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Combination of the loss of $\text{cmnm}^5\text{U}_{34}$ with the lack of s^2U_{34} modifications of tRNA^{Lys} , tRNA^{Glu} and tRNA^{Gln} altered mitochondrial biogenesis and respiration

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Abstract

Yeast *Saccharomyces cerevisiae* *MTO2*, *MTO1* and *MSS1* genes encoded highly conserved tRNA modifying enzymes for the biosynthesis of $\text{cmnm}^5\text{s}^2\text{U}_{34}$ in mitochondrial tRNA^{Lys} , tRNA^{Glu} and tRNA^{Gln} . In fact, *Mto1p* and *Mss1p* are involved in the biosynthesis of the cmnm^5 group ($\text{cmnm}^5\text{U}_{34}$), while *Mto2p* is responsible for the 2-thiouridylation (s^2U_{34}) of these tRNAs. Previous studies showed that partial modifications at U_{34} in mitochondrial tRNA enabled *mto1*, *mto2* and *mss1* strains to respire. In this report, we investigated the functional interaction between *MTO2*, *MTO1* and *MSS1* genes by using the *mto2*, *mto1* and *mss1* single, double and triple mutants. Strikingly, the deletion of *MTO2* was synthetically lethal with a mutation of *MSS1* or deletion of *MTO1* on medium containing glycerol, but not on medium containing glucose. Interestingly, there were no detectable levels of 9 tRNAs including tRNA^{Lys} , tRNA^{Glu} and tRNA^{Gln} in *mto2/mss1*, *mto2/mto1* and *mto2/mto1/mss1* strains. Furthermore, *mto2/mss1*, *mto2/mto1* and *mto2/mto1/mss1* mutants exhibited extremely low levels of *COX1* and *CYTb* mRNA, 15S and 21S rRNA as well as the complete loss of mitochondrial protein synthesis. The synthetic enhancement combinations likely resulted from the completely abolished modification at U_{34} of tRNA^{Lys} , tRNA^{Glu} and tRNA^{Gln} , caused by the combination of eliminating the 2-thiouridylation by the *mto2* mutation with the absence of the $\text{cmnm}^5\text{U}_{34}$ by the *mto1* or *mss1* mutation. The complete loss of modifications at U_{34} of tRNAs altered mitochondrial RNA metabolisms, causing a degradation of mitochondrial tRNA, mRNA and rRNAs. As a result, failures in mitochondrial RNA metabolisms were responsible for the complete loss of mitochondrial translation. Consequently, defects in mitochondrial protein synthesis caused the instability of their mitochondrial genomes, thus producing the respiratory deficient phenotypes. Therefore, our findings demonstrated a critical role of modifications at U_{34} of tRNA^{Lys} , tRNA^{Glu} and tRNA^{Gln} in maintenance of mitochondrial genome, mitochondrial RNA stability, translation and respiratory function.

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Keywords

Mitochondrial tRNA; nucleotide modification; respiration; biogenesis; metabolism

INTRODUCTION

All tRNA species from living organisms contain modified nucleotides, which are derivatives of the four normal nucleotides adenosine (A), guanosine (G), uridine (U) and cytidine (C).¹ To date, more than 80 different modifications have been identified in tRNA from various organisms.³ Of these, the nucleotide at the position 34 (wobble position of anticodon) of tRNA is more prone to modification than those at other positions of tRNA.⁴ In *Escherichia coli*, the uridine at position 34 of tRNA is always modified, and the modification is either xo⁵U-type (derivatives of 5-hydroxyuridine) or xm⁵(s²)U(m)-type (derivatives of 5-methyluridine, and 5-methyl-aminomethyl-2-thio-uridine).⁴ In particular, the nucleoside 5-methyl-aminomethyl-2-thio-uridine (mnm⁵s²U34) occurs at the wobble position 34 of bacterial tRNA^{Glu}, tRNA^{Lys} and tRNA^{Gln}.^{4,5,6} The synthesis of mnm⁵s²U34 is a multiple step process catalyzed by an enzyme complex.^{7,8} Of these, MnmE (homolog of Mss1/Gtpbp3),^{8,9} GidA (homolog of Mto1)^{7,10} and MnmA/TrmU (homolog of Mto2)^{11,12} are components of the enzyme complex responsible for the biosynthesis of mnm⁵s²U34. In fact, MnmE and GidA assemble in a functional complex involved in the formation of carboxymethylaminomethyl (cmnm) group at position 5 of U₃₄ of several tRNAs including tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}.^{7,8,13,14,15} while MnmA, together with other proteins, catalyzes the thiolation at position 2 of U₃₄ of tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}.^{11,16,17} This modified nucleotide plays a pivotal role in the structure and function of tRNAs including the stabilization of anticodon structure, the ribosome binding ability to tRNA and the improvement of reading frame maintenance.^{1,2,3,4}

