Mitochondrial translation requires folate-dependent tRNA methylation

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Folates enable the activation and transfer of one-carbon units for the biosynthesis of purines, thymidine and methionine1–3. Antifolates are important immunosuppressive1 and anticancer agents5. In proliferating lymphocytes6 and human cancers7,8, mitochondrial folate enzymes are particularly strongly upregulated. This in part reflects the need for mitochondria to generate one-carbon units and export them to the cytosol for anabolic metabolism2,9. The full range of uses of folate-bound one-carbon units in the mitochondrial compartment itself, however, has not been thoroughly explored. Here we show that loss of the catalytic activity of the mitochondrial folate enzyme serine hydroxymethyltransferase 2 (SHMT2), but not of other folate enzymes, leads to defective oxidative phosphorylation in human cells due to impaired mitochondrial translation. We find that SHMT2, presumably by generating mitochondrial 5,10-methylenetetrahydrofolate, provides methyl donors to produce the taurinomuramyl base at the wobble position of select mitochondrial tRNAs. Mitochondrial ribosome profiling in SHMT2-knockout human cells reveals that the lack of this modified base causes defective translation, with preferential mitochondrial ribosome stalling at certain lysine (AAG) and leucine (UUG) codons. This results in the impaired expression of respiratory chain enzymes. Stalling at these specific codons also occurs in certain inborn errors of mitochondrial metabolism. Disruption of whole-cell folate metabolism, by either folate deficiency or antifolate treatment, also impairs the respiratory chain. In summary, mammalian mitochondria use folate-bound one-carbon units to methylate tRNA, and this modification is required for mitochondrial translation and thus oxidative phosphorylation.

The major source of folate one-carbon (1C) units in mammalian cells is the amino acid serine1–3. Transfer of the 1C unit of serine to tetrahydrofolate (THF) can occur in either the cytosol or the mitochondrion, via the enzyme SHMT1 or SHMT2, respectively10 (Fig. 1a). Evidence from stable isotope tracing indicates that cancer cells predominantly use SHMT2 to catabolize serine, exporting the resulting 1C units to the cytosol to support nucleotide synthesis2,9. The extent to which 1C unit production via SHMT2 is also important to support mitochondrial health has yet to be determined.

When characterizing a set of human HCT116 colon cancer CRISPR-deletion cell lines that lack folate 1C enzymes, we discovered that the loss of SHMT2 induces a change in media colour indicative of enhanced extracellular acidification (Extended Data Fig. 1a). Quantitative analysis of the media confirmed increased glucose uptake and lactate secretion. This effect was specific to SHMT2: loss of other core 1C enzymes, including SHMT1 and mitochondrial enzymes such as MTHFD2 and MTHFD1L, did not induce glycolysis (Fig. 1b, Extended Data Fig. 1b).

A common cause of increased glycolytic flux is respiratory deficiency11. Loss of SHMT2 reduced both basal respiration and maximal respiratory capacity and decreased the NAD+/NADH ratio in several HCT116 (Fig. 1c, Extended Data Fig. 1c) and HEK293T SHMT2-knockout clones. Knockout cell lines lacking other core folate enzymes did not show impaired respiration (Extended Data Fig. 1d, e). Consistent with respiratory chain deficiency, the loss of SHMT2 decreased glucose flux into the tricarboxylic acid (TCA) cycle intermediate citrate, with an increased fraction of citrate instead being produced by reductive carboxylation12. As reported recently in other models of mitochondrial damage13,14, the pool size of TCA cycle metabolites and associated amino acids was also decreased (Extended Data Fig. 1f, g). To identify the cause of respiratory deficiency, we examined the abundances of several mitochondrial proteins, and found decreased abundances of complex I, IV and V subunits with retained levels of complexes II and III and markers of mitochondrial mass (Fig. 1d and Extended Data Fig. 1h, i). Thus, SHMT2 is required to maintain the levels of several mitochondrial respiratory chain proteins.

Given that the loss of SHMT2, but not of the immediate downstream enzymes of mitochondrial 1C metabolism, caused impaired oxidative phosphorylation, we wondered whether the phenotype reflected a requirement for the catalytic activity of SHMT2, or alternatively a non-catalytic role of SHMT2, perhaps related to its reported interaction with the mitochondrial nucleoid15. Accordingly, in the SHMT2-knockout background, we stably re-expressed catalytically inactive SHMT2 (p.Glu98Leu/p.Tyr106Phe), pyridoxal 5′-phosphate (PLP)-binding mutant SHMT2 (p.Lys280Gln) or wild-type SHMT2 protein (Extended Data Figs 2, 3a). Re-expression of wild-type protein, but not the catalytically inactive mutants, rescued the oxidative phosphorylation defect (Fig. 1e and Extended Data Fig. 3b) and normalized glycolytic flux (Extended Data Fig. 3c). In addition, SHMT2 re-expression rescued the growth defect of SHMT2-knockout cells6 (Extended Data Fig. 3d) and normalized 1C metabolism (Extended Data Fig. 3e, f). Thus, mitochondrial SHMT catalytic activity is crucial to sustain oxidative phosphorylation.

Two compartment-specific uses of mitochondrial folate 1C units have been reported: the local biosynthesis of deoxythymidime triphosphate (dTTP)16,17 and of N-formylmethionine (f-Met)18,19 (Fig. 2a). The production of dTTP requires 5,10-methylene-THF (methylene-THF), whereas f-Met requires 10-formyl-THF (formyl-THF). SHMT2 is upstream of both compounds. By contrast, MTHFD2 sits between methylene-THF and formyl-THF. The lack of an oxidative phosphorylation phenotype with MTHFD2 knockout led us to hypothesize that methylene-THF is the required 1C species. Consistent with this, SHMT2-knockout cell lines showed unchanged N-terminal f-Met levels of the mitochondrially translated COX1 peptide (encoded by the MT-CO1 gene)19 (Extended Data Fig. 4a). To confirm that methylene-THF is the required species, we generated SHMT2/ MTHFD2 double-deletion cells and supplemented them with methylglycine (sarcosine), which can produce mitochondrial methylene-THF.
in which drainage of methylene-THF to formyl-THF is blocked (Fig. 2b and Extended Data Fig. 4b). Thus, mitochondrial methylene-THF is required to maintain respiratory capacity.

Depletion of mitochondrial dTTP and/or accumulation of uridine nucleotides have been shown to induce mitochondrial respiratory chain deficiency by promoting mitochondrial DNA damage. However, SHMT2-knockout cells showed no evidence of altered mitochondrial DNA copy number (Fig. 2c and Extended Data Fig. 4c), deletions (Extended Data Fig. 4d), or mutations (Extended Data Fig. 4g, h and Supplementary Table 1). Moreover, whole-cell RNA sequencing revealed normal transcript levels for both nuclear and mitochondrial-encoded respiratory chain protein subunits (Fig. 2d, Extended Data Fig. 4e, f). Thus, the dependence of oxidative phosphorylation on SHMT2 reflects a requirement for mitochondrial methylene-THF for a purpose other than supplying local dTTP to maintain mitochondrial DNA.

