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The 2-thiouridylase function of the human MTU1 (TRMU) enzyme is dispensable for mitochondrial translation

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MTU1 (TRMU) is a mitochondrial enzyme responsible for the 2-thiolation of the wobble U in $tRNA^{Lys}$, $tRNA^{Glu}$ and $tRNA^{Gln}$, a post-transcriptional modification believed to be important for accurate and efficient synthesis of the 13 respiratory chain subunits encoded by mtDNA. Mutations in *MTU1* are associated with acute infantile liver failure, and this has been ascribed to a transient lack of cysteine, the sulfur donor for the thiouridylation reaction, resulting in a mitochondrial translation defect during early development. A mutation in $tRNA^{Lys}$ that causes myoclonic epilepsy with ragged-red fibers (MERRF) is also reported to prevent modification of the wobble U. Here we show that mitochondrial translation is unaffected in fibroblasts from an MTU1 patient, in which MTU1 is undetectable by immunoblotting, despite the severe reduction in the 2-thiolation of mitochondrial $tRNA^{Lys}$, $tRNA^{Glu}$ and $tRNA^{Gln}$. The only respiratory chain abnormality that we could observe in these cells was an accumulation of a Complex II assembly intermediate, which, however, did not affect the level of the fully assembled enzyme. The identical phenotype was observed by siRNA-mediated knockdown of MTU1 in HEK 293 cells. Further, the mitochondrial translation deficiencies present in myoblasts from mitochondrial encephalomyopathy, lactic acidosis and stroke-like episode and MERRF patients, which are associated with defects in post-transcriptional modification of mitochondrial tRNAs, did not worsen following knockdown of MTU1 in these cells. This study demonstrates that MTU1 is not required for mitochondrial translation at normal steady-state levels of tRNAs, and that it may possess an as yet uncharacterized function in another sulfur-trafficking pathway.

INTRODUCTION

The 2008 update of MODOMICS, a database of RNA modification pathways, identified 119 different posttranscriptional modifications in all types of RNA, the largest number of which are present in tRNAs (1). The uridine at position 34 (U34), the first (wobble) position of the anticodon in the tRNAs for Lys, Glu and Gln, is almost universally modified at carbons 2 and 5. While carbon 2 is modified exclusively through thiolation (s^2), various methyl derivatives can be found at carbon 5 (xm^5), examples of which include methylaminomethyl (mnm^5) and carboxymethylaminomethyl ($cmnm^5$) in the bacterial tRNAs, methoxycarbonylmethyl (mcm^5) in the cytoplasmic tRNAs of eukaryotes (2) and taurinomethyl (τm^5) in the mammalian mitochondrial tRNAs (3).

The tRNAs for Lys, Glu and Gln recognize each a set of two codons which are part of split codon boxes (coding for two amino acids) and which end in a purine. The xm^5s^2 modification was postulated to confer conformational rigidity to the U34 wobble base of these tRNAs, leading to preferential pairing with purines, and preventing misreading of the near-cognate codons ending in pyrimidines (4). In contrast, a study in *E. coli* demonstrated increased misreading by wild-type (fully modified), when compared with hypomodified, $tRNA^{Lys}$ of the near-cognate codons which end in a pyrimidine and which code for asparagine (5). In this case, the hypomodified forms of $tRNA^{Lys}$ were carrying either the mnm^5U34 or the s^2U34 modification. An alternative role for the U34 wobble base modifications in increasing the efficiency, rather than the accuracy of translation, was suggested by

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several studies, including the one in which the lethal phenotype of a yeast strain containing a double deletion for enzymes involved in the modifications of the cytoplasmic tRNAs at carbons 2 and 5 was rescued by overexpression of the unmodified forms of $tRNA^{Lys}$ and $tRNA^{Gln}$ (6).

Aside from directly modulating the accuracy or efficiency of translation, the 2-thio group has been shown to be important for the recognition of $tRNA^{Glu}$ by the *E. coli* glutamyl-tRNA synthetase (7), for ribosome binding of *E. coli* $tRNA^{Lys}$ at the appropriate codons (8), for promoting bacterial growth at higher temperatures through thermal stabilization of tRNAs (9) and for improving reading frame maintenance (10).

The biosynthesis of s^2U34 in *E. coli* requires the 2-thiouridylase MnmA and the cysteine desulfurase IscS (11), as well as a complex sulfur-relay system composed of the products of five genes: *tusA*, *tusB*, *tusC*, *tusD* and *tusE* (12). Recently, the sulfur-relay system required for 2-thiouridine biosynthesis at the wobble position of cytoplasmic tRNAs in *Saccharomyces cerevisiae* was described (13). Given the lack of conservation of the Tus proteins even across bacteria (12), it is not surprising that the sulfur mediators and reaction mechanism are distinct in yeast and bacteria.

The mitochondrial tRNA-specific 2-thiouridylase, MTU1 (also known as TRMU or MTO2) is the homolog of the bacterial MnmA (14). Although the official symbol for the human gene is *TRMU*, for 'tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase', we selected for the purpose of this work the symbol *MTU1*, which describes accurately the function of this enzyme, namely thiouridylase, rather than methyltransferase. The enzyme responsible for 2-thiouridylation of tRNAs in the eukaryotic cytoplasm, TUC1/NCS6, is the homolog of the *E. coli* TtcA enzyme, which is required for the synthesis of 2-thiocytidine at position 32 in a few bacterial tRNA species, a modification absent in yeast (6). Studies in yeast have demonstrated that deletion mutants of *MTU1* display impaired 2-thio modification of the mitochondrial tRNAs for Lys, Glu and Gln (14) and a decrease in steady-state levels of these and other mitochondrial tRNAs (15), resulting in a severe defect in mitochondrial translation and impaired growth on non-fermentable carbon sources (14,16). Mutations in *MTU1* have also recently been reported in acute infantile liver failure resulting from deficiencies in multiple respiratory chain enzymes (17,18).

Lack of post-transcriptional modifications at the wobble positions of mitochondrial tRNAs for Leu and Lys has been associated with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and myoclonic epilepsy with ragged-red fibers (MERRF), respectively (19). In MELAS, lack of the taurine modification at the wobble position of $tRNA^{Leu}$ harboring either the A3243G or the T3271C mutation leads to severely reduced translation of UUG (but not UUA) codons (20). In MERRF, both s^2 and τm^5 modifications are absent in the $tRNA^{Lys}$ bearing the A8344G mutation (21), leading to impaired decoding of both codons for lysine (22). Based on studies in *E. coli* showing the pivotal role of 2-thiolation at the wobble position of $tRNA^{Lys}$ in efficient decoding of lysine codons (8), it has been suggested that the lack of the s^2 modification in the mutant A8344G $tRNA^{Lys}$ might be central to the decoding deficiency in MERRF. However, this has not been tested directly. One study performed in human cells showed that HeLa cells in which MTU1 was knocked down display reduced 2-thiolation of the mitochondrial $tRNA^{Lys}$, decreased oxygen consumption and defective membrane potential (14).