In the yeast *Saccharomyces cerevisiae*, *MSS1*, *MTO1* and *MTO2* genes encoded highly conserved tRNA modifying enzymes for the biosynthesis of cmnm⁵s²U34 of mitochondrial tRNAs.^{12,18,19,20} Mutations in these genes altered modifications in mitochondrial tRNAs, thereby causing a failure in tRNA metabolisms.^{20,21,22} The deletion of *MTO2* in the *S. cerevisiae* led to the complete loss of 2-thiouridylation in the mitochondrial tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}, while the inactivation of *MTO1* and *MSS1* conferred the complete loss of 5-carboxymethylaminomethyluridine (cmnm⁵U) of mitochondrial tRNA^{Lys}.²⁰ The *mto1*, *mto2* and *mss1* strains were still able to grow on glycerol medium, indicating that *mto1*, *mto2* or *mss1* mutation by themselves were insufficient to produce the respiratory deficient phenotype.^{12,18,19,20} However, the *mto1*, *mto2* and *mss1* null mutants expressed the respiratory deficient phenotype in the context of mitochondrial 15S rRNA C1409G mutation,^{12,18,19} corresponding to human deafness-associated 12S rRNA C1494T mutation.²³ Furthermore, *mto1*, *mto2* and *mss1* mutants altered mitochondrial protein synthesis and expression of mitochondrial genes, especially in *CYTB* and *COX1* in the presence of 15S rRNA C1409G allele.^{12,18,19} These strongly indicate that products of *MTO1*, *MTO2* and *MSS1* functionally interact with the decoding region of 15S rRNA, particularly at site of C1409G mutation. Subsequent investigations indicated that the human mutated *TRMU*, acting as a modifier factor, modulated the phenotypic manifestation of the deafness-associated mitochondrial 12S rRNA mutations.²⁴ To further investigate the functional interactions among *MTO1*, *MTO2* and *MSS1*, *mto2/mto1*, *mto2/mss1*, *mss1/mto1* double mutants and *mto2/mto1/mss1* triple mutant were constructed by crossing one *mto1* null strain with one *mto2/mss1*-18 strain. The resultant strains were characterized by examining the growth properties, the 2-thiouridine modification, the steady-state level and aminoacylation of tRNAs, the stability of mRNA and rRNAs and mitochondrial protein synthesis.

RESULTS

The combination of *mto2* allele with *mto1* or *mss1* allele led to a respiratory-deficient phenotype

The previous investigations showed that *mto1*, *mss1* and *mto2* null mutants were able to grow in glycerol medium.^{12,18,19} To examine if there are functional interactions among these genes, *mto2/mto1*, *mto2/mss1*, *mss1/mto1* double mutants and *mto2/mto1/mss1* triple mutant were constructed by crossing one *mto1* null strain with one *mto2/mss1*-18 ρ^0 strain isolated by treating *mto2/mss1*-18 (ρ^R) cells¹² with ethidium bromide to cause the loss of mtDNA (ρ^0).²⁵ The resulting diploids were sporulated and products of meiosis were dissected onto glucose medium. Meiotic progeny derived from the cross was examined for the genotype and then tested for the growth on glucose and glycerol media. As shown in Figure 1, *mto1*, *mss1* and *mto2* null mutant strains and *mto1/mss1* double mutant were able to grow on glycerol medium, indicating that these cells were respiratory-competent. Conversely, *mto2/mto1*, *mto2/mss1* double mutants and *mto2/mto1/mss1* triple mutant were unable to grow in glycerol medium, indicating that these cells were respiratory-deficient. These data strongly suggested that the expression of respiratory deficient phenotype in the *mto1* and *mss1* null mutants was fully dependent on the presence of the *mto2* allele.

