

Mutations in *MTFMT* Underlie a Human Disorder of Formylation Causing Impaired Mitochondrial Translation

Elena J. Tucker,^{1,3,15} Steven G. Hershman,^{4,5,6,15} Caroline Köhrer,^{7,15} Casey A. Belcher-Timme,^{4,5,6} Jinal Patel,⁶ Olga A. Goldberger,^{4,5,6} John Christodoulou,^{8,9,10} Jonathon M. Silberstein,¹¹ Matthew McKenzie,¹² Michael T. Ryan,^{13,14} Alison G. Compton,¹ Jacob D. Jaffe,⁶ Steven A. Carr,⁶ Sarah E. Calvo,^{4,5,6} Uttam L. RajBhandary,⁷ David R. Thorburn,^{1,2,3,*} and Vamsi K. Mootha^{4,5,6,*}

¹Murdoch Childrens Research Institute

²Genetic Health Services Victoria

Royal Children's Hospital, Melbourne, VIC 3052, Australia

³Department of Paediatrics, University of Melbourne, Melbourne, VIC 3052, Australia

⁴Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA 02114, USA

⁵Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA

⁶Broad Institute, Cambridge, MA 02142, USA

⁷Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142, USA

⁸Genetic Metabolic Disorders Research Unit, Children's Hospital at Westmead, Sydney, NSW 2006, Australia

⁹Discipline of Paediatrics and Child Health

¹⁰Discipline of Genetic Medicine

University of Sydney, Sydney, NSW 2006, Australia

¹¹Department of Neurology, Princess Margaret Hospital for Children, Perth, WA 6008, Australia

¹²Centre for Reproduction and Development, Monash Institute of Medical Research, Monash University, Melbourne, VIC 3168, Australia

¹³Department of Biochemistry

¹⁴ARC Centre of Excellence for Coherent X-Ray Science

La Trobe University, Melbourne, VIC 3086, Australia

¹⁵These authors contributed equally to this work

*Correspondence: david.thorburn@mcri.edu.au (D.R.T.), vamsi@hms.harvard.edu (V.K.M.)

DOI 10.1016/j.cmet.2011.07.010

SUMMARY

The metazoan mitochondrial translation machinery is unusual in having a single tRNA^{Met} that fulfills the dual role of the initiator and elongator tRNA^{Met}. A portion of the Met-tRNA^{Met} pool is formylated by mitochondrial methionyl-tRNA formyltransferase (MTFMT) to generate N-formylmethionine-tRNA^{Met} (fMet-tRNA^{Met}), which is used for translation initiation; however, the requirement of formylation for initiation in human mitochondria is still under debate. Using targeted sequencing of the mtDNA and nuclear exons encoding the mitochondrial proteome (MitoExome), we identified compound heterozygous mutations in *MTFMT* in two unrelated children presenting with Leigh syndrome and combined OXPHOS deficiency. Patient fibroblasts exhibit severe defects in mitochondrial translation that can be rescued by exogenous expression of MTFMT. Furthermore, patient fibroblasts have dramatically reduced fMet-tRNA^{Met} levels and an abnormal formylation profile of mitochondrially translated COX1. Our findings demonstrate that *MTFMT* is critical for efficient human mitochondrial translation and reveal a human disorder of Met-tRNA^{Met} formylation.

INTRODUCTION

Of the ~90 protein components of the oxidative phosphorylation (OXPHOS) machinery, 13 are encoded by the mitochondrial DNA (mtDNA) and translated within the organelle. Defects in mitochondrial protein synthesis lead to combined OXPHOS deficiency. Although the mtDNA encodes the ribosomal and transfer RNAs, all remaining components of the mitochondrial translational machinery are encoded by nuclear genes and imported into the organelle. To date, mutations in more than ten different nuclear genes have been shown to cause defective mitochondrial translation in humans. However, molecular diagnosis by sequencing these candidates in patients with defects in mitochondrial translation is far from perfect (Kemp et al., 2011), underscoring the need to identify additional pathogenic mutations underlying these disorders.

Translation within metazoan mitochondria is reminiscent of the bacterial pathway, initiating with N-formylmethionine (fMet) (Kozak, 1983). Unlike bacteria, which encode distinct tRNA^{Met} molecules for translation initiation and elongation, metazoan mitochondria express a single tRNA^{Met} that fulfills both roles (Anderson et al., 1981). After aminoacylation of tRNA^{Met}, a portion of Met-tRNA^{Met} is formylated by mitochondrial methionyl-tRNA formyltransferase (MTFMT) to generate fMet-tRNA^{Met}. The mitochondrial translation initiation factor (IF2^{mt}) has high affinity for fMet-tRNA^{Met}, which is recruited to the ribosomal P site to initiate translation (Spencer and Spremulli, 2004).