Whereas the vast majority of the approximately 1,100 mitochondrial proteins are imported from the cytosol, 13 essential respiratory chain subunits are locally transcribed and translated. These include components of complexes I, III, IV and V, but not complex II. On the basis of the normal mitochondrial transcript abundances and complex II protein levels, we hypothesized that mitochondrial methylene-THF is required for local translation. Indeed, [35S]methionine incorporation assays showed decreased synthesis of certain complex I and IV subunits (Extended Data Fig. 5a). To probe mitochondrial translation further, we developed a protocol for mitochondrial ribosome profiling based on digesting unprotected mRNA with micrococcal nuclease, enriching the 5′SS mitochondrial ribosome, and sequencing the protected footprints (Fig. 3a and Extended Data Fig. 5b). This approach achieved more than 90% average mitochondrial transcript sequence coverage, with an average depth of at least 80 reads per codon (Supplementary Table 3 and Extended Data Fig. 5c). In SHMT2-knockout cells, the distribution of ribosome-protected footprints showed pronounced stalling at defined codon positions (Fig. 3b and Extended Data Fig. 6a). This resulted in relatively fewer actively translating ribosomes (that is, bound and not stalled) for certain subunits of respiratory chain complexes I, IV and V (Extended Data Fig. 6b). Consistent with the ribosome profiling data, enzymatic assays revealed decreased activity of complexes I, IV and V (Extended Data Fig. 6c).

We next aimed to determine the cause of ribosomal stalling. The aminoacyl-tRNA acceptor-site (A-site) coordinates of stalled ribosomes revealed notable ribosome accumulation in SHMT2-knockout cells at particular lysine and leucine codons: LysAAG and LeuUUG (Fig. 3c).

**Figure 1** | Mitochondrial respiratory chain function is dependent on SHMT2 catalytic activity. a, 1C pathway and known mitochondrial products. b, Lactate secretion of HCT116 knockout cell lines (n = 6). ∆SHMT2-A and ∆SHMT2-B denote two separate SHMT2-knockout lines. WT, wild type. c, Oxygen consumption rate measured by Seahorse XF analyser (n = 3), FCCP denotes a mitochondrial uncoupling agent. Oligom., oligomycin; Rot./antim., rotenone/antimycin. d, Immunoblot for mitochondrial respiratory complex I and II (CI and CII) proteins (NDUFS4 and SDHA, respectively), 1C enzymes, and a marker of mitochondrial mass (VDAC1). e, Basal respiration (n = 3) upon re-expression of wild-type or catalytically deficient mutant forms of SHMT2 in HEK293T knockout cell lines. Data are mean ± s.e.m. n indicates the number of biological replicates, which for the Seahorse experiments refers to independent plates on separate days. *P < 0.01, two-tailed Student’s t-test (see Supplementary Table 7 for exact P values). Cat. inact., catalytically inactive SHMT2; PLP bind., PLP binding-deficient SHMT2.

**Figure 2** | SHMT2-knockout-induced respiratory chain deficiency is caused by mitochondrial methylene-THF depletion but is unrelated to dTTP synthesis. a, Sarcosine serves as an SHMT2-independent source of mitochondrial methylene-THF. b, NAD+/NADH ratio (n = 6) and NDUFS4 (complex I) protein expression upon sarcosine supplementation (1 mM) in SHMT2 single-knockout (∆SHMT2) and SHMT2/MITFHD2 double-knockout (∆SHMT2/∆MITFHD2) cell lines compared to wild-type cells. c, d, Functional readouts for mitochondrial dTTP status based on mitochondrial DNA (mtDNA) levels (n = 3) determined by quantitative PCR (qPCR; c) and gene expression determined by RNA-seq (d) in SHMT2-knockout and wild-type HEK293T cells. RPKM, reads per kilobase per million mapped reads. In d, each data point represents the mean gene expression of two biological replicates of two independent knockout clones (n = 4) and two wild-type replicates (n = 2). Genes linked to OXPHOS function are highlighted in red (nuclear-encoded) or blue (mitochondrial-encoded). Significantly differentially expressed genes are shown in Supplementary Table 2. Data are mean ± s.e.m. n indicates the number of independent biological replicates. *P < 0.01, two-tailed Student’s t-test (see Supplementary Table 7 for exact P values).
This did not seem to reflect a shortage of these amino acids or tRNAs in mitochondria, as much less stalling was observed at the corresponding Lys\(^{\text{AAA}}\) and Leu\(^{\text{UAU}}\) codons. Instead, it appeared to relate to difficulty in reading the 3’ codon guanosine of certain codons: those in which the 3’ position identity (purine versus pyrimidine) determines the encoded amino acid (‘split codon boxes’; Extended Data Fig. 6d). Increased codon occupancy was observed also for Trp\(^{\text{UGG}}\), Glu\(^{\text{GAG}}\) ribosome density along selected mitochondrial transcripts. Additional transcripts were sequenced. Cells were lysed and RNA was digested using micrococcal nuclease was halted using chloramphenicol and immersion into liquid nitrogen, and protected fragments were sequenced.

Codon occupancy due to defective tRNA modification.

Oxidative phosphorylation defect is a consequence of impaired trans-

mitochondrial ribosome translation.

is dependent on mitochondrial methylene-THF and that the observed methionine codons are highlighted in blue and show no increased codon occupancy. The insert shows mean normalized ribosome density relative to UUG and AAG codon position. aa, amino acids. Data in b and c represent two technical replicates of two independent samples.

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of $\text{m}^5\text{U}$ (Extended Data Fig. 7e). Mitochondrial ribosome profiling of these engineered MTO1-deletion cells showed an increase in codon occupancy at the same codons as with SHMT2 knockout (Fig. 4c).

In both cases, the strongest stalling occurred at Lys$^{AAG}$ and Leu$^{UUG}$, potentially because these codons have, in addition to the wobble base pairing, only A–U base pairings versus the stronger C–G base pairings in Glu$^{GAG}$, Glu$^{CUG}$ and Trp$^{UAG}$ codons. Mitochondrial ribosome profiling of primary fibroblasts of two patients carrying different MTO1 missense mutations revealed increased codon occupancy selectively at Lys$^{AAG}$, but not Leu$^{UUG}$. Thus, these hypomorphic mutations seem to cause a selective defect in taurinomethylation of the Lys tRNA. Of all adenosine-ending codons, Lys$^{AAA}$ showed the greatest increase in codon occupancy across all cell lines, being most pronounced in the MTO1 patients (Fig. 4c).