Alterations in mitochondrial genomes

To examine for the presence of mtDNA in these respiratory-deficient cells, Southern blot analysis were performed by isolating total mitochondrial DNA from yeast strains, digesting with restriction enzyme *Cfo*I, separating them by electrophoresis, blotting and hybridizing with non-radioactive DIG-labeled *CYTB*, *COX1*, *COX2*, *COX3*, *ATP6*, *ATP9*, and 21S rRNA, respectively. As an internal control, DNA blots were stripped and rehybridized with a DIG-labeled nuclear 25S rRNA probe. As shown in Fig. 2, the patterns and levels of mtDNAs in *mto2*, *mto1* and *mss1* strains appeared to be comparable with those of wild strain M12. In *mto1/mss1* strains, the levels of bands corresponding to six genes were comparable with those in control strain, while the band corresponding to *COX3* exhibited very lower level. By the contrast, the deletions and very lower levels of mitochondrial genomes occurred in the *mto2/mss1*, *mto2/mto1* and *mto2/mto1/mss1* strains. In particular, the bands corresponding to *COX1* and *ATP6* were not detected in these mutant strains. Quantification of the hybridization was carried out by the Image-Quant program. For comparison of the data from different blots, the values obtained for the each strain in each blot were normalized to the values obtained for the M12 sample in the same blot. After normalization to the level of 25S rDNA, the average levels of mtDNA in *mto2*, *mto1*, *mss1* and *mto1/mss1* strains were 85%, 85%, 86% and 77% of control levels (M12), respectively. On the other hand, the average levels of mtDNA in *mto2/mss1*, *mto2/mto1* and *mto2/mto1/mss1* strains were 28%, 31% and 34% of control levels (M12) after normalization to 25S rRNA, respectively. These data suggested that the respiratory deficient phenotypes in the *mto2/mss1*, *mto2/mto1* and *mto2/mto1/mss1* strains resulted from the loss and decrease of their mtDNAs.

Defect in 2-thiouridine modification at position 34 in mitochondrial tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}

To investigate if the *mto2*, *mto1* and *mss1* mutations impair 2-thiouridine modification at position 34 in mitochondrial tRNAs, the 2-thiouridylation levels of tRNAs were determined by isolating total mitochondrial RNA from eight strains, and qualifying the 2-thiouridine modification by the retardation of electrophoresis mobility in polyacrylamide gel containing 0.05 mg/ml [(N-acryloylamino)phenyl] mercuric chloride (APM)^{26–28} and hybridizing specific probes for the tRNA^{Lys}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Met} and tRNA^{His}. As shown in Fig. 3, the upper band represented the thiouridylated tRNA, and the lower band was unthiouridylated tRNA. In this system, the mercuric compound can specifically interact with

the tRNAs containing thiocarbonyl group including tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}, thereby retarding tRNA migration.^{26,27} However, no 2-thiouridylation occurs in other tRNAs lacking thiocarbonyl group such as tRNA^{Leu}, tRNA^{Met} and tRNA^{His}. The proportions of the 2-thiouridylation levels of the tRNA in the wild type strain were 92%, 85% and 92% in the tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}, respectively, whereas the *mto2* null strain exhibited the complete loss of the 2-thiouridylation in tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}. In addition, the 2-thiouridylation levels of the tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln} in *mss1-18*, *mto1* and *mss1/mto1* double mutants were comparable with those in wild type strain. However, there was no detectable thiouridylated and unthiouridylated tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln} as well as tRNA^{Leu}, tRNA^{Met} and tRNA^{His} in the *mto2/mto1*, *mto2/mss1* double mutants and *mto2/mto1/mss1* triple mutant.

