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NAR Breakthrough Article

Metabolic and chemical regulation of tRNA modification associated with taurine deficiency and human disease

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ABSTRACT

Modified uridine containing taurine, 5-taurinomethyluridine (τm^5U), is found at the anticodon first position of mitochondrial (mt-)transfer RNAs (tRNAs). Previously, we reported that τm^5U is absent in mt-tRNAs with pathogenic mutations associated with mitochondrial diseases. However, biogenesis and physiological role of τm^5U remained elusive. Here, we elucidated τm^5U biogenesis by confirming that 5,10-methylene-tetrahydrofolate and taurine are metabolic substrates for τm^5U formation catalyzed by MTO1 and GTPBP3. *GTPBP3*-knockout cells exhibited respiratory defects and reduced mitochondrial translation. Very little τm^5U34 was detected in patient's cells with the *GTPBP3* mutation, demonstrating that lack of τm^5U results in pathological consequences. Taurine starvation resulted in downregulation of τm^5U frequency in cultured cells and animal tissues (cat liver and flatfish). Strikingly, 5-carboxymethylaminomethyluridine ($cmnm^5U$), in which the taurine moiety of τm^5U is replaced with glycine, was detected in mt-tRNAs from taurine-depleted cells. These results indicate that tRNA modifications are dynamically regulated via sensing of intracellular metabolites under physiological condition.

INTRODUCTION

RNA molecules contain a wide variety of chemical modifications that are introduced post-transcriptionally. RNA modifications confer chemical diversity on simple RNA molecules, endowing them with a wider range of biological

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functions. To date, >130 species of RNA modifications have been reported in various RNA molecules from all domains of life (1–4). Many of these modifications were discovered in transfer RNAs (tRNAs), and tRNA modifications are involved in various molecular and physiological functions (5,6). In particular, tRNA modifications at the first (wobble) position of the anticodon (position 34) play critical roles in regulating the codon–anticodon interaction at the A-site of ribosome during the decoding process in translation (7).

The mitochondrion is a eukaryotic intracellular organelle that produces adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS). Mitochondria contain their own genome, called mitochondrial DNA (mtDNA), which encodes 13 essential subunits of the respiratory chain complexes, two mt-rRNAs and 22 mt-tRNAs. The mammalian mitochondrial genetic code deviates from the universal code by using four dialectal codons: AUA for Met, UGA for Trp and AGA and AGG (AGR; R = A or G) for the stop signal. The 22 species of mt-tRNAs participate in translation of 13 proteins encoded in the mtDNA. RNA modifications in mt-tRNAs play critical roles in accurate and efficient translation in mitochondria (8). Previously, we mapped all modifications in 22 species of mammalian mt-tRNAs, and found 15 species of modified nucleosides at 118 positions (9). Three of the modifications, 5-formylcytidine (f^5C), 5-taurinomethyluridine (τm^5U) and 5-taurinomethyl-2-thiouridine (τm^5s^2U) are specific to mt-tRNAs.

f^5C is present at the first position of the anticodon in mt-$tRNA^{Met}$ (10), and f^5C34 plays an essential role in translation of the dialectal AUA codon as Met (8,11,12). We and other groups, recently demonstrated that f^5C34 is synthesized via consecutive reactions, initiated by 5-methylcytidine (m^5C) formation catalyzed by NSUN3 (13,14), followed by hydroxylation and oxidation of m^5C to generate f^5C, mediated by ALKBH1 (15,16). Both enzymes are required for efficient mitochondrial translation and respiratory activity. We also identified two pathogenic point mutations in mt-$tRNA^{Met}$ that impair f^5C34 formation (13). In addition, loss-of-function mutations in *NSUN3* were detected in a patient with symptoms of mitochondrial disease (14). Together, these observations indicate that loss of f^5C34 results in pathological consequences.

Two taurine-containing uridines are present at the wobble positions of five mt-tRNAs: τm^5U in the mt-tRNAs for Leu(UUR) and Trp, and τm^5s^2U in the mt-tRNAs for Glu, Lys and Gln (9,17) (Figure 1A). These modifications promote accurate decoding of purine-ending codons (NNR; N = U, C, A or G) and prevent misreading of pyrimidine-ending codons (NNY; Y = U or C) (8). During decoding, τm^5U34 stabilizes U–G wobble pairing by assuming Watson–Crick geometry, thus engaging in a favorable stacking interaction with the 3′ adjacent base (A35) (18). We found that $\tau m^5(s^2)U$ modifications do not occur in mutant tRNAs isolated from cells of patients with mitochondrial encephalomyopathies (8,19). The mt-$tRNA^{Leu(UUR)}$ harboring one of five pathogenic mutations associated with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) lacks τm^5U34 (20,21). Biochemical studies revealed that the hypomodified mt-$tRNA^{Leu(UUR)}$ lacking τm^5U34 decodes the UUA codon normally, but failed to efficiently decode the UUG codon efficiently (22), strongly suggesting that inefficient decoding by the hypomodified tRNA results in defective translation of protein subunits of respiratory chain complexes. In addition, τm^5s^2U34 is absent from mt-$tRNA^{Lys}$ with the A8344G point mutation associated with MERRF (myoclonus epilepsy associated with ragged red fibers) (23,24). The hypomodified mt-$tRNA^{Lys}$ also lacks the ability to decode its cognate codons, leading to defective mitochondrial translation and respiratory activity (25). The pathogenic point mutations associated with MELAS and MERRF are thought to prevent recognition by the tRNA-modifying enzymes responsible for τm^5U formation. These findings represent the first instance of human disease caused by aberrant RNA modifications (19). Hence, we propose 'RNA modopathy' as a distinct category of human disease.

A metabolic labeling experiment revealed that extracellular taurine supplied in the medium is directly incorporated to 5-taurinomethyl groups in mt-tRNAs (17). However, a comprehensive understanding of τm^5U biogenesis remains elusive. Based on a genetic study in yeast (26), we previously predicted the tRNA modifying enzymes responsible for τm^5s^2U34. MTO1 and GTPBP3 are likely to be involved in the formation of the 5-taurinomethyl group, while MTU1 is responsible for the 2-thio group of τm^5s^2U34 in mt-tRNAs. Genetic mutations in *MTU1* were identified in patients of reversible infantile liver failure (RILF) (27–29). Liver-specific knockout of *Mtu1* mice exhibited severe impairment of mitochondrial translation and respiratory activities in the hepatocytes, demonstrating that mitochondrial dysfunction due to loss of 2-thiolation of τm^5s^2U34 is a primary cause of RILF (30). Regarding 5-taurinomethyl modification, exome sequencing identified pathogenic mutations in both *MTO1* (31–33) and *GTPBP3* (34,35) associated with hypertrophic cardiomyopathy and lactic acidosis, indicating that lack of taurine modification results in mitochondrial dysfunction via a mechanism similar to those of MELAS and MERRF. However, it has not been directly demonstrated that the frequency of $\tau m^5(s^2)U34$ is reduced in patients' cells or tissues.

In regard to the biogenesis of τm^5U34, we demonstrate here that the β-carbon of L-serine is a source of the methylene group of $\tau m^5(s^2)U34$ via 5,10-methylene-tetrahydrofolate (THF) in one-carbon (1C) metabolism. This finding is supported by the observation that the level of $\tau m^5(s^2)U34$ was reduced in cells harboring mutations in serine hydroxymethyltransferase 2 (SHMT2) and mitochondrial folate transporter (MFT). In addition, we found that the τm^5U34 frequency in mt-tRNAs was down-regulated by taurine starvation. Intriguingly, we detected 5-carboxymethylaminomethyluridine ($cmnm^5U$), in which the taurine moiety of τm^5U is replaced with glycine, in mt-tRNAs of taurine-starved cells, indicating that tRNA modification is dynamically regulated by sensing of intracellular metabolites under physiological condition. In addition, in the cells of domestic cat (*Felis catus*) and Japanese flounder (*Paralichthys olivaceus*), neither of which have the biosynthetic pathway for taurine, we observed that the τm^5U34 frequency in mt-tRNAs decreased upon taurine starvation during feeding, indicating that dietary taurine deficiency decreases the abundance of the taurine modification in