Notably, in both SHMT2 and MTO1 deletion cells, stalling did not affect all AAG and UUG codons uniformly, but occurred most strongly at the same specific gene locations (Extended Data Fig. 7g). Mapping of AAG and UUG codons at stalling sites relative to mRNA secondary structure did not reveal any clear pattern (Extended Data Fig. 8a, b and Supplementary Tables 4, 5). Mapping onto the structure of the protein being synthesized (Extended Data Fig. 8c), however, showed a trend towards stalling at transitions between transmembrane helices and non-membrane domains (Supplementary Table 5). Thus, stalling due to the defective codon–anticodon interaction might be exacerbated by particular protein sequence features.

Defined mutations in the Lys and Leu1 mitochondrial tRNAs (which decode the most strongly affected Lys$^{AAG}$ and Leu$^{UUG}$ codons) result in a tRNA-specific $\text{m}^5\text{U}$ modification defect, causing the human mitochondrial disorders MERRF and MELAS. Mitochondrial ribosome profiling of fibroblasts from two patients with MELAS due to the m.3243A>G mutation in the MT-TL1 gene revealed, as expected, increased occupancy at Leu$^{UUG}$ but not Lys$^{AAG}$ or Leu$^{UUG}$ (Fig. 4d). For unknown reasons, increased ribosome occupancy for either Ser$^{ACG}$ or Thr$^{ACG}$ was also observed in individual patients (Extended Data Fig. 9a).

The extent of stalling and complex I depletion was less than or ThrACG was also observed in individual patients (Extended Data Fig. 9b). Collectively these observations highlight a common biochemical mechanism that links mitochondrial folate metabolism with a considerable fraction of inborn errors of mitochondrial metabolism.
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METHODS

Cell lines and growth conditions. HCT116 (CCL-247) and HEK293T/17 (CRL-11268) were purchased from ATCC. Generation of a subset of clonal CRISPR-Cas9 knockout cell lines and detailed characterization has been reported previously. Additional clonal knockout cell lines (Supplementary Table 6a) were established following the protocol published previously. In brief, exon-targeting guide RNAs (Supplementary Table 6b) were designed against genes of interest and cloned into an expression vector containing the double nicking Cas9 variant (Addgene). Cells were transiently transfected using Lipofectamine 2000 (Life Technologies) (HEK293T) or Fugene HD (Promega) (HCT-116) and selected for 48 h with 2 μg/ml puromycin. Single clones were isolated using serial dilution into 96-well plates.

Stable SHMT2 re-expression was achieved by transfecting HEK293T knockout cell lines with NM_005412.5 DNA (GE Healthcare) cloned into pCMV-Tag8 vector (Agilent) and selection for three weeks with 200 μg/ml hygromycin B (Sigma-Aldrich). Catalytic inactive (p.Glu98Leu/p.Tyr106Phe) and PLP-binding deficient (p.Lys280Gln) mutants were obtained following the QuickChange II protocol (Agilent). Knockout and re-expression cell lines were functionally verified by immunoblotting followed by targeted genomic resolution sequencing (Supplementary Fig. 9) and, in the case of SHMT2 cell lines, also by tracing of [2,3,3-2H]serine labelling into [14C]TTP. MTO1 and MT-TL1 protein fibroblasts and controls were provided by the Department of Paediatrics, Salzburger Landeskiniken and Paracelsus Medical University, Salzburg. Studies with primary human cell lines were approved by the local ethics-committee and informed consent was obtained from all subjects. The genotypes of MTO1-deficient patients (GenBank NM_011223.3) were as follows: patient A: c.1261-5T>G [I430G>A], (p.[3]:[Arg77His]); patient B: c.1222T>A; c.1227T>A, (p.[Ile408Phe]; [Ile408Phe]). Patient B MTO1 has been reported before. Both MELAS patients carried the commonly accepted T324C > A G mutation with the heteroplasmy.

Immunoblotting. Cells were transiently transfected using Lipofectamine 2000 (Life Technologies) and dried using a 443 Slab Dryer (BioRad). The dried gel was exposed to a phosphor screen (GE Healthcare) and imaged on a Typhoon FLA 9500 (GE Healthcare). Equal sample loading was confirmed by Coomassie brilliant blue staining (BioRad).

Metabolite concentrations and labelling patterns. Cells were grown in 6-cm dishes for at least 48 h and collected at 75% confluency. Media was replaced every 24 h and additionally 6 h before collection. Metabolism was quenched and metabolites were extracted by aspirating media and immediately adding 1 ml of 80:20 methanol:water at −80 °C. Plates were kept on ice, scraped and non-soluble debris was pelleted at 18,000 g for 10 min. Samples were directly analysed by hydrophilic interaction chromatography coupled with negative-mode electrospray-ionization high resolution mass spectrometry on a quadrupole-orbitrap scanning from m/z 73 to 1,000 at 1 Hz and 140,000 resolution (Q Exactive Plus, Thermo-Fisher). Liquid chromatography separation was achieved on an XBridge BEH Amide column (2.1 mm × 150 mm, 2.5 μm particle size, 130 Å pore size; Waters) using a gradient of solvent A (20 mM ammonium acetate + 20 mM ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45) and solvent B (acetonitrile). Flow rate was 150 μl/min. The gradient was: 0 min, 85% B; 2 min, 85% B; 3 min, 60% B; 9 min, 60% B; 9.5 min, 35% B; 12 min, 35% B; 12.5 min, 0% B; 18 min, 0% B; 18.5 min, 85% B; 23 min, 85% B. Data were processed and analysed using MAVEN software.

Analysis of mitochondrial specific translation. [35S]-labelling of mitochondrial proteins was performed following the method described previously. In brief, cells were grown on 6 cm plates for 48h to sub-confluence. Media was changed to DMEM with 10% dFBS without methionine (MP Biomedicals). After a 30 min incubation, cystosolic translation was inhibited by emetine hydrochloride (0.1 mg/ml) Sigma-Aldrich) and labelling was conducted for 1 h after the addition of 500 μCi [35S]methionine (EasyTag [35S]- Metionine, Perkin Elmer). Protein lysates (30 μg) were then separated on a 15% polyacrylamide gel (8.3 × 7.3 cm) and dried using a 443 Slab Dryer (BioRad). The gel was then scanned using a storage phosphor screen (GE Healthcare) and imaged on a Typhoon FLA 9500 (GE Healthcare). Equal sample loading was confirmed by Coomassie brilliant blue staining (BioRad).

Mitochondrial DNA content and integrity. To analyse mitochondrial DNA content, total DNA was extracted from 7 × 106 cells using Gentra Puregene Cell Kit (Qiagen) after freezing the cell pellet at −80 °C for 1 h and overnight digestion with Proteinase K (Roche Diagnostics). qPCR (ViiA 7, Applied Biosystems) was performed using primers targeting the mitochondrial ND2 locus (also known as MT-ND2 pseudogene) (forward: 5′−TGGTTGTGTTACCTCCTGGCACTA-3′; reverse: 5′−CCTGCAAAATGTTAGTATAGTAGTA-3′) and a nuclear ALU repeat sequence (forward: 5′−CTTCGATGTACCCGAGATT−3′; reverse: 5′−GAGGACGG GTCTCGGCTCTG-3′) as published earlier. The relative mitochondrial DNA content was determined using the ΔΔCt method. Each independent sample given in the figures represents the mean of 6 technical replicates.