Decreases in the steady-state levels of mitochondrial tRNAs

To examine if the defective nucleotide modification alters the stability of mitochondrial tRNAs, the steady-state levels of nine tRNAs were determined by isolating total mitochondrial RNA from eight yeast strains, separating them by a 10% polyacrylamide/7 M urea gel, electroblotting and hybridizing with non-radioactive DIG-labeled oligodeoxynucleotide probes specific for tRNA^{Lys}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Gly}, tRNA^{Met}, tRNA^{Arg}, tRNA^{Phe} and tRNA^{His}, respectively. For comparison, the average levels of each tRNA in the various control or mutant yeast strains were then normalized to the average levels in the same strain for the mitochondrial 21S rRNA. As shown in Fig. 4 and Table 2, the average steady-state levels of tRNAs, except tRNA^{His}, were significantly decreased in the *mto2* mutant relative to the wild type strain. The average levels of tRNAs in the *mto2* strain varied from 22% in the tRNA^{Gln} to 58% in tRNA^{Met} of control levels. Similarly, the average levels of tRNAs in *mto1* strain, except tRNA^{Met} and tRNA^{His}, ranged from 43% in the tRNA^{Arg} to 74% in tRNA^{Phe}, of control levels. Furthermore, the average levels of those tRNAs in *mss1-18* strain, except tRNA^{Lys} and tRNA^{Leu}, were comparable to those in the wild type strain. In particular, the average levels of tRNA^{Lys} and tRNA^{Leu} in *mss1-18* strain were 66% and 64% of wild type strain, respectively. Surprisingly, the average levels of those tRNAs in *mss1-18/mto1* double mutant strain appeared to increase, as compared with those in the wild type strain. On the other hand, there were no detectable mitochondrial tRNAs in the *mto2/mto1*, *mto2/mss1* double mutants and *mto2/mto1/mss1* triple mutant.

Alteration in aminoacylation of mitochondrial tRNAs

It has been shown that the mnm⁵-s²U34 serves as a determinant for tRNA recognition by cognate aminoacyl-tRNA synthetases in *E. coli*.^{29,30} This led us to test whether the *mto1*, *mto2* and *mss1* mutations affect the aminoacylation of mitochondrial tRNAs. For this purpose, the steady state levels of aminoacylation in tRNA^{Lys}, tRNA^{Arg} and tRNA^{His} in wild-type and mutant strains were carried out by the use of electrophoresis in an acid polyacrylamide/urea gel system to separate un-charged tRNA species from the corresponding charged tRNA.^{21,22} As shown in Fig. 5, the upper band represented the charged tRNA, and the lower band was uncharged tRNA. As shown in Table 3, the proportions of aminoacylation of the tRNA^{Lys}, tRNA^{Arg} and tRNA^{His} in the wild type strain were 46%, 40% and 44%, respectively, while the proportions of aminoacylation of the tRNAs in strains carrying *mto2*, *mto1*, *mss1* and *mto1/mss1* alleles: were 29%, 28%, 43% and 38% of tRNA^{Lys}, 21%, 22%, 35% and 36% of tRNA^{Arg}, 43%, 41%, 47% and 44% of tRNA^{His}, respectively. The steady state levels of aminoacylation in these tRNAs were significantly decreased in *mto2* and *mto1* cells, relative to the wild type cells ($P=0.01-0.05$). By contrast, there was no effect in levels of aminoacylation in tRNA^{His} among these control and mutant cells. Furthermore, the proportions of aminoacylation of the tRNA^{Lys}, tRNA^{Arg} and tRNA^{His} in *mss1-18* and *mto1/mss1-18* mutant strains were comparable with those in the wild type strain. However, there was no detectable tRNA^{Lys},

tRNA^{Arg} and tRNA^{His} in the *mto2/mto1*, *mto2/mss1* double mutant and *mto2/mto1/mss1* triple mutant strains.