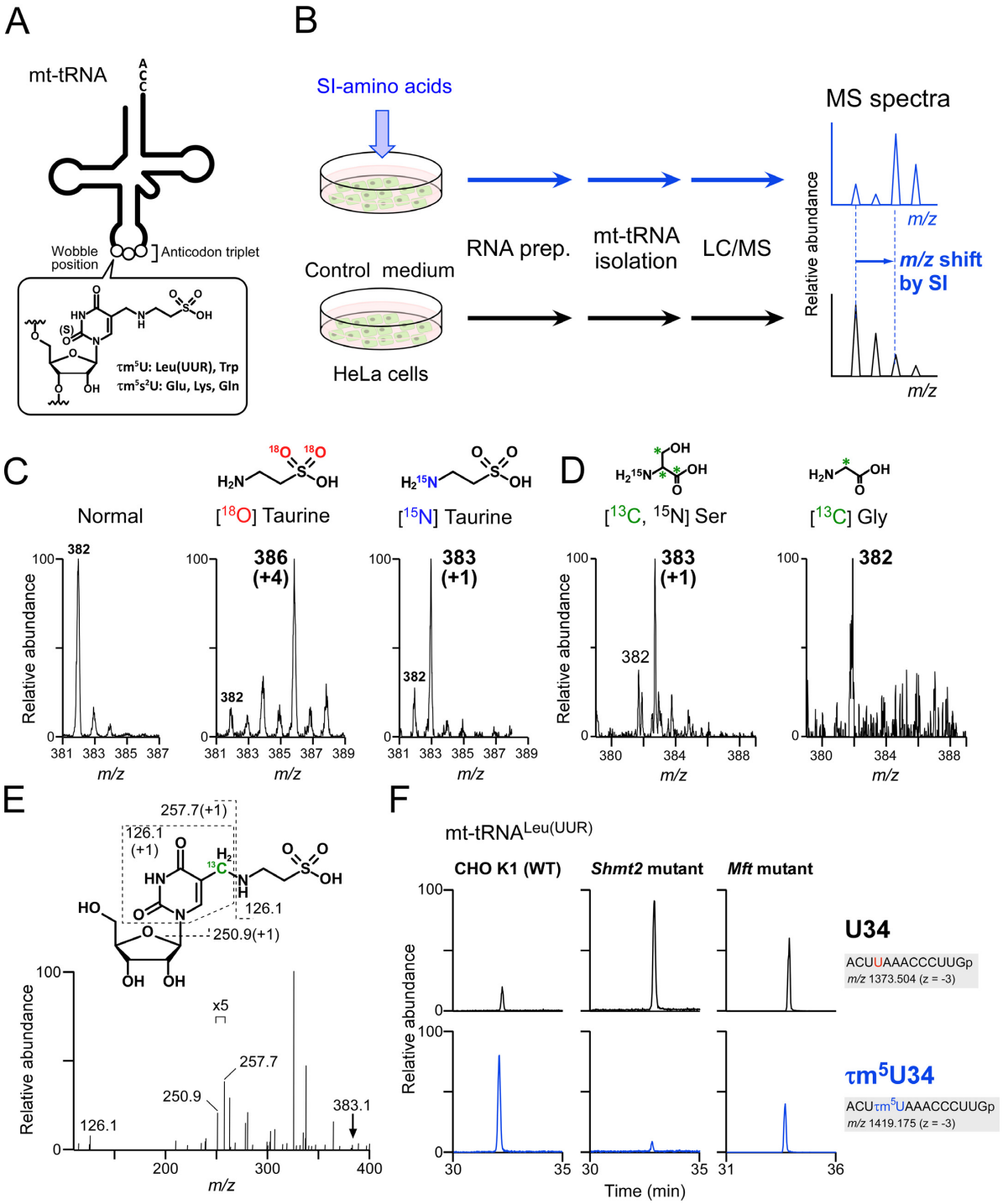

Figure 1. Metabolic labeling of mt-tRNAs to determine metabolic sources of τm^5U. (**A**) Chemical structure of $\tau m^5(s^2)U$ which is present at the wobble (first) position of anticodon of five mt-tRNAs. (**B**) Scheme for metabolic labeling experiments. HeLa cells were cultured with stable isotope (SI)-labeled or non-labeled amino acids. Total RNA was extracted from each culture, and individual mt-tRNAs were isolated and digested into nucleosides, followed by liquid chromatography–mass spectrometry (LC/MS) analysis to detect SI-labeled $\tau m^5(s^2)U$. (**C**) Mass spectra of τm^5U in mt-tRNA^{Trp} isolated from HeLa cells cultured in normal medium (first from the left), [^{18}O] taurine–supplemented medium (second) and [^{15}N] taurine–supplemented medium (third). Labeled atoms are colored as indicated. Increased m/z values of τm^5U due to the presence of SI-atoms are shown in parentheses. (**D**) Mass spectra of τm^5U in mt-tRNAs for Trp and Leu(UUR) isolated from HeLa cells cultured in [^{15}N, $^{13}C_3$] Ser–supplemented medium (left) and [^{13}C] Gly–supplemented medium (right). ^{13}C atoms in their chemical structures are indicated by asterisks. (**E**) Collision-induced dissociation (CID) spectrum of [^{13}C]-labeled τm^5U nucleoside. The precursor ion (m/z 383.1) for CID is marked by an arrow. Product ions in the CID spectrum are assigned in the chemical structure of τm^5U. (**F**) Mitochondrial 1C metabolism involved in τm^5U biosynthesis (see Figure 7). Extracted mass chromatograms (XICs) of anticodon-containing RNase T_1-digested fragments bearing U34 (upper panels) or τm^5U34 (lower panels) from mt tRNA$^{Leu(UUR)}$ isolated from Chinese hamster ovary (CHO) wild-type (WT) (left panels), *Shmt2* mutant (center panels) and *Mft* mutant (right panels) cells. Sequence of the detected fragment, with its m/z value and charge state, is indicated on the right.

mt-tRNAs, leading to pathological consequences including cardiomyopathy and other manifestation of mitochondrial diseases. The physiological importance of the taurine modifications was demonstrated by the finding that *GTPBP3*-knockout cells exhibited respiratory defects and reduced mitochondrial translation. We here clearly demonstrated that $\tau m^5(s^2)U34$ was absent from mt-tRNAs isolated from *GTPBP3* KO cells, *Mto1* KO cells, as well as fibroblasts of a patient with pathogenic mutations in *GTPBP3*. Finally, we successfully reconstituted τm^5U34 formation in mt-tRNA *in vitro* in a system consisting of GTPBP3 and MTO1 in the presence of taurine, the methylene-THF, guanosine triphosphate (GTP) and other co-factors.

MATERIALS AND METHODS

Cell culture and stable-isotope labeling

HeLa and HEK293T cells were cultured at 37°C in 5% CO_2 in Dulbecco's modified eagle's medium (DMEM) (high-glucose) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. *GTPBP3* KO cells were maintained at 37°C in 5% CO_2 in DMEM (high-glucose) supplemented with 0.11 g/l sodium pyruvate, 0.05 g/l uridine, 10% FBS and 1% penicillin–streptomycin.

For stable isotope (SI) labeling with taurine, HeLa cells were cultured with taurine-$^{18}O_2$ or taurine-^{15}N (Aldrich) as described (17). To determine the 1C carbon source, HeLa cells were cultured at 37°C in 5% CO_2 for 48 h with a synthetic medium containing 1× minimal essential media (GIBCO), 10% dialyzed FBS (GIBCO), 20 mM HEPES-KOH (pH 7.5), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.5 mM sodium pyruvate and 0.1 mM each of amino acids (L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-methionine and L-proline), supplemented with 0.5 mM Gly-2–^{13}C (Taiyo Nippon Sanso) or Ser-^{15}N, $^{13}C_3$ (Taiyo Nippon Sanso).

For taurine starvation medium, 10% dialyzed FBS was used instead of 10% FBS. To restore taurine, 40 mM taurine (final concentration) was added to the taurine starvation medium.

Chinese hamster ovary (CHO) K1 cells were obtained from RIKEN BioResource Center (RCB2869). The glycine auxotrophic CHO *glyA* (*Shmt2*) and *glyB* (*Mft*) mutant strains (36) were kindly provided by Dr Lawrence Chasin (Columbia University). CHO strains were cultured at 37°C in 5% CO_2 with DMEM (high-glucose) containing 10% FBS and 1% penicillin–streptomycin.

Mto1 heterozygous mice were generated by conventional homologous recombination to replace exons 1 and 2 of *Mto1* with the neomycin-resistance cassette (37). Mouse ES cell clones were prepared by *in vitro* fertilization of male and female *Mto1* heterozygous mice. The Animal Ethics Committee of Kumamoto University reviewed and approved all animal procedures (Approval ID: A27−037 R1). ES cells were cultured under feeder-free conditions in DMEM supplemented with 1% FBS (Hyclone), 15% knockout serum replacement (Invitrogen), CHIR99021 (WAKO Pure Chemical Industries) and PD184352 (WAKO Pure Chemical Industries).

Neonatal human dermal fibroblasts (NHDFs, Takara Bio) and patient fibroblasts (Pt751) were cultured as described (35). RNA analyses using these fibroblasts were approved by the ethics committee of the Saitama Medical University.

Construction of *GTPBP3* KO cells

GTPBP3 KO cells were generated using the CRISPR/Cas9 system as described previously (13,38). In short, sense and antisense oligonucleotides encoding a single guide RNA (sgRNA) (Supplementary Table S1) were cloned into pX330 vector (Addgene plasmid 42230) (39). HEK293T cells seeded in 24-well plates were transfected using FuGENE HD (Promega) with 300 ng of the pX330 containing the designed sgRNA sequence, 100 ng of pEGFP-N1 (Clontech) and 100 ng of a modified pLL3.7 containing the puromycin-resistance gene. One day after transfection, cells were seeded at low density, and transfectants were selected with 1 μg/ml puromycin. Transfection efficiency was monitored by enhanced green fluorescent protein (EGFP) fluorescence. Eight days after transfection, several colonies were picked and further grown for several days. The target region of the genome in each clone was polymerase chain reaction (PCR)-amplified using primers listed in Supplementary Table S1 and sequenced.

Animal models

Animal experiments using domestic cats were approved by the Ethics Committee of Tokyo University of Agriculture and Technology (protocol 29–18). Domestic cats were obtained from Saitama Experimental Animals Supply Co., Ltd, Saitama, Japan. A custom-ordered taurine deficient cat diet was purchased from Bio-Serv, Inc. An individual cat fed the taurine-deficient diet was maintained for 6 months. Blood taurine concentration decreased from 169.7 to 42.9 nmol/ml in the first month, as described (40). At the end of the 6 months period, the animal was sacrificed and its organs were harvested. A healthy cat liver was obtained from a cat incidentally sacrificed due to a traffic accident.

For the flatfish experiment, the formulation of the taurine-deficient diet is described in Supplementary Table S2. All ingredients were mixed well, moistened by addition of tap water, formed into pellets using a mincer, freeze-dried and stored at −20°C until use. Japanese flounder *P. olivaceus* juveniles were purchased from a private hatchery (Nisshin Marinetech, Japan) and fed at the Nansei Laboratory, National Research Institute of Aquaculture. Initially, they were fed a commercial fishmeal-based flounder feed (Higashimaru, Japan) in a 500 l round tank with sand-filtered sea water. Then, 45 flatfish (mean weight, 16.3 g) were fed the taurine-deficient diet to apparent satiation four times a day. The water temperature was 21.7 ± 1.1°C (mean ± SD). Sixty days after the feeding, ten flatfish were anesthetized with 2-phenoxyethanol (200 ppm) and weighed (mean weight, 49.2 g). Control flatfish fed the commercial feed were taken from the 500 l tank. Individual muscles were dissected out, frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA from isolated liver or muscle was prepared as described below.

RNA preparation and tRNA isolation

Total RNA from each cell or tissue was prepared using the TriPure Isolation Reagent (Roche Life Science). Individual mt-tRNAs were isolated by the reciprocal circulating chromatography method, as described (41). DNA probes for the target mt-tRNAs are listed in Supplementary Table S1. For nucleoside analysis, 450 μg of total RNAs extracted from HeLa cells cultured under taurine-depleted or taurine-restored conditions were subjected to an anion exchange resin (DEAE Sepharose Fast Flow, GE Healthcare) with low buffer [10 mM Hepes-KOH (pH 7.5), 250 mM NaCl and 2 mM dithiothreitol (DTT)] and small RNA fractions were eluted with high buffer [10 mM Hepes-KOH (pH 7.5), 1 M NaCl and 2 mM DTT].

Mass spectrometry

The isolated tRNAs were digested with RNase T_1 (Ambion) and analyzed by capillary liquid chromatography (LC)/nano-electron spray ionization mass spectrometry (ESI-MS), as described (9,42,43). SI-labeled nucleoside analysis by LC/MS was performed as described (44,45). For nucleoside analysis of HeLa cells cultured under taurine-depleted condition, 5 μg of tRNA fraction was digested as follows: 10 μl reaction mixture consisting of 20 mM ammonium acetate (pH 5.3) and 0.01 units/μl nuclease P1 (Wako Pure Chemical Industries) was incubated at 37°C for 30 min, followed by adding 0.5 μl of 1 M ammonium bicarbonate (pH 8.0) and 0.25 units of phosphodiesterase I (Worthington Biochemical Corporation) and incubated at 37°C for 30 min, then 0.1 units of alkaline Phosphatase (*Escherichia coli* C75) (Takara Bio) was added and further incubated at 37°C for 30 min. The digests were subjected to hydrophilic interaction liquid chromatography (HILIC)/MS analysis as described (46). For detection of THF derivatives bound to *Thermotoga maritima* MnmE, 175 μg of recombinant protein was directly injected into the LC/MS under the same conditions used for the nucleoside analysis.

Northern blotting

Northern blotting analysis of mt-tRNAs was conducted essentially as described (43). The DNA probes used in this study are listed in Supplementary Table S1.

Oxygen consumption rate

An XF24 extracellular flux analyzer (Seahorse Bioscience) was used to measure oxygen consumption rate (OCR). Wild-type (WT) HEK293T and *GTPBP3* KO cells were seeded in three wells each (3×10^5 cells per well) of an XF24 cell culture miniplate pre-coated with collagen, and then cultured for 24 h. The medium was then replaced with a solution consisting of 25 mM glucose and 1.25 mM pyruvic acid (adjusted to pH 7.4 with NaOH). The OCR of each well was measured over the course of programmed injections of oligomycin (f.c. 2.5 μg/ml), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, f.c. 1 μM) and rotenone/antimycin A (f.c. 2 μM/4 μM).

