

MITOMAP: <http://www.mitomap.org>

MSeqDR: <https://mseqdr.org>

Online Mendelian Inheritance in Man: <https://www.omim.org/>

Seqr: <https://github.com/macarthur-lab/seqr>

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Figure Titles and Legends

Figure 1. Identification of *MRPS34* mutations in six subjects from four families.

- (A) Pedigrees and genotype of subjects with *MRPS34* variants. ‘-’ denotes a mutant allele.
- (B) Sequencing chromatograms confirming the *MRPS34* variants in affected subjects, and the carrier status of family members with DNA available.
- (C) Protein sequence alignment of human MRPS34 with its homologs in 9 other vertebrate species showing the conservation of the p.Glu13 residue mutated in family 4. Asterisks (*) depict conserved amino acids.

Figure 2. Characterisation of MRPS34 in affected subjects with *MRPS34* mutations.

- (A) PCR amplicons of *MRPS34* exons 1-3 generated from control and subject 1 fibroblast cDNA +/- cycloheximide (CHX). The amplicon detected in subject 1 was smaller than the control amplicon.
- (B) Sequence analysis of the *MRPS34* cDNA PCR amplicon detected in subject 1 identified a 24bp deletion corresponding to the utilisation of an upstream donor site in exon 1. This splicing mutation therefore produces a shortened transcript that results in an in-frame deletion of 8 amino acids, p.Val100_Gln107del.
- (C) Schematic diagram depicting the abnormal transcript generated from the c.312+1G>T variant (family 1) and two abnormal plus residual wild-type transcript generated from the c.322-10G>A variant (families 2 and 3). The distribution of the three transcripts generated from the c.322-10G>A variant in S2a fibroblasts (40 clones sequenced) and lymphoblasts (40 clones sequenced) is additionally described in the table. The red line indicates the position of the variant. The diagram solid black bars represent exons, while the open bars represent untranslated region.
- (D) Protein sequence alignment of human MRPS34 with its homologs in 9 other vertebrate species. Asterisks (*) depict conserved amino acids. The 8 amino acids missing from the MRPS34 protein produced in subject 1 are highly conserved across the species examined.
- (E) SDS-PAGE western blot of MRPS34 and Complex II 70kDa subunit SDHA (loading control) from control (C1 and C2) and subject 1 fibroblasts and liver showed the absence of wildtype MRPS34

protein in subject 1. Long exposures revealed faint double banding in subject 1 fibroblast samples probed with MRPS34 antibody.

(F and G) SDS-PAGE western blot of MRPS34 in fibroblasts and lymphoblasts revealed a substantial decrease in MRPS34 levels in subjects 2a, 2b and 4 relative to controls (C1 and C2) and to parental samples (I-2 and I-1 from family 2). Complex II subunits SDHA and SDHB, VDAC1 and citrate synthase represent loading controls.

Figure 3. Evidence of combined OXPHOS deficiency and reduced mitochondrial translation in affected subjects with *MRPS34* mutations.

(A, B and C) SDS-PAGE western blot of protein from fibroblasts and lymphoblasts showed reduced levels of complex I (CI) and complex IV (CIV) subunits in subjects 1, 2a, 2b and 4 relative to controls (C1-C3) and to parental samples (I-2 and I-1 from family 2). Complex II subunits (SDHA and SDHB) are indicative of loading.

(D and E) BN-PAGE western blot of fibroblast protein showed reduced levels of CI and CIV in subjects 1 and 4 relative to controls (C1 and C2). Complex II (SDHA and SDHB) is indicative of loading.

(F and G) Protein synthesis in cell lysates was measured by pulse incorporation of ³⁵S-labelled methionine and cysteine. Equal amounts of cellular protein were separated by SDS-PAGE and visualized by autoradiography. The *in vitro* pulse labelling of mitochondrial translation products revealed decreased levels of mtDNA-encoded subunits in subject 1 and subject 4 relative to controls (C1 and C2). The Coomassie stain represents relative loading.