In the present study, we knocked down MTU1 in different human cell types and assessed the effect on 2-thiolation of the three s^2 -modified mitochondrial tRNAs and on mitochondrial translation in these cells and in fibroblasts from a patient carrying a pathogenic mutation in the *MTU1* gene.

RESULTS

MTU1 is not essential for mitochondrial translation in HEK 293 cells

To investigate directly the involvement of human MTU1 in mitochondrial protein synthesis, we knocked down MTU1 in HEK 293 cells using three different siRNA constructs, and then pulse-labeled the mitochondrial translation products in these cells with ^{35}[S]-(methionine/cysteine). All siRNA constructs tested were similarly efficient in knocking down MTU1 below the detectable levels, as shown by immunoblot analysis with a specific anti-human MTU1 antibody (Fig. 1B). Surprisingly, this had no measureable effect on mitochondrial translation, which was similar in control and MTU1-knocked down cells (Fig. 1A).

Next, we assessed the steady-state levels and the extent of 2-thiouridylation of the mitochondrial tRNAs for Lys, Glu and Gln in the cells in which MTU1 had been knocked down, using $tRNA^{Trp}$ for normalization of the signal. To this end, we performed [(*N*-acryloylamino)phenyl]mercuric chloride (APM)-northern blot analysis, in which the organomercuric compound APM (23) is polymerized in the gel, resulting in specific retardation of thio-modified tRNAs through its binding to the sulfur in the tRNA. While the tRNAs for Lys, Glu and Gln are almost completely modified (>90%) in HEK 293 cells, knocking down MTU1 resulted in a severe reduction in the 2-thiouridylation of all three mitochondrial tRNAs, with residual levels of <10% of total for $tRNA^{Lys}$, 20–25% of total for $tRNA^{Glu}$ and 30–40% for $tRNA^{Gln}$ (Figs 2B and 3B). As confirmed by analysis of gels from which APM was excluded, the steady-state levels of $tRNA^{Gln}$ were normal, while those of $tRNA^{Lys}$ and $tRNA^{Glu}$ were decreased to 70–80% of control (Figs 2A and 3A). We conclude that MTU1 is dispensable for mitochondrial translation in HEK 293 cells, although it is clearly involved in the synthesis of 2-thiouridine at the wobble position of the mitochondrial tRNAs for Lys, Glu and Gln.

Blue native polyacrylamide gel electrophoresis analysis of oxidative phosphorylation assembly in MTU1-depleted HEK 293 cells

Given the role of MTU1 in sulfur trafficking, we tested whether the human enzyme might be further involved in the biogenesis of the oxidative phosphorylation (OXPHOS) complexes containing iron-sulfur clusters by blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis. On first inspection, the overall assembly of all five OXPHOS complexes appeared to be normal in HEK 293 cells in which MTU1 was knocked down. Longer exposure of the blot

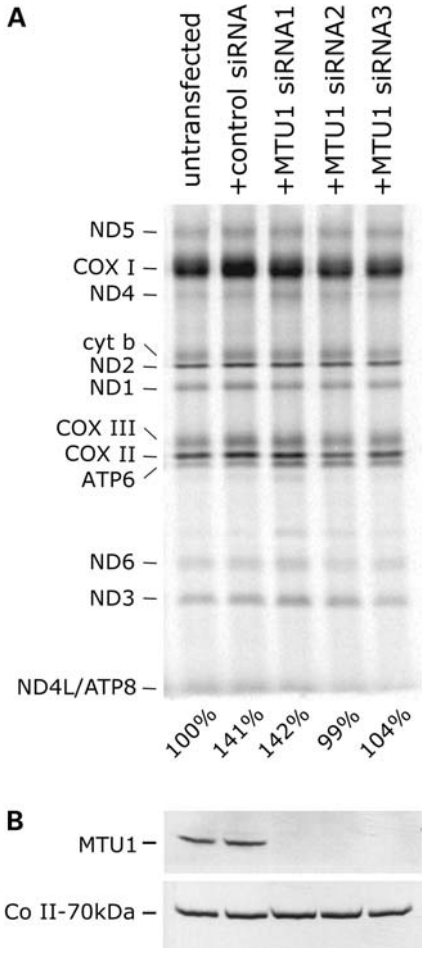

Figure 1. Mitochondrial translation in HEK 293 cells is unaffected by the knockdown of MTU1. (**A**) HEK 293 cells were transiently transfected either with three different siRNA constructs specific to MTU1 (siRNA1–3) or a control siRNA, after which mitochondrial translation products were pulse-labeled with [^{35}S]-(Met/Cys) in the presence of emetine and 50 μg total protein of each sample were then run on a 15–20% polyacrylamide gradient gel. The seven subunits of Complex I (ND), one subunit of Complex III (cyt b), three subunits of Complex IV (COX) and two subunits of Complex V (ATP) are indicated at the left of the gel. Total mitochondrial translation levels expressed as percentages of the levels in untransfected cells (set arbitrarily at 100%) are indicated at the bottom of the gel. (**B**) Immunoblot analysis of MTU1 protein in knockdown cells using the Complex II–70 kDa subunit as a loading control.