RNA sequencing. RNA was isolated from cell lines using RNeasy Plus kit (Qiagen) according to the manufacturer’s recommendation. After the depletion of ribosomal RNA, libraries were prepared according to the TruSeq Stranded Total RNA protocol (Illumina) and sequencing was performed on a HiSeq 2500 (Illumina). Analysis was performed using the Galaxy system and the R software package.

DNA extraction and analysis. DNA samples were extracted from 10^6 cells per well. Total DNA was extracted from 7 × 10^6 cells using Qiagen after freezing the cell pellet at −80 °C for 1 h and overnight digestion with Proteinase K (Roche Diagnostics). qPCR (ViiA 7, Applied Biosystems) was performed using primers targeting the mitochondrial ND2 locus (also known as MT-ND2 pseudogene) (forward: 5′−TGGTTGTGTTACCTCCTGGCACTA-3′; reverse: 5′−CCTGCAAAATGTTAGTATAGTAGTA-3′) and a nuclear ALU repeat sequence (forward: 5′−CTTCGATGTACCCGAGATT−3′; reverse: 5′−GAGGACGG GTCTCGGCTCTG-3′) as published earlier. The relative mitochondrial DNA content was determined using the ΔΔCt method. Each independent sample given in the figures represents the mean of 6 technical replicates.
The MT-TL1 mutation load was determined using primers specifically spanning the m.3243 position for targeted enrichment (forward: 5′-AATGATACGGCCGAC CACCAGATCTACACNNNNNGCTCCCTCGGTTAAGT3′-reverse: 5′-CAACAGAAACCGCCGATCGAATGCAAGCTGCTGTTTGTAGT3′) followed by sequencing on a MiSeq nano flow cell using a custom sequencing primer (sequence: TATTTAACCCACACCACCGAGAGTTGTTTGAAG). Alignment to GRCh38 was performed using Bowtie2 (Galaxy Version 0.6) at default settings and position-specific mutation load was derived from the Integration Mapping tool.

Mitochondrial ribosome profiling. Development of our ribosome profiling method was based on concepts reported previously57–59. For mitochondrial ribosome profiling, cell lines were grown on 15-cm plates to 70–85% confluency. Sarcosine rescue of ribosome stalling in the SHMT2/MTFH2 double knockout background was assessed after growth in the presence of 1 mM sarcosine for 5 days. After removal of media, plates were rapidly rinsed with ice-cold PBS containing chloramphenicol (100 μg ml−1) (Sigma-Aldrich) and cycloheximide (100 μg ml−1) (Sigma-Aldrich) followed by immediate immersion into liquid nitrogen. Plates were then transferred to wet ice and 1 ml of 1.5 × lysis buffer was added and the lysate was collected using a cell scraper. Lysis buffer contained the following: 1.5% Triton X-100 (Sigma-Aldrich), 0.15% NP40 (Sigma-Aldrich), 1 × complete protease- and phosphate inhibitors (Roche), and 30 μl−1 DNase I (Roche) in buffer base (20 mM Tris–HCl pH 7.8, 100 mM KCl (Ambion), 10 mM MgCl2 (Ambion), 100 μg ml−1 chloramphenicol, 100 μg ml−1 cycloheximide). 1.6–1.8 ml were recovered per plate and homogenized by passing three times through a 32G needle at 4 °C. Non-soluble debris was pelleted at 5,000 × g for 10 min and 1,520 μl supernatant was used for digestion with 7,500 U ml−1 micrococcal nuclease (Roche) after adding 40 μl SUPERaseIN (Ambion) and 5 mM CaCl2 (Ambion). Digestion was stopped after 1 h gentle shaking at 25 °C using a final concentration of 6 mM EDTA.

Buffer base was used to make 5%–45% sucrose gradients (Gradient Master, Biocomp). After cooling to 4 °C, samples were separated in an ultracentrifuge using the SW-41Ti rotor at 210,000g for 2.5h. Live UV absorption at 254 nm was used to track the mitochondrial 55S monosome enriched fractions (Extended Data Fig. 5b). The 55S fractions were pooled and mixed with 57 μl 20% SDS per millilitre sample before performing acid phenol chloroform RNA extraction. RNA was precipitated using 300 mM sodium acetate pH 5.5 and equal volume isopropanol and run on a 1% TBE-urea gel (Invergent) at 210 V for 1 h for size selection. Gels were stained with Sybr Gold (Invitrogen) and RNA fragments corresponding to mitochondrial ribosome footprints (approximately 28–40 nucleotides) were cut and recovered from the gel using the crush and soak method. After sodium acetate/isopropanol precipitation, library preparation was conducted following the TruSeq Ribosome (Illumina) protocol.

Sequencing of ribosome protected footprints (RPFs) was performed on a HiSeq 2500 in rapid mode followed by adaptor trimming using Cutadapt (Galaxy Version 1.6)60. Reads were mapped to the human genome reference GRCh38 using BWA (Galaxy Version 0.9)48 with those mapping to the mitochondrial protein-coding genes included in the subsequent analysis. The Platistad package61 and customized Phython and R62 scripts were used for analysing mitochondiral ribosome profiling data. Alignment was performed from 3′ of reads which was reported to yield superior results after digestion with Micrococcal nuclease61,62. Each read, corresponding to a mitochondrial ribosome protected fragment (mtRPF), was assigned to a nucleotide position representing the respective ribosomal A-site as determined by metagenome analysis62. mtRPF counts were then normalized to reads per million (RPM) mapped reads within each sample and single nucleotide positions were grouped by codon index. This transformation allows for relative quantification of bound ribosomes for each nucleotide triplet along a transcript. Stalling plots were created by plotting the mean cumulative mtRPF count along each mitochondrial group (codon triplet), against the nucleotide at the 5′ end of the transcript. The mean value from each position was plotted. Codons were defined as stalling sites when the normalized counts mapped to the specific codon (mtRPF codon/mtRPF median) exceeded 2 s.d. from all codons in the genome. The relative abundance of actively translating ribosomes (that is, not stalled) was calculated by subtracting mtRPF counts in stalled regions from the total sum of ribosome footprints for each gene as ∑mtRPFactive − ∑mtRPFstalled. Then the gene specific ratio was plotted as ∑mtRPFactive/shmt2/ ∑mtRPFactive/WT. Stalling sites specific to the SHMT2-knockout condition were identified using the ratio of occupancy at each codon position relative to wild type. Specifically, codons were defined as SHMT2-specific stalling sites when the normalized counts in the mutant relative to wild type (mtRPFmutant/wt) exceeded 2 s.d. (or, as indicated, 3 s.d.) from this ratio as determined for all codons in the genome, and the site also met the general stalling site criterion.