(H) Examination of mitochondrial protein synthesis in control (C) and subject 1 fibroblasts by [³⁵S]-methionine radiolabelling. Isolated mitochondria were subject to BN-PAGE, following which the complexes were visualised by autoradiography. A slower formation of complex IV, was observed in subject 1 relative to control. SDHA was used as a loading control. * denotes a non-specific band.

Figure 4. MRPS34 mutations are associated with reduced protein levels of small mitoribosomal subunits and destabilization of the mitoribosome.

(A and B) SDS-PAGE western blot of protein from fibroblasts showed reduced protein levels of small mitoribosomal subunit proteins in subjects 1 (A) and 4 (B) relative to controls (C1 and C2). The abundance of large mitoribosomal proteins in subjects 1 and 4 were comparable to controls. Complex II subunit SDHA, VDAC1 and GAPDH were used as loading controls.

(C) A continuous 10-30% sucrose gradient was used to determine the distribution of the small and large ribosomal subunit and the monosome in mitochondria isolated from control (C) and subject 1 cells. Mitochondrial ribosomal protein markers of the small (MRPS16 and MRPS35) and large (MRPL11 and MRPL37) ribosomal subunits were detected by immunoblotting with specific antibodies. The data are representative of results from three independent biological experiments. The dashed vertical lines denote the relevant fractions as indicated.

Figure 5. Quantitative proteomic analysis of fibroblasts from an affected subject with *MRPS34* mutations identifies a general decrease in small mitoribosomal and OXPHOS subunit proteins.

(A) Quantitative mass spectrometry of mitochondrial proteins in control and subject 1 fibroblasts demonstrates downregulation of small mitoribosome subunits (red dots), as well as OXPHOS subunits (blue dots), in subject 1. In contrast, the levels of large mitoribosome subunits (yellow dots) in subject 1 are generally unaffected. Proteins examined in this study by SDS-PAGE and observed to have reduced levels are indicated by the text labels. The horizontal line within the volcano plot represents a significance value of $p=0.05$, where the levels of proteins represented above the horizontal $p=0.05$ line was regarded as significantly different from control. The two dashed vertical lines indicate Log_2 changes of >0.5 up- or downregulation relative to controls.

(B) OXPHOS and mitoribosome protein levels in subject 1 represented as a ratio of the control mean. Hashes denote groups that were significantly reduced in subject 1 relative to control (all with p -value < 0.0001). The middle bar represents the mean value, while the upper and lower bars represent the 95% confidence interval of the mean value. Each dot represents a single protein.

(C) Changes in mitoribosome protein levels between subject 1 relative to controls mapped to the structure of the human mitoribosome. As per the inset scale, proteins coloured in blue are decreased,

and those coloured in red are increased, in subject 1 relative to controls. Grey indicates no data; yellow indicates *MRPS34*.

(D) Changes in OXPHOS subunit levels for complexes I-IV mapped to homologous subunits of the relevant structure. Colour scale as per (C).

Figure 6. Lentiviral-mediated expression of wild-type *MRPS34* rescues the defect in mitochondrial translation in cells from affected subjects.

(A) Fibroblasts from control, subject 1 and a subject with pathogenic *MRPS7* variants were transduced with wild-type *MRPS34* cDNA. Representative SDS-PAGE western blot demonstrates an increase in protein levels of CI (NDUFB8) and CIV (COXII) subunits in subject 1 fibroblasts transduced with *MRPS34* relative to untransduced cells. VDAC1 was used as a loading control.

(B and C) Densitometry analysis revealed that the increase in CI subunit NDUFB8 (B) and CIV subunit COXII (C) observed in subject 1 fibroblasts transduced with *MRPS34* relative to untransduced cells was significant ($p = 0.0049$ and 0.036 respectively). Results were normalized to VDAC1 and presented as the percent of average untransduced control cells. The data represents the mean of three independent transfections \pm SEM.

