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

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SUMMARY

The metazoan mitochondrial translation machinery is unusual in having a single tRNA\textsuperscript{Met} that fulfills the dual role of the initiator and elongator tRNA\textsuperscript{Met}. A portion of the Met-tRNA\textsuperscript{Met} pool is formylated by mitochondrial methionyl-tRNA formyltransferase (MTFMT) to generate N-formylmethionine-tRNA\textsuperscript{Met} (fMet-tRNA\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{Met} molecules for translation initiation and elongation, metazoan mitochondria express a single tRNA\textsuperscript{Met} that fulfills both roles (Anderson et al., 1981). After aminocacylation of tRNA\textsuperscript{Met}, a portion of Met-tRNA\textsuperscript{Met} is formylated by mitochondrial methionyl-tRNA formyltransferase (MTFMT) to generate fMet-tRNA\textsuperscript{Met}. The mitochondrial translation initiation factor (IF2\textsubscript{mt}) has high affinity for fMet-tRNA\textsuperscript{Met}, which is recruited to the ribosomal P site to initiate translation (Spencer and Spremulli, 2004).
In contrast, the mitochondrial elongation factor (EF-Tu mt) specifically recruits Met-tRNA\textsuperscript{Met} to the ribosomal A site to participate in polypeptide elongation. Synthesized proteins can then be deformedylated by a mitochondrial peptide deformylase (PDF) and demethionylated 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 (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 \textsuperscript{35}S-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.

**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 \textsuperscript{35}S-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 \(\rightarrow\) 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 (GTGTGT) (Wang et al., 2004). Skipping of exon 4 results in a frameshift and premature stop codon (p.R128X). The second mutation in P1 is a nonsense mutation (c.382C \(\rightarrow\) T, p.R128X), while the second mutation in P2 changes a highly conserved serine to leucine in the catalytic core of MTFMT (c.374C \(\rightarrow\) T, p.S125L).
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<sub>Met</sub> Pools Are Abnormal in Patient Fibroblasts

To directly analyze the mitochondrial tRNA<sub>Met</sub> 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<sub>Met</sub>.

Figure 2. Identification of Pathogenic Compound Heterozygous Mutations in MTFMT

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

(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 mutation and to lack 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.

MTFMT Mutations Impair Mitochondrial Translation

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

Figure 3. Mitochondrial tRNA<sub>Met</sub> Pools Are Abnormal in Patient Fibroblasts

(1) Representative western blots showing reduced COX2 and NDUFB8 in patient fibroblasts and restoration of protein levels with MTFMT cDNA or control C8orf38 cDNA.

(2) 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.

(3) 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.
**Cell Metabolism**

*MTFMT* Mutations Impair Mitochondrial Translation

Figure 3. Patient Fibroblasts Have a Defect in Met-tRNA<sup>Met</sup> Formylation

(A) In metazoan mitochondria, a single tRNA<sup>Met</sup> species acts as both initiator and elongator tRNA<sup>Met</sup>. After aminoacylation of tRNA<sup>Met</sup> by the mitochondrial methionyl-tRNA synthetase (MetRS<sub>mt</sub>), a portion of Met-tRNA<sup>Met</sup> is formylated by MTFMT to generate fMet-tRNA<sup>Met</sup>. fMet-tRNA<sup>Met</sup> is used by the mitochondrial IF2 (IF2<sub>mt</sub>) to initiate translation, whereas Met-tRNA<sup>Met</sup> is recognized by the mitochondrial EF-Tu (EF-Tu<sub>mt</sub>) 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<sup>Met</sup> 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<sup>Met</sup> showed constant signal throughout (Figure 3B, bottom panel). The analysis of the mitochondrial tRNA<sup>Met</sup> pools clearly shows a defect in tRNA<sup>Met</sup> 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<sup>Met</sup> that is rapidly consumed in translation initiation and, therefore, undetectable by Northern blot analyses and/or (2) the human IF2<sub>mt</sub> recognizing, albeit weakly, the nonformylated Met-tRNA<sup>Met</sup> 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<sup>Met</sup> 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<sup>Met</sup> pools in patient fibroblasts revealed severe MTFMT dysfunction. To our knowledge, the human mitochondrial tRNA<sup>Met</sup> profile has not been previously reported. It is interesting to note that control fibroblasts lack detectable Met-tRNA<sup>Met</sup>, suggesting that it is utilized as quickly as it is produced; either converted to fMet-tRNA<sup>Met</sup> or used to donate Met to the growing polypeptide chain. Strikingly, patient fibroblasts lack detectable levels of fMet-tRNA<sup>Met</sup> and contain mostly Met-tRNA<sup>Met</sup>.

Drastically decreased fMet-tRNA<sup>Met</sup> 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.

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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 Spremulli, 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 into the genetic basis of complex mitochondrial disorders.

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**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 MFADRWLFSNTHK.

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

(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 (Köhrer and RajBhandary, 2008). Experiments 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**
MitDNA-encoded proteins in patient fibroblasts were labeled with [35S]-methionine/35S-cysteine (EXPRESS 35S 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 Lombers, Paris), α-complex IV subunit I monoclonal antibody (Invitrogen, 459600), α-complex IV subunit II monoclonal antibody (Invitrogen, A6404), Total OXPHOS Human WB Antibody Cocktail containing αNDUFB8 and αCOX2 (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 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 [Durbín 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 cloned into the 4-hydroxytamoxifen-inducible lentiviral vector, pF_Sx_UAS_MCS_SV40_puroGEV16-W (Yap et al., 2010).

**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 to targeted MS/MS for m/zs corresponding to the z = 3 states of the unformylated, formylated and des-Met species of the N-terminal peptide of COX1 (MFADRWLFSSTNHK).

**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.

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