Short peptides from leucyl-tRNA synthetase rescue disease-causing mitochondrial tRNA point mutations

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Abstract

Mutations in mitochondrial (mt) genes coding for mt-tRNAs are responsible for a range of syndromes, for which no effective treatment is available. We recently showed that the carboxy-terminal domain (Cterm) of human mt-leucyl tRNA synthetase rescues the pathologic phenotype associated either with the m.3243A>G mutation in mt-tRNALeu(UUR) or with mutations in the mt-tRNAIle, both of which are aminoacylated by Class I mt-aminoacyl-tRNA synthetases (mt-aaRSs). Here we show, by using the human transmitochondrial cybrid model, that the Cterm is also able to improve the phenotype caused by the m.8344A>G mutation in mt-tRNALys, aminoacylated by a Class II aaRS. Importantly, we demonstrate that the same rescuing ability is retained by two Cterm-derived short peptides, β30_31 and β32_33, which are effective towards both the m.8344A>G and the m.3243A>G mutations. Furthermore, we provide in vitro evidence that these peptides bind with high affinity wild-type and mutant human mt-tRNALeu(UUR) and mt-tRNAlys, and stabilize mutant mt-tRNAleu(UUR). In conclusion, we demonstrate that small Cterm-derived peptides can be effective tools to rescue cellular defects caused by mutations in a wide range of mt-tRNAs.

Introduction

Mitochondrial (mt) diseases are multi-system disorders due to mutations in nuclear or mtDNA genes. Among the latter, >50% are located in transfer RNA (tRNA) genes [(1); URL: http://www.mitomap.org] and are responsible for a wide range of clinical phenotypes, such as the severe Mitochondrial Encephalopathy with...
Lactic Acidosis and Stroke like Episodes (MELAS) and Myoclonic Epilepsy with Ragged-Red Fibres (MERRF) syndromes, for which no effective treatments are available at present (2).

Evidence provided by our group and others has shown that overexpression of mt-aminoacyl-tRNA synthetases (mt-aaRSs) attenuates the detrimental effects of point mutations in cognate mt-tRNAs both in human cells (3–6) and in a yeast model (7,8). Our group and others extended these results by showing that class I human mt-leucyl-tRNA synthetase (mt-LeuRS) and mt-valyl-tRNA synthetase (mt-ValRS) are able to rescue defects due to pathogenic mutations in non-cognate, as well as cognate, mt-tRNAs, in both human transmitochondrial cybrid cells (herein named cybrids) (9,10) and yeast (11).

We further demonstrated that the rescuing activity of human mt-LeuRS resides in a relatively small (<70 amino acids) protein region: the carboxy-terminal domain (Cterm) (9,10). Cterm overexpression in human cybrids rescued both relatively mild defects caused by two different homoplasmic mutations in the non-cognate mt-tRNAIle gene (MTTI) and severe biochemical and clinical phenotypes associated with mt disease, leading to a wide spectrum of clinical manifestations. These are partly determined by the level of heteroplasmy and include maternally inherited diabetes and deafness and the severe biochemical and clinical phenotypes ascribed to MELAS (12,13). We showed that the Cterm is both necessary and sufficient for the entire rescuing activity. It is more effective than the full-length enzyme on all mtDNA mutations tested, whereas the activity of a mt-LeuRS variant deleted of the Cterm is negligible or missing (9). We proposed that the Cterm rescuing activity is mediated by a chaperone-like mechanism: the Cterm would directly interact with the mutated mt-tRNAs and stabilize a conformation suitable to establish interactions with cognate aaRS, or other molecular partners required to perform tRNA function. This hypothesis is supported by in vitro experiments demonstrating that the Cterm directly interacts with both the cognate human mt-tRNAIle(UUR), with high affinity and stability, and with the non-cognate human mt-tRNAIle, although with lower affinity (9).

To identify the minimal Cterm regions responsible for the rescue, we designed short peptides comprising the Cterm regions that interact with tRNAIle in the available three-dimensional (3D) structures (14,15). These peptides were named β30_31 (15 aa) and β32_33 (16 aa), since they encompass β-strands 30 and 31, and β-strands 32 and 33, respectively. Both peptides were endowed with even higher rescuing activity than the full Cterm when directed to a large panel of yeast mt-tRNA mutants (14,15).

In the work here presented, we have investigated the rescuing ability of β30_31 and β32_33 peptides in human cybrids bearing either the aforementioned m.3243A>G mutation in MTTI, or the m.3844A>G MERRF mutation in mt-tRNAIle(UUR). Both peptides are able to ameliorate viability and energetic proficiency of human cells carrying either mutation and to interact in vitro with mutant human mt-tRNAIle(UUR) and mt-tRNAIle(UUR). In the case of the ‘cognate’ mt-tRNAIle(UUR) m.3243A>G mutant, both peptides also restore wild-type (WT) levels of thermal stability and structure, which are strongly impaired by the mutations.