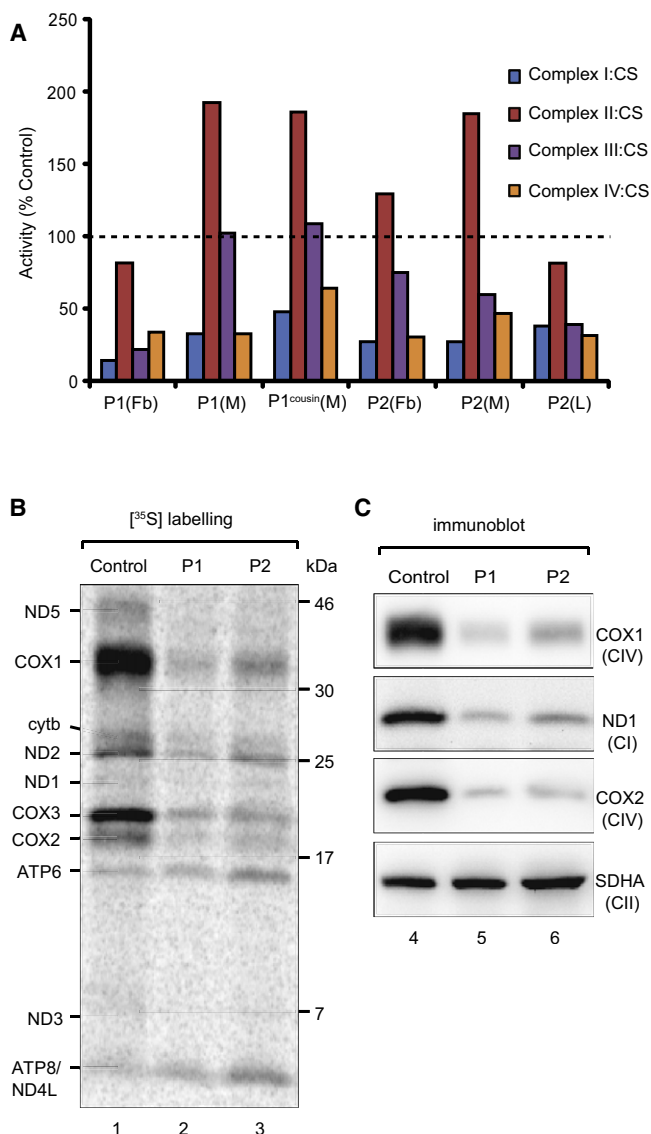


Figure 1. Combined OXPHOS Deficiency Due to a Defect in Mitochondrial Translation

(A) Biochemical analysis of OXPHOS complexes relative to citrate synthase (CS) in fibroblasts (Fb), muscle (M), or liver (L), expressed as a percent of mean from healthy controls.

(B) SDS-PAGE analysis of ³⁵S-methionine-labeled mtDNA-encoded proteins from control and patient fibroblasts. MtDNA-encoded subunits of complex I (ND1, ND2, ND3, ND5, ND4L), complex III (cytb), complex IV (COX1, COX2, COX3), and complex V (ATP6, ATP8) are shown.

(C) The gel in (B) was immunoblotted with antibodies against mtDNA-encoded ND1, COX1, COX2 and nuclear-encoded SDHA (complex II; loading control).

In contrast, the mitochondrial elongation factor (EF-Tu_m) specifically recruits Met-tRNA^{Met} to the ribosomal A site to participate in polypeptide elongation. Synthesized proteins can then be deformylated by a mitochondrial peptide deformylase (PDF) and demethylated by a mitochondrial methionyl aminopeptidase (MAP1D) (Serero et al., 2003; Walker et al., 2009).

Here, we applied targeted exome sequencing to two unrelated patients with Leigh syndrome and combined OXPHOS deficiency to discover pathogenic mutations in *MTFMT*. Fibroblasts from these patients have impaired Met-tRNA^{Met} formylation, peptide formylation, and mitochondrial translation. Despite studies in yeast suggesting that *MTFMT* is not essential for mitochondrial translation (Hughes et al., 2000; Li et al., 2000; Vial et al., 2003), we show that in humans this gene is required for efficient mitochondrial translation and function.

RESULTS

Mitochondrial Translation Is Impaired in Two Unrelated Patients with Leigh Syndrome

We studied two unrelated patients with Leigh syndrome and combined OXPHOS deficiency (Figure 1A). Clinical summaries for patient 1 (P1) and patient 2 (P2) are provided in the Supplemental Results (available online). Patient fibroblasts had reduced synthesis of most mtDNA-encoded proteins as assayed by [³⁵S]-methionine labeling in the presence of inhibitors of cytosolic translation (Figure 1B). This correlated with reduced steady state protein levels as detected by immunoblotting (Figure 1C), and, at least for ND1, was not due to reduced mRNA (Figure S1). Collectively, these data suggest a defect in translation of mtDNA-encoded proteins.

MitoExome Sequencing Identifies *MTFMT* Mutations

To elucidate the molecular basis of disease in P1 and P2, we performed next-generation sequencing of coding exons from 1034 nuclear-encoded mitochondrial-associated genes and the mtDNA (collectively termed the “MitoExome”). DNA was captured via an in-solution hybridization method (Gnirke et al., 2009) and sequenced on an Illumina GA-II platform (Bentley et al., 2008). Details are provided in the Supplemental Results and Table S1.

We identified ~700 single-nucleotide variants (SNVs) and short insertion or deletion variants (indels) in each patient relative to the reference genome, and prioritized those that may underlie a severe, recessive disease (Figure 2A). We first filtered out likely benign variants present at a frequency of >0.005 in public databases which left ~20 variants in each patient. We then prioritized variants that were predicted to have a deleterious impact on protein function (Calvo et al., 2010), leaving ~12 variants. Focusing on genes that fit autosomal recessive inheritance, having either homozygous variants or two different variants in the same gene, only one candidate gene, *MTFMT*, remained in each patient (Figure 2A).

We identified three distinct heterozygous variants in our patients (Figure 2B). Both patients harbor a c.626C → T mutation. The c.626C site is 20 bp upstream of the 3' end of exon 4 and is predicted to eliminate two overlapping exonic splicing enhancers (GTCAAG, TCAAGA) (Fairbrother et al., 2002) and to generate an exonic splicing suppressor (GTTGTT) (Wang et al., 2004). Skipping of exon 4 results in a frameshift and premature stop codon (p.R181SfsX5). The second mutation in P1 is a nonsense mutation (c.382C → T, p.R128X), while the second mutation in P2 changes a highly conserved serine to leucine in the catalytic core of *MTFMT* (c.374C → T, p.S125L).