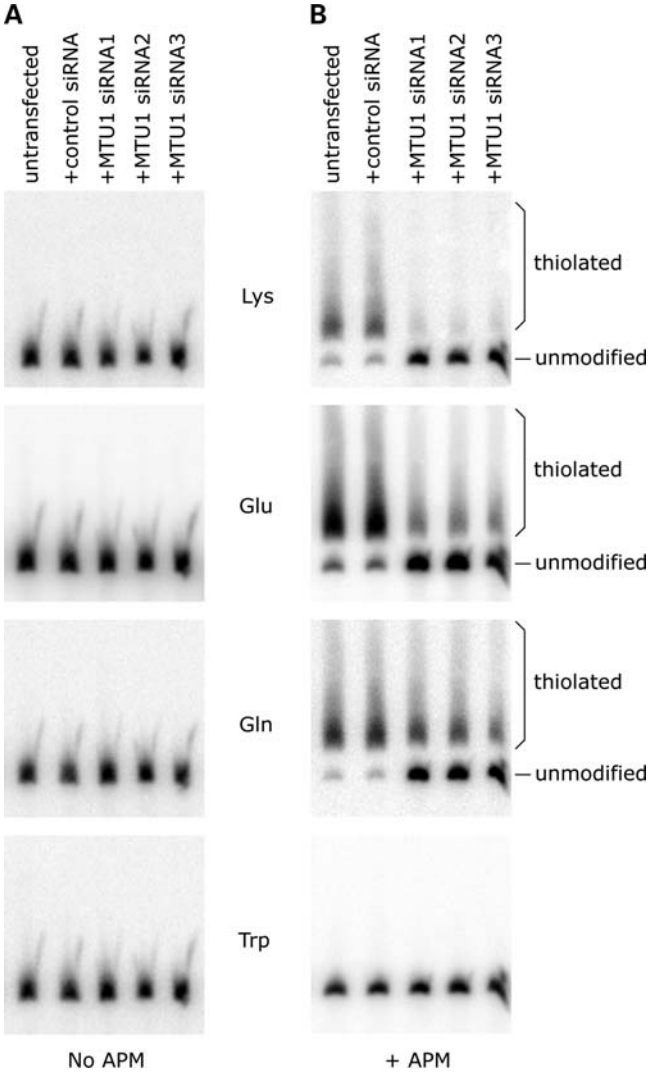

Figure 2. Knockdown of MTU1 in HEK 293 cells impairs 2-thiouridylation of the wobble position in mitochondrial tRNAs for Lys, Glu and Gln. Total RNA was extracted from HEK 293 cells transiently transfected with the siRNA constructs used in Figure 1, and 5 μg total RNA/sample were run on a 10% polyacrylamide gel containing 7 M urea, in the absence (**A**) or the presence (**B**) of 1 μg/ml APM. After transfer to a nitrocellulose membrane, hybridization was performed with oligonucleotide probes complementary to mitochondrial tRNAs for Lys, Glu and Gln, using tRNA^{Trp} as a loading control. The thiolated and unmodified forms are indicated at the right of the figure.

showed a subcomplex of Complex II, an enzyme complex containing two Fe–S clusters (Fig. 4), using an antibody against the 70 kDa subunit. When Complex II was detected with an antibody against its 30 kDa subunit, the specific absence of a different subcomplex was revealed in the MTU1-knocked down cells. This, however, did not result in a reduced amount of fully assembled Complex II.

MTU1 is not essential for mitochondrial translation in human fibroblasts

To test the validity of our findings in other cell types, we analyzed patient fibroblasts in which a compound heterozygous mutation in the *MTU1* gene was described previously (17,24). We confirmed that, of the two mutations described in this case, only the 9 bp allele in-frame insertion is expressed. The other, a 1 bp insertion causing a frameshift and premature stop probably leads to nonsense-mediated mRNA decay. Mitochondrial translation in patient fibroblasts appeared to be normal (Fig. 5A), despite the complete absence of immunodetectable MTU1 protein (Fig. 5B). BN-PAGE analysis revealed normal assembly of the five OXPHOS complexes in patient fibroblasts, as well as the presence of a subcomplex of Complex II of the same appearance and size as that described above in HEK 293 cells (Fig. 5C).

Next, we assessed the steady-state levels and the extent of 2-thiouridylation of the mitochondrial tRNAs for Lys, Glu

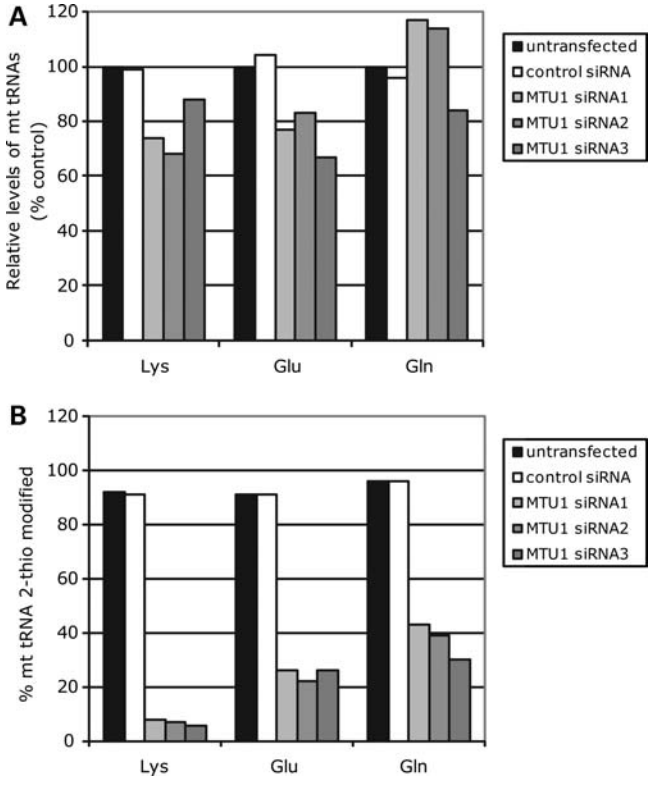

Figure 3. Quantification of the total levels (**A**) and the percentage of thiouridylated (**B**) mitochondrial tRNAs for the experiment in Figure 2. For each sample, the signal corresponding to the amount of tRNA for Lys, Glu or Gln was normalized to the signal corresponding to the amount of $tRNA^{Trp}$. The total levels of each of the three thio-modified tRNAs in the untransfected cells were set arbitrarily to 100% (A). The values in the histogram (A) are averages of two measurements, one corresponding to the signal from the gel without APM, the other, to the total signal (thiolated plus unmodified), from the gel containing APM.

and Gln in the patient fibroblasts, as done in the HEK 293 cells (Fig. 5D). Similar to the observations made in HEK 293 cells, 90% of the tRNAs for Lys, Glu and Gln are 2-thio modified in control fibroblasts, while in patient fibroblasts the modification is either completely ($tRNA^{Lys}$) or almost completely ($tRNA^{Glu}$ and $tRNA^{Gln}$) abolished (Fig. 5D and E). The total levels of these tRNAs are similar in patient and control fibroblasts (Fig. 5E). Thus, a lack of immunodetectable MTU1 protein in patient fibroblasts, which essentially prevents the 2-thio modification, has no effect on mitochondrial protein synthesis.

