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

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

Citation

As Published
http://dx.doi.org/10.1016/j.cmet.2011.07.010

Publisher
Elsevier

Version
Final published version

Accessed
Wed Dec 19 07:10:39 EST 2018

Citable Link
http://hdl.handle.net/1721.1/92287

Terms of Use
Article is made available in accordance with the publisher’s policy and may be subject to US copyright law. Please refer to the publisher’s site for terms of use.

Detailed Terms
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 Jonathan 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. Mootha4,5,6,*

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 mitochondrial 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 aminoclylation 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).
MTFMT Mutations Impair Mitochondrial Translation

Here, we applied targeted exome sequencing to two unrelated patients with Leigh syndrome and combined OXPHOS deficiency (Figure 1). 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.

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 (GGTGT) (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).
MTFMT Mutations Impair Mitochondrial Translation

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\(^{\text{Met}}\) Pools Are Abnormal in Patient Fibroblasts

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

Figure 2. Identification of Pathogenic Compound Heterozygous Mutations in MTFMT

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

**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**

Met-tRNA<sup>Met</sup> and fMet-tRNA<sup>Met</sup> from total RNA isolated from fibroblasts and to show that two independent wild-type cell lines contained uncharged tRNA<sup>Met</sup> and fMet-tRNA<sup>Met</sup>, but very little Met-tRNA<sup>Met</sup> (Figure 3B, lanes 1–7). In striking contrast, fibroblasts from P1 and P2 lacked detectable fMet-tRNA<sup>Met</sup> and contained mostly Met-tRNA<sup>Met</sup> along with traces of the uncharged tRNA<sup>Met</sup> (Figure 3B, compare lanes 8–13 to control lanes 5–7). We also observed a 2.7-fold increase of the overall 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.

**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> (top) and the cytoplasmic initiator tRNA<sup>Met</sup> (bottom) were detected 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.
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 fibro-
blasts, 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. Formyla-
tion is not essential in all bacteria (Newton et al., 1999) and in
yeast disruption of FMT1 causes no discernible defect in mito-
chondrial 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 indi-
cate 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 mitochon-
drial 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 trans-
lation (Hughes et al., 2000; Li et al., 2000; Vial et al., 2003), we
show here that in humans this gene is required for efficient mito-
chondrial 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 pep-
tide (VFSWTLHGSNMRK).

(E) Fractional ion current of the three N-terminal states of COX1 from immu-
noprecipitated 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**

Mitochondrial 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 Lombre, Paris), α-complex IV subunit I monoclonal antibody (Invitrogen, 459660), α-complex IV subunit II monoclonal antibody (Invitrogen, A6404), Total OXPHOS Human WB Antibody Cocktail containing nNDUF8 and aCOX2 (MitoSciences, MS601), and either α-mouse or α-rabbit IgG horseradish peroxidase (HRP; DakoCytoMation).

**MitofExome Sequencing**

We used an in-solution hybridization capture method (Gnirke et al., 2009) to MitoExome Sequencing to capture mitochondrial DNA (mtDNA). The 4.1 Mb of targeted DNA included the 16 kb mtDNA puroGEV16-W (Yeap et al., 2010). 4-hydroxytamoxifen-inducible lentiviral vector, pF_5x_UAS_MCS_SV40_Lentiviral Transduction previously (Calvo et al., 2010).

**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. 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.

**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 this article online at doi:10.1016/j.cmet.2011.07.010.

**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.

**Accession Numbers**

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