Deficient expression of mitochondrial genes

We examined whether the combination of *mto2* null mutation with *mss1* and *mto1* null mutation also affects the expression of mitochondrial genes by Northern blot analysis. For this purpose, RNA blots were hybridized with DIG-labeled probes for exon regions of *CYTB*, *COX1*, 15S rRNA and 21S rRNA probes, respectively. In these mitochondrial genes, *CYTB* consists of six exons and five introns, *COX1* contains 8 exons and 7 introns, 21S rRNA harbors two exons and one intron, but 15S rRNA lacks intron.³¹ As shown in Fig. 6, the amount of mature *CYTB* mRNA was decreased in the *mto2*, *mto1* and *mss1-18* strains, compared with the wild type strain, while the amount of mature *COX1* mRNA were decreased in the *mto2* and *mto1* strains but not in *mss1-18* strain, compared with the wild type strain. On the other hand, there were no detectable mature *COX1* and *CYTB* mRNAs in *mto2/mto1*, *mto2/mss1-18*, *mto1/mss1-18* and *mto2/mto1/mss1-18* strains. Furthermore, there were the similar levels of 15S rRNA and 21S rRNA between wild-type strain and *mto2*, *mto1*, *mss1-18*, or *mto1/mss1-18* strains. However, levels of 15S rRNA and 21S rRNA were extremely lower or undetectable in *mto2/mto1*, *mto2/mss1-18* and *mto2/mto1/mss1-18* strains, as compared with those in the wild type strain.

Mitochondrial protein synthesis defects

To examine if a failure in RNA metabolism alters mitochondrial protein synthesis, mutant and control strains were labeled for 2.5 min with [³⁵S] methionine in a methionine-free medium in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Fig. 7 shows SDS-PAGE electrophoretic patterns of the organelle-specific products of mutant and wild-type strains. Interestingly, the patterns of the mitochondrial translational products from *mto2*, *mto1* and *mto1/mss1-18* mutants differed from those of the wild-type strain. There was a marked reduction in the overall labeling of the mitochondrial translation products in the *mto2* and *mto1* mutants, when compared with wild type strain. In particular, the Cox1 and Cytb polypeptides were less labeled in *mto2*, *mto1* and *mto1/mss1-18* mutants than in wild type strain. The patterns of the mitochondrial translational products from *mss1-18* mutant strain are similar to those in wild type strain. However, the mitochondrial translation was completely abolished in the *mto2/mto1*, *mto2/mss1-18* and *mto2/mto1/mss1-18* strains.

DISCUSSION

In this study, we have investigated the genetic interactions among *MTO1*, *MTO2* and *MSS1* genes encoding tRNA modifying enzymes responsible for the biosynthesis of cmnm⁵s²U₃₄ in the mitochondrial tRNAs. Mto1p and Mss1p are involved in the biosynthesis of the cmnm⁵ group (cmnm⁵U₃₄), while Mto2p is responsible for the 2-thiouridylation (s²U₃₄).^{20,21,22,32} In particular, Mss1p and Mto1p form a functional complex that would catalyze the production of an unknown intermediate group (X) at position 5.^{7,18,32} Glycine would be subsequently incorporated into tRNA by unidentified transferases to produce cmnm⁵U₃₄.^{20,32} On the other hand, U₃₄ undergoes thiolation at position 2 catalyzed by Mto2p, together with other proteins including Nfs1/IscS.^{7,11,20,32} The deletion of *MTO2* abolished the 2-thiouridylation in mitochondrial tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}, while the inactivation of *MTO1* and *MSS1* caused the complete loss of cmnm⁵U₃₄ in tRNA^{Lys}.²⁰ These implied that the modifications at positions 2 and 5 of U₃₄ in these tRNAs proceed independently. Despite the absence of cmnm⁵s²U₃₄ of these tRNAs, the presence of cmnm⁵U₃₄ or s²U₃₄ makes *mto2*, *mto1*, *mss1* or *mss1/mto1* strains to respire. However, the combination of the loss of cmnm⁵U₃₄ caused by the *mss1* or *mto1* mutation with the absence of s²U₃₄ caused by the *mto2* mutation completely abolished nucleotide modification at U₃₄ of tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln} in