These results indicate that β30_31 and β32_33 peptides, derived from the Cterm fragment of mt-LeuRS, are new tools to effectively correct defective phenotypes caused by mutations in mt-tRNA genes. Hence, they represent attractive lead molecules for the development of compounds aimed at therapeutic applications against syndromes associated with pathogenic mt-tRNA mutations.

Results
Pathological phenotype of m.8344A>G MTTK mutant cybrids
First, we investigated the Cterm ability to rescue the pathological phenotype of cybrid cells bearing a mutation in mt-tRNAIle, which is aminoacylated by class II LysRS. To this end, we manipulated m.8344A>G osteosarcoma-derived cybrids (kind gift from Valeria Tiranti) using ethidium bromide (EtBr) to obtain different cell lines bearing either high or low levels of mutated mtDNA (see the Materials and Methods section). The assessment of m.8344A>G mutation load was performed by quantitative pyrosequencing. The selected clones showed 34% (L-8344, low-mutation percentage) and 86% (H-8344, high-mutation percentage) m.8344A>G mutation loads, respectively. To investigate the phenotype of mutant cell lines, cybrids were grown in glucose-free medium supplemented with galactose (galactose medium), a condition forcing cells to rely on the mt respiratory chain for ATP synthesis. In this condition, cell growth of H-8344 cybrids was severely impaired whereas L-8344 cybrids were indistinguishable from WT cells (Supplementary Material, Fig. S1A), providing us with an isogenic control for further experiments. Decreased viability of H-8344 cybrids was associated with an increase in apoptosis and impaired respiratory chain activity, as compared with isogenic L-8344 cybrids (Supplementary Material, Fig. S1A–C). To evaluate the effect of the m.8344A>G mutation on mt-tRNAIle(UUR) steady-state levels we performed high-resolution northern blot analysis. We showed that high-mutation levels are associated with a 50% decrease in mt-tRNAIle(UUR) steady-state levels relative to the amount of SSrRNA (Supplementary Material, Fig. S1D).

The carboxy-terminal domain of human mt-LeuRS is able to rescue the phenotype of m.8344A>G MTTK mutant cybrids
We transiently expressed the Cterm in H-8344 and L-8344 cybrids. This resulted in increased cell viability of H-8344 cybrids after 24 h incubation in galactose medium (up to 1.5-fold as compared to mock transformants) and decreased apoptosis after 6 h (up to 1.6-fold when compared with mock transformants) (Fig. 1A and B). Moreover, Cterm transformants showed a significant increase in oxygen consumption when compared with mock transformants (up to 1.4-fold increase) (Fig. 1C). Conversely, Cterm overexpression did not affect viability of isogenic L-8344 cybrids (Fig. 1A and C). For each experiment, we measured transfection efficiency by quantitative real-time polymerase chain reaction (PCR) (Fig. 1D).

Cterm-derived peptides are endowed with rescuing ability towards both m.3243A>G MTTK1 and m.8344A>G MTTK mutant cybrid phenotypes
The structural basis of the interaction between the Cterm of human mt-LeuRS and the cognate tRNA can be inferred from the analysis of the experimentally determined 3D structures of LeuRS-tRNAIle complexes from the bacteria Thermus thermophilus (14) and Escherichia coli (15) available from the Protein Data Bank [PDB; (18)]. As shown in Figure 2, two pairs of β-strands in the carboxy-terminal domain of bacterial LeuRS (whose human homologues were named β30_31 and β32_33 (16) comprise most of the residues that are involved in contacts with the elbow region of tRNAIle(UUR). Thus, we tested the hypothesis that the short peptides from the human enzyme were responsible for the Cterm ability
to interact with, and have rescuing activity towards, mutations in human cognate mt-tRNALeu(UUR) and non-cognate mt-tRNALys.

The constructs designed for transfection experiments comprise: the mt-targeting sequence (MTS) of human COX8a gene, to ensure mt localization; the nucleotide sequence coding for peptide β30_31, β32_33 or Cterm (as a positive control) and a FLAG domain (for more details see ‘Materials and Methods’ section).

We transiently overexpressed either β30_31 or β32_33 peptide in cybrid lines, which were subsequently stressed by galactose medium incubation. In these conditions, transfected cells showed: (i) increased viability (up to 1.8- and 1.7-fold for m.3243A>G and m.8344A>G cybrids, respectively, when compared with their mock transfomers); and (ii) decreased apoptotic cell death (up to 1.6- and 2-fold for m.3243A>G and m.8344A>G cybrids, respectively, when with to their mock transformers) (Fig. 3A and B). Conversely, peptide overexpression had no effect on the viability of WT and L-8344 cybrids (Supplementary Material, Fig. S3) as well as intense cytoplasmic granular stain with specific anti-FLAG antibodies which was evident in about 50% of transfected cells (Fig. 4A). In addition, mt localization following transient transfection was verified by using a specific anti-FLAG antibody in combination with Mito-tracker Red CMXRos (see Materials and Methods section). As shown in Figure 4B, the peptide-specific immunofluorescence pattern (green) perfectly superimposes the mt network (red).