Respiratory complex activities

For isolation of mitochondrial fraction, about 1 mg of WT or GTPBP3 KO cells was homogenized in hypotonic buffer [80 mM sucrose and 10 mM 3-morpholinopropanesulfonic acid (MOPS) (pH 7.2)], followed by addition of an equal volume of hypertonic buffer [250 mM sucrose and 10 mM MOPS (pH 7.2)]. After centrifugation at 600 × *g* for 5 min, the supernatant was collected, homogenized again and centrifuged at 10 000 × *g* for 20 min. The precipitate was suspended in 100 μl of hypertonic buffer. Respiratory complex activities were measured as described (47). Absorbance at the indicated wave length was monitored on a UV–visible spectrophotometer V-630 equipped with a temperature control unit PAC-743R (JASCO).

Western blotting

The mitochondrial fraction was isolated from WT and *GTPBP3* KO cells (1×10^7 cells) using the Mitochondria Isolation Kit (Miltenyi Biotec). Subunit proteins of mitochondrial respiratory chain complexes were detected by immunoblotting using Total OXPHOS Rodent WB Antibody Cocktail (1:250, ab110413, Abcam), anti-ND2 antibody (1:200, 19704–1-AP, Proteintech) and anti-mt-VDAC1 antibody (1:500, 10886–1-AP, Proteintech) as a control. HRP-conjugated anti-rabbit IgG (1:20000, 715–035-152, Jackson ImmunoResearch) was used as the secondary antibody.

Pulse-labeling of mitochondrial protein synthesis

The pulse-labeling experiment was performed essentially as described basically performed as described (13). WT and *GTPBP3* KO cells (2.0×10^6 cells) were cultured at 37°C for 15 min in methionine/cysteine-free medium [D0422 (Sigma) with 10 mM HEPES-KOH (pH 7.5), 2 mM L-glutamine, 133 μM L-cysteine and 10% FBS] containing 50 μg/ml emetine to inhibit cytoplasmic protein synthesis, followed by addition of 7.4 MBq (0.2 mCi) of [^{35}S]-methionine/[^{35}S]-cysteine (EXPRE^{35}S^{35}S Protein Labeling Mix, [^{35}S]-, PerkinElmer), and then cultured for 1 h to specifically label mitochondrial translation products. Cell lysates (100 μg of total proteins) were resolved by Tricine-sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (16.5%), and the gel was Coomassie brilliant blue (CBB)-stained and dried on a gel drier (AE-3750 RapiDry, ATTO). The radiolabeled mitochondrial protein products were visualized on an imaging plate (BAS-MS2040, Fujifilm) by a laser scanner imaging system (FLA-7000, Fujifilm).

Expression and isolation of the GTPBP3–MTO1 complex

The cDNAs of human *GTPBP3* and *MTO1* were cloned into pDEST12.2 (Invitrogen) and transferred to vector pEFh SBP vector (kindly provided by Dr Yamashita of Yokohama City University) (48) to construct expression vectors pEFh-GTPBP3 (without tag) and pEFh-MTO1-FLAG (C-terminal FLAG tag) using primers listed in Supplementary Table S1. The In-Fusion HD Cloning Kit (Clontech) was used for vector conversion.

For transient expression of GTPBP3 and MTO1, HEK293T cells (2.0×10^6) were transfected with 6 μg each of pEFh-GTPBP3 and pEFh-MTO1-FLAG using FuGENE HD (Promega), and then cultured for 48 h. The cells were harvested and suspended with 1 ml of lysis buffer [100 mM KCl, 10 mM Tris–HCl (pH8.0), 2.5 mM $MgCl_2$, 1 mM DTT, 0.05% Triton X-100, 10% glycerol and 1× Complete EDTA-free protease inhibitor cocktail (Roche Life Science)] and lysed on ice by 20 passages through a 25G syringe needle. The lysate was centrifuged twice at 20 000 × *g* for 20 min to remove cell debris. The supernatant was pre-cleared by mixing with 30 μl of Mouse IgG-Agarose (Sigma), and immunoprecipitated with 30 μl of anti-FLAG beads (ANTI-FLAG M2 affinity gel, Sigma). The beads were washed four times with 1 ml of lysis buffer, and the GTPBP3–MTO1 complex was eluted with FLAG peptide in elution buffer [100 mM KCl, 10 mM Tris–HCl (pH 8.0), 2.5 mM $MgCl_2$, 1 mM DTT, 0.05% Triton X-100, 10% glycerol and 250 μg/ml FLAG peptide]. To remove the FLAG peptide, the eluent was gel-filtered using Centri Sep spin columns (Princeton Separations) and concentrated using an Amicon Ultra-0.5 ml device [nominal molecular weight limit: 30 kDa]. The GTPBP3–MTO1 complex was quantified based on SDS-PAGE band intensity after staining with SYPRO Ruby (Thermo Fisher Scientific), using a BSA standard.

In vitro reconstitution of cmnm^{5}U

MnmG (*E. coli*) and MnmE (*E. coli* and *T. maritima*) were cloned into pET28b (Novagen) to construct expression vectors for N-terminally His-tagged proteins. Expression and purification of the recombinant proteins were performed basically as described (49). Purified recombinant proteins were dialyzed and stored in 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 7 mM β-mercaptoethanol (for MnmG) or 10 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM DTT for (*E. coli* MnmE) or 10 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT (for *T. maritima* MnmE).

The DNA template for *in vitro* transcription of tRNA was prepared using a set of synthetic DNAs shown in Supplementary Table S1. *Escherichia coli* $tRNA^{Gly}_{UCC}$ was transcribed by T7 RNA polymerase essentially as described (50). The transcripts were gel-purified by 10% denaturing PAGE.

For *in vitro* reconstitution of cmnm^{5}U, a reaction mixture (20 μl) consisting of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 100 mM KCl, 2.5 mM FAD, 2.5 mM GTP, 2.5 mM NADH, 2 mM 5,10-CH_2-THF (or 5-formyl-THF), 2.5 mM glycine (or taurine), 0.2 μM *E. coli* $tRNA^{Gly}_{UCC}$ transcript and 10 μM each recombinant *E. coli* MnmG and MnmE was incubated at 37°C for 1.5 h. As controls, the same reaction was performed in the presence of THF or 5-formyl-THF instead of 5,10-CH_2-THF, or without the enzymes. After the reaction, the tRNA was recovered using the QIAquick nucleotide removal kit (QIAGEN) and dialyzed for 1 h on a 0.025 μm VSWP filter (Millipore) floating on Milli-Q water. Modification frequency was measured by mass spectrometric analysis as described above.

In vitro reconstitution of τm^{5}U

The reaction mixture (20 μl) consisting of 50 mM Tris–HCl (pH8.0), 10 mM $MgCl_2$, 5 mM DTT, 100 mM KCl, 2.5 mM FAD, 1 mM ATP, 2.5 mM NADH, 2.5 mM NADPH, 2 mM 5,10-CH_2-THF and 0.19 μM hypomodified mt-$tRNA^{Leu(UUR)}$ (isolated from *GTPBP3* KO cells), 2.5 mM GTP, 2.5 mM taurine and 2 μM GTPBP3–MTO1 complex was incubated at 37°C for 1 h. As negative controls, the same reaction was performed without GTP and taurine, or without enzyme complex. After the reaction, the mt-$tRNA^{Leu(UUR)}$ was extracted from the mixture by phenol–chloroform treatment, cleaned up by with Q Sepharose High Performance (GE Healthcare), and recovered by two rounds of ethanol precipitation. Modification frequency was measured by mass spectrometric analysis as described above.

RESULTS

Identification of the metabolic sources of τm^{5}U

To identify metabolic substrates for the 5-taurinomethyl group of τm^{5}U in five mt-tRNAs (Figure 1A), we carried out metabolic labeling experiments in human cell culture. As illustrated in Figure 1B, HeLa cells were cultured in a defined medium with or without SI-labeled metabolite to label τm^{5}U in mt-tRNAs. Total RNA was extracted from the cells, and mt-tRNAs were isolated and digested to nucleosides, which were then analyzed by LC/MS to detect the labeled τm^{5}U with increased molecular mass derived from the SI-labeled metabolite.

As reported previously (17), when cells were cultured with [^{18}O]-labeled taurine, we observed a proton adduct of τm^{5}U nucleoside in mt-$tRNA^{Trp}$ with molecular mass (m/z 386) 4-Da larger than that of the natural nucleoside (m/z 382), confirming that the sulfonic acid moiety of the labeled taurine was incorporated in τm^{5}U (Figure 1C). To demonstrate that the entire molecule of taurine was incorporated into τm^{5}U, the cells were cultured with [^{15}N] taurine. We detected τm^{5}U with molecular mass (m/z 383) 1 Da larger than the natural nucleoside (Figure 1C), demonstrating that free taurine is a direct metabolic substrate for τm^{5}U biogenesis.

We still needed to determine the carbon source of the methylene group of τm^{5}U. In bacteria, yeast and nematode mitochondria, cmnm^{5}U (see Figure 6F) is present at the wobble position in tRNAs. cmnm^{5}U is a modified uridine in which the taurine moiety of τm^{5}U is replaced with Gly. Genetic and biochemical studies revealed that MnmE and MnmG employ glycine and one-carbon (1C) folate derivatives as substrates, and catalyze cmnm^{5}U formation in the presence of essential co-factors including GTP, K^+ and FAD (51–53). Given that GTPBP3 and MTO1 are mammalian homologs of bacterial MnmE and MnmG, respectively, we speculated that the methylene group of τm^{5}U comes from a folate derivative in 1C metabolism. To determine which folate derivative is actually involved in cmnm^{5}U formation, we conducted LC/MS analysis of THF derivatives bound to *T. maritima* MnmE recombinantly expressed in *E. coli*. We detected 5,10-methylene(CH_2)-THF, along with THF, but no formyl-THF, indicating that 5,10-CH_2-