mto2/mto1, *mto2/mss1* or *mto2/mss1/mto1* strains. As a result, the *mto2* allele, acting in synergy with the *mto1* or *mss1* mutation, manifested the respiratory phenotype, as in the case of 15S rRNA C1409G mutation.^{18,19} Thus, Mto2p appeared to act as a hub of a strong set of synthetic genetic interactions.

The $\text{cmnm}^5\text{s}^2\text{U}_{34}$ modified nucleotide plays a pivotal role in the structure and function of tRNAs including stability, turnover, aminoacylation of tRNAs and codon-recognition.^{4,7,29,33} Thus, the deficiency of this U_{34} modification may affect the structure and function of these mitochondrial tRNAs. In fact, the *mto2*, *mto1* and *mss1* mutations caused a large proportion of incompletely modified mitochondrial tRNAs.²⁰ These unmodified tRNAs likely leave the tRNAs more exposed to degradation, thus reducing the levels of mitochondrial tRNAs.^{21,22,24} Here, the lowered levels of tRNA^{Lys} , tRNA^{Glu} , tRNA^{Gln} in *mto2* and *mto1* strains likely resulted from the deficient uridine modification at position 34 of these tRNAs. On the other hand, the reduced levels of tRNA^{Lys} but not tRNA^{Glu} and tRNA^{Gln} in *mss1* strain implied that Mss1p is involved in uridine modification at position 34 of tRNA^{Lys} but not tRNA^{Glu} and tRNA^{Gln} . Of other six tRNAs, the steady-state levels of tRNA^{Leu} , tRNA^{Gly} , tRNA^{Met} , tRNA^{Arg} , tRNA^{Phe} in *mto2* mutant cells, tRNA^{Leu} , tRNA^{Gly} , tRNA^{Met} , tRNA^{Arg} , tRNA^{Phe} in *mto1* strains and only tRNA^{Leu} in *mss1* mutant cells were decreased, as compared with those in the wild type strain. The lowered levels of those tRNAs likely result from directly or indirectly transcriptional/translational defects caused by mutations in *MTO1*, *MTO2* and *MSS1*. Alternatively, the reduced levels of tRNA^{Leu} , tRNA^{Gly} , tRNA^{Met} , tRNA^{Arg} , tRNA^{Phe} could be attributed to preferential deletion of the ρ^- cells containing particular segments of mtDNA carrying these tRNA genes.²² Furthermore, the deficient nucleotide modification altered the aminoacylation of tRNAs.^{29,30,34} Here, the tRNA^{Lys} and tRNA^{Arg} were uncharged to a significantly high degree in vivo in *mto2* and *mto1* mutants, compared to the wild type strain. However, the proportions of aminoacylation of the tRNA^{Lys} and tRNA^{Arg} in *mss1* strain were comparable with those in the wild type strain. These clearly indicated that the loss of synthesis of s^2U_{34} by the deletion of *MTO2* and the loss of $\text{cmnm}^5\text{U}_{34}$ synthesis by *mto1* mutation impaired the aminoacylation of tRNA^{Lys} . Subsequently, the destabilization of tRNAs caused by the loss of $\text{cmnm}^5\text{U}_{34}$ resulted from *mto1* or *mss1* mutation was worsened when coupled with the lack of s^2U_{34} caused by the *mto2* mutation. Indeed, the rapid tRNA decay was observed in yeast cells lacking nucleotide modifications.^{35,36} As a result, the complete loss of modification in U_{34} in tRNA^{Lys} , tRNA^{Glu} and tRNA^{Gln} caused a rapid mitochondrial tRNA degradation in the *mto2/mto1*, *mto2/mss1* and *mto2/mto1/mss1* strains.