To determine the relative abundance of mitochondrial ribosomes bound to each nucleotide triplet, codon-specific occupancy ratios were calculated. For each codon (codon, i–64), the gene-specific ratio between experimentally measured ribosome density and expected density (which is proportional to codon frequency) was calculated. Codon occupancy (COi−1–64) for each codon is the mean of the ratios from all 13 genes. The relative codon occupancy (COi−1–64/shmt2) was plotted with error bars representing s.d. across replicates after error propagation. To investigate the ribosome distribution relative to the major stalled codons (AAG and UUG), ribosome densities flanking the codons of interest within 25 amino acids were selected. Each selected fragment was first normalized to its total count so every codon of interest from the genome is weighted equally. The mean value from each position was plotted.

Mitochondrial enzyme activities. Activities of individual OXPHOS complexes I–IV, ATP synthase and citrate synthase (which is a nuclear encoded and thus not a mitochondrial enzyme) were spectrophotometrically measured in vivo as a marker of mitochondrial mass) were spectrophotometrically measured as described in the manuscript for nuclear mitochondrial supercomplexes. In addition, a hidden Markov model based algorithm for transmembrane helices (TMHMM 2.0)63 was used to predict α-helical transmembrane domains in the Homo sapiens sequences. This method assigns each codon a probability for transmembrane helix localization which was then used for genome wide assessment of AAG and UUG localization relative to transmembrane helices. AAG and UUG codons were defined to be at a transition between a transmembrane helix and a non-membrane region if, within the five flanking codons, probabilities >0.5 and <0.5 for being in a transmembrane helix are found. AAG and UUG codons were defined as stalling sites based on the 3 s.d. cut-off as per Extended Data Fig. 8b. In total, 4 out of 5 stalling AAG and UUG codons, and 7 out of 23 non-stalling AAG and UUG codons were at a membrane transition (P = 0.04 by chi-square test).

Evaluation of mRNA secondary structure effects on ribosome stalling. To study a potential effect of mRNA secondary structure64 (that is, base pairing) on ribosome stalling, we used the previously published dimethyl sulfate sequencing datasets on human K562 cell lines65 to identify structured regions in mitochondrial transcripts. Following the methods described in the manuscript for nuclear transcripts, identification of sites with secondary structure was performed on mitochondrial transcript data14. In brief, FASTQ files (accession numbers GSM1297495 and GSM1297493) were retrieved from sequence read archive and mapped to GRCh38 with BWA (Galaxy Version 0.9)48. Reads were assigned to the nucleotide at the 5′ end with no offset using Plastid60. R values (cut-off 0.75) and Gini differences (cut-off 0.01) between the in vivo and denatured dataset were calculated for the complete mitochondrial transcriptome for a window size of 50 adenosine/cytosine nucleotides and a step size of 10. This provided a list of structured mitochondrial transcript regions, with most mRNA regions unstructured. The list of structured regions was compared to the SHMT2-specific stalling sites for potential co-localization. No stalling site mapped to a structured region.

Mitochondrial enzyme activities. Activities of individual OXPHOS complexes I–IV, ATP synthase and citrate synthase (which is nuclear encoded and was used as a marker of mitochondrial mass) were spectrophotometrically measured as previously described (Uvicron 922, Kontron)66–70. Measurements were performed with 2 μl of mitochondria isolated by differential centrifugation71 (except for complex I, in which 10 μl was used). Citrate synthase (EC 2.3.1.31) activity was determined following extraction directly from 5 ml, indicating the cleavage of Elman's reagent (0.2 mM) after addition of oxaloacetate (0.5 mM) to the buffer reaction solution containing acetyl-CoA (0.15 mM). Rotenone-sensitive complex I (NADH-decyliubiquinone oxidoreductase, EC 1.6.5.3) activity was measured by adding NADH (0.2 mM) and monitoring at 340 nm for the reduction of decyl-ubiquinone (50 μM). Complex II (succinate:ubiquinone-oxidoreductase, EC 1.3.5.1) was measured at 600 nm by monitoring the reduction of 2,6-dichlorophenol-indophenol (80 μM) after addition of succinate (10 mM). The reaction mixture to determine complex III activity (coenzyme Q:cytochrome c oxidoreductase, EC 1.10.2.2) contained cytochrome c (100 μM) and decyl-ubiquinol (200 μM) and was measured at 550 nm. After inhibition by addition of antimycin A (1 μM), the insensitive activity was subtracted to calculate specific complex III activity. The enzyme activity of complex IV (ferrocychrome c oxidoreductase, EC 1.9.3.1) was read as the oxidation rate of reduced cytochrome C (60 μM) at 550 nm. Complex V (F1F0 ATP synthase, EC 3.6.3.14) was indirectly measured as oligomycin-sensitive ATPase activity in a reaction mixture containing 0.5 mM ATP. Formed ADP was coupled
to a pyruvate kinase reaction, using phosphoenolpyruvate (2 mM) to generate ATP and pyruvate. The latter is then used by lactate dehydrogenase in the oxidation of NADH (0.2 mM) that served as readout (340 nm). Reagents were obtained from Sigma-Aldrich.

**Mitochondrial RNA modifications.** HCT116 or HEK293T cell lines and subclones were grown to 70–85% confluency and collected for mitochondrial extraction (1 × 10^7–2 × 10^9). Mitochondrial RNAs were extracted using the MirVana miRNA Isolation Kit (Ambion) for isolation of small RNAs followed by 10% TBE-urea gel purification (see Supplementary Table 3). In addition, acetylation, formylation and loss of methionine were specified as potential modifications at the N terminus of proteins. The resulting cysteine was used as fixed modification and oxidation of methionine as dynamic modification. MS1 and 0.6 Da MS2 mass tolerances were specified. Carbamidomethylation of cysteine was added to the list. Samples were loaded directly onto a 45 cm × 0.4 cm × 0.25 μm C18 nano-capped column for separation. For detailed information, see Supplementary Table 7. Small filled circles are individual data points. All values for individual comparisons are given as mean ± standard error of the mean (SEM). Significance was determined using the Student’s t-test or 2-way ANOVA with a Bonferroni correction for multiple comparisons. Unless stated otherwise, all experiments were performed in triplicate. The results were representative of at least two independent experiments. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

**Code availability.** All code used to generate the data in this manuscript is publicly available from GitHub (https://github.com/R-J-Morschel).

**Data availability.** RNA sequencing and protein expression data from this study are available from the Sequence Read Archive (SRA) under BioProject accession number PRJNA419990. Source data for Figs 1c, 3c, 4c, Extended Data Figs 3d, 6e are provided with the paper. Unprocessed versions of blots are provided in Supplementary Figs 1–8; Sanger sequencing traces are provided in Supplementary Fig. 9. Other data that support the findings of this study are available from the corresponding author upon request.