We also investigated whether transfection with either peptide affected the steady-state levels of mt-tRNA mutants. High-resolution northern blot analysis was performed in WT and m.3243A>G MTTL1 or m.8344A>G MTTK mutant cybrids transiently transfected with peptide β30_31 or β32_33. Values were normalized for 5S rRNA. Consistent with our previous report on m.3243A>G MTTL1 mutant cybrids transfected with the Cterm (9), transient overexpression of either peptide did not result in a significant increase of the mutated mt-tRNALeu(UUR) or mt-tRNAlys steady-state levels (Supplementary Material, Fig. S4), although these changes were at the lower limits of detection for northern experiments.

Figure 1. The carboxy-terminal domain of human mt-LeuRS (Cterm) is able to rescue the phenotype of m.8344A>G MTTK mutant cybrids. (A) Viability of mock (M) and Cterm (pCterm) transformants evaluated after 24 h incubation in galactose medium. The number of viable cells in galactose is normalized to the number of viable cells in glucose at the same time point. (B) Apoptotic cell death of M and pCterm evaluated after 6 h incubation in galactose medium. (C) A respiration rate measured in M and pCterm. (D) Relative expression levels of Cterm in transformant cybrids with respect to 18S gene. Gene expression levels are normalized to the gene expression level of the relative mock. L-8344 and H-8344 low- and high-percentage m.8344A>G mutant cells. Results are the mean ± SEM of at least triplicate transfection experiments. *P < 0.05, **P < 0.01 for H-8344 mock cells versus L-8344 mock cells. °P < 0.05, °°P < 0.01 for transformants versus mock cells (T-test).
Cterm-derived peptides directly interact with both human mt-tRNALeu(UUR) m.3243A>G and mt-tRNALys m.8344A>G mutants with high affinity in vitro

The ability of peptides β30_31 and β32_33 to interact with the WT and mutated tRNAs under investigation was evaluated by surface plasmon resonance (SPR) experiments. These were carried out by immobilizing N-biotinylated peptides onto BioCap sensorchips and adding human mt-tRNALeu(UUR) (WT or m.3243A>G mutant), or human mt-tRNALys (WT or m.8344A>G mutant) as analytes. Figure 5 shows the sensorgrams of each of the eight evaluated interactions (A–D), a logarithmic plot of all experiments with peptides β30_31 (E) and β32_33 (F), and a table with the $K_D$ values of each peptide for each tRNA, obtained from Scatchard analysis of all the experiments (G).

Both peptides interact directly and with high affinity with all four tRNAs, although the strength of the interactions differs substantially. Unsurprisingly, the most efficient interactions are with the cognate human mt-tRNALeu(UUR) forms. Peptide β30_31 shows $K_D = 34$ and 44 µm with WT and m.3243A>G mutant, respectively, while peptide β32_33 interacts with even higher affinity with both forms ($K_D = 1.0$ and 1.6 µm with WT and m.3243A>G mutant). Remarkably, these values are very similar to the affinity of the whole Cterm for mt-tRNALeu(UUR) [i.e. 0.6 µm; (9)], indicating that peptide β32_33 comprises most of the Cterm residues required for cognate mt-tRNA recognition. Interestingly, peptide β32_33 has higher affinity than β30_31 towards non-cognate human mt-tRNA as well (Fig. 5). These results suggest that peptides β30_31 and β32_33 might be able to recognize general tRNA features and, therefore, bind additional non-cognate tRNA molecules.

Cterm-derived peptides compensate structure and thermal stability impairments of both human mt-tRNA mutants in vitro