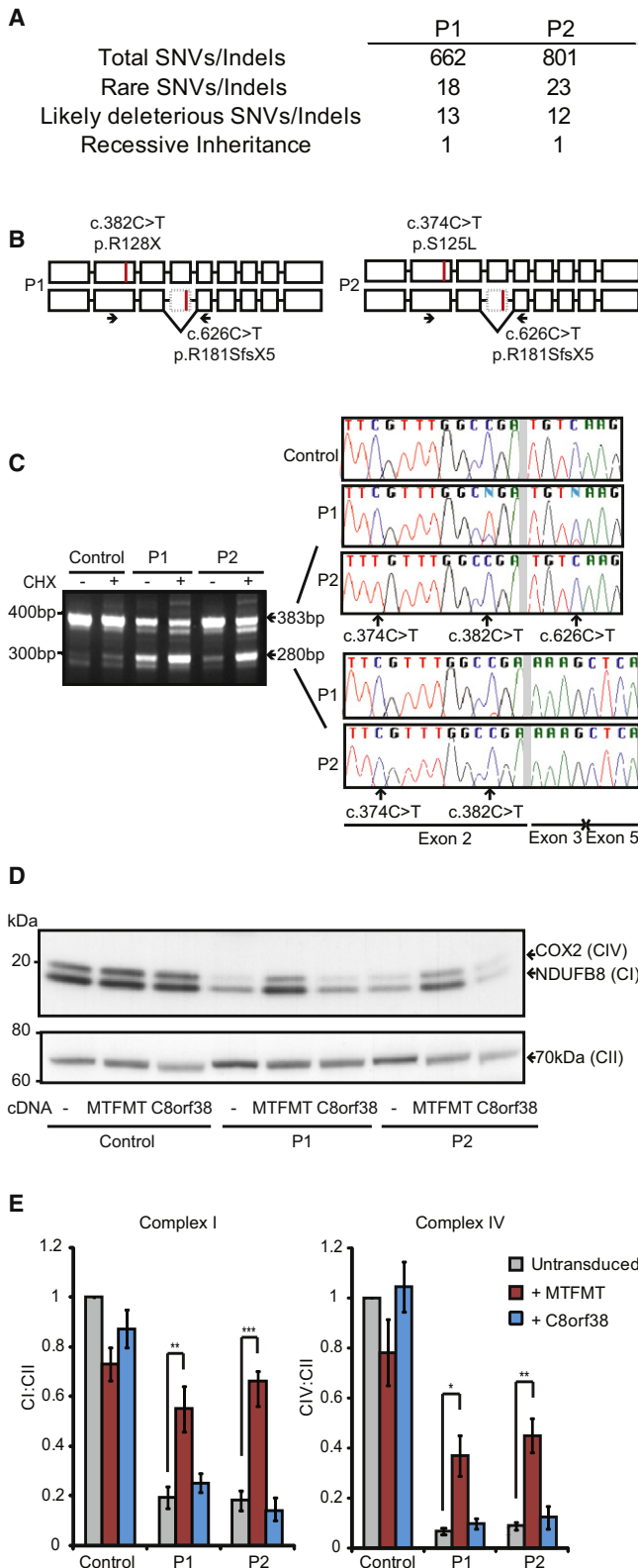


Figure 2. Identification of Pathogenic Compound Heterozygous Mutations in *MTFMT*

(A) Number of MitoExome variants that pass prioritization filters.

(Figure S2). An affected cousin of P1 also carries the c.382C → T and c.626C → T mutations.

As predicted by in silico analysis, the shared c.626C → T mutation caused skipping of exon 4 (Figure 2C). qRT-PCR analysis revealed that P1 had only 9% full-length *MTFMT* transcript compared to controls (Figure S2), the majority of which carries the c.382C → T nonsense mutation and lacks the c.626C → T splicing mutation (Figure 2C). P2 had 56% full-length *MTFMT* transcript (Figure S2), all of which appears to carry the c.374C → T mutation and to lack the c.626C → T splicing mutation (Figure 2C). Collectively, these results confirm compound heterozygosity of the *MTFMT* mutations and almost complete exon skipping due to the c.626C → T mutation.

Mitochondrial Translation Is Rescued in Patient Fibroblasts by Exogenous *MTFMT*

We used complementary DNA (cDNA) complementation to prove that the translation defect in these patients is due to mutations in *MTFMT*. Fibroblasts from both patients showed reduced levels of the mtDNA-encoded complex IV subunit, COX2, consistent with a defect in mitochondrial translation, and of the nuclear-encoded complex I subunit, NDUFB8, reflecting instability of complex I in the absence of mtDNA-encoded proteins (Figure 2D). Lentiviral transduction of *MTFMT* cDNA caused a significant increase of COX2 and NDUFB8 in both patients (Figure 2E). In contrast, lentiviral transduction of a control cDNA, *C8orf38*, caused no change of these subunits (Figure 2E). These data confirm that an *MTFMT* defect is responsible for the combined OXPHOS deficiency in these patients.

Mitochondrial tRNA^{Met} Pools Are Abnormal in Patient Fibroblasts

To directly analyze the mitochondrial tRNA^{Met} pools (Figure 3A), we used a modified protocol of acid-urea PAGE followed by northern blotting (Enríquez and Attardi, 1996; Köhrer and RajBhandary, 2008; Varshney et al., 1991) (Figure 3B). We were able to separate the mitochondrial uncharged tRNA^{Met},

(B) Schematic diagram of *MTFMT* showing the location of mutations in P1 and P2 (red bars), exon skipping (gray boxes), and primers for RT-PCR (forward and reverse arrows).

(C) Electrophoresis of RT-PCR products demonstrates a smaller cDNA species (280 bp) in P1 and P2 that is particularly prominent in cells grown in the presence of cycloheximide (+CHX). Top: Sequence chromatograms of full-length *MTFMT* RT-PCR products (-CHX) to confirm compound heterozygosity. Bottom: Sequence chromatograms of the smaller RT-PCR products (+CHX) shows patient cDNA lacks the c.382C → T (P1) or c.374C → T (P2) mutations and skips exon 4, which carries the shared c.626C → T mutation.

(D and E) Patient and control fibroblasts were transduced with *MTFMT* cDNA or control *C8orf38* cDNA. (D) Representative SDS-PAGE western blot shows reduced COX2 and NDUFB8 in patient fibroblasts and restoration of protein levels with *MTFMT* but not *C8orf38* transduction. The 70kDa complex II subunit acts as a loading control.