Mitochondrial translation levels are unchanged following knockdown of MTU1 in myoblasts derived from patients with MELAS and MERRF

We next tested whether knocking down MTU1 in cells derived from patients with an existing defect in mitochondrial protein synthesis would lead to a further decrease in their levels of mitochondrial translation products. For this purpose, we used homoplasmic mutant myoblast clones derived from a MERRF patient with the A8344G mutation and a MELAS patient with the A3243G mutation, both of which are known to be deficient in post-transcriptional modifications of their mitochondrial tRNAs. At the same time, this analysis allowed us to assess the consequences of knocking down MTU1 in additional human cell types and compare them to the effects observed in the HEK 293 cells and fibroblasts.

Unexpectedly, the steady-state levels of MTU1 protein in MERRF and MELAS myoblasts were decreased when compared with wild-type cells on immunoblot analysis (Fig. 6B). A further decrease in the levels of MTU1 could be achieved using siRNA knockdown in mutant (especially MELAS) myoblasts, while in the wild-type cells, the knockdown lowered MTU1 levels to the levels observed in mutant cells (Fig. 6B). Despite this reduction in parental levels of MTU1, mitochondrial translation appeared to be normal in the wild-type myoblasts, and there was no evidence of a further decrease in the levels of mitochondrial translation products synthesized in the mutant cells (Fig. 6A and D). The assembly of all OXPHOS complexes was equally unaffected by the knockdown of MTU1 in wild-type and mutant myoblasts, as shown by BN-PAGE analysis (Fig. 6C). Longer exposure of the blots did not reveal a subcomplex of Complex II, perhaps because of the less-efficient knockdown of MTU1 in myoblasts when compared with HEK 293 cells or the MTU1 patient fibroblasts.

Next, we used APM-northern blotting analysis to assess the 2-thiouridylation status and the levels of mitochondrial tRNAs for Lys, Glu and Gln in wild-type and mutant myoblasts (Figs 7 and 8). Similar to HEK 293 cells and fibroblasts, ~90% of the mitochondrial $tRNA^{Gln}$ is 2-thio modified in myoblasts; however, only 70% of the $tRNA^{Lys}$ and 80% of the $tRNA^{Glu}$ appear to be thiouridylated at the wobble position in myoblasts. As expected, both the total levels and the percentage of thiouridylated $tRNA^{Lys}$ are decreased in the MERRF myoblasts: the total amount in the mutant is between 20 and 50% of the amount in wild-type cells, of which only 30–40% is thio modified.

Knockdown of MTU1 in wild-type and MELAS myoblasts resulted in a decrease in the thiouridylated fraction of $tRNA^{Lys}$ from 70 to 30–40% of total, which is similar to the percentage observed in the MERRF myoblasts (Figs 7 and 8B). The total levels of $tRNA^{Lys}$ were unaffected by the knockdown in the wild-type cells and decreased to 70% of the transfection control in the MELAS myoblasts (Figs 7 and 8A). No further decrease in total levels or the thyouridylated fraction could be observed for $tRNA^{Lys}$ in MERRF myoblasts following knockdown of MTU1. Although the effects of the knockdown on the 2-thio modification of the $tRNA^{Glu}$ and $tRNA^{Gln}$ were more subtle than for $tRNA^{Lys}$, they were, nevertheless, observed consistently in all the cells tested: the 2-thio-modified fraction decreased from 80 to, on average, 60% of total for $tRNA^{Glu}$, and from 90 to, on average, 80% of total for $tRNA^{Gln}$ (Figs 7 and 8B). The total levels of the $tRNA^{Glu}$ and $tRNA^{Gln}$ were, depending on the line tested, either unaffected or slightly decreased by the knockdown of MTU1.

DISCUSSION

In this study, we demonstrate that mitochondrial translation in three different human cell types is unaffected by the lack of

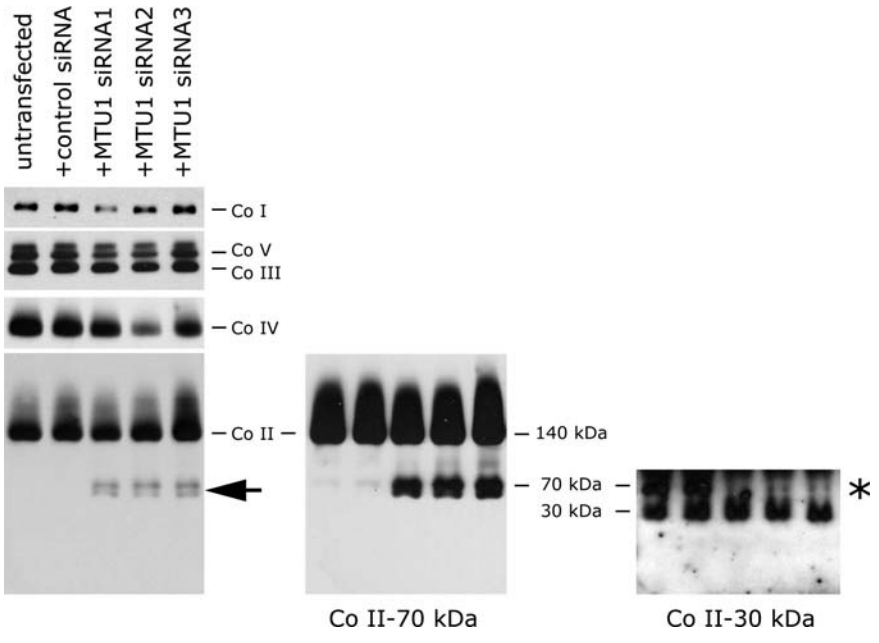

Figure 4. BN-PAGE analysis of the oxidative phosphorylation complexes in HEK 293 cells in which MTU1 was knocked down. Each of the five OXPHOS complexes (Complexes I–V) was visualized with a subunit-specific antibody. The blots in the center and at the right are longer exposures of the blot at the left, probed with antibodies against either the 70 or the 30 kDa subunits of Complex II, as indicated. The arrow points to a subcomplex of Complex II observed only in knockdown cells, while the asterisk indicates the absence of a subcomplex of Complex II from the knockdown cells.