Thermal denaturation (TD) experiments were carried out to elucidate whether, and to what extent, the interaction with peptides β30_31 and β32_33 reverts the structure and thermal stability alterations that occur in human mt-tRNALeu(UUR) and mt-tRNALys as a consequence of the m.3243A>G and m.8344A>G mutations, respectively. To this end, tRNAs were synthesized as molecular beacons (MB-tRNAs) with chemical groups able to emit and quench fluorescence at the 5′ and 3′ ends, respectively. In all experiments, low fluorescence was observed at low temperature, due to the vicinity of the 6-carboxyfluorescein (FAM) fluorophore and DABCYL quencher (at 5′ and 3′ end, respectively), and high fluorescence upon thermal melting, due to decreased fluorescence quenching caused by tRNA unfolding. Figure 6A–H
shows the results of thermal shift assays (average of three experiments) performed on human MB-mt-tRNA Leu(UUR) WT and m.3243A>G mutant (A–D), and MB-mt-tRNA Lys WT and m.8344A>G mutant (E–H), in the presence of different concentrations of peptide β30_31 or β32_33. Figure 6I reports the values of MB-tRNAs melting temperature (Tm) and percentage of folding at 37% (%Folded37) calculated for each series of experiments. The thermal and structural stability of MB-mt-tRNA Leu(UUR) m.3243A>G mutant are lower than the WT in the absence of peptides (Tm = 48°C for both forms but %Folded37 = 66% for the mutant and 82% for the WT) and increase significantly upon addition of peptide β30_31 and β32_33 up to 2 µM. Consistent with the results of tRNA-binding affinity, peptide β32_33 has the highest stabilization effect (Tm increases by 4°C versus 1°C and %Folded37 increases by 8 versus 5%). As expected, WT MB-mt-tRNA Leu(UUR) undergoes only small changes in thermal and structural stability in the presence of peptide β30_31 up to 3 µM and β32_33 up to 2 µM (see Fig. 6). Intriguingly, the structures of MB-mt-tRNA Lys WT and m.8344A>G mutant are slightly stabilized by peptide β30_31 but not by the addition of peptide β32_33.
Figure 4. The Cterm- and short-peptide derivatives are over-expressed and colocalize to mitochondria. (A) Representative images showing the over-expression of MTS-Cterm-FLAG, MTS-β30_31-FLAG and MTS-β32_33-FLAG constructs in transformant cybrids. Immunofluorescence shows M, pCterm, pβ30_31 and pβ32_33 stained with a specific anti-FLAG antibody. Nuclei are stained with 4',6-diamidino-2-phenylindole (scale bar: 20 µm). (B) Stain of mock, Cterm, β30_31 and β32_33 transformants (M, pCterm, pβ30_31 and pβ32_33) showing colocalization of the antibody against the FLAG and the Mitotracker Red CMXRos. Overlap is shown in yellow. WT cells were examined and imaged with an Olympus IX50 fluorescence microscope (scale bar: 20 µm).
Figure 5. Short peptides derived from the Cterm interact directly and with high affinity with both human mt-\textit{tRNA}\textsubscript{Leu(UUR)} m.3243A>G and mt-\textit{tRNA}\textsubscript{Lys} m.8344A>G mutants in vitro. (A–D) Sensorgrams of the interaction between N-biotinylated \(\beta_{30-31}\) (A and C) or \(\beta_{32-33}\) (B and D) peptide, immobilized on BioCap sensorchips, with human WT (left panels) or mutant (right panels) mt-tRNAs. (A) \textit{mt-tRNA}\textsubscript{Leu(UUR)} (left) and \textit{mt-tRNA}\textsubscript{Leu(UUR)} m.3243A>G mutant (right); tRNA concentrations: 1.5; 3.0; 6.0; 12; 24 and 48 \(\mu\text{M}\). (B) \textit{mt-tRNA}\textsubscript{Leu(UUR)} (left; concentrations = 0.8; 1.5; 3.0; 6.0 and 12 \(\mu\text{M}\)) and \textit{mt-tRNA}\textsubscript{Leu(UUR)} m.3243A>G mutant (right; concentrations: as in (A). (C) \textit{mt-tRNA}\textsubscript{Lys} (left) and \textit{mt-tRNA}\textsubscript{Lys} m.8344A>G mutant (right); tRNA concentrations: 0.75; 1.5; 3.0; 6.0; 12; 24 and 48 \(\mu\text{M}\) at 0–20; 21–40; 41–60; 61–80; 81–100; 101–120 and 121–130 s, respectively. (D) \textit{mt-tRNA}\textsubscript{Lys} (left) and \textit{mt-tRNA}\textsubscript{Lys} m.8344A>G mutant (right); tRNA concentrations: 0.37 \(\mu\text{M}\); 0.75 \(\mu\text{M}\); 1.5 \(\mu\text{M}\); 3.0 \(\mu\text{M}\); 6.0 \(\mu\text{M}\); 12 \(\mu\text{M}\); 24 \(\mu\text{M}\) at the same time points as in (C). (E and F) Logarithmic plot of the interaction of N-biotinylated \(\beta_{30-31}\) (E) and \(\beta_{32-33}\) (F) peptide with human WT \textit{mt-tRNA}\textsubscript{Leu(UUR)} (white circle), \textit{mt-tRNA}\textsubscript{Leu(UUR)} m.3243A>G mutant (black circle), human WT \textit{mt-tRNA}\textsubscript{Lys} (white square) and \textit{mt-tRNA}\textsubscript{Lys} m.8344A>G mutant (black square). The maximal response is plotted versus the logarithm of tRNA concentration. (G) \(K_D\) values of the interactions between peptides and tRNAs.
All TD experiments were highly reproducible. Melting curves measured in the same experimental conditions from different samples are entirely superimposable, with standard deviations for $T_m$ values below 1°C for each measurement.