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Extended Data Figure 1 | SHMT2 deletion-induced respiratory chain dysfunction in different cellular backgrounds and clones. **a**, Change in media colour after 48 h cell growth. **b, c**, Lactate secretion (b) and normalized NAD+/NADH ratio (c) of HCT116 knockout cell lines (n = 6). **d, e**, Basal respiration as measured by Seahorse XF analyser (n = 3) (d) and normalized NAD+/NADH ratio (n = 3) (e) of HEK293T folate 1C gene CRISPR–Cas9 knockout cell lines. **f**, Normalized levels of TCA cycle and associated metabolites (n = 3). **g**, Steady-state labelling fraction into citrate from [U-13C]substrates glutamine (left) and glucose (right) (n = 3). **h**, Immunoblot of extracted mitochondria for subunits of respiratory chain complexes I–V (CI–CV) and markers of mitochondrial mass. **i**, Mitochondrial complex I levels (NDUFS4) in independent HCT116 folate 1C gene knockout clones. Data are mean ± s.e.m. n indicates the number of biological replicates. *P < 0.01, two-tailed Student’s t-test (see Supplementary Table 7 for exact P values).
Extended Data Figure 2 | Catalytically deficient SHMT2 constructs. a, Mapping of mutated amino acid residues on human SHMT1 (PDB code 1BJ4) using iCn3D and alignment of *E. coli* serine hydroxymethyltransferase (GLYA), *H. sapiens* mitochondrial serine hydroxymethyltransferase 2 (GLYM) and cytosolic serine hydroxymethyltransferase 1 (GLYC). Positions for GLYM are given with reference to GenBank NM_005412.5. b, Sanger sequencing traces of mutant constructs. c, Immunoblot for mitochondrial complex I levels (NDUFS4) in cell lines re-expressing catalytically deficient forms of SHMT2.
Extended Data Figure 3 | Restoring SHMT2 catalytic activity normalizes 1C flux, respiratory chain expression, glycolytic activity, and cell growth. 

**a**, Immunoblot of re-expression of catalytically active SHMT2 (left) and the effects of its re-expression on mitochondrial complex I and II levels (right). 

**b**, Normalized NAD⁺/NADH ratio (n = 6).

**c**, Lactate secretion and glucose uptake (n = 6).

**d**, Cell proliferation (n = 6).

**e**, Purine biosynthesis intermediate 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) levels (n = 4) as an indicator of cytosolic folate 1C status.

**f**, [2,3,3⁻²H]serine tracing to differentiate cytosolic from mitochondrial folate 1C unit production for incorporation into deoxythymidine triphosphate (n = 3). Data are mean ± s.e.m. *n* indicates the number of biological replicates. *P < 0.01, two-tailed Student’s t-test (see Supplementary Table 7 for exact P values).
Extended Data Figure 4 | Oxidative phosphorylation defect is caused by a post-transcriptional mechanism independent of methionine formylation. a, Fraction of initiating amino acid (formylmethionine versus methionine) of mitochondrial-expressed COX1 peptide determined by high-resolution LC–MS (wild type \( n = 4 \), ΔSHMT2 \( n = 3 \), ΔMTHFD2 \( n = 2 \)). b, Lactate secretion \( (n = 3) \) upon sarcosine supplementation (1 mM). c, Relative mtDNA levels in HEK293T cells \( (n = 3) \). d, Agarose gel of mtDNA long-range PCR products of HCT116 and HEK293T knockout cell lines. e, Relative mRNA levels of mtDNA-encoded respiratory chain subunits in the HEK293T background \( (n = 3) \). f, Gene expression levels in SHMT2-knockout cell lines compared to SHMT2 wild-type re-expressed lines by total RNA sequencing. Each dot represents mean gene expression as derived from two biological replicates of two independent knockout clones and matched re-expressed lines \( (n = 4) \). Genes linked to human OXPHOS function\(^3\) are highlighted in red. Significantly differentially expressed genes are listed in Supplementary Table 2. g, Position-dependent next-generation sequencing coverage of mtDNA in HEK293T wild-type, SHMT2-knockout and MTHFD2-knockout cell lines supports the absence of deletions due to SHMT2 loss. h, Corresponding variant position and frequency. Variant list is provided in Supplementary Table 1. Data are mean ± s.e.m. \( n \) indicates the number of biological replicates. *\( P < 0.01 \), two-tailed Student’s \( t \)-test (see Supplementary Table 7 for exact \( P \) values).
Extended Data Figure 5 | Impairment of mitochondrial translation due to loss of SHMT2. 

a, SDS–PAGE of [35S]methionine-labelled mitochondrially translated proteins in wild-type (lane 1) and two SHMT2-knockout (lane 2 and 3) HEK293T cell lines. Decreased synthesis of COX1 and COX2/3 are evident upon short exposure and reduced synthesis of ND5 and ND6 is more easily visualized upon longer exposure.

b, Absorbance at 254 nm upon sucrose gradient fractionation of cell lysates digested by micrococcal nuclease (Fig. 3a). Fractions corresponding to 4 and 5 were collected for mitochondrial ribosome enrichment as shown on the matched immunoblot for mitochondrial ribosome subunit MRPL11.

c, Read length distribution (top) and read length-dependent sub-codon read phasing (bottom) across the 13 mitochondrial protein-coding transcripts. Data in c are based on the mitochondrial ribosome profiling experiment in Fig. 3, and represent the mean of two technical replicates of two independent samples.
Extended Data Figure 6 | Mitochondrial ribosome stalling at guanosine-ending split codon box nucleotide triplets suggests deficient 5-taurinomethyluridine modification. a, Expanded version of Fig. 3b, showing the mean cumulative ribosome protected fragments of all mitochondrial protein-coding genes. b, Mean relative density of actively translating (that is, not stalled) ribosomes for mitochondrial transcripts. Data in a and b represent two technical replicates of two independent samples. c, Enzymatic activities of citrate synthase and individual mitochondrial respiratory chain complexes from mitochondrial extracts (n = 5). Data are mean ± s.e.m. *P < 0.01, two-tailed Student's t-test (see Supplementary Table 7 for exact P values). d, Mitochondrial genetic code table with split codon boxes depending on taurinomethylated tRNAs for translation highlighted in red. Codons decoded by anticodon formylcytidine-containing tRNA^Met are highlighted in blue. e, Mean codon-specific mitochondrial ribosome occupancy of HCT116 SHMT2/MTHFD2 double-knockout cell lines supplemented with sarcosine (1 mM). Codons highlighted in red are decoded by tRNAs carrying a 5-taurinomethyluridine modification. The supplementation with sarcosine prevents the stalling normally observed with SHMT2 deletion (n = 2).
Extended Data Figure 7 | tRNA modification status in ΔSHMT2 and effects of 5-taurinomethyluridine modification loss caused by human disease gene MTO1. a, Total ion chromatogram of 5-formylcytidine monophosphate in digested mitochondrial tRNAs upon loss of SHMT2. The same samples were analysed for 5-taurinomethyluridine monophosphate (p-τm5U) in Fig. 4b. The combined data demonstrate that SHMT2 deletion causes loss of τm5U but not 5-formylcytidine. b, Levels of τm5U, 5-taurinomethyl-2-thiouridine monophosphate (p-τm5s2U) and 2-thiouridine monophosphate (p-s2U) in wild-type HCT116 and SHMT2 deletion lines normalized to 5-formylcytidine monophosphate (p-f5C) (n = 3). c, Taurine levels in HCT116 wild-type and SHMT2-knockout cells (n = 3). d, τm5U levels in digested mitochondrial tRNAs upon re-expression of SHMT2 (n = 1). e, τm5U, τm5s2U and s2U levels normalized to f5C in HCT116 SHMT2/MTHFD2 knockout lines after sarcosine supplementation and HCT116 upon loss of MTO1 (n = 2). For all panels, data are mean ± s.e.m. or individual data points only. f, Labelling pattern of 5-taurinomethyluridine and 5-formylcytidine monophosphate extracted from mitochondrial tRNAs after growth in media containing either [3-13C]serine or [U-13C]methionine. g, Mean cumulative count of ribosome protected fragments (RPF) mapping to mitochondrial protein coding transcripts upon ribosome profiling in HCT116 MTO1-knockout cell lines. Data were normalized to RPM (n = 2); n indicates the number of biological replicates. *P < 0.01, two-tailed Student’s t-test (see Supplementary Table 7 for exact P values).
Extended Data Figure 8 | Investigation of mRNA and protein secondary structure effects on mitochondrial ribosome stalling sites. 