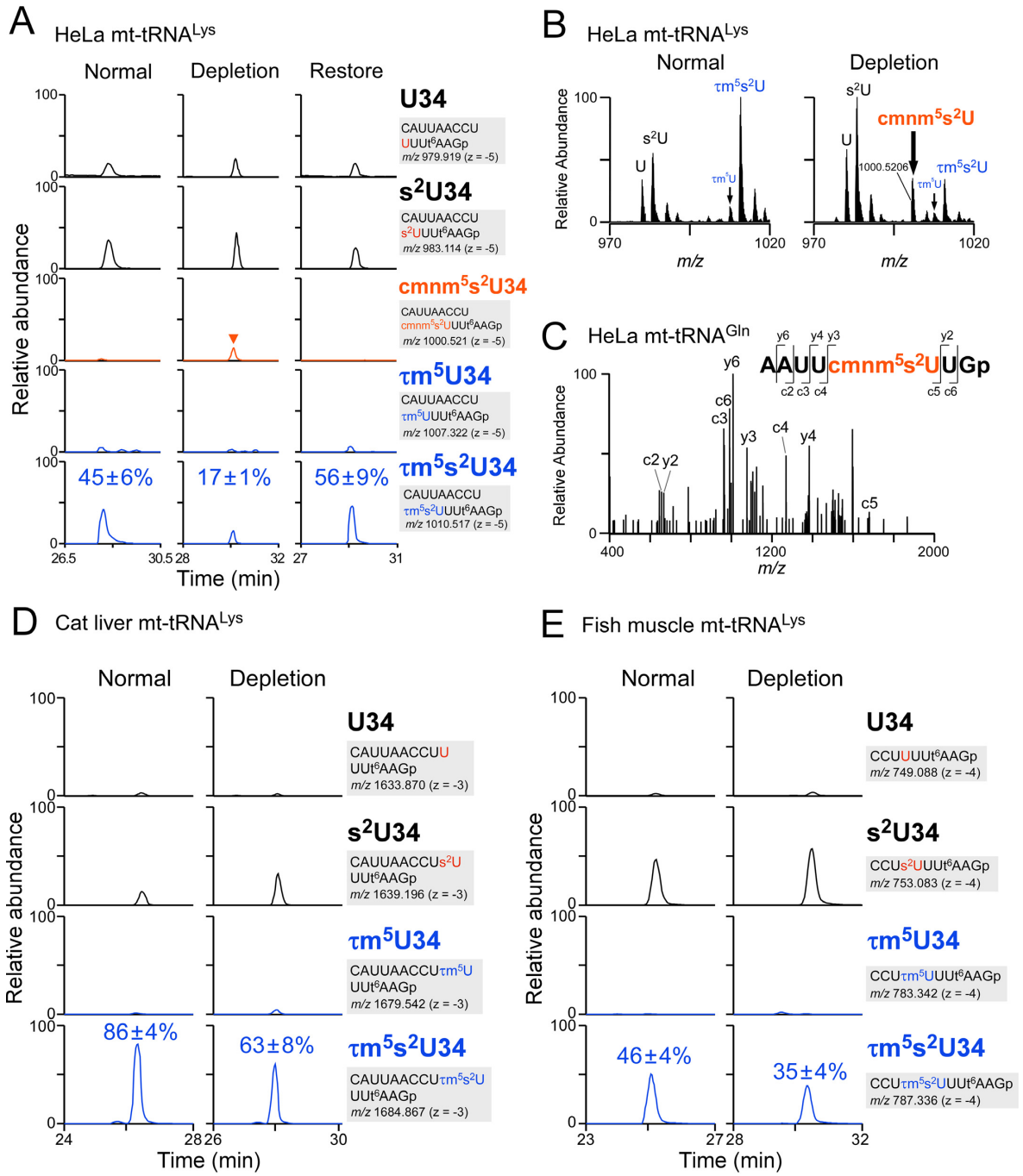

Figure 2. Downregulation of $\tau m^5(s^2)U$ in culture cells and animal tissues upon taurine starvation. (**A**) XICs of anticodon-containing RNase T_1-digested fragments with different wobble modifications of mt-$tRNA^{Lys}$ isolated from HeLa cells cultured in normal (left panels), taurine-depleted (center panels) and taurine-restoring (right panels) conditions. Sequence of the detected fragment, with its *m/z* value and charge state, is indicated on the right. Relative $\tau m^5(s^2)U$ frequencies indicated in the bottom chromatograms are averaged values with standard deviations, calculated from the peak height ratio of the multiply charged negative ions (−3 to −7) for RNA fragments with different modifications. s^2U, 2-thiouridine; t^6A, N^6-threonylcarbamoyladenosine. (**B**) Averaged mass spectra of the anticodon-containing fragments with different wobble modifications of mt-$tRNA^{Lys}$ isolated from HeLa cells cultured in normal (left panel) and taurine-depleted (right panel) conditions. (**C**) CID spectrum of the anticodon-containing RNase T_1-digested fragment bearing $cmnm^5s^2U$ of mt-$tRNA^{Gln}$ isolated from HeLa cells cultured under taurine-depleted conditions. The c- and y-series of the product ions are assigned on the sequence. (**D**) XICs of anticodon-containing RNase T_1-digested fragments with different wobble modifications of mt-$tRNA^{Lys}$, isolated from livers of cats fed a normal diet (left panels) or a taurine-depleted diet (right panels). Sequence of the detected fragment, with its *m/z* value and charge state, is indicated on the right. Relative $\tau m^5(s^2)U$ frequencies indicated in the bottom chromatograms are averaged values with standard deviations, calculated from the peak height ratio of the multiply charged negative ions (−3 to −7) for RNA fragments with different modifications. (**E**) XICs of anticodon-containing RNase T_1-digested fragments with different wobble modifications of mt-$tRNA^{Lys}$, isolated from muscles of flatfish fed a normal diet (left panels) or a taurine-depleted diet (right panels). Sequence of the detected fragment, with its *m/z* value and charge state, is indicated on the right. Relative $\tau m^5(s^2)U$ frequencies indicated in the bottom chromatograms are averaged values with standard deviations, calculated from the peak height ratio of the multiply charged negative ions (−2 to −4) for RNA fragments with different modifications.

THF is a *bona fide* substrate for cmnm^{5}U formation (Supplementary Figure S1A and B). We then reconstituted cmnm^{5}U on *E. coli* tRNA with recombinant MnmE and MnmG in the presence of a series of folate derivatives and Gly, and found that 5,10-CH_2-THF served as a substrate for cmnm^{5}U formation (Supplementary Figure S1C).

To identify the carbon source for the methylene group in τm^{5}U biogenesis, we cultured HeLa cells in a medium supplemented with SI-labeled Gly or Ser, because the α-carbon of Gly or β-carbon of Ser is a source of 1C folate derivative (Figure 1B). Then, mt-tRNA^{Trp} and mt-tRNA$^{Leu(UUR)}$ were isolated from each culture and subjected to nucleoside analysis by LC/MS (Figure 1D). When the cells were cultured with the SI-labeled Ser, we clearly detected a 1 Da larger species (*m/z* 383) of τm^{5}U, which we then further probed by collision-induced dissociation (CID). Based on assignment of the product ions generated by CID (Figure 1E), the ^{13}C atom derived from SI-labeled Ser resided in the product ion composed of uracil base and C5-carbon. Because uridines and pseudouridines contained no ^{13}C derived from the SI-labeled Ser (data not shown), the ^{13}C atom was attributed to the methylene group of τm^{5}U. On the other hand, when the cells were cultured with SI-labeled Gly, no carbon derived from this amino acid could be detected in τm^{5}U (Figure 1D). This result demonstrated that the β-carbon of Ser is a metabolic source of the methylene group of τm^{5}U, via a 1C folate derivative that is presumably 5,10-CH_2-THF.

Requirement of 1C metabolism in τm^{5}U biosynthesis

In 1C metabolism in mammalian mitochondria, 5,10-CH_2-THF is generated via two independent pathways mediated by SHMT2 and the glycine cleavage system (GCS) (see Figure 7). SHMT2 catabolizes Ser to Gly and transfers the β-carbon of Ser as a 1C unit to 5,10-CH_2-THF, whereas GCS digests Gly into CO_2 and NH_3 and provides the α-carbon of Gly as a 1C unit to 5,10-CH_2-THF. Judging from our metabolic labeling experiment, SHMT2-mediated 5,10-CH_2-THF formation is a major mitochondrial pathway in HeLa cells, as in other cancer cells (54). To confirm whether 1C metabolism is actually involved in τm^{5}U formation, we analyzed τm^{5}U status in CHO *glyA* and *glyB* mutant cell lines, which exhibit glycine auxotrophy due to reduced Shmt2 and Mft activities, respectively (36,55,56). The mt-tRNA$^{Leu(UUR)}$ was isolated from WT and mutant CHO cells, digested with RNase T_1 and subjected to RNA–MS to analyze τm^{5}U status (Figure 1F). In the WT cells, about 80% of mt-tRNA$^{Leu(UUR)}$ contained τm^{5}U, whereas in *Shmt2* mutant cells, the τm^{5}U frequency dropped sharply to 9%, demonstrating that loss of 5,10-CH_2-THF due to impairment of SHMT2 activity results in τm^{5}U deficiency. In addition, we observed hypomodification of τm^{5}U (48%) in the *Mft* mutant strain, indicating that folate transport to mitochondria is required for efficient τm^{5}U formation. Taken together, these observations indicate that τm^{5}U biogenesis in mammalian cells requires 5,10-CH_2-THF generated by SHMT2 in mitochondrial 1C metabolism.

Taurine starvation downregulates τm^5(s^2)U in HeLa cells and animal tissues

Taurine is an essential nutrient for carnivores, including cat and fox, which have little endogenous taurine biogenesis activity. In these animals, deficiency of dietary taurine causes developmental defects, central retinal degradation, hepatic lipidosis and dilated cardiomyopathy (57,58), which are also major manifestations of human mitochondrial encephalomyopathies (59). In humans and possibly primates, infants and young children have low capacity for taurine biosynthesis, so dietary taurine is essential for normal development (60).

These facts prompted us to investigate whether τm^{5}U frequency could be affected by extracellular taurine concentration. In this experiment, HeLa cells were cultured in a medium containing dialyzed FBS, which does not contain taurine and other small compounds, and mt-tRNAs were isolated for analysis of τm^{5}U status by RNA–MS. In HeLa cells cultured in a normal medium, the frequency of τm^{5}s^{2}U34 (and τm^{5}U34) in mt-tRNA^{Lys} was 45% (Figure 2A and Supplementary Table S3). When the cells were cultured in a medium with dialyzed FBS, the τm^{5}U frequency in mt-tRNA^{Lys} dropped to 17%. When 40 mM taurine was added back to medium containing dialyzed FBS, the τm^{5}U frequency in mt-tRNA^{Lys} was restored to 56%. This result was reproducible (Supplementary Figure S2 and Supplementary Table S3). We also analyzed the other four mt-tRNAs isolated from cells cultured with dialyzed FBS, and found that the τm^{5}U frequency was significantly reduced in each of them (Supplementary Figure S3 and Supplementary Table S3). These results indicate that the τm^{5}U frequency in mt-tRNAs is dynamically regulated by extracellular taurine concentration.

Upon taurine depletion, we happened to detect 5-carboxymethylaminomethyl-2-thiouridine (cmnm^{5}s^{2}U), in which the taurine moiety of τm^{5}s^{2}U is replaced with Gly in mt-tRNA^{Lys} (Figure 2A and B; Supplementary Figure S2). We also detected cmnm^{5}U in mt-tRNAs for Leu(UUR) and Trp (Supplementary Figures S3A,B and S4A,B), and cmnm^{5}s^{2}U in mt-tRNAs for Gln and Glu (Supplementary Figures S3A,B and S4A,B), only in cells cultured under taurine-depleted conditions. We confirmed presence of cmnm^{5}U (*m/z* 332) and cmnm^{5}s^{2}U (*m/z* 348) by conducting LC/MS nucleoside analyses of total RNAs extracted from HeLa cells cultured under taurine-depleted condition (Supplementary Figure S5). When taurine was restored to the medium, cmnm^5(s^2)U completely disappeared, while τm^5(s^2)U increased clearly. As a negative control, no signal of cmnm^5(s^2)U as well as τm^5(s^2)U was detected in *GTPBP3* knockout cells (see below), indicating that cmnm^5(s^2)U are synthesized by the same pathway of τm^5(s^2)U biogenesis. Sequence analysis of the anticodon-containing fragment by CID revealed that cmnm^{5}s^{2}U was present at position 34 (Figure 2C). Judging from the mass-chromatographic intensities, 5–23% of mt-tRNAs contained cmnm^{5}U or cmnm^{5}s^{2}U (Figure 2A; Supplementary Figures S2 and S3). This result suggests that cmnm^5(s^2)U34 is synthesized by utilizing Gly instead of taurine under taurine-depleted conditions.