(E) Protein expression was quantified by densitometry and bar charts show the level of complex I (NDUFB8) or complex IV (COX2) relative to complex II (70 kDa) normalized to control, before and after transduction. Error bars show the mean of three biological replicates and error bars indicate ± 1 standard error of the mean (SEM). Asterisks indicate p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).

See also Figure S2 and Table S1.

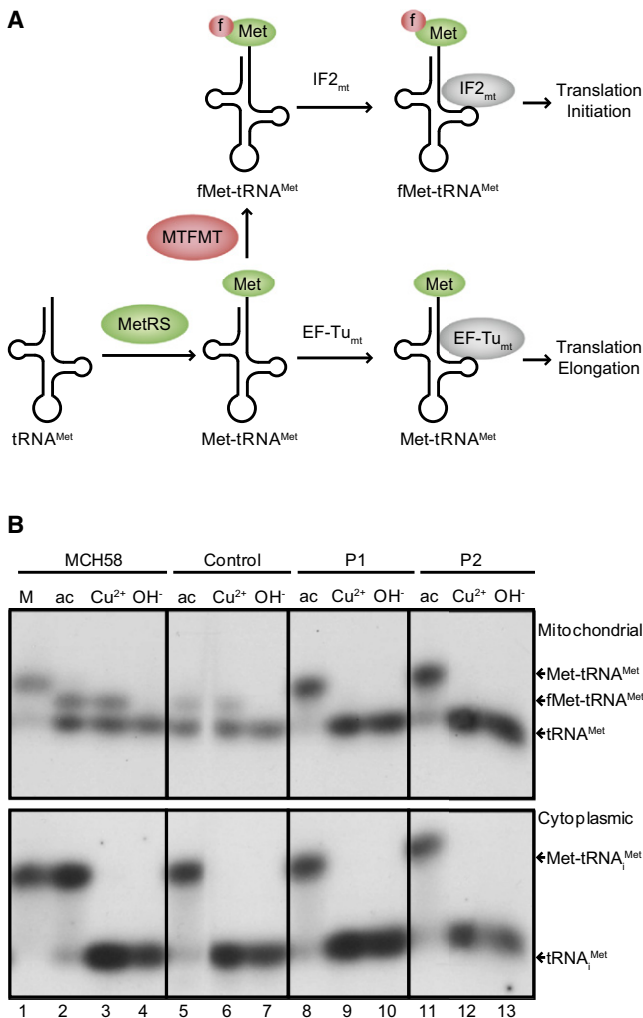


Figure 3. Patient Fibroblasts Have a Defect in Met-tRNA^{Met} Formylation

(A) In metazoan mitochondria, a single tRNA^{Met} species acts as both initiator and elongator tRNA^{Met}. After aminoacylation of tRNA^{Met} by the mitochondrial methionyl-tRNA synthetase (MetRS_{mt}), a portion of Met-tRNA^{Met} is formylated by MTFMT to generate fMet-tRNA^{Met}. fMet-tRNA^{Met} is used by the mitochondrial IF2 (IF2_{mt}) to initiate translation, whereas Met-tRNA^{Met} is recognized by the mitochondrial EF-Tu (EF-Tu_{mt}) for the elongation of translation products. (B) Total RNA from control (lanes 5–7) and patient fibroblasts (P1, lanes 8–10; P2, lanes 11–13) was separated by acid-urea PAGE. Total RNA from MCH58 cells is shown as a reference (lanes 1–4). The mitochondrial tRNA^{Met} (top) and the cytoplasmic initiator tRNA_i^{Met} (bottom) were detected by northern hybridization. Total RNA was isolated under acidic conditions, which preserves both the Met-tRNA^{Met} and fMet-tRNA^{Met} (ac); tRNAs were treated with copper sulfate (Cu²⁺), which specifically deacylates Met-tRNA^{Met} but not fMet-tRNA^{Met}, or with base (OH⁻), which deacylates both Met-tRNA^{Met} and fMet-tRNA^{Met}. Base-treated tRNA was reaminoacylated in vitro with Met using MetRS generating a Met-tRNA^{Met} standard (M).

Met-tRNA^{Met}, and fMet-tRNA^{Met} from total RNA isolated from fibroblasts and to show that two independent wild-type cell lines contained uncharged tRNA^{Met} and fMet-tRNA^{Met}, but very little Met-tRNA^{Met} (Figure 3B, lanes 1–7). In striking contrast, fibroblasts from P1 and P2 lacked detectable fMet-tRNA^{Met} and contained mostly Met-tRNA^{Met} along with traces of the uncharged

tRNA^{Met} (Figure 3B, compare lanes 8–13 to control lanes 5–7). We also observed a 2.7-fold increase of the overall mitochondrial tRNA^{Met} signal in patient fibroblasts compared to control (Figure 3B, top panel; compare lanes 8 and 11 to control lane 5), while the cytoplasmic initiator tRNA_i^{Met} showed constant signal throughout (Figure 3B, bottom panel). The analysis of the mitochondrial tRNA^{Met} pools clearly shows a defect in tRNA^{Met} formylation.

COX1 Protein Formylation Is Decreased in Patient Fibroblasts

Although fibroblasts from P1 and P2 have severely impaired mitochondrial translation, they do retain residual activity (Figure 1B). This residual activity could be due to (1) low activity of mutant MTFMT generating a small amount of fMet-tRNA^{Met} that is rapidly consumed in translation initiation and, therefore, undetectable by Northern blot analyses and/or (2) the human IF2_{mt} recognizing, albeit weakly, the nonformylated Met-tRNA^{Met} species to support translation initiation. Translation through the first mechanism would produce formylated protein, while translation through the second mechanism would produce unformylated protein.

To investigate these two possibilities, we used semiquantitative mass spectrometric analysis to simultaneously measure three possible N-terminal states of mitochondrially translated COX1: formylated (Figure 4A), unformylated (Figure 4B), and demethionylated (des-Met) (Figure 4C). We applied this method to complex IV immunoprecipitated from fibroblasts from P1 and P2 and two independent wild-type cell lines (Figure 4D). Although no fMet-tRNA^{Met} was detected in patient fibroblasts by northern blotting (Figure 3B), the dominant COX1 peptide in all four samples is the formylated species as estimated from total ion current of each form (Figure 4E). The expression of mitochondrial *PDF* and *MAP1D* was normal in patient fibroblasts (Figure S3). These semiquantitative analyses clearly demonstrate that patient fibroblasts retain residual MTFMT activity.