a, Identification of mitochondrial RNA secondary structure based on analysis of the mitochondrial transcript data from the dimethyl sulfate sequencing dataset published previously. R values and Gini differences were calculated to detect changes in nucleotide reactivity between the in vivo and denatured condition for the complete mitochondrial transcriptome. Coloured points indicate structured regions given in Supplementary Table 4.

b, Determination of ribosome stalling sites in SHMT2-knockout HCT116 cell lines. Data points represent individual codons of all 13 mitochondrial protein-coding transcripts. For each codon, the y axis indicates the ribosome counts normalized to the gene median in RPM. The x axis indicates the ratio of normalized counts in SHMT2-knockout to normalized counts in wild-type HCT116. Two and three s.d. above the mean of all codons in the genome are indicated by the grey and black dotted line, respectively. Highlighted in red are codons with greater than 2 s.d. c, Mapping of AAG and UUG codons from SHMT2 knockout-specific ribosome stalling sites (>3 s.d.) on protein structures. For b and c, analysis is based on ribosome profiling data in Fig. 3, with two technical replicates of two independent samples. A list of identified codons and mapped AAG and UUG sites is provided in Supplementary Table 5.
Extended Data Figure 9 | Mitochondrial transcript codon occupancy from ribosome profiling of individual patient lines. a, Codon-specific mitochondrial ribosome occupancy ratio (patient/control fibroblasts) in individual patient derived cell lines (n = 1 for each individual patient, normalized to mean of n = 2 control fibroblast lines). Patients either had nuclear MTO1 missense mutations (patient A c.[1261-5T>G];[1430G>A], patient B c.[1222T>A];[1222T>A]) or were diagnosed with MELAS and carry the recurrent point mutation m.3243A>G in the mitochondrial gene for tRNA Leu1 (MT-TL1). b, Next-generation sequencing of mtDNA mutation load m.3243A>G (MT-TL1) in control fibroblasts and MELAS patient cell lines. Each bar shows one biological replicate for control and patient cell lines. Integrative genomics viewer sequencing raw data are shown on the right.
Extended Data Figure 10 | Effects of targeting 1C metabolism on mitochondrial function. 

**a.** Mitochondrial complex I and II levels after growth in the absence of folate for five passages or in the presence of the indicated methotrexate concentration for 96 h. Ethidium bromide (250 nM) was used as a positive control.

**b.** Cellular mtDNA levels in HCT116 cells after folate depletion (with or without 100 μM hypoxanthine and 16 μM thymidine (HT) as rescue agents) or in the presence of methotrexate for 96 h (n = 3).

**c.** To determine whether the decrease in respiration due to methotrexate arises from methotrexate depleting mitochondrial DNA, impairing mitochondrial translation, or a combination, in HCT116 cells we compared the effects of methotrexate (50 nM) to ethidium bromide (250 nM = 100 ng ml⁻¹), which is classically used to deplete mitochondrial DNA, and to chloramphenicol (310 μM = 100 μg ml⁻¹), which blocks mitochondrial translation. After 48 h of treatment, methotrexate and ethidium bromide both decreased oxygen consumption and DNA content. Importantly, despite ethidium bromide depleting mitochondrial DNA much more strongly, methotrexate had an equivalent effect on oxygen consumption, consistent with the effect of methotrexate on oxygen consumption being in part via mitochondrial translation inhibition. Data are normalized and compared to untreated control (all n = 3; except oxygen consumption methotrexate 96 h n = 6 and control n = 4). Data are mean ± s.e.m. n indicates the number of biological replicates. *P < 0.01, two-tailed Student's t-test (see Supplementary Table 7 for exact P values).
Experimental design

1. Sample size
   Describe how sample size was determined.
   No statistical methods were used to predetermine sample size. Sample size was determined based on experimental feasibility, sample availability, and N necessary to obtain definitive results.

2. Data exclusions
   Describe any data exclusions.
   None.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   Experimental findings were successfully replicated at least twice.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Samples were allocated into experimental groups by the confirmed genetic modification of the cell line they were derived from (CRISPR/Cas9 deletion) or the respective drug treatment. This design does not allow for randomization, as the origin of samples is critical. However, whenever possible samples were analyzed in a randomized order (i.e. when run on liquid-chromatography mass spectrometry).

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   This study did not involve animals or human research participants. The researchers were not blinded during data collection and/or analysis.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).
   Confirmed
   X The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   X A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   X A statement indicating how many times each experiment was replicated
   X The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   X A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   X The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   X A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   X Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study.

The following software was used to analyze data in our study:

Next-Generation sequencing raw data was processed using the Galaxy system with software-packages and version numbers given below:
- Cutadapt (Galaxy version 1.6)
- TopHat (Galaxy Version 0.9)
- htseq-count (Galaxy Version 0.6.1galaxy1)
- Bowtie2 (Galaxy Version 0.6)
- DeepTools bamCoverage (Galaxy Version 2.3.6.0)
- Freebayes (Galaxy Version 0.4.1)
- BWA (Galaxy Version 0.9)

Ribosome profiling data was additionally processed using scripts from the plastid package version 0.4.8

For downstream analysis R the software for statistical computing was used (Version 3.3.1) with software-packages and version numbers given below:
- DESeq2 package (Version 1.12.3)
- Gviz package (Version 1.18.0)
- dplyr (Version 0.5.0)
- ggplot (Version 2.2.1)
- tibble (Version 1.2)
- tidyr (Version 0.6.1)

Data from high resolution mass-spectrometry was analyzed using the Metabolomic Analysis and Visualization Engine (MAVEN build 682)