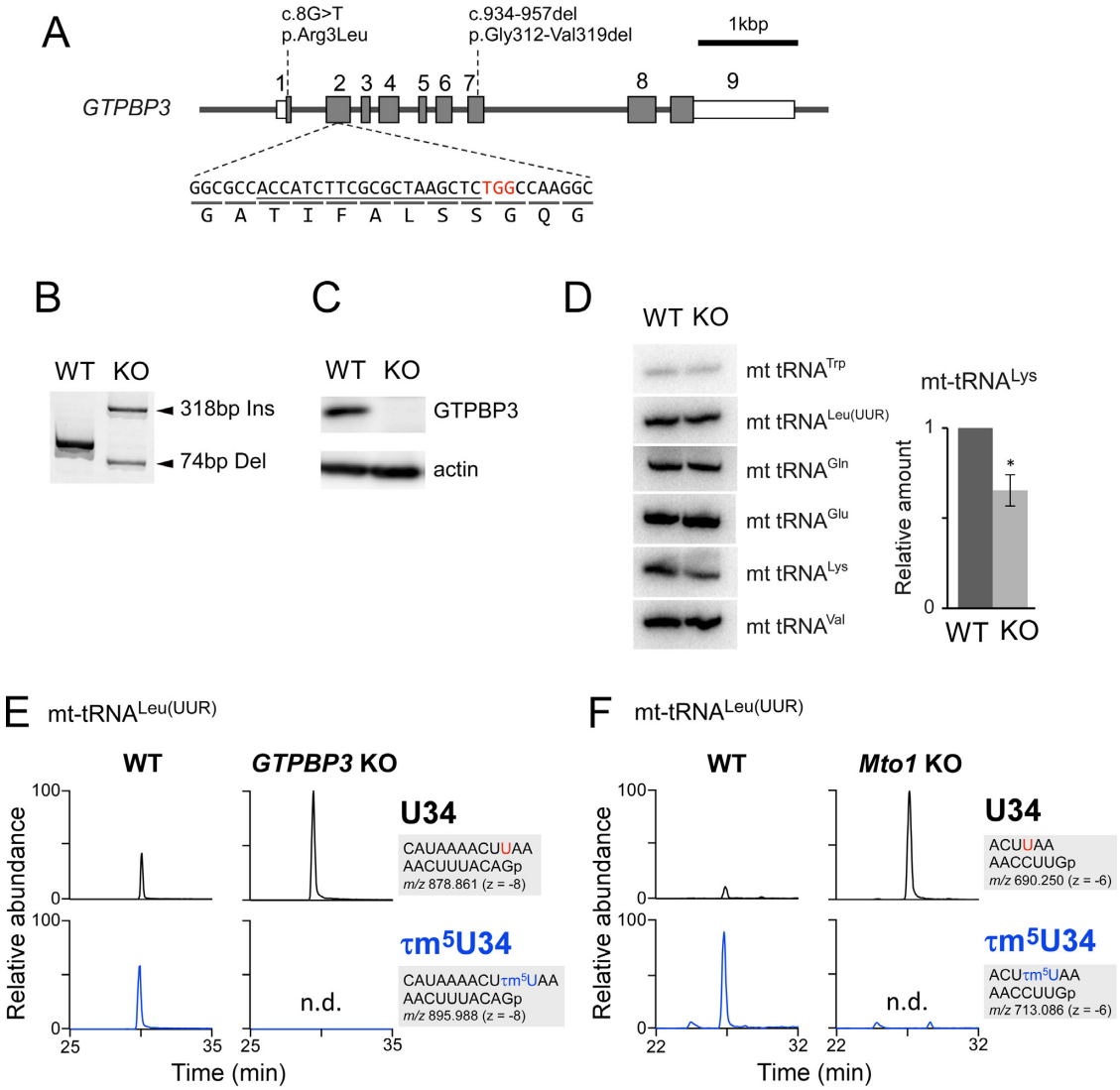

Figure 3. GTPBP3 and MTO1 are essential for τm^5U biogenesis. (**A**) A diagram of the human *GTPBP3* gene and the sites of mutations introduced by the CRISPR–Cas9 system. Shaded boxes indicate coding regions, open boxes indicate untranslated regions of exons and lines indicate introns. Heterozygous mutations found in the patient with mitochondrial disease are indicated above the diagram (34,35). The sgRNA sequence is underlined; the PAM sequence is in red. Reading frame and translated amino acids are shown below the sequence. (**B**) Genomic PCR products covering the sgRNA target site from WT HEK293T and *GTPBP3* KO cells. One allele of the KO cell has a 318 bp insertion, and the other has a 74 bp deletion. (**C**) Western blotting of GTPBP3 and actin proteins in WT and KO cells. (**D**) Steady-state levels of six mt-tRNAs from WT and KO cells, evaluated by northern blotting. The bar graph shows relative amounts of mt-tRNA^{Lys} from WT and KO cells, quantified in three independent replicates. Error bars indicate standard deviation. *p = 0.02, Student's paired t-test. (**E**) XICs of anticodon-containing RNase T_1-digested fragments with U34 (upper panels) and τm^5U34 (lower panels) of mt tRNA$^{Leu(UUR)}$, isolated from WT (left panels) and *GTPBP3* KO cells (right panels). Sequence of the detected fragment, with its m/z value and charge state, is indicated on the right. (**F**) XICs of anticodon-containing RNase T_1-digested fragments with U34 (upper panels) and τm^5U34 (lower panels) of mt tRNA$^{Leu(UUR)}$, isolated from WT (left panels) and *Mto1* KO mES cells (right panels). Sequence of the detected fragment, with its m/z value and charge state, is indicated on the right.

Given that cats do not have the biosynthetic pathway for taurine, we asked whether the τm^5U frequency in cat mt-tRNAs could be regulated by taurine starvation. For this purpose, we raised two domestic cats (*Felis catus*), fed either a control or taurine-deficient diet. mt-tRNA^{Lys} was isolated from livers of those cats and subjected to RNA–MS for analysis of τm^5U status, which revealed that the frequencies of τm^5s^2U34 (and τm^5U34) in mt-tRNAs^{Lys} were 86% and 63% in the the control and taurine-deprived animals, respectively (Figure 2D and Supplementary Table S3). Thus, the τm^5U frequency in cat liver was slightly but significantly decreased upon taurine depletion.

The Japanese flounder *P. olivaceus* has little taurine biogenesis activity. Accordingly, in aquaculture production, taurine promotes growth of the juvenile flatfish (61,62). To examine the effect of dietary taurine level on τm^5U frequency, we cultivated *P. olivaceus* for 2 months on a control or taurine-deficient diet. mt-tRNA^{Lys} was isolated from the flatfish muscle tissues and analyzed by RNA-MS. The τm^5s^2U frequencies of mt-tRNAs^{Lys} isolated from the flatfish cultivated on the control and taurine-deficient diets

were 46% and 35%, respectively (Figure 2E and Supplementary Table S3). Thus, dietary taurine regulates τm^5U frequency in flatfish mt-tRNAs.

GTPBP3 and MTO1 are essential for τm^5U biogenesis

Based on our previous finding that MSS1 and MTO1 are responsible for $cmnm^5U$ formation of mt-tRNAs in yeast mitochondria (26), we investigated the involvement of their human homologs, GTPBP3 and MTO1, in τm^5U biosynthesis. First, we knocked out the *GTPBP3* gene in HEK293T cells using the CRISPR–Cas9 system (Figure 3A), and obtained a knockout cell line in which both alleles harbor indel mutations (Figure 3B). Endogenous GTPBP3 was not detectable by western blotting in *GTPBP3* KO cells (Figure 3C). We then performed northern blotting to compare the steady-state levels of six mt-tRNAs, including five τm^5U34-containing tRNAs, between WT HEK293T and *GTPBP3* KO cells (Figure 3D). The results revealed that the level of mt-$tRNA^{Lys}$ decreased moderately, whereas the levels of the other five tRNAs were unchanged. We then isolated five τm^5U-containing mt-tRNAs from WT and KO cells, digested them with RNase T_1 and analyzed the RNA fragments by RNA-MS. The $\tau m^5(s^2)U34$-containing fragments detected in mt-tRNAs isolated from the WT cells completely disappeared, and were converted to the U34 or s^2U34-containing fragments, in *GTPBP3* KO cells (Figure 3E and Supplementary Figure S6).

Next, to examine the role of *MTO1*, we obtained *Mto1* KO ES cells and isolated five mt-tRNAs from the cells. RNA–MS analysis revealed that all five mt-tRNAs isolated from the *Mto1* KO ES cells lacked $\tau m^5(s^2)U34$ and remained unmodified at this position (Figure 3F and Supplementary Figure S7). Together, these results clearly demonstrated that *GTPBP3* and *MTO1* are responsible for τm^5U34 formation of five mt-tRNAs in mammalian cells.

Mitochondrial dysfunction in *GTPBP3* KO cells

We then assessed mitochondrial function in *GTPBP3* KO cells. The OCR of the KO cells, measured using a flux analyzer, was significantly lower than that of WT cells (Figure 4A). Adding of an uncoupler, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, barely increased OCR in the KO cells, suggesting that knockout of *GTPBP3* results in loss of electron transfer activity. We then compared the biochemical activities of respiratory complexes between *GTPBP3* KO and WT cells (Figure 4B). In *GTPBP3* KO cells, we observed severe reduction in complex I, mild reduction in complex IV and no significant change in complex III. On the other hand, the activity of complex II, all of whose components are encoded in the nuclear genome, was elevated in *GTPBP3* KO cells, probably to compensate for the reduction in complex I activity. We then analyzed steady-state levels of protein components in the respiratory complexes by western blotting (Figure 4C), and found severe reductions in the levels of ND2 and NDUFB8, both of which are subunit proteins in complex I, indicating defective assembly of complex I in *GTPBP3* KO cells.

To evaluate mitochondrial protein synthesis, we conducted a pulse-labeling experiment (Figure 4D). Cytoplasmic translation was inhibited by addition of emetine, and mitochondrial translation products were labeled with [^{35}S] Met and Cys. Mitochondrial protein synthesis was drastically lower in *GTPBP3* KO cells than in WT cells; in particular, the levels of CO1 and ND2 were markedly reduced in *GTPBP3* KO cells. Given that $\tau m^5(s^2)U34$ in mt-tRNAs plays a critical role in the decoding process of mitochondrial translation, these phenotypes can be explained by the hypomodification of mt-tRNAs in *GTPBP3* KO cells. Reduced activity of complex I in *GTPBP3* KO cells explains complex I deficiency as a clinical symptom of patients harboring pathogenic mutations of the *GTPBP3* gene (34,35) and mtDNA mutations associated with MELAS (63).

Hypomodification of the taurine modification in a *GTPBP3* patient

To determine whether pathogenic mutations in the *GTPBP3* gene actually impair $\tau m^5(s^2)U34$ in mt-tRNAs, we isolated five mt-tRNAs from the fibroblasts of a 2-year-old female patient harboring pathogenic mutations in *GTPBP3* genes (Figure 3A) (35), and analyzed $\tau m^5(s^2)U$ status by RNA–MS (Figure 5). No $\tau m^5(s^2)U34$ was detected in three mt-tRNAs (for Glu, Trp and Lys), whereas the frequencies of $\tau m^5(s^2)U$ in mt-$tRNA^{Leu(UUR)}$ and mt-$tRNA^{Gln}$ were 2.5% and 1.1%, respectively, indicating that most GTPBP3 enzymatic activity was lost due to the pathogenic mutations. This phenomenon could represent a primary cause of disease.

In vitro reconstitution of τm^5U

Although pathogenic point mutations in mt-tRNAs associated with MELAS and MERRF might act as negative determinants that prevent $\tau m^5(s^2)U34$ formation mediated by GTPBP3 and MTO1, no direct evidence confirming this prediction has been provided to date. *In vitro* reconstitution of τm^5U34 formation would help us to directly examine the effect of these pathogenic mutations on τm^5U34 formation.