MTU1 protein, despite a resulting defect in the 2-thiouridylation of the wobble position of the mitochondrial tRNAs for Lys, Glu and Gln. In contrast, yeast Δ*MTU1* deletion strains, which also show impaired 2-thiouridylation of the three mitochondrial tRNAs, have a severe defect in mitochondrial protein synthesis (14,16). One likely explanation for this discrepancy is that Δ*MTU1* mutant yeast have an additional, significant reduction in the steady-state levels of the total amount of the three 2-thio-modified mitochondrial tRNAs, with residual levels between 19 and 37% of control (15), whereas the total steady-state tRNA levels (thiolated and unmodified) in human cells are only slightly reduced. This logic supports a role for the 2-thio modification in increasing tRNA-decoding efficiency.

Several studies suggest a central role for post-transcriptional modifications, in particular 2-thiouridylation of tRNAs, in increasing the efficiency of translation. Viability of a yeast strain lacking the mcm^5 and s^2 modifications at the wobble positions of their cytoplasmic tRNAs can be restored by the over-expression of the unmodified $tRNA^{Lys}$ and $tRNA^{Gln}$ (6). Indeed, in this context, if post-transcriptional modifications were important in preventing misreading, over-expression of the unmodified forms of the tRNAs might have been detrimental, and not beneficial to the cell. In another study, deletion of any one of the three genes required for the formation of either the mcm^5 or the s^2 modifications in the *S. cerevisiae* cytoplasmic tRNAs resulted in similar phenotypes, including defects in transcription, chromatin remodeling and exocytosis, all of which could be suppressed by elevated levels of hypomodified $tRNA^{Lys}$ and $tRNA^{Gln}$ (containing either only the s^2 or only the mcm^5 modification, respectively) (25). Another report demonstrated the presence of the $cmnm^5s^2U$ modification at the wobble position of the tRNAs for Leu and Trp and the absence of 2-thiolation at the wobble base of tRNAs for Lys, Glu and Gln in nematode mitochondria (26). The mitochondrial genes of nematodes contain an unusually high percentage of leucine codons, and infrequent codons for Lys, Glu and Gln (27,28), suggesting a role for post-transcriptional modification in increasing the efficiency of tRNAs decoding high usage codons.

Other cellular functions apart from mitochondrial translation might be affected by the knockdown of MTU1. We hypothesized that MTU1 might be involved in the assembly of enzyme complexes containing iron–sulfur clusters. We identified a subcomplex of Complex II of the respiratory chain in HEK 293 cells and in fibroblasts lacking immunodetectable MTU1 protein as a result of either knockdown or the pathogenic mutation. The subcomplex appeared as a doublet at ~70 kDa. Since the detection was performed with an antibody against the 70 kDa subunit of Complex II, the doublet might indicate an early assembly intermediate containing this particular subunit. When Complex II was detected with an antibody against its 30 kDa subunit, an assembly intermediate of ~70 kDa normally found in HEK 293 cells was absent upon knockdown of MTU1. Since the 30 kDa subunit contains the Fe–S clusters of Complex II, it is conceivable that the lack of MTU1 interferes with the addition of the clusters onto this subunit. Fully assembled Complex II (140 kDa) likely results from the assembly of the subcomplexes detected with the antibodies against the 30 kDa and the 70 kDa subunits, as each is ~70 kDa. Thus, in cells in which MTU1 is knocked down, the absence of the subcomplex containing the 30 kDa subunit would result in unassembled subcomplex containing the 70 kDa subunit. Although fully assembled Complex II was present in normal amounts in these cells, the presence of the subcomplex containing the 70 kDa subunit was specifically