DISCUSSION

Here, we report human patients with mutations in *MTFMT*, a gene that has not been previously linked to human disease. We verified the causal mutations by rescuing the mitochondrial translation defects in patient fibroblasts via lentiviral transduction of *MTFMT*. Analysis of the tRNA^{Met} pools in patient fibroblasts revealed severe MTFMT dysfunction. To our knowledge, the human mitochondrial tRNA^{Met} profile has not been previously reported. It is interesting to note that control fibroblasts lack detectable Met-tRNA^{Met}, suggesting that it is utilized as quickly as it is produced; either converted to fMet-tRNA^{Met} or used to donate Met to the growing polypeptide chain. Strikingly, patient fibroblasts lack detectable levels of fMet-tRNA^{Met} and contain mostly Met-tRNA^{Met}.

Drastically decreased fMet-tRNA^{Met} levels prevent efficient mitochondrial translation as demonstrated by the reduced translation observed in patient fibroblasts. Although fibroblasts from P1 and P2 have severely impaired mitochondrial translation, they do retain some residual activity. To understand the origin of this activity, we measured the relative distribution of three possible N-terminal states of mitochondrially translated COX1

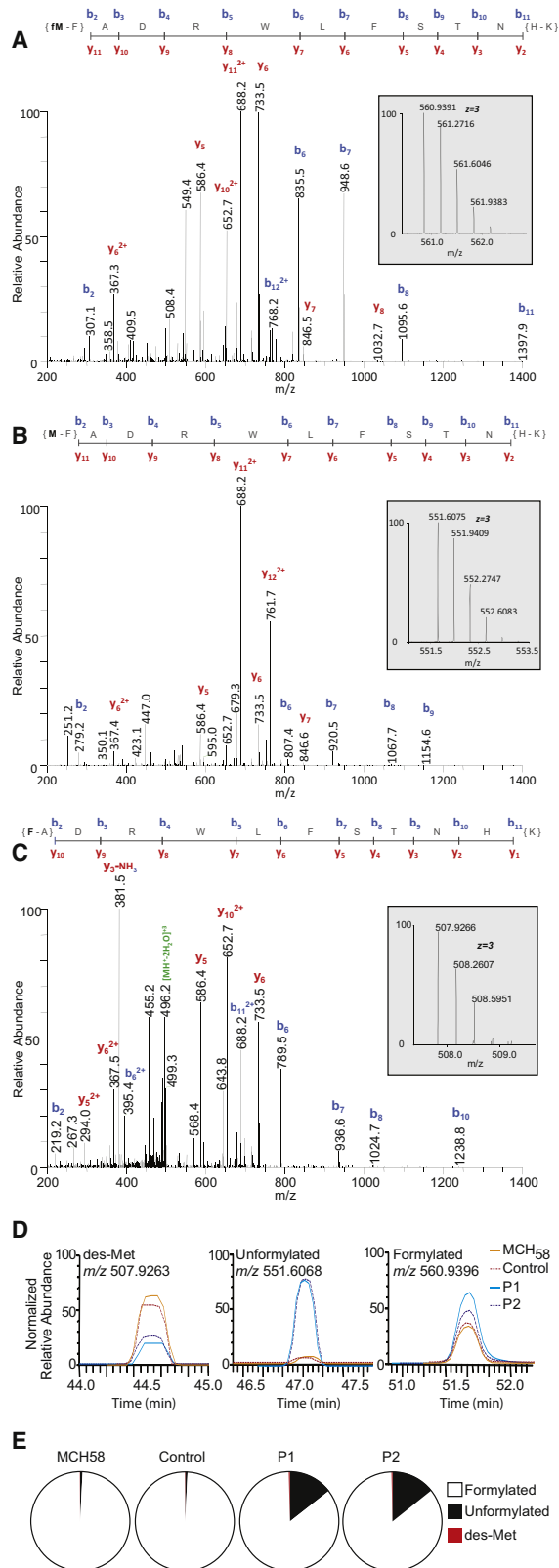


Figure 4. Analysis of the COX1 N Terminus in Patient Fibroblasts (A–C) Annotated MS/MS spectra confirming correct targeting of the three possible N termini of COX1. The sequence of the peptide is MFADRWLFSTNHK

by mass spectrometry. While previous studies have interrogated the formylation status of the N terminus of COX1 (Escobar-Alvarez et al., 2010), our study interrogates all three modification states and demonstrates mitochondrial methionine excision activity, which is detectable albeit weak.

Formylated COX1 is the dominant species in patient fibroblasts, indicating residual MTFMT activity. Assuming P1’s nonsense mutation has a full loss of function, then the allele harboring the shared c.626C → T mutation must confer MTFMT activity. Transcript that has not undergone skipping of exon 4 encodes an MTFMT variant harboring a p.S209L missense mutation. Residue p.S209 is moderately conserved and lies on the periphery of MTFMT based on homology with the bacterial enzyme. Similarly, P2’s residual MTFMT activity must originate from enzyme variants carrying the p.S209L mutation and/or the p.S125L mutation located in the active site.

Studies in bacteria and yeast have raised questions about the absolute requirement for Met-tRNA^{Met} formylation. Formylation is not essential in all bacteria (Newton et al., 1999) and in yeast disruption of *FMT1* causes no discernible defect in mitochondrial protein synthesis or function (Hughes et al., 2000; Li et al., 2000; Vial et al., 2003). Additionally, bovine IF2_{mt} is able to restore respiration in a yeast mutant lacking both IF2_{mt} and *FMT1* (Tibbetts et al., 2003), suggesting that bovine IF2_{mt}, like yeast IF2_{mt}, can initiate protein synthesis without fMet-tRNA^{Met}. However, a number of studies in mammals indicate that formylation of mitochondrial Met-tRNA^{Met} is required for translation initiation. Bovine IF2_{mt} has a 25- to 50-fold greater affinity for fMet-tRNA^{Met} than for Met-tRNA^{Met} in vitro (Spencer and Spemulli, 2004) and 12 of the 13 bovine mtDNA-encoded proteins retain fMet at the N terminus (Walker et al., 2009).