Small scale data was processed using GraphPad Prism 7.02.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There are no restrictions on availability of materials used in this study.
Antibodies were used in accordance to the manufacturer’s directions. Antibodies against folate 1C enzymes used in this study were cross-validated in this study by the loss of the specific band in sequence verified CRISPR/Cas9 knockout cell lines.

| Antibodies from Cell Signaling | Validation Details                                                                 |
|-------------------------------|-----------------------------------------------------------------------------------|
| Anti-SHMT1 (12612; Lot: 01/2017) | validated for immunoblotting by provider; validated for use in human samples; references: PMID:27307216, PMID:27110680, validated by CRISPR/Cas9 knockout |
| Anti-SHMT2 (12762; Lot:07/2016) | validated for immunoblotting by provider; validated for use in human samples; references: PMID: 27604570, PMID:27126896, validated by CRISPR/Cas9 knockout |
| Anti-MRPL11 (2066; Lot: 09/2015) | validated for immunoblotting by provider; validated for use in human samples; reference: PMID: 22829971, Anti-S6RP (5G10; Lot: 08/2015) validated for immunoblotting by provider; validated for use in human samples; references: PMID: 28367235, PMID: 28348518 |
| Anti-β-actin HRP (5125; Lot: 04/2016) | validated for immunoblotting by provider; validated for use in human samples; references: PMID: 28358054, PMID: 27649272 |

Antibodies used from Abcam Inc.:

| Antibodies from Abcam Inc. | Validation Details                                                                 |
|----------------------------|-----------------------------------------------------------------------------------|
| Anti-MTHFD2 (ab151447; GR145284-1) | validated for immunoblotting by provider; validated for use in human samples; references: PMID: 27732838, validated by CRISPR/Cas9 knockout |
| Anti-NDUFS4 (ab139178; Lot: YJ052508CS) | validated for immunoblotting by provider; validated for use in human samples; reference: PMID: 26053068, knockout validated by supplier |
| Anti-SDHA (ab147175; Lot: GR192960-6) | validated for immunoblotting by provider; validated for use in human samples; references: PMID: 27392540, PMID: 27974379 Anti-SDHB (ab14714; Lot: L6978) validated for immunoblotting by provider; validated for use in human samples; references: PMID: 28414270, PMID: 27345149 Anti-VDAC1 (ab14734; Lot: GR183548-26) validated for immunoblotting by provider; validated for use in human samples; references: PMID: 27974379, PMID: 28028439 Anti-ND1 (ab222892; Lot: GR3184720-1) validated for immunoblotting by provider; validated for use in human samples Anti-NDUF8 (ab110242; Lot: L6978) validated for immunoblotting by provider; validated for use in human samples; references: PMID: 28414270, PMID: 27345149 Anti-NDUF5 (ab110246; Lot: GR255750-5) validated for immunoblotting by provider; validated for use in human samples; references: PMID: 27181046, PMID: 24492964, PMID: 24815183 Anti-CO1 (ab14705; Lot: GR291384-3) validated for immunoblotting by provider; validated for use in human samples; references: PMID: 26971449, PMID: 27828948 Anti-CO2 (ab200615; GR219672-1) validated for immunoblotting by provider; validated for use in human samples Anti-COX4 (ab14744; GR281421-5) validated for immunoblotting by provider; validated for use in human samples; references: PMID: 21444675, PMID: 20805355 Anti-ATPSA (ab176569; GR303070-2) validated for immunoblotting by provider; validated for use in human samples; references: PMID: 28426667, PMID: 27590850 Anti-mtTFA (ab176558; GR137920-5) validated for immunoblotting by provider; validated for use in human samples; references: PMID: 25561980, PMID: 28714949 Antibodies used from Sigma-Aldrich:

| Antibodies from Sigma-Aldrich | Validation Details                                                                 |
|-------------------------------|-----------------------------------------------------------------------------------|
| Anti-MTHFD1L (HPA029041; Lot: R31922) | verified and developed by Atlas Antibodies; validated for use in human samples; validated by CRISPR/Cas9 knockout |
| Anti-ND6 (SAB2108622; Lot: QC48692) | validated for immunoblotting by provider; validated for use in human samples |
| Anti-CYB (HPA068400; Lot: R100268) | verified and developed by Atlas Antibodies; validated for use in human samples |
| Anti-UQRC2 (HPA019146; Lot: RO8218) | verified and developed by Atlas Antibodies; validated for use in human samples; references: PMID 24130818, PMID 23281071 |
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used.
      All cell lines were purchased from ATCC. MTO1 and MT-TL1 patient fibroblasts and
      controls were provided by the Department of Pediatrics, Salzburger Landeskliniken
      and Paracelsus Medical University, Salzburg.
   b. Describe the method of cell line authentication used.
      Sequencing to confirm genetic alterations.
   c. Report whether the cell lines were tested for
      mycoplasma contamination.
      All cells have been tested negative for mycoplasma contamination.
   d. If any of the cell lines used are listed in the database
      of commonly misidentified cell lines maintained by
      ICLAC, provide a scientific rationale for their use.
      No such cell lines were used.

▶ Animals and human research participants

Policy information about studies involving animals: when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived
   materials used in the study.
   No animals were used.

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population
   characteristics of the human research participants.
   MTO1 and MT-TL1 patient fibroblasts and controls were provided by the
   Department of Pediatrics, Salzburger Landeskliniken and Paracelsus Medical
   University, Salzburg. Studies with primary human cell lines were approved by the
   local ethics-committee and informed consent was obtained from all subjects.
   Genotype of MTO1 deficient patients (GenBank NM_012123.3) was as follows:
   pat_a c.[1261-5T>G];[1430G>A], (p.[?];[Arg477His]); pat_b c.[1222T>A];[1222T>A],
   (p.[Ile408Phe]; [Ile408Phe]). Patient MTO1_pat_b has been reported before. Both
   MELAS patients carried the common MT-TL1 m.3243A>G mutation with the
   heteroplasmy rate reported in this study. Further description of human research
   participants:
   MT-TL1 patient 1: sex: m; age at presentation: 2 week; clinical features:
   cardiomyopathy, muscle weakness; enzymatic phenotype: complex I+IV defect in
   muscle biopsy
   MT-TL1 patient 2: sex: f; age at presentation: 6 years; clinical features:
   developmental delay, sensorineural hearing loss, muscle weakness, microcytic
   anemia, stroke-like episodes; enzymatic phenotype: complex I defect in muscle
   biopsy
   MTO1 patient 1: sex: f; age at presentation: intrauterine; clinical features: brain
   cysts at 18th weeks gestation, hypertrophic cardiomyopathy, muscular hypotonia,
   partial agenesis of corpus calosum, microcephaly, hypertelorism, low set ears;
   enzymatic phenotype: complex I+IV defect in muscle biopsy
   MTO1 patient: sex: m; age at presentation: 1 day; hypertrophic cardiomyopathy,
   perinatal asphyxia, severe global developmental delay; enzymatic phenotype:
   complex I+IV defect in muscle biopsy