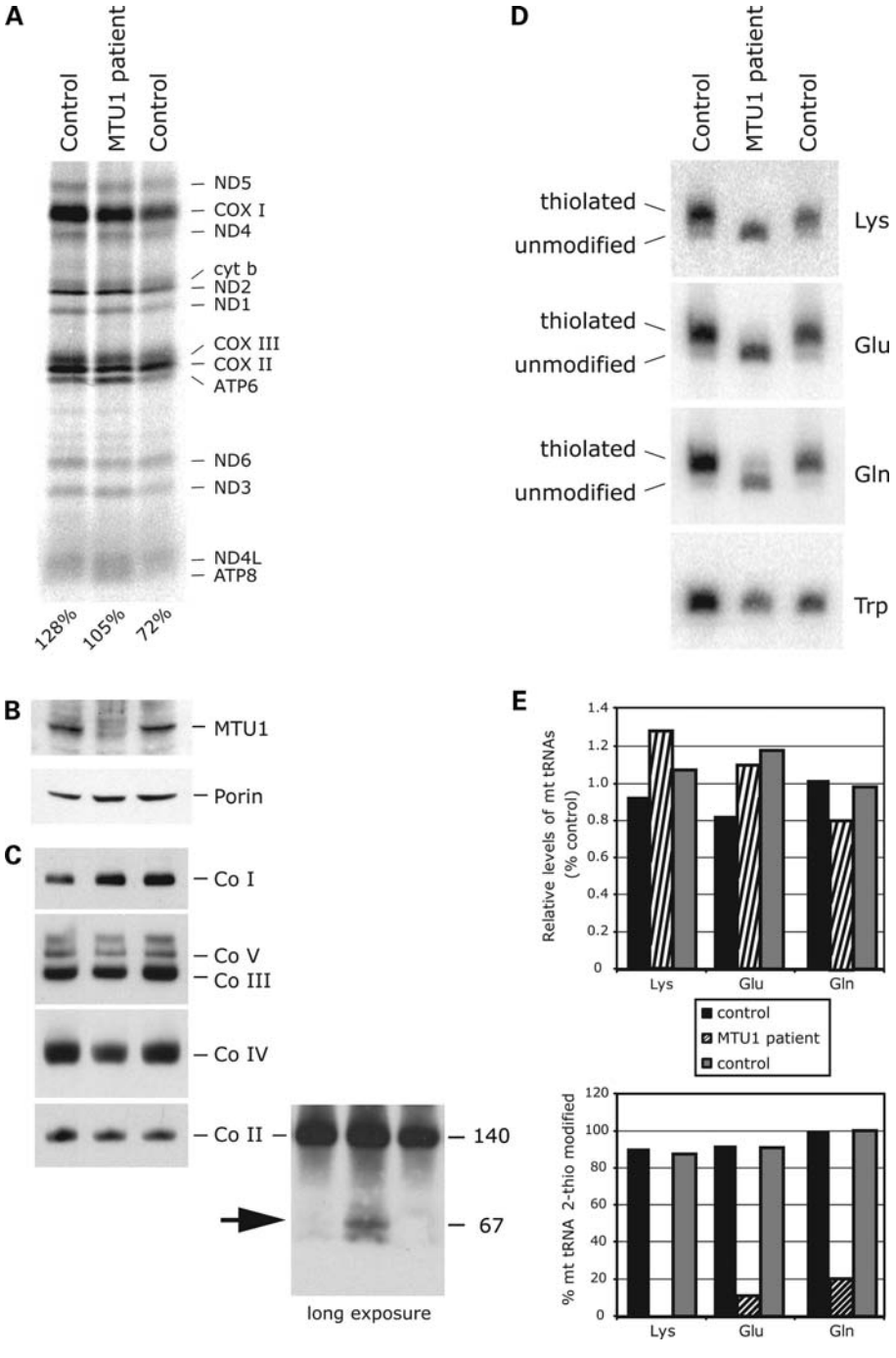

Figure 5. Analysis of mitochondrial translation and OXPHOS assembly in fibroblasts from a MTU1 patient. (**A**) Mitochondrial translation products in fibroblasts from the patient and two controls were pulse-labeled and analyzed as in Figure 1. Total mitochondrial translation levels expressed as percentages of the average of the two controls are indicated at the bottom of the gel. (**B**) The levels of MTU1 protein in fibroblasts from the patient and two controls were visualized by immunoblotting with an antibody raised against MTU1 using porin as a loading control. (**C**) Fibroblasts from the patient and two controls were analyzed by BN-PAGE, and each of the five OXPHOS complexes (Complex I–V) was visualized with a subunit-specific antibody. The arrow indicates the presence of a subcomplex of Complex II in patient fibroblasts, and the blot at the right is a longer exposure. (**D**) APM-northern blotting analysis and (**E**) quantification (single estimates) of the 2-thiouridilation status of the mitochondrial tRNAs.

and consistently observed in patient fibroblasts and with each of the three siRNA constructs in HEK 293 cells, suggesting that MTU1 could play some other role in sulfur trafficking.

Bifunctional enzymes involved in post-transcriptional modifications of tRNAs have already been described; for instance, the *E. coli* methyltransferase MnmC was shown to catalyze two reactions in the same pathway through two distinct domains with independent enzymatic functions (29,30). Another bacterial methyltransferase, the *trmA* gene product, which is involved in the synthesis of 5-methyluridine, was

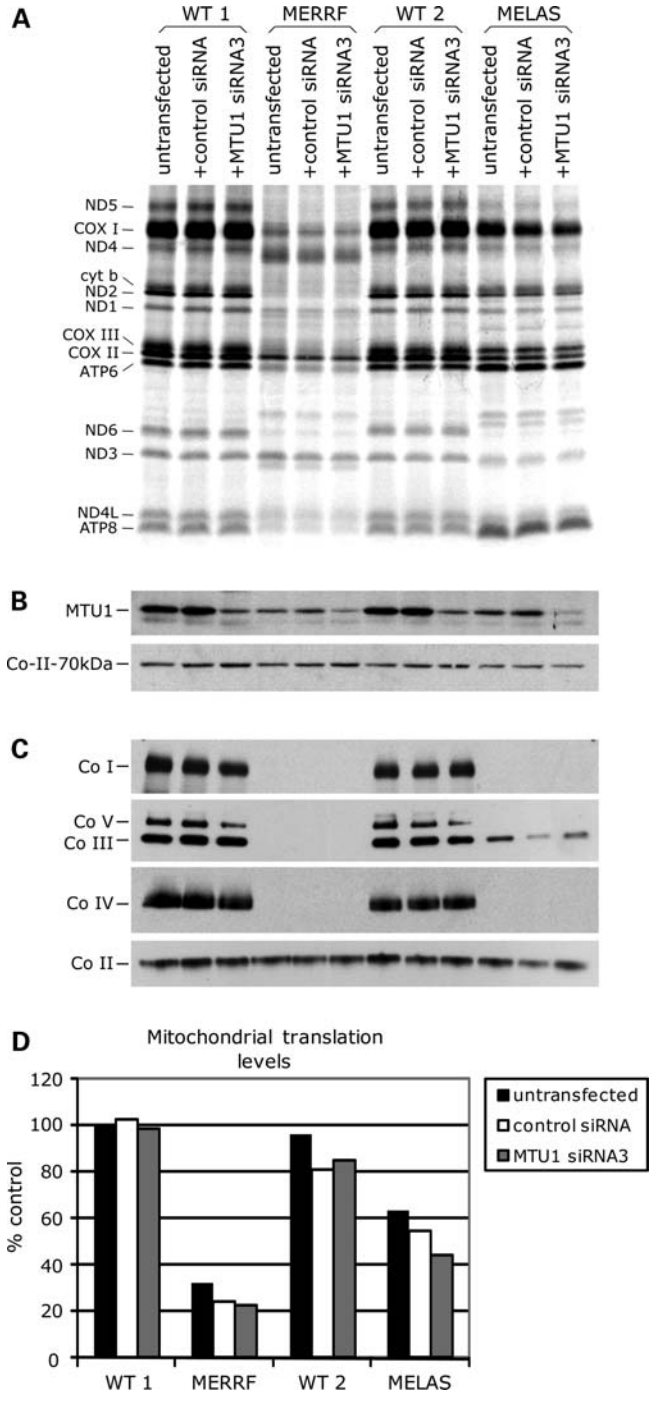

Figure 6. Analysis of wild-type and mutant (MELAS and MERRF) myoblasts following knockdown of MTU1. (**A**) Homoplasmic wild-type (WT 1 and WT 2) and homoplasmic mutant (MELAS and MERRF) myoblasts were transiently transfected with the siRNA constructs, and pulse-labeled to assess mitochondrial protein synthesis as in Figure 1. WT 1 is a homoplasmic wild-type myoblast clone derived from the MERRF patient, whereas WT 2 is a similar clone from the MELAS patient. (**B**) Knockdown of MTU1 protein levels was confirmed through immunoblotting using the 70 kDa subunit of Complex II as a loading control (**C**). BN-PAGE analysis of the experiment in (A). (**D**) Quantification of the total mitochondrial translation levels in wild-type and mutant myoblasts following MTU1 knockdown. The results are expressed as percentages of the total mitochondrial translation levels in the homoplasmic wild type WT 1, which were arbitrarily set at 100%.