Given that $cmnm^5U$ is a structural homolog of τm^5U in which the taurine moiety is replaced with glycine, we first tried to reconstitute τm^5U on *E. coli* tRNA using recombinant *E. coli* MnmE and MnmG in the presence of 5,10-CH_2-THF and taurine as substrates. The products were digested with RNase T_1 and analyzed by RNA–MS. In a positive control reaction, $cmnm^5U$ was efficiently reconstituted in the presence of glycine (Figure 6A and Supplementary Figure S1C). When taurine was added instead of glycine to the reaction mixture, a small amount of τm^5U was introduced into the tRNA (Figure 6A). The exact mass of the fragment containing τm^5U was consistent with the calculated m/z value (m/z 1494.1889, $z = -2$), with an error rate of 0.2 ppm (Figure 6B). This fragment was further probed by CID to determine the position of τm^5U reconstituted *in vitro*. The data confirmed that τm^5U resided at the wobble position, as determined by assignment of the product ions of CID (Figure 6C). This result demonstrated that *E. coli* MnmE and MnmG have a limited ability to synthesize τm^5U on *E. coli* tRNA.

Next, we tried to reconstitute τm^5U using human GTPBP3 and MTO1. Because yeast Mss1p and Mto1p

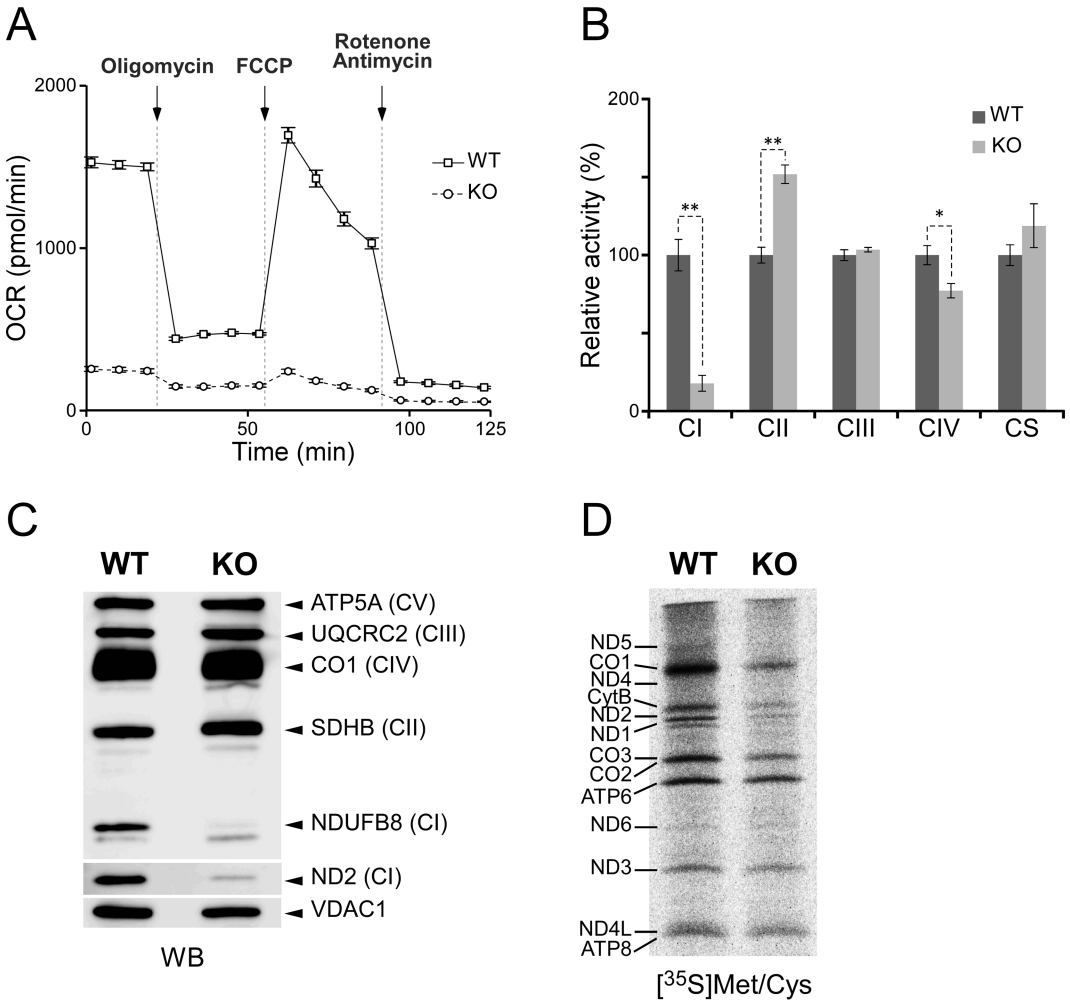

Figure 4. Mitochondrial dysfunction in *GTPBP3* KO cells. (**A**) Oxygen consumption rate in HEK293T WT (square) and *GTPBP3* KO (circle) cells, measured using a flux analyzer. Each plot represents the mean value of three independent samples. Error bars indicate standard deviations. (**B**) Relative activities of respiratory chain complexes I–IV and citrate synthase of WT (black bar) and *GTPBP3* KO (gray bar) cells. Error bars indicate standard deviation. *$p < 0.05$, **$p < 0.01$, Student's *t*-test. (**C**) Western blotting of subunit proteins (indicated on the right) of respiratory chain complexes in isolated mitochondria from WT and *GTPBP3* KO cells. The CBB-stained gel images are shown in Supplementary Figure S8A. (**D**) Pulse-labeling of mitochondrial protein synthesis. WT and *GTPBP3* KO cells were labeled with [^{35}S] Met and [^{35}S] Cys after cytoplasmic protein synthesis was halted by emetine. Assignment of mitochondrial proteins is indicated on the left. The CBB-stained gel images are shown in Supplementary Figure S8B.

formed a stable complex in mitochondria (64), we first examined the direct interaction between GTPBP3 and MTO1 in the cell. For this purpose, C-terminally FLAG-tagged MTO1 (MTO1-FLAG) and GTPBP3 without tag were transiently expressed in HEK293T cells. MTO1-FLAG was immunoprecipitated with anti-FLAG antibody, and the precipitants were resolved by SDS-PAGE. As shown in Figure 6D, GTPBP3 was co-precipitated with MTO1. The processed form of GTPBP3 lacking the N-terminal mitochondrial targeting sequence (MTS) was detected, indicating that the MTS is cleaved off by a mitochondrial processing protease after import into the mitochondria. Both proteins are predominantly localized to mitochondria (65,66), and they form a stable complex involved in τm^5U34 formation. Because tRNA modifications other than τm^5U34 might be required for efficient τm^5U34 formation, as a substrate, we prepared mt-tRNA$^{Leu(UUR)}$ lacking τm^5U34 isolated from *GTPBP3* KO cells. Based on our finding that 5,10-CH_2-THF is employed as a substrate employed by MnmE (Supplementary Figure S1C), we conducted *in vitro* reconstitution of τm^5U34 on mt-tRNA$^{Leu(UUR)}$ with the GTPBP3–MTO1-FLAG complex in the presence of taurine, 5,10-CH_2-THF and co-factors including GTP, FAD and potassium ion. In negative control reactions, no product was formed without the enzyme complex or in the absence of GTP and taurine (Figure 6E). In the presence of all components, the τm^5U34-containing fragment was clearly detected (Figure 6E). Judging from the ratio of modified (τm^5U34) versus unmodified (U34) fragments, 3.3% of mt-tRNA$^{Leu(UUR)}$ was modified. The observed m/z value of the τm^5U-containing fragment (m/z 895.9884, $z = -8$) was nearly identical to the calculated one (m/z 895.9881, $z = -8$), with a small error of 0.33 ppm, suggested that τm^5U was successfully reconstituted in this reaction (Figure 6F). This is the first demonstration that GTPBP3–MTO1 complex can form τm^5U34 on mt-tRNA.

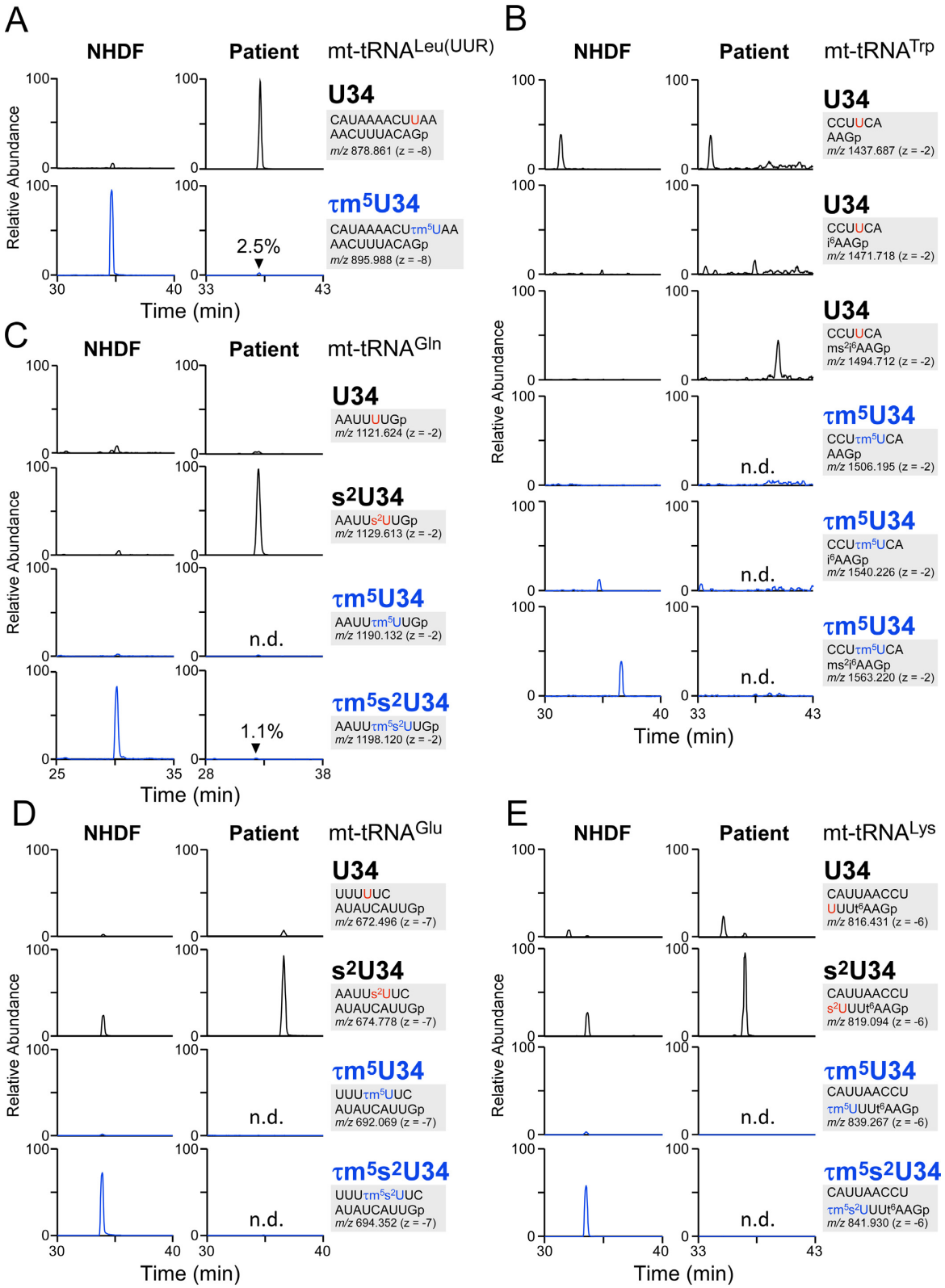

Figure 5. Hypomodification of the taurine modification in a patient with pathogenic mutations in *GTPBP3*. XICs of anticodon-containing RNase T_1-digested fragments with different modifications of mt-tRNAs for Leu(UUR) (**A**), Trp (**B**), Gln (**C**), Glu (**D**) and Lys (**E**), isolated from neonatal human dermal fibroblast (NHDF) (left panels) and patient fibroblasts (right panels). Sequence of the detected fragment, with its *m/z* value and charge state, is indicated on the right. Relative frequencies of $\tau m^5(s^2)U$ in the patient sample are indicated in the bottom chromatograms. n.d., not detected. ms^2i^6A, 2-methylthio-N^6-isopentenyladenosine; i^6A, N^6-isopentenyladenosine.