Supplementary Material, Figure S5 section shows gel electrophoresis of 1 µM mt-tRNALeu(UUR) m.3243A>G samples incubated in 20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM MgCl$_2$ and 0.1 mM spermine (Spm) for 15′ in the presence of 1 mM Mg$^{2+}$, 0.1 mM spermine (Spm) and different concentrations of β30_31 or β32_33 peptides. The interaction with 10 µM peptides decreases by ∼7–10% the amount of mt-tRNALeu(UUR) m.3243A>G mutant in the dimer form (upper band), first reported by Wittenhagen and Kelley [19], whereas in the same conditions WT mt-tRNALeu(UUR) is fully monomeric.

**Discussion**

In the present study, we expand recent results previously reported by our group and others about the ability of human mt-LeuRS Cterm to interact with both cognate and non-cognate mt-tRNAs, and rescue pathological phenotypes arising from mt-tRNA mutations in human cells [9,10]. While the Cterm had been shown to interact with a number of non-cognate mt-tRNAs aminoacylated by both class I and II aminoacyl-tRNA synthetases (aaRSs) [10], its rescuing ability had been demonstrated only towards mt-tRNAs aminoacylated by aaRSs belonging, like LeuRS, to class Ia [9]. Since aaRSs belonging to the same class and subclass have higher structure and sequence similarity to one another than to other aaRSs, whether the therapeutic properties of the Cterm could be extended to mutations in mt-tRNAs aminoacylated by aaRSs other than those in class Ia remained an open question.

Here, we show that, remarkably, the Cterm is able to ameliorate the phenotype of cybrid cells carrying the m.8344A>G mutation in mt-tRNALys, which has the following features: (i) the cognate LysRS belongs to class II and thus is not evolutionarily related to class I enzymes; (ii) it is one of the most prevalent among disease-causing mt-tRNA mutations; (iii) it is responsible for a severe syndrome (MERRF). Together with recent observations in yeast [16,17], these results strongly support the hypothesis that the Cterm may rescue defects due to mutations in a number of additional mt-tRNAs aminoacylated by aaRSs belonging to both classes. This makes the Cterm a promising candidate as ‘universal suppressor’ for mt-tRNA mutation-associated diseases.

Importantly, we demonstrate here that not only LeuRS C-terminal domain but also two short peptides derived from it, β30_31 and β32_33, are endowed with rescuing ability towards two mutations: m.3243A>G in mt-tRNA$^{Leu(UUR)}$ and m.8344A>G.
in mt-tRNA<sup>Leu(UUR)</sup>. The ability of both peptides to rescue pathological phenotypes due to mutations in a number of mt-tRNAs, which are aminoacylated by aaRSs belonging to different classes and subclasses, has been recently suggested in yeast (17). Our results on the activities of peptides β<sub>30_31</sub> and β<sub>32_33</sub>, together with the overall LeuRS sequence conservation from eukaryote mitochondria to bacterial cells indicate that, in spite of the evolutionary distance and different cellular compartment, the modality of tRNA recognition and regions involved in tRNA binding are largely conserved.

The overall rescuing activity of peptides β<sub>30_31</sub> and β<sub>32_33</sub> is manifested by increased cell viability and decreased apoptotic cell death, as is the case with the Cterm (9). Intriguingly, while peptide β<sub>32_33</sub> restores oxygen consumption of both mutant cybrids, upon transfection with peptide β<sub>30_31</sub> this effect is observed only in m.8344A>G cybrids, suggesting a different effect of this peptide on the respiratory chain function affected by either mutation.

Despite the significant increase in cell viability, we did not detect an increase of either mutant mt-tRNA<sub>Leu(UUR)</sub> or mt-tRNA<sup>Leu(UUR)</sup> steady-state levels upon transient overexpression of either peptide β<sub>30_31</sub> or β<sub>32_33</sub>. These results are in line with our previous observations on m.3243A>G cybrids transiently transfected with the Cterm (9) and suggest that, in agreement with our hypothesis of a chaperonic mechanism of action, the effect of our molecules is mainly to improve the function of the mutant mt-tRNAs, rather than preventing them from degradation. Although we cannot exclude that the effect of β<sub>32_33</sub> peptide on mt-tRNA<sup>Leu(UUR)</sup> stability depends on peptide binding to regions different from those recognized in ‘cognate’ mt-tRNA<sub>Leu(UUR)</sub>, the ability of the β<sub>32_33</sub> peptide to rescue defects due to mutations in a large panel of mt-tRNA mutants previously demonstrated in yeast (17) supports the hypothesis that common structural features of tRNA molecules are at least partially involved in the interaction.