(D and E) Complex I (D) and complex IV (E) activity was measured in fibroblasts from control, subject 1 and a subject with pathogenic *MRPS7* variants that were transduced with wild-type *MRPS34* cDNA. Complex I and complex IV activity was significantly increased in subject 1 cells transduced with wild-type *MRPS34* relative to untransduced cells (both $p < 0.0001$). Complex IV was significantly decreased in control cells transduced with wild-type *MRPS34* relative to untransduced cells ($p = 0.0004$). Results were normalized to citrate synthase and presented as the percent of average untransduced control cells. The data represents the mean of three independent transfections \pm SEM.

(F) The level of small mitoribosomal subunit proteins MRPS5 and MRPS18B was examined in fibroblasts from controls (C1 and C2) and subject 4 transduced with either *RFP* or wild-type *MRPS34* by SDS-PAGE western blotting. Fibroblasts from subject 4 that had been transduced with wild-type *MRPS34* had increased levels of MRPS5 and MRPS18B relative to cells transduced with

RFP. β -actin was used as a loading control. The blot shown is representative of 3 independent experiments.

Supplemental Video. Change in protein abundance between subject 1 and controls mapped to the human mitoribosome structure. The colour scale is as per Figure 5C.

Table 1. Biochemical and Clinical Characteristics in Individuals with *MRPS34* Variants.

Subject details			<i>MRPS34</i> Variants	OXPHOS Enzyme Activities ^a		Clinical Summary		
ID	Sex	Ethnicity	cDNA (GenBank: NM_023936.1) Protein (GenBank: NP_076425.1)	Tissue	Deficient Enzymes	Age of Onset	Clinical Course	Clinical Features and Relevant Family History
S1	Male	Italian	c.[321+1G>T];[321+1G>T], p.[Val100_Gln107del];[Val100_Gln107del]	Muscle Liver Fibroblasts	CI, CIII, CIV CI, CIV CI, CIV	4 months	Died at 9 months	Leigh syndrome ^b , hyperlacticacidemia, microcephaly
S2a	Female	Puerto Rican	c.[322-10G>A];[322-10G>A], p.[Asn108Leufs*12, Asn108Glyfs*50, =]; [Asn108Leufs*12, Asn108Glyfs*50, =]	Muscle (19 months) Muscle (12 years)	Not deficient CI, CIII, CIV	6 months	Alive at 17 years	Leigh syndrome ^b , non-verbal, microcephaly, horseshoe kidney, mild coarsening of facial features
S2b	Female	Puerto Rican	c.[322-10G>A];[322-10G>A], p.[Asn108Leufs*12, Asn108Glyfs*50, =]; [Asn108Leufs*12, Asn108Glyfs*50, =]	Not performed		6 months	Alive at 14 years	Leigh-like syndrome, abnormal MRI, non-verbal, microcephaly, mild coarsening of facial features, sibling of S2a
S3a	Female	Puerto Rican	c.[322-10G>A];[322-10G>A], p.[Asn108Leufs*12, Asn108Glyfs*50, =]; [Asn108Leufs*12, Asn108Glyfs*50, =]	Muscle	CI, CII, CIII, CIV	6 months	Alive at 7 years	Leigh syndrome ^b , non-verbal, suspected sleep apnea, dysmorphic facies, precocious adrenarche
S3b	Female	Puerto Rican	c.[322-10G>A];[322-10G>A], p.[Asn108Leufs*12, Asn108Glyfs*50, =]; [Asn108Leufs*12, Asn108Glyfs*50, =]	Not performed		6 months	Alive at 2 years	Leigh syndrome ^b , suspected sleep apnea, sibling of S3a
S4	Male	French	c.[37G>A];[94C>T], p.[Glu13Lys];[Gln32*]	Muscle Fibroblasts	CIV CIV	~10 days	Died at 8.5 months	Leigh syndrome ^b , transient metabolic acidosis, hemodynamic instability related to tubulopathy

^aActivities of OXPHOS enzyme complexes I, II, III, IV (CI, CII, CIII, CIV) were measured in skeletal muscle, liver or skin fibroblasts; for details see Table S2.

^bDiagnoses of Leigh syndrome include compatible neuroimaging or postmortem findings.