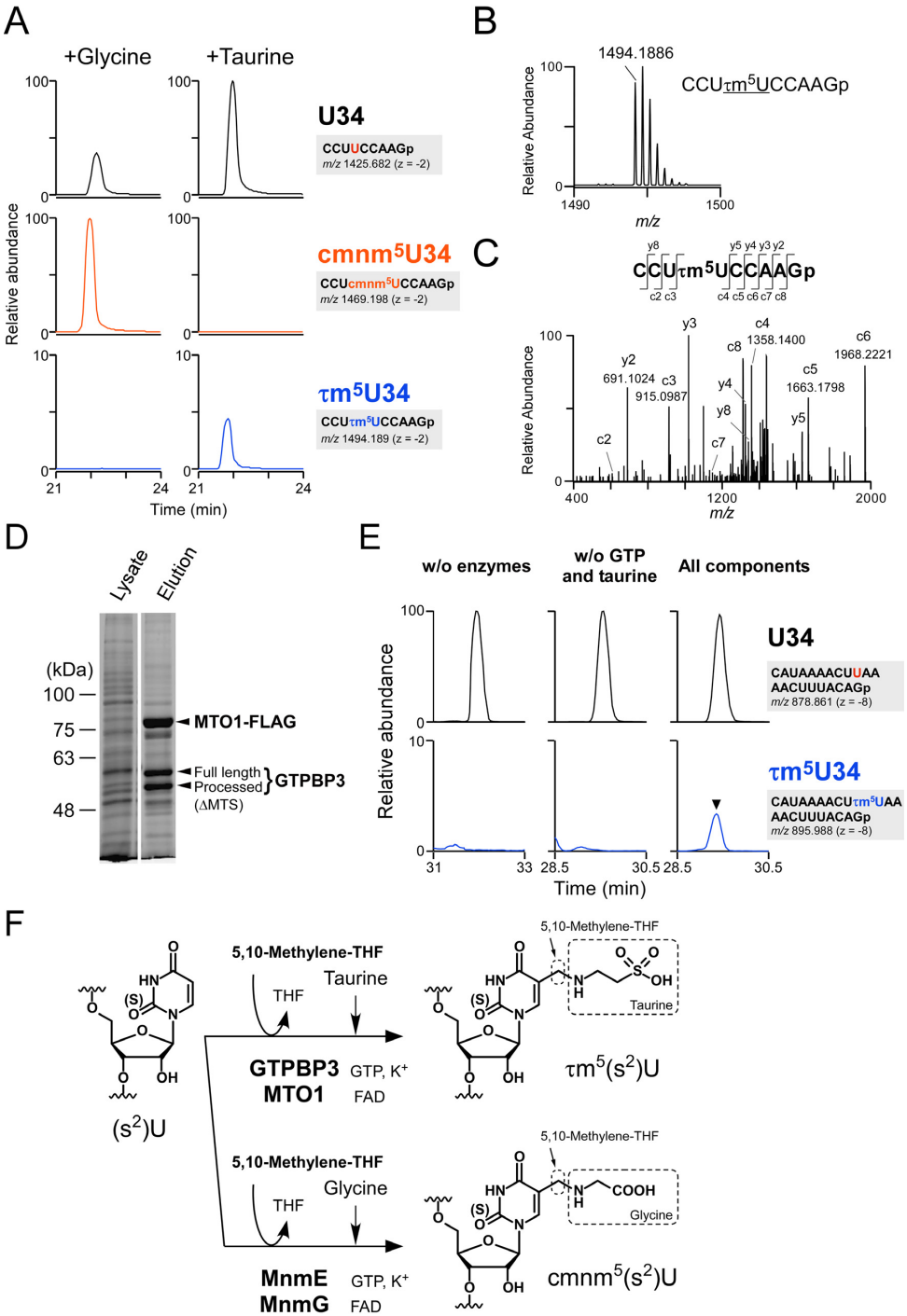

Figure 6. *In vitro* reconstitution of τm^5U34. (**A**) *In vitro* reconstitution of $cmnm^5U34$ and τm^5U34 on *E. coli* $tRNA^{Gly}$(UCC) in the presence of Gly (left panels) or taurine (right panels) along with other substrates and co-factors. XICs of anticodon-containing RNase T_1-digested fragments with U34 (top panels), $cmnm^5U34$ (middle panels) and τm^5U34 (bottom panels) of *E. coli* $tRNA^{Gly}$(UCC) after the reaction are shown. Sequence of the detected fragment, with its *m/z* value and charge state, is indicated on the right. (**B**) Mass spectrum of the anticodon-containing RNase T_1-digested fragment of *E. coli* $tRNA^{Gly}$(UCC) containing τm^5U34, reconstituted *in vitro*. The exact mass of the mono-isotopic ion is indicated. (**C**) CID spectrum of the above fragment with τm^5U34. Assigned c- and y-product ions are indicated. (**D**) Co-immunoprecipitation of GTPBP3 and MTO1-FLAG complex. Both proteins were transiently co-expressed in HEK293T cells, pulled down with anti-FLAG antibody–agarose and eluted with FLAG peptide. Whole lysate and eluate were resolved by SDS-PAGE and stained with a fluorescent dye. MTS stands for mitochondrial targeting sequence. (**E**) *In vitro* reconstitution of τm^5U on mt-$tRNA^{Leu(UUR)}$ without enzymes (left panels), without GTP and taurine (middle panels), or with all components including substrates and co-factors (right panels). XICs of anticodon-containing RNase T_1-digested fragments with U34 (top panels) and τm^5U34 (bottom panels) of mt-$tRNA^{Leu(UUR)}$ after the reaction. Sequence of the detected fragment, with its *m/z* value and charge state, is indicated on the right. (**F**) Scheme of τm^5U and $cmnm^5U$ biosynthesis. τm^5U is synthesized by GTPBP3/MTO1 complex using 5,10-CH_2-THF and taurine as substrates while glycine is used in place of taurine for $cmnm^5U$ by MnmE–MnmG complex. Co-factors including GTP, FAD and potassium ion are essential to $cmnm^5U$ formation, suggesting that they are also required for τm^5U.

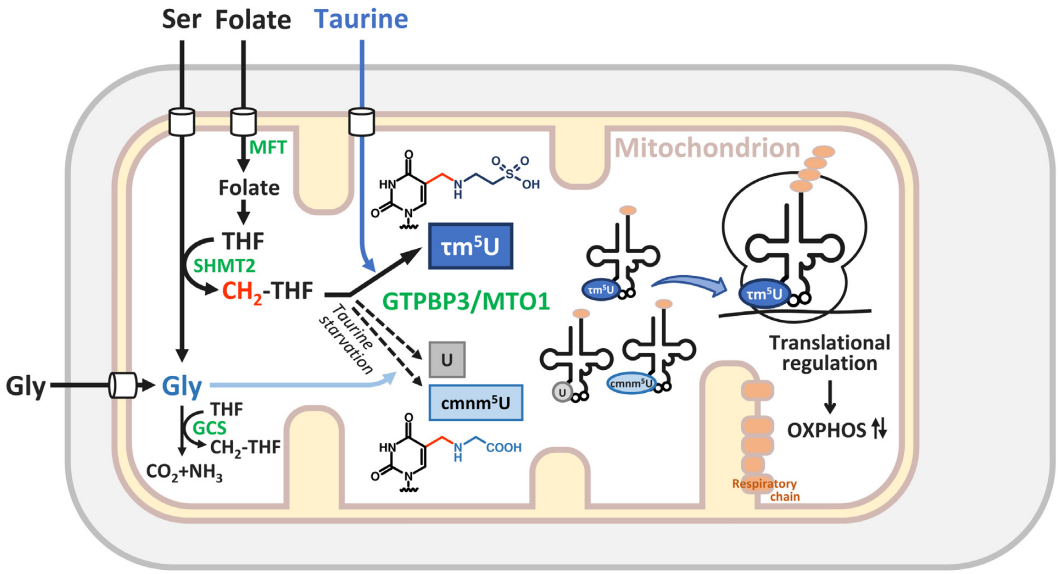

Figure 7. Metabolic regulation and chemical switching of tRNA modification in mitochondria. Schematic depiction of 1C metabolism, τm^5U biogenesis and potential translational regulation in mitochondria. τm^5U is biosynthesized from CH_2-THF and taurine catalyzed by GTPBP3–MTO1 complex. τm^5U in mt-tRNAs is required for efficient mitochondrial translation to synthesize the respiratory chain complexes essential for OXPHOS on the mitochondrial inner membrane. Taurine starvation decreases τm^5U frequency and increases unmodified U. In this condition, $cmnm^5U$, in which the taurine moiety of τm^5U is replaced with glycine, is also introduced in mt-tRNAs. The tRNA modification dynamically regulated in response to metabolic status implies translational regulation in a codon-specific manner under physiological conditions. Abbreviations are as follows: THF, tetrahydrofolate; SHMT2, serine hydroxymethyltransferase 2 (mitochondrial SHMT); MFT, mitochondrial folate transporter; GCS, glycine cleavage system.

DISCUSSION

In this study, a metabolic labeling experiment revealed that the taurinomethyl group of τm^5U34 is synthesized by taurine and the β-carbon of Ser which is a carbon source for 1C metabolism mediated by the folate pathway (Figure 1D). τm^5U was down-regulated in glycine auxotroph CHO cells bearing mutations in *Shmt2* and *Mft* (Figure 1F), indicating that 5,10-CH_2-THF and mitochondrial folate metabolism are involved in τm^5U formation. We clearly showed that $cmnm^5U$ on tRNA could be efficiently reconstituted by bacterial MnmE and MnmG utilizing 5,10-CH_2-THF and Gly as substrates (Supplementary Figure S1C and Figure 6A). Moreover, we directly detected 5,10-CH_2-THF bound to *T. maritima* MnmE (Supplementary Figure S1A). Based on these results, we conclude that 5,10-CH_2-THF is a *bona fide* substrate for τm^5U formation. In fact, we successfully reconstituted a small amount of τm^5U on mt-$tRNA^{Leu(UUR)}$ *in vitro* using the GTPBP3/MTO1 complex in the presence of taurine, 5,10-CH_2-THF and co-factors (Figure 6E).