In conclusion, we provide evidence that small peptides derived from the mt-LeuRS Cterm can be effective tools to rescue cellular defects caused by mutations in mt-tRNAs. As a consequence, these peptides represent an important advance in the quest for therapeutic agents against diseases caused by mutations in mt-tRNAs. The whole Cterm is amenable to gene therapy-based approaches [see for example (22,23)], while small peptides could be more directly administered, possibly aided by suitable carrier molecules and/or mitochondria-targeting agents. Additionally, small peptides may serve as a basis for the development of non-peptide synthetic compounds, particularly if the rescuing activity can be mapped to even smaller peptide regions.

Strikingly, the ability of the peptide to rescue defects due to mutations in ‘non-cognate’ in addition to ‘cognate’ mt-tRNAs suggests that they may be used to develop wide-spectrum therapeutics towards mt-tRNA mutation-associated diseases.

### Materials and Methods

#### Cybrid cell lines

For this study, we used previously established osteosarcoma-derived (143B.TK) cybrid cell lines from patients (24) and controls (25); generous gift from Dr Valeria Tiranti and Dr Valerio Carelli [Supplementary Material, Table S1]. The m.8344A>G cybrids were established in Valeria Tiranti’s laboratory as previously described (26). We then manipulated the ratio of mutant and WT mt-DNA of the m.8344A>G cybrids using EtBr. Cybrid cells were maintained in glucose medium supplemented with 20 ng/ml of EtBr for 10 days as previously described (27) and replated to repopulate mitochondria at low density in glucose medium. Individual cell clones (~50) were isolated 10–20 days later using glass cylinders and tested for the mutation load. For the present study, we selected two clones, showing high (86%) and low (34%) levels of m.8344A>G mutation, respectively.

#### Assessment of m.8344 A>G mutation load

Accurate m.8344A>G heteroplasmy levels were obtained by quantitative pyrosequencing using a Pyromark Q24 platform. The PyroMark assay design software v2.0 (Qiagen, Hilden, Germany) and the current mtDNA reference sequence (Genbank accession number: NC_012920.1) were used to design mutation-specific pyrosequencing primer trios [5’ biotinylated forward: m. 8240-8264; reverse: m. 8364-8387 and reverse pyrosequencing primer: m.8348-8367 (IDT, Coralville, IA, USA)]. The allele quantification application of the proprietary Q24 software was used to calculate mtDNA heteroplasmy levels using standard parameters.
Cell culture
Cybrid cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 4.5 g/l d-glucose, 10% fetal bovine serum (FBS), 2 mm l-glutamine, 50 µg/ml uridine, 100 U/ml penicillin and 100 mg/ml streptomycin (referred to as glucose vine serum (FBS), 2 m
medium (DMEM), supplemented with 4.5 g/l d-glucose, 10% fetal bo-

tional region of human mt-LeuRS (named pCterm) or the short

cybrids did not change upon transfec-

Bioinformatics analysis
Structure visualization and analysis were performed using PyMol (http://www.pymol.org/). Cterm residues interacting with tRNA^Luu in experimentally determined 3D structures (14,15) were calculated using the FACE2FACE server developed by the authors (http://apps.ibpm.cnr.it/f2f/index). The multiple sequence alignment shown in Figure 2C was obtained by merging the alignments of human and yeast mt-LeuRS to LeuRS from T. thermophilus and E. coli obtained using Phyre2 (28).

Plasmid construction
Cybrid cells were transiently transfected with either the C-ter-
terminal region of human mt-LeuRS (named pCterm) or the short peptides derived from it (named p30_31 and p32_33) cloned into pcDNA3.2-TOPO vector (Invitrogen, Harbor, ME, USA) and 46 bps.

Transient and stable transfections
Cybrids were plated in 60 mm dishes, incubated in glucose me-
dium for 24 h, and then transfected with 2.5 µg of the full or empty vector (mock) by using LIPOFECTINE 3000 (Life Technolo-
gies Italia), according to the manufacturer’s protocol. Stable clones were selected and maintained in glucose medium supplemented with G418 (300 µg/ml). Prior to testing the effect of our constructs, we verified that the mutation load of m.3243A>G and m.8344A>G mutant cybrids did not change upon transfection (Supplementary Material, Fig. S6). To assess the relative expression of the target gene was evalu-

Evaluation of transient transfection efficiency by real-time PCR
About 24 h after transfection total RNA was isolated, measured and reverse-transcribed to cDNA as previously described (9). To assess the relative expression of MTS-Cterm-FLAG we used Taq-

Evaluation of constructs expression levels by immunofluorescence
About 24 h after transfection cells were fixed with 4% formalde-
hyde freshly prepared from paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) with 0.1% Triton X-100. Mouse monoclonal anti-FLAG (Sigma, Saint Louis, MO, USA) was used as primary antibody and visualized using secondary fluorescein isothiocyanate-conjugated antibody (Jackson Laboratories, Bar Harbor, ME, USA). Cells were examined and imaged with an Olympus IX50 Fluorescence microscope.