tRNA nucleotide modifications are essential for mitochondrial translation, gene expression and genome maintenance. It has been shown that the deficient tRNA nucleotide modifications affected the fidelity and efficiency of mitochondrial translation.^{37–41} In the present investigation, the reduced levels of mitochondrial tRNAs, caused by the loss of s^2U_{34} or $\text{cmnm}^5\text{U}_{34}$, most probably accounts for a marked decrease in the overall rate of mitochondrial translation in *mto2* and *mto1* strains. Conversely, it appears that the reduced levels of tRNA^{Lys} and tRNA^{Leu} in the *mss1* strain did not severely affect the mitochondrial protein synthesis, suggesting that these tRNAs are not limiting factors. However, the lack of both $\text{cmnm}^5\text{U}_{34}$ and s^2U_{34} modifications of tRNA^{Lys} , tRNA^{Glu} and tRNA^{Gln} completely abolished the mitochondrial translation in *mto2/mto1*, *mto2/mss1* and *mto2/mto1/mss1* strains. Perturbations in mitochondrial translations affected the synthesis of the maturases encoded by introns of *CYT8* and *COX1* genes.^{31,42,43}, thereby affecting the processing of polycistronic precursors and the stability of mature transcripts. The incomplete removal of introns reduced the levels of mature *CYT8* and *COX1* mRNAs in these *mto2*, *mto1* or *mss1* single, double or triple mutant strains. Alternatively, these transcriptional/translational defects can also lower the steady-state levels of *CYT8* and *COX1* mRNAs and those of *COX2*, *COX3*, *ATP6* and *ATP9* lacking introns.³¹ Strikingly, the combination of *mto2* with *mto1* or *mss1* mutation produced synthetic phenotypes for 15S rRNA and 21S rRNA. Therefore, the *mto2* mutation,

acting in synergy with *mtol* or *mss1* mutations, significantly perturbed mitochondrial mRNA and rRNA metabolism. Furthermore, it has been shown that mutations in genes affecting mitochondrial protein synthesis caused the instability of mtDNA.^{44–46} In fact, a failure in RNA metabolism promoted the petite formation, thereby accounting for slight reductions in levels of mtDNA in the *mtol2*, *mtol1* and *mss1* cells.^{21,22} Strikingly, mtDNAs in the *mtol2/mtol1*, *mtol2/mss1* or *mtol2/mss1/mtol1* mutants were by themselves very unstable. Their mtDNA levels in these mutant cells, unlike the cytoplasmic petites,²⁵ were only 30% of wild type level (comprising large deletions or loss of entire genomes). The instability of their mtDNA is likely a secondary effect by the reduced levels of transcripts or unstable transcripts caused by the impairment of translation. It appears that the instability of mtDNA is preferentially in some genes. In particular, the *COX1* and *ATP6* genes, whose transcripts were processed from the same polycistronic precursor,⁴² were not detectable in these double and triple mutants. This instability of these specific genes may be related to the loss of introns such as the intron *ai5α* in *COX1* gene⁴⁷ or a transcription-dependent DNA traction.⁴⁸ However, this conclusion may not be extended to mammalian mitochondria.⁴⁹

In summary, *MTO2*, *MTO1* and *MSS1* encoded enzymes responsible for the biosynthesis of $\text{cmnm}^5\text{s}^2\text{U}_{34}$ in of mitochondrial tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}. The unmodified mitochondrial tRNAs caused by the mutations in *MTO2*, *MTO1* and *MSS1* genes likely makes the tRNA more unstably, thus reducing the levels of mitochondrial tRNAs. The unmodified mitochondrial tRNAs are poor substrates for cognate aminoacyl-tRNA synthetases, thereby decreasing the aminoacylation levels of mitochondrial tRNAs. As shown in Table 4, the loss of $\text{cmnm}^5\text{U}_{34}$ by *mtol1* and *mss1* mutations or s^2U_{34} by *mtol2* mutation was insufficient to produce a respiratory deficient phenotype. On the other hand, the loss of both $\text{cmnm}^5\text{U}_{34}$ and s^2U_{34} modifications lead to very significant failures in mitochondrial RNA metabolism, complete loss of mitochondrial protein synthesis, instability of mitochondrial genome and subsequent respiratory deficient phenotype. These data demonstrated the functional interaction between *MTO2* and *MTO1* or *MSS1* and the critical role of modifications at U_{34} of tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln} in mitochondrial functions.