What are the factors that could allow nonformylated Met-tRNA^{Met} to initiate mitochondrial translation? In *Salmonella typhimurium*, amplification of initiator tRNA genes compensates for a lack of methionyl-tRNA formyltransferase activity and allows translation initiation without formylation of the initiator tRNA (Nilsson et al., 2006). The “upregulation” of the mitochondrial tRNA^{Met} in patient fibroblasts (Figure 3B) could, in principle, be a compensatory response due to limited fMet-tRNA^{Met}.

In summary, we have used MitoExome sequencing to identify *MTFMT* as a gene underpinning combined OXPHOS deficiency associated with Leigh syndrome. We have shown that patient fibroblasts have a striking deficiency of fMet-tRNA^{Met} leading to impaired mitochondrial translation. Despite studies in yeast suggesting that *MTFMT* is not essential for mitochondrial translation (Hughes et al., 2000; Li et al., 2000; Vial et al., 2003), we show here that in humans this gene is required for efficient mitochondrial translation and function. More generally, this study demonstrates how MitoExome sequencing can reveal insights

where the Met residue may be (A) formylated, (B) unformylated, or (C) absent (des-Met). Insets show high-resolution, high-mass accuracy precursors from which the fragmentation spectra were derived. Given their sequence similarity, peptides are expected to have similar ionization efficiencies.

(D) Extracted ion chromatograms (XICs) of three N-terminal states of COX1 ([fMet, Met, des-Met]FADRWLFSTNHK), normalized to an internal COX1 peptide (VFSWLATLHGNSNMK).

(E) Fractional ion current of the three N-terminal states of COX1 from immunoprecipitated complex IV of patients and controls.

See also Figure S3.

into basic biochemistry and the molecular basis of mitochondrial disease.

EXPERIMENTAL PROCEDURES

Cell Culture

Cells were grown at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen).

Biochemical Analysis

Spectrophotometric analysis of mitochondrial OXPHOS activity was performed as described previously (Kirby et al., 1999). Investigations were performed with informed consent and in compliance with ethics approval by the Human Research Ethics Committee of the Royal Children's Hospital, Melbourne.

Translation Assays

MtDNA-encoded proteins in patient fibroblasts were labeled with ³⁵S-methionine/³⁵S-cysteine (EXPRE³⁵S³⁵S Protein Labeling Mix; Perkin Elmer Life Sciences) prior to mitochondrial isolation and analysis of translation products by SDS-PAGE as previously described (McKenzie et al., 2009).

SDS-PAGE and Immunoblotting

Immunoblotting was performed as previously described (Calvo et al., 2010). Proteins were detected with the following antibodies: complex II α -70 kDa subunit monoclonal antibody (MitoSciences, MS204), ND1 polyclonal antibody (kind gift from Anne Lombes, Paris), α -complex IV subunit I monoclonal antibody (Invitrogen, A459600), α -complex IV subunit II monoclonal antibody (Invitrogen, A6404), Total OXPHOS Human WB Antibody Cocktail containing α NDUFB8 and α COX2 (MitoSciences, MS601), and either α -mouse or α -rabbit IgG horseradish peroxidase (HRP; DakoCytomation).

MitoExome Sequencing

We used an in-solution hybridization capture method (Gnirke et al., 2009) to isolate target DNA, which was sequenced on the Illumina GA-II platform (Bentley et al., 2008). The 4.1 Mb of targeted DNA included the 16 kb mtDNA and all coding and untranslated exons of 1381 nuclear genes, including 1013 mitochondrial genes from the MitoCarta database (Pagliarini et al., 2008), 21 genes with recent strong evidence of mitochondrial association, and 347 additional genes. All analyses were restricted to the mtDNA and coding exons of the 1034 genes with confident evidence of mitochondrial association (1.4 Mb). Detailed methods for target selection, sequencing, alignment and variant detection are submitted elsewhere (S.E.C., unpublished data).

Variant Prioritization

Differences in DNA sequence between each individual and the GRCh37 human reference assembly were identified. Nuclear variants that passed quality control metrics were prioritized according to three criteria: (1) SNV allele frequency <0.005 in public databases (dbSNP [Sherry et al., 2001] version 132 and the 1000 genomes project [Durbin et al., 2010] released November 2010) or indels absent in the 1000 genomes data, (2) variants predicted to modify protein function as previously described (Calvo et al., 2010), and (3) variants consistent with recessive inheritance (homozygous variants or two heterozygous variants in the same gene). We also prioritized mtDNA variants annotated as pathogenic in MITOMAP (Ruiz-Pesini et al., 2007). Detailed methods are submitted elsewhere (S.E.C., unpublished data).

Sanger DNA Sequencing

DNA isolation, RNA isolation, cDNA synthesis, inhibition of nonsense mediated decay, and sequencing of PCR products were performed as described previously (Calvo et al., 2010).

Lentiviral Transduction

The *MTFMT* open reading frame (ORF) was purchased in a pCMV-SPORT6 vector (Clone ID: BC033687.1, Open Biosystems) and was cloned into the 4-hydroxytamoxifen-inducible lentiviral vector, pF_{5x}UAS_MCS_SV40_puroGEV16-W (Yeap et al., 2010).

MTFMT viral particles were generated and patient fibroblasts were transduced as described previously (Calvo et al., 2010). Three independent transductions were performed and cells were harvested after 10–12 days selection with 1 μ g/ml puromycin.