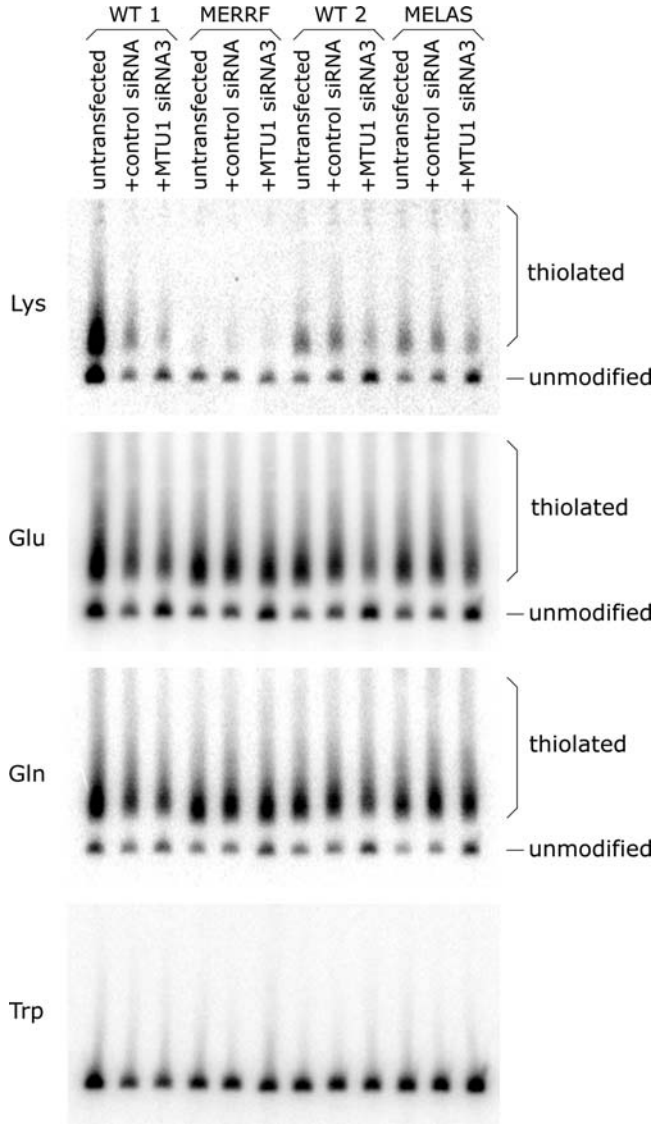

Figure 7. Investigation of the 2-thiouridylation status of the wobble U in mitochondrial tRNAs for Lys, Glu and Gln in wild-type (WT 1 and WT 2) and mutant (MELAS and MERRF) myoblasts in which MTU1 was knocked down. The experiment was done as in Figure 2.

shown to be essential for viability in *E. coli*; however, a strain containing a mutation in this gene is viable despite the lack of the 5-methyluridine modification in its tRNA, suggesting that the enzyme has a second function essential for viability (31). Similarly, deletion of the *truB* gene encoding pseudouridine synthase in *E. coli* results in growth defects that can be rescued by a *truB* gene containing a mutation in the active side of the enzyme (32), again pointing to an additional function of this particular enzyme. In the latter two examples, the authors speculate that this additional function could be that of RNA chaperone, either for RNA components of the ribosome or for tRNAs.

Another way in which cellular functions apart from mitochondrial translation could be affected by the knockdown of MTU1 is if the 2-thiolated mitochondrial tRNAs serve

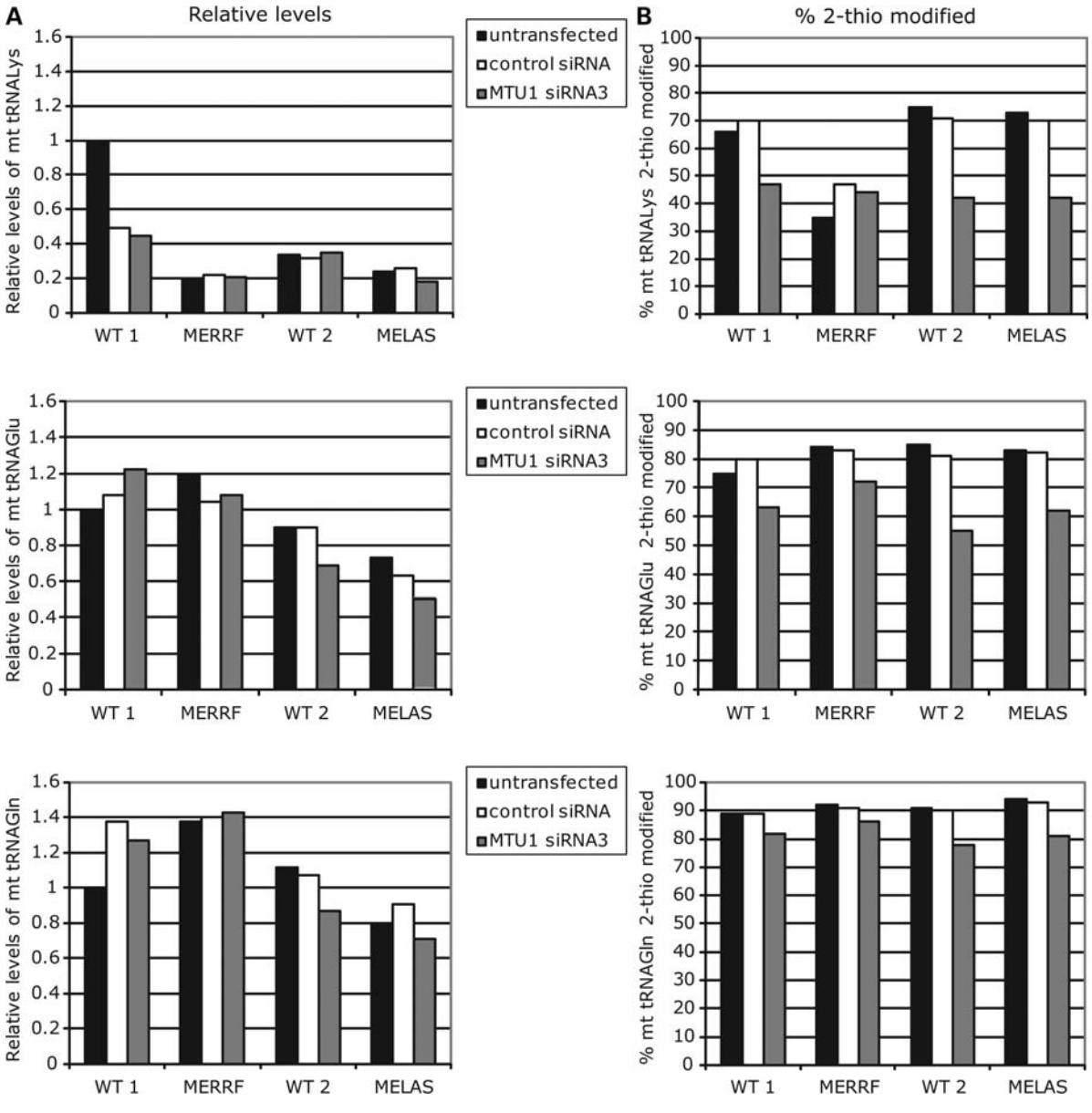

Figure 8. Quantification of the relative, total levels (**A**) and the percentage of thiouridylation (**B**) of the mitochondrial tRNAs for Lys, Glu and Gln in wild-type (WT 1 and WT 2) and mutant (MELAS and MERRF) myoblasts in which MTU1 was knocked down (Fig. 7).