Mt localization of constructs
About 24 h after transfection cells were incubated with 100 nm Mitotracker red CMXRos (Life Technologies Italia) for 20 min at 37°C and then fixed with 4% formaldehyde freshly prepared from paraformaldehyde in PBS (pH 7.4) for 15 min at 37°C. Subsequently cells were incubated with mouse monoclonal anti-FLAG (Sigma) and visualized by mean of secondary Alexa fluor goat anti-mouse 488 (Life Technologies Italia).

Cell proliferation assay
To test growth capability, transformed cells were maintained in glucose medium for 24 h and then harvested and seeded at 30 × 10^4 in 60 mm dishes in glucose or galactose. Cell viability was measured by the Trypan blue dye exclusion assay. Cells were harvested after 24 h with 0.25% trypsin and 0.2% ethylene-diaminetetraacetic acid, washed, suspended in PBS in the pres-

Rate of apoptosis by flow cytometer
To test the rate of apoptosis transformed cells were maintained in glucose medium for 24 h and then harvested and seeded at 0.5–1 × 10^6 in glucose or galactose for 6–24 h. Cells were washed and harvested in binding buffer 1× (BD Biosciences, Franklin Lakes, NJ, USA) by scraper to minimize potentially high annexin V background levels in adherent cells. Transfected cells were
stained with APC-conjugated annexin V (BD Biosciences) and analysed on a FACS-Calibur flow cytometer (BD Biosciences) (32).

Respirometry assay

Oxygen consumption rate (OCR) of transfected cybrids was evaluated with Clark type oxygen electrode (Hansatech Instruments, Norfolk UK). After transfection control and mutant cybrids were maintained in glucose medium for 48 h, then OCR was measured in intact cells (3 x 10^6) in 1 ml DMEM lacking glucose supplemented with 10% sodium pyruvate, as previously described (33).

High-resolution northern

To determine mt-tRNA^{Leu(UUR)} WT and mt-tRNA^{Leu(UUR)} m.8344A>G steady-state levels cybrids were maintained in glucose medium for 12 h and then collected for RNA extraction. Total RNA was obtained using Trizol reagent (Life Technologies, Paisley, UK). RNA (2 µg) was electrophoresed through 15% denaturing polyacrylamide gel, electoblotted onto Hybond N+ membrane (GE Healthcare Life Sciences, Little Chalfont, UK) in 0.25× TBE and immobilized by ultraviolet cross-linking. Hybridization with radiolabelled probes was performed as previously described (34). Primers for generating the mt-tRNA^{Leu(UUR)} and mt-tRNA^{Leu(UUR)} probes were L3200 (positions 3200–3219; GenBank accession number: NR_023379.1); primers for generating the 5S probe were F1 (positions 8390–8407) and L3200 (positions 8371–8380), respectively. Steady-state mt-tRNA levels were normalized to that of 5S rRNA (RN5S17; GenBank accession number: NR_023379.1); primers for generating the 5S probe were F1 (positions 1–23) and R121 (positions 121–101).

SPR experiments

SPR experiments were carried out with a SensiQ Pioneer apparatus. N-biotinylated p30_31 and p32_33 peptides (see Fig. 2) were synthesized by Biomatik (purity > 90%). Streptavidin-coated BioCap sensorchips were chemically activated by a 35 µl injection of N-biotinylated β30_31 and (i) 0.37; (ii) 0.75; (iii) 1.5; (iv) 3.0; (v) 6.0; (vi) 12 µM; (vii) 24 µM with peptide p30_31 and (i) 0.37; (ii) 0.75; (iii) 1.5; (iv) 3.0; (v) 6.0; (vi) 12 µM; (vii) 24 µM with peptide p32_33. The increase in RU relative to baseline indicates complex formation between the immobilized N-biotinylated peptide and tRNA. The plateau region represents the steady-state phase of the interaction. The decrease in RU (after 100 s for WT and mutant mt-tRNA^{Leu(UUR)} and 130 s for WT and mutant mt-tRNA^{Lys}) indicates tRNA dissociation from the immobilized peptide after buffer injection. A response change of 1000 RU typically corresponds to 1 ng/mm^2 change in analyte concentration on the sensor chip.

As a negative control, sensor chips were treated as described above in the absence of immobilized N-biotinylated peptides. Values of the plateau signal at steady-state (R_neq) were calculated from kinetic evaluation of the sensorgrams using the Qdat 4.0 programme. The equilibrium constant of ligand–analyte interaction was evaluated by Scatchard analysis of the dependence of R_neq on analyte concentration.