Acid-Urea PAGE and Northern Blotting

Total RNA was isolated from frozen cells under acidic conditions with TRIzol (Invitrogen) according to the manufacturer's instructions. Acid-washed glass beads (0.5 mm diameter; Sigma) were added during extraction. Total RNAs were separated by acid-urea PAGE as described previously (Enriquez and Attardi, 1996; Köhrer and RajBhandary, 2008; Varshney et al., 1991) with modifications. In brief, 0.1 A₂₆₀ units of each RNA sample were loaded onto a 6.5% polyacrylamide gel containing 7 M urea and 0.2 M sodium acetate (pH 5.0). Individual tRNAs were detected by northern blotting (Köhrer and RajBhandary, 2008) with the following hybridization probes: 5'TAGTACGG GAAGGTATAA3' (mitochondrial tRNA^{Met}) and 5'TTCCACTGCACCACT CTGCT3' (cytoplasmic initiator tRNA^{Met}). Northern blots were quantified by PhosphorImager analysis with ImageQuant software (Molecular Dynamics). Experiments were performed in duplicate.

Aminoacyl-tRNAs and formylaminoacyl-tRNAs were deacylated by base treatment in 0.1 M Tris-HCl (pH 9.5) at 65°C for 5 min, followed by incubation at 37°C for 1 hr. Aminoacyl-tRNAs were selectively deacylated by treatment with copper sulfate as described (Schofield and Zamecnik, 1968). Total RNA was aminoacylated in vitro with methionine using *E. coli* MetRS as previously described with minor modifications (Köhrer and RajBhandary, 2008).

Mass Spectrometric Analysis of COX1 N Termini

In brief, complex IV was immunoprecipitated from control and patient fibroblasts with MitoSciences' complex IV Immunocapture kit (MS-401) and separated by gel electrophoresis on a NuPAGE 4%–12% Bis-Tris gel (Invitrogen). A band corresponding to the MW of COX1 was excised and subjected to in-gel Lys-C digestion (Kinter and Sherman). Extracted peptides were separated on C18 column with a 1200-Series nano-LC pump (Agilent) and run on a LTQ-Velos-Orbitrap mass spectrometer (ThermoFisher) set to scan and to targeted MS/MS for *m/z*s corresponding to the *z* = 3 states of the unformylated, formylated and des-Met species of the N-terminal peptide of COX1 (MFADRWLFSTNHK).

Extracted ion chromatographs (XICs) were generated from the Orbitrap survey scans based on the *z* = 3 states of the unformylated, formylated and des-Met species of the N-terminal peptide of COX1 using XCalibur software (ThermoFisher Scientific). The identities of the peaks corresponding to these species were verified with the accompanying static MS/MS spectra (Figures 4A–4C). The areas under these peaks were integrated with the Genesis peak detection algorithm included in XCalibur with all standard defaults. Peak areas were further normalized to the peak area of a distal peptide of COX1 (VFSWLATLHGSNMK, *m/z* 795.9085, *z* = 2) to ensure that comparisons allowed for the different amounts of COX1 in control and patient samples. Full methods are in the Supplemental Experimental Procedures.

Statistical Analysis

Two-way repeated-measures analysis of variance (ANOVA) was used for comparisons of groups followed by post hoc analysis via the Bonferroni method.

ACCESSION NUMBERS

The GenBank accession number for the *MTFMT* sequence reported in this paper is NM_139242.3.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at doi:10.1016/j.cmet.2011.07.010.

ACKNOWLEDGMENTS

We thank J. Silke and P. Ekert for providing the pF_{5x}UAS_MCS_SV40_puroGEV16-W vector, C. Guiducci, C. Sougnez, L. Ambrogia, and J. Wilkinson,

for assistance with sample preparation and sequencing, T. Fennel, M. DePristo, E. Banks, and K. Garimella for assistance with bioinformatic analysis, S. Flynn for assistance with IRBs, and the subjects and referring physicians who participated in the study. This work was supported by an Australian Postgraduate Award to E.J.T., a National Defense Science and Engineering Graduate Fellowship to S.G.H., an Australian National Health and Medical Research Council (NHMRC) Career Development Award to M.M., an NHMRC Principal Research fellowship to D.R.T., the Victorian Government's Operational Infrastructure Support Program, and grants from the Ramaciotti Foundation and the James and Vera Lawson Trust to M.M., the NHMRC to M.M., M.T.R. and D.R.T., and the National Institutes of Health to U.L.R. (GM17151) and to V.K.M. (GM077465 and GM097136).