other functions in addition to their role in protein synthesis. A few such examples have, in fact, been described: aminoacyl-tRNAs have been shown to participate in N-terminal protein modification (33) which signals protein degradation (34,35), in porphyrin biosynthesis (36,37), in the aminoacylation of phospholipids in the cell membrane (38) and in the crosslinking of peptidoglycan in the cell walls of Gram-positive bacteria (39). Chloroplast $tRNA^{Glu}$ containing the mnm^5s^2 modification at the wobble position is required in the first step of chlorophyl biosynthesis (40), and it is possible that post-transcriptional modifications of tRNAs are relevant to such processes.

Mutations in *MTU1* were recently shown (17,18) to be associated with acute infantile liver failure with a reversible outcome. In one study (18), the authors demonstrated a reduction in the extent of 2-thiolation of mitochondrial tRNAs for Lys, Glu and Gln to 20–30% of control and modest, nonspecific decreases in the total levels of mitochondrial tRNAs in patient fibroblasts. Symptomatic individuals had multiple OXPHOS enzyme deficiencies in liver and muscle, which were, however, reversible. The limited availability of cysteine, the sulfur donor for MTU1, during the neonatal period, was suggested as a possible pathogenic mechanism. The gradual increase in the enzymes involved in the endogenous synthesis of cysteine over the first months of life would then explain the reversibility of the biochemical phenotype. The present study shows that lack of MTU1 does not impair mitochondrial translation in three different cell types, including myoblasts, but we cannot of course exclude such a defect in the neonatal liver. It seems unlikely that a

defect in Fe–S biosynthesis is responsible for the enzyme deficiencies in the patients as Complex IV, which does not contain an Fe–S center, is affected in the patients. What then could be the mechanism? One possible explanation would be limiting steady-state levels of total mitochondrial tRNAs during early development. If, as we have argued above, the primary role of wobble U thiolation is to increase tRNA-decoding efficiency, a translation defect would only be observed in the absence of MTU1 when the tRNAs that are modified become limiting. This idea could be tested by measuring total tRNA levels in affected tissues from MTU1 patients, or by reducing the amount of charged tRNAs in cell culture models of MTU1 deficiency.

MATERIALS AND METHODS

Cell culture and siRNA transfection

Fibroblasts and myoblasts were immortalized by transduction with a retroviral vector expressing the HPV-16 E6 and E7 genes and with another one expressing the catalytic component of human telomerase (htert) (41). Cells were grown at 37°C and 5% CO_2, either in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (fibroblasts and HEK 293 cells) or, for myoblasts, in supplemented growth medium as described previously (42).

Stealth RNAi duplexes 118697H06–118697H11 (Invitrogen) or the fluorescent oligo control Block-iT™ Alexa Fluor®Red (Invitrogen) were transiently transfected at a final concentration of 10 nM using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's specifications. Transfection was repeated on Day 3, and cells were harvested and analyzed on Day 6.

Anti-MTU1 antibody production, purification and immunoblot analysis

The synthetic peptide CESPSDSPED was conjugated to Imject® Maleimide Activated mcKLH (Pierce) as specified by the manufacturer, and then injected into rabbits. Rabbits were boosted three times at intervals of 3–4 weeks, bled periodically and screened for antibody response by immunoblot analysis. Anti-MTU1 polyclonal antibodies were affinity-purified from serum of immunized animals with the SulfoLink® Kit (Pierce), then used for immunoblotting at a dilution of 1:1000.

For immunoblotting, protein extracts were prepared in 1.5% *n*-dodecyl maltoside/PBS from the following: whole HEK 293 cells, a mitochondria-enriched fraction of fibroblasts prepared by differential centrifugation or digitonized myoblasts [1.2 mg digitonin/mg protein (43)]. Ten to twenty micrograms of protein/sample were used for Tris–glycine SDS–PAGE, then transferred to a nitrocellulose membrane and used for detection of MTU1; of the 70 kDa subunit of Complex II, with a monoclonal antibody from Molecular Probes; or of porin, with a monoclonal antibody from Sigma (St. Louis, MO, USA).

Pulse-labeling of mitochondrial translation products and BN-PAGE

Pulse-labeling of mitochondrial translation products and BN-PAGE were carried out as described in detail elsewhere (44).

APM-northern blotting analysis of the 2-thiolation status of the mitochondrial tRNAs

[(*N*-acryloylamino)phenyl]mercuric chloride (APM)-northern blotting analysis of the 2-thiolation status of the mitochondrial tRNAs was performed essentially as described previously (14). Briefly, 5 μg (HEK 293 cells) or 7 μg (fibroblasts or myoblasts) of total RNA were run on a 10% polyacrylamide gel containing 7 M urea and 1 μg/ml APM (a kind gift from T. Suzuki, University of Tokyo), followed by transfer to Hybond N+ membrane (GE Healthcare). Pre-hybridization and hybridization were carried out in EXPRESSHyb solution (Clontech) according to the manufacturer's instructions. The oligonucleotides used for the generation of the ^{32}P-labeled probes had the following sequences: 5′-tggtattctcgcacggactacaac-3′ for $tRNA^{Glu}$; 5′-tggctaggactatgagaatcgaac-3′ for $tRNA^{Gln}$; 5′-tgg tcactgtaaagaggtgttggt-3′ for $tRNA^{Lys}$ and 5′-tggcagaaa ttaagtattgcaact-3′ for $tRNA^{Trp}$. Quantification of the radioactive signal was performed with the ImageQuant software from Molecular Dynamics/GE Healthcare.

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