MATERIALS AND METHODS

Yeast strains, media and genetic techniques

The genotypes and sources of strains of *S. cerevisiae* used in this investigation are listed in Table 1. The media used to grow yeast have been described elsewhere.⁵⁰ Standard procedures were used for crossing and selecting diploids, including sporulation and dissecting tetrads.⁵¹

Southern blot analysis

Yeast genomic DNA was extracted as detailed elsewhere.⁵¹ Equal amounts (10 µg) of total DNA were digested with restriction enzyme *CfoI*, fractionated by electrophoresis through a 1.8% agarose gel, transferred onto a positively charged membrane (Roche Applied Science), and hybridized with digoxigenin (DIG)-labeled probes specific for *CYT6*, *COX1*, *COX2*, *COX3*, *ATP6*, *ATP9*, and 21S rRNA,^{12,22} respectively. As an internal control, DNA blots were stripped and rehybridized with DIG-labeled nuclear 25S rRNA probe.¹² The hybridization was performed as detailed elsewhere.^{21,22} Quantification of density in each band was made as detailed elsewhere.^{21,22}

APM Gel electrophoresis to quantify the 2-thiouridine modification in tRNAs

The presence of the thiouridine modification in the tRNAs was verified by the retardation of electrophoretic mobility in a polyacrylamide gel that contains 0.05 mg/ml (N-acryloylamino) phenylmercuric chloride (APM).^{26–28} Total mitochondrial RNAs were obtained using TOTALLY RNA™ kit (Ambion) from mitochondria isolated from yeast cells, as described

previously.^{21,22} Equal amounts (2 µg) of total mitochondrial RNA were separated by polyacrylamide gel electrophoresis and blotted onto positively charged membrane (Roche Applied Science). Each tRNA fraction was detected with a specific non-radioactive digoxigenin-(DIG) oligodeoxynucleoside probe at the 3' termini according to the method as described elsewhere.^{27,28,29,50} Following oligodeoxynucleosides were used:

5'-TGGTGAGAATAGCTGGAGTTGAAC-3' (tRNA^{Lys});

5'-TGGTAACCTTAATCGGAATCGGAATCGAAC-3' (tRNA^{Glu});

5'-TGGTTGAATCGGTTTGATTGGAAC-3' (tRNA^{Gln});

5'-TGGTGCTATTTAAAGGACTTGAACCTT-3' (tRNA^{Leu});

5'-TGGTACTTGTAGAAGGAATTGAAC-3' (tRNA^{Met});

5'-TGGGGTGAATACTGAGAATCGAACTCA-3' (tRNA^{His}) (GenBank accession no: AJ011856).³¹ DIG-labeled oligodeoxynucleosides were generated by using the DIG oligonucleoside Tailing kit (Roche). APM gel electrophoresis and quantification of 2-thiouridine modification in tRNAs were conducted as detailed elsewhere.²⁶⁻²⁸

Mitochondrial tRNA Northern-blot analysis

Two µg of total mitochondrial RNA were electrophoresed through a 10% polyacrylamide/7 M urea gel in Tris-borate-EDTA buffer (after heating the sample at 65°C for 10 min), and then was electroblotted onto a positively charged nylon membrane (Roche) for the hybridization analysis with specific oligodeoxynucleoside mitochondrial tRNA probes.

Oligodeoxynucleosides used for (DIG) labeled probes of tRNA^{Lys}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Met} and tRNA^{His} were described as above. Oligodeoxynucleosides specific for tRNA