Received: April 22, 2011

Revised: July 6, 2011

Accepted: July 26, 2011

Published: September 6, 2011

REFERENCES

- Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., et al. (1981). Sequence and organization of the human mitochondrial genome. *Nature* 290, 457–465.
- Bentley, D.R., Balasubramanian, S., Swerdlow, H.P., Smith, G.P., Milton, J., Brown, C.G., Hall, K.P., Evers, D.J., Barnes, C.L., Bignell, H.R., et al. (2008). Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* 456, 53–59.
- Calvo, S.E., Tucker, E.J., Compton, A.G., Kirby, D.M., Crawford, G., Burt, N.P., Rivas, M., Guiducci, C., Bruno, D.L., Goldberger, O.A., et al. (2010). High-throughput, pooled sequencing identifies mutations in NUBPL and FOXRED1 in human complex I deficiency. *Nat. Genet.* 42, 851–858.
- Durbin, R.M., Abecasis, G.R., Altshuler, D.L., Auton, A., Brooks, L.D., Gibbs, R.A., Hurles, M.E., and McVean, G.A.; 1000 Genomes Project Consortium. (2010). A map of human genome variation from population-scale sequencing. *Nature* 467, 1061–1073.
- Enriquez, J.A., and Attardi, G. (1996). Analysis of aminoacylation of human mitochondrial tRNAs. *Methods Enzymol.* 264, 183–196.
- Escobar-Alvarez, S., Gardner, J., Sheth, A., Manfredi, G., Yang, G., Ouerfelli, O., Heaney, M.L., and Scheinberg, D.A. (2010). Inhibition of human peptide deformylase disrupts mitochondrial function. *Mol. Cell. Biol.* 30, 5099–5109.
- Fairbrother, W.G., Yeh, R.F., Sharp, P.A., and Burge, C.B. (2002). Predictive identification of exonic splicing enhancers in human genes. *Science* 297, 1007–1013.
- Gnirke, A., Melnikov, A., Maguire, J., Rogov, P., LeProust, E.M., Brockman, W., Fennell, T., Giannoukos, G., Fisher, S., Russ, C., et al. (2009). Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nat. Biotechnol.* 27, 182–189.
- Hughes, T.R., Marton, M.J., Jones, A.R., Roberts, C.J., Stoughton, R., Armour, C.D., Bennett, H.A., Coffey, E., Dai, H., He, Y.D., et al. (2000). Functional discovery via a compendium of expression profiles. *Cell* 102, 109–126.
- Kemp, J.P., Smith, P.M., Pyle, A., Neeve, V.C.M., Tuppen, H.A.L., Schara, U., Talim, B., Topaloglu, H., Holinski-Feder, E., Abicht, A., et al. (2011). Nuclear factors involved in mitochondrial translation cause a subgroup of combined respiratory chain deficiency. *Brain* 134, 183–195.
- Kirby, D.M., Crawford, M., Cleary, M.A., Dahl, H.H., Dennett, X., and Thorburn, D.R. (1999). Respiratory chain complex I deficiency: an underdiagnosed energy generation disorder. *Neurology* 52, 1255–1264.
- Köhler, C., and RajBhandary, U.L. (2008). The many applications of acid urea polyacrylamide gel electrophoresis to studies of tRNAs and aminoacyl-tRNA synthetases. *Methods* 44, 129–138.
- Kozak, M. (1983). Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. *Microbiol. Rev.* 47, 1–45.
- Li, Y., Holmes, W.B., Appling, D.R., and RajBhandary, U.L. (2000). Initiation of protein synthesis in *Saccharomyces cerevisiae* mitochondria without formylation of the initiator tRNA. *J. Bacteriol.* 182, 2886–2892.
- McKenzie, M., Lazarou, M., and Ryan, M.T. (2009). Chapter 18 Analysis of respiratory chain complex assembly with radiolabeled nuclear- and mitochondrial-encoded subunits. *Methods Enzymol.* 456, 321–339.
- Newton, D.T., Creuzenet, C., and Mangroo, D. (1999). Formylation is not essential for initiation of protein synthesis in all eubacteria. *J. Biol. Chem.* 274, 22143–22146.
- Nilsson, A.I., Zorzet, A., Kanth, A., Dahlström, S., Berg, O.G., and Andersson, D.I. (2006). Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. *Proc. Natl. Acad. Sci. USA* 103, 6976–6981.
- Pagliarini, D.J., Calvo, S.E., Chang, B., Sheth, S.A., Vafai, S.B., Ong, S.E., Walford, G.A., Sugiana, C., Boneh, A., Chen, W.K., et al. (2008). A mitochondrial protein compendium elucidates complex I disease biology. *Cell* 134, 112–123.
- Ruiz-Pesini, E., Lott, M.T., Procaccio, V., Poole, J.C., Brandon, M.C., Mishmar, D., Yi, C., Kreuziger, J., Baldi, P., and Wallace, D.C. (2007). An enhanced MITOMAP with a global mtDNA mutational phylogeny. *Nucleic Acids Res.* 35 (Database issue), D823–D828.
- Schofield, P., and Zamecnik, P.C. (1968). Cupric ion catalysis in hydrolysis of aminoacyl-tRNA. *Biochim. Biophys. Acta* 155, 410–416.
- Serero, A., Giglione, C., Sardini, A., Martinez-Sanz, J., and Meinel, T. (2003). An unusual peptide deformylase features in the human mitochondrial N-terminal methionine excision pathway. *J. Biol. Chem.* 278, 52953–52963.
- Sherry, S.T., Ward, M.-H., Kholodov, M., Baker, J., Phan, L., Smigielski, E.M., and Sirotkin, K. (2001). dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res.* 29, 308–311.
- Spencer, A.C., and Spremulli, L.L. (2004). Interaction of mitochondrial initiation factor 2 with mitochondrial fMet-tRNA. *Nucleic Acids Res.* 32, 5464–5470.
- Tibbetts, A.S., Oesterlin, L., Chan, S.Y., Kramer, G., Hardesty, B., and Appling, D.R. (2003). Mammalian mitochondrial initiation factor 2 supports yeast mitochondrial translation without formylated initiator tRNA. *J. Biol. Chem.* 278, 31774–31780.
- Varshney, U., Lee, C.P., and RajBhandary, U.L. (1991). Direct analysis of aminoacylation levels of tRNAs in vivo. Application to studying recognition of *Escherichia coli* initiator tRNA mutants by glutamyl-tRNA synthetase. *J. Biol. Chem.* 266, 24712–24718.
- Vial, L., Gomez, P., Panvert, M., Schmitt, E., Blanquet, S., and Mechulam, Y. (2003). Mitochondrial methionyl-tRNA^{fMet} formyltransferase from *Saccharomyces cerevisiae*: gene disruption and tRNA substrate specificity. *Biochemistry* 42, 932–939.
- Walker, J.E., Carroll, J., Altman, M.C., and Fearnley, I.M. (2009). Chapter 6 Mass spectrometric characterization of the thirteen subunits of bovine respiratory complexes that are encoded in mitochondrial DNA. *Methods Enzymol.* 456, 111–131.
- Wang, Z., Rolish, M.E., Yeo, G., Tung, V., Mawson, M., and Burge, C.B. (2004). Systematic identification and analysis of exonic splicing silencers. *Cell* 119, 831–845.
- Yeap, Y.Y., Ng, I.H., Badrian, B., Nguyen, T.V., Yip, Y.Y., Dhillon, A.S., Mutsaers, S.E., Silke, J., Bogoyevitch, M.A., and Ng, D.C. (2010). c-Jun N-terminal kinase/c-Jun inhibits fibroblast proliferation by negatively regulating the levels of stathmin/oncoprotein 18. *Biochem. J.* 430, 345–354.