TD experiments on tRNA-MBs

Samples of human WT mt-tRNA^{Leu(UUR)} WT, mt-tRNA^{Leu(UUR)} m.3243A>G, WT mt-tRNA^{Lys} and mt-tRNA^{Lys} m.8344A>G were synthesized by Trilink Biotechnologies. All tRNAs used for TD experiments were synthesized as MB-tRNAs by using a 3′-DABCYL cation was added support for tRNA synthesis, and 5′ FAM modification was added using phosphoramidite chemistry. Dual polyacrylamide gel electrophoresis/high-pressure (or high-performance) liquid chromatography purification was performed for all samples, followed by a desalting step and subsequent lyophilization of the final compound. For all tested samples, purity was >95%. p30_31 and p32_33 peptides were synthesized by Biomatik (purity > 90%).

To determine mt-tRNA^{Leu(UUR)} WT and mt-tRNA^{Leu(UUR)} m.3243A>G, WT mt-tRNA^{Lys} and mt-tRNA^{Lys} m.8344A>G were synthesized as MB-tRNAs by using a 3′-DABCYL cation was added to support for tRNA synthesis, and 5′ FAM modification was added using phosphoramidite chemistry. Dual polyacrylamide gel electrophoresis/high-pressure (or high-performance) liquid chromatography purification was performed for all samples, followed by a desalting step and subsequent lyophilization of the final compound. For all tested samples, purity was >95%. p30_31 and p32_33 peptides were synthesized by Biomatik (purity > 90%).

Samples of human WT mt-tRNA^{Leu(UUR)} WT, mt-tRNA^{Leu(UUR)} m.3243A>G, WT mt-tRNA^{Lys} and mt-tRNA^{Lys} m.8344A>G were synthesized by Trilink Biotechnologies. All tRNAs used for TD experiments were synthesized as MB-tRNAs by using a 3′-DABCYL cation was added support for tRNA synthesis, and 5′ FAM modification was added using phosphoramidite chemistry. Dual polyacrylamide gel electrophoresis/high-pressure (or high-performance) liquid chromatography purification was performed for all samples, followed by a desalting step and subsequent lyophilization of the final compound. For all tested samples, purity was >95%. p30_31 and p32_33 peptides were synthesized by Biomatik (purity > 90%).

To determine mt-tRNA^{Leu(UUR)} WT and mt-tRNA^{Leu(UUR)} m.3243A>G, WT mt-tRNA^{Lys} and mt-tRNA^{Lys} m.8344A>G were synthesized by Trilink Biotechnologies. All tRNAs used for TD experiments were synthesized as MB-tRNAs by using a 3′-DABCYL cation was added support for tRNA synthesis, and 5′ FAM modification was added using phosphoramidite chemistry. Dual polyacrylamide gel electrophoresis/high-pressure (or high-performance) liquid chromatography purification was performed for all samples, followed by a desalting step and subsequent lyophilization of the final compound. For all tested samples, purity was >95%. p30_31 and p32_33 peptides were synthesized by Biomatik (purity > 90%).

TD experiments were performed with a MX3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA) in PCR-96-microplates, using 25 µl sample volume, 0.14 µM MB-tRNAs in 20 µM HEPES pH 7.4 + 150 mM NaCl + 1 mM MgCl2 + 0.1 mM spermine (Spm), and different concentrations of p30_31 or p32_33 peptide. At least two experiments were performed for each condition, and triplicate samples were used within the same experiment. Sample temperature was increased from 25 to 90°C at 0.5°C/min rate. Fluorescence was measured at each temperature step (0.5°C) using FAM filter set (excitation: 492 nm, emission: 516 nm). As negative controls we used sample wells with 20 µM HEPES pH 7.4 + 150 mM NaCl + 1 mM MgCl2 + 0.1 mM spermine alone or in the presence of either MB-tRNA or different concentration of p30_31 or p32_33 peptide.

Normalized fluorescence values of the traces [relative fluorescence (F)] were obtained by dividing the fluorescence intensity of each sample by the maximum fluorescence of the same trace. For each temperature (T), the differential fluorescence (df) value of each trace was obtained as follows: df = F(T) – F(37°C) – F(37°C) = F_min[1 – F_min], where F_min is the minimum value of F for the group of experiments.
and 0.1 mm Spm for 15’ in the presence of different amounts of β30_31 or β32_33 peptides was carried out in 12% native electrophoresis gel, and stained using SYBR Green. Dimerization percentage in each lane was calculated using the Fiji suite.

Statistical analysis
All data are expressed as mean ± SEM. Data were analysed by standard paired t-test procedures. The significance was considered at P < 0.05. Numerical estimates were obtained with the Graphpad InStat 3 version (Graphpad, Inc., San Diego, CA, USA).

Supplementary Material
Supplementary Material is available at HMG online.

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