In mitochondria, SHMT2 digests Ser into Gly, and transfers the β-carbon of Ser to THF to synthesize 5,10-CH_2-THF (Figure 7). SHMT2 is involved in cellular growth regulation by producing Gly for *de novo* nucleotide synthesis (54). The 1C carbon of 5,10-CH_2-THF is redundantly derived from the β-carbon of Ser mediated by SHMT2 or the α-carbon of Gly mediated by the GCS pathway. Both pathways are active in stem cells, whereas little GCS pathway activity can be detected in most cancer cells, indicating that 5,10-CH_2-THF is mainly produced via the SHMT2 pathway in cancer cells (54). Indeed, *SHMT2* expression is elevated in various cancer cells, and high expression defines poor-prognosis subtypes (67). Becasue downregulation of *SHMT2* suppresses tumorigenesis in human hepatocellular carcinoma (68), *SHMT2* is regarded to be a potential therapeutic target of cancers. Moreover, *SHMT2* plays a critical role in activation and survival of T cells by stimulating mitochondrial 1C metabolism (69). Intriguingly, the mitochondrial respiratory defect in elderly humans can be partly explained by down regulation of SHMT2 (70). Consistent with this, the OCR was decreased by *SHMT2* knockdown. Considering the fact that hypomodification of τm^5U was observed in *Shmt2* mutant CHO cells in this study (Figure 1F), it is likely that age-associated downregulation of *SHMT2* decreases the frequency of τm^5U, leading to mitochondrial dysfunction and respiratory defects.

Taurine is one of the most abundant amino acids, and ubiquitously distributed in various organs and tissues in mammals. Taurine is involved in a wide variety of cellular functions, including bile salt synthesis and cholesterol metabolism, antioxidation, osmoregulation and modulation of neuronal excitability (71,72). Mammals not only take up taurine from the diet but also synthesize it *de novo* from cysteine. Carnivores like cat and fox do not synthesize taurine *de novo* due to low activity of cysteine sulfinate decarboxylase, and therefore must acquire taurine through the diet. Taurine deficiency in cats leads to dilated cardiomyopathy and blindness due to retina failure (57,73). Because human infants have low *de novo* taurine synthesis activity, dietary taurine is essential for normal human development (60). Juvenile flatfish do not synthesize taurine *de novo*, and therefore require it as a nutrient for rapid growth in aquaculture production (61,74). We clearly showed that extracellular taurine is incorporated to the cells and used as

a direct metabolic substrate for τm^5U in mt-tRNAs (17). The lack of τm^5U34 results in a decoding deficiency of mt-tRNAs and decreases the rate of mitochondrial translation (75). Thus, if τm^5U frequency is modulated by intracellular taurine concentration, taurine depletion could downregulate the corresponding mt-tRNA modification, resulting in impaired mitochondrial translation and respiratory activity. In this study, we observed a marked reduction in the $\tau m^5(s^2)U$ frequency in mt-tRNAs isolated from HeLa cells cultured under taurine-depleted conditions (Figure 2A; Supplementary Figures S2 and S3). When taurine was added back to the medium, the $\tau m^5(s^2)U$ frequency was restored (Figure 2A and Supplementary Figure S2). These results demonstrated that $\tau m^5(s^2)U$ frequency can be dynamically regulated by cellular taurine concentration, indicating that taurine supplied from the extracellular environment is necessary for maintaining a high frequency of $\tau m^5(s^2)U$, even though human cells can biosynthesize taurine from cysteine *de novo*. Downregulation of $\tau m^5(s^2)U$ was also observed in animal models under taurine-depleted conditions. Specifically, we observed a reduction in the $\tau m^5(s^2)U$ frequency in mt-tRNAs isolated from the livers of cats fed a taurine-deficient diet (Figure 2D). This result revealed the physiological importance of taurine, which is key to maintaining a high $\tau m^5(s^2)U$ frequency in cat. Moreover, we observed that the level of $\tau m^5(s^2)U$ decreased in mt-tRNAs isolated from flatfish cultivated on taurine-deficient diets for 2 months (Figure 2E). Although the reduction rate of $\tau m^5(s^2)U$ was not large in either animal model, mitochondrial translation could nonetheless be affected by alteration of the $\tau m^5(s^2)U$ frequency following taurine depletion. These findings suggest that tRNA modification is dynamically regulated by sensing of cellular metabolic status, challenging accepted notions that tRNA modifications are stable, static and rarely regulated.

Taurine plays a critical role in mitochondrial translation and respiratory activity by maintaining $\tau m^5(s^2)U$ in mt-tRNAs, and at least one other physiological connection between taurine and mitochondria has been reported. Taurine-upregulated gene 1 (Tug1), a long non-coding RNA induced by taurine (76), physically interacts with PGC1α, a master transcription factor involved in mitochondrial biogenesis and facilitates the binding of PGC1α to its own promoter, leading to activation of mitochondrial biogenesis in mouse kidney (77). Hence, taurine synchronizes mitochondrial protein synthesis by maintaining $\tau m^5(s^2)U34$ and mitochondrial biogenesis via PGC1α activation.

$cmnm^5U$, a structural homolog of τm^5U in which taurine is replaced with Gly, is used as the wobble modification in bacterial tRNAs, as well as in yeast and nematode mt-tRNAs (26,78), suggesting that taurine took over for Gly as s structural component of the wobble modification over the course of animal mitochondrial evolution. Intriguingly, we happened to detect $cmnm^5(s^2)U$ modification of mt-tRNAs in HeLa cells cultured under taurine-depleted conditions (Figure 2A, B and C; Supplementary Figures S2, S3 and S4): this is the first observation of $cmnm^5(s^2)U$ detected in mammalian cells. This finding suggests that the GTPBP3–MTO1 complex retains an intrinsic specificity for Gly, which is a substrate of the bacterial MnmE–MnmG complex. Curiously, bacterial MnmE–MnmG complex had a limited ability to use taurine to synthesize τm^5U *in vitro* (Figure 6A), implying that the GTPBP3–MTO1 complex gradually acquired taurine specificity over the course of mitochondrial evolution. $\tau m^5(s^2)U34$ is primarily synthesized when the taurine concentration is high enough in mitochondria, whereas $cmnm^5(s^2)U34$ is synthesized by utilizing Gly instead of taurine under taurine-starved conditions, indicating that $cmnm^5(s^2)U$ could be used as a molecular marker for taurine starvation in mammals. In a sense, $cmnm^5(s^2)U34$ formation can be regarded as a backup system for mitochondrial translation under taurine-depleted conditions. However, as the decoding efficiency of $cmnm^5(s^2)U34$ in mammalian mitochondria has not been characterized, functional and physiological impact of this modification remains unclear. According to a chemical characterization of these nucleosides (79), the electron density of the uracil rings and the acidity of the N3H protons in these compounds are modulated by different C5-substituents, suggesting that the decoding efficiency and fidelity of NNG codons is affected by mt-tRNAs bearing $cmnm^5(s^2)U34$. High Gly concentration in blood is a characteristic feature of nonketotic hyperglycinemia, a type of mitochondrial disease caused by pathogenic mutations in the mitochondrial GCS pathway (80). The levels of $cmnm^5(s^2)U34$ might be elevated in patients with this disease, and the modification status of mt-tRNAs might be involved in its molecular pathogenesis.

Several groups studied the physiological impact of *GTPBP3* and *MTO1* using genetic approaches, and observed mitochondrial dysfunction when either protein was depleted (81–83). However, none of these studies provided direct evidence that these genes are actually involved in τm^5U34 formation. In this study, we clearly showed that τm^5U34 was absent from five mt-tRNAs in *GTPBP3* KO cells (Figure 3E and Supplementary Figure S6), as well as in *Mto1* KO cells (Figure 3F and Supplementary Figure S7). *GTPBP3* KO cells exhibited marked reductions in OCR and mitochondrial translation. We also observed reduced levels of complex I subunit proteins and deficient complex I activity (Figure 4A and B). Taken together, our findings demonstrate that τm^5U34 is essential for efficient mitochondrial translation and respiratory activity. Because $\tau m^5(s^2)U34$ is present in five mt-tRNAs whose codons are widely distributed throughout all genes encoded in mtDNA, lack of the taurine modification is predicted to result in global reduction of mitochondrial protein synthesis. To modulate respiratory complex assembly, mitochondrial protein synthesis is coordinated with inner membrane insertion on the matrix side (84,85). Thus, repression of mitochondrial translation due to lack of τm^5U34 in *GTPBP3* KO might disrupt maturation and stability of respiratory chain complexes, leading to defective assembly of complex I. We also observed defective mitochondrial translation and respiratory activity in *NSUN3* KO and *ALKBH1* KO, in which formation of f^5C34 in mt-$tRNA^{Met}$ is impaired (13,15). However, the pulse-labeling pattern of mitochondrial translation and respiratory activities in these KO cells are quite different from those observed in *GTPBP3* KO cells, indicating that a codon-specific decoding defect mediated by different

tRNA species arises in each system. In particular, because f^5C34 is essential for AUA decoding (11), translational defects caused by lack of f^5C34 could be related to the AUA codon distribution. Likewise, the distribution of codons decoded by five mt-tRNAs bearing $\tau m^5(s^2)U$ should be associated with the characteristic phenotype and translational deficiency observed in *GTPBP3* KO cells. To obtain mechanistic insights into the decoding disorder caused by deficiency in wobble modification, mitoribosome profiling of each type of KO cell could provide a snapshot of the decoding status at single-codon resolution (86).

Several approaches to restoring mitochondrial dysfunction in MELAS patients have been explored at both the basic research and clinical levels (87–92). Considering that lack of $\tau m^5(s^2)U$ in mutant mt-tRNA is a primary cause of MELAS and MERRF, restoration of the taurine modification represents a promising therapeutic approach for treatment of these diseases. The pathogenic point mutations found in MELAS and MERRF are thought to prevent mutant tRNA recognition by GTPBP3–MTO1 complex, hampering $\tau m^5(s^2)U$ formation. Although we showed here that GTPBP3–MTO1 complex is responsible for τm^5U formation in all five mt-tRNA species, these tRNAs contain no consensus sequence or characteristic structural features, indicating that tRNA recognition by the GTPBP3–MTO1 complex has broad specificity. Nevertheless, it is puzzling that only one point mutation in mt-tRNAs associated with MELAS and MERRF impaired $\tau m^5(s^2)U$ formation. To obtain mechanistic insights into $\tau m^5(s^2)U$ formation and the tRNA specificity of GTPBP3–MTO1 complex, it is necessary to recapitulate τm^5U formation. *In vitro* τm^5U34 formation would be a powerful tool for directly examining the impact of pathogenic point mutations associated with MELAS and MERRF. In this study, we successfully introduced τm^5U in mt-tRNA *in vitro* using the GTPBP3–MTO1 complex expressed in HEK293T cells in the presence of taurine, 5,10-CH_2-THF, GTP and co-factors (Figure 6E), although the efficiency was quite low in comparison with that of *in vitro* $cmnm^5U34$ formation by bacterial MnmE–MnmG complex (Figure 6A). To advance these studies, it will be necessary to optimize the reaction conditions to improve the efficiency as much as possible, such as adequate salt concentration, optimal pH, divalent cations, coenzymes and so on. Otherwise, some accessory proteins and/or unidentified co-factors might be required for efficient τm^5U formation. In this study, we used monoglutamate form of CH_2-THF for *in vitro* reconstitution. But, polyglutamate form is a major folate derivative in mitochondria (93). It's worth examining polyglutamate form of CH_2-THF for further study. Using *in vitro* τm^5U34 formation, it might be possible to investigate other pathogenic mutations in mt-tRNAs that impair τm^5U formation. We could also analyze enzymatic activities of GTPBP3 and MTO1 with the reported pathogenic mutations associated with respiratory deficiency. In the future, it could be possible to engineer a GTPBP3–MTO1 complex that is capable of recognizing mt-tRNAs with pathogenic mutations and introducing τm^5U into these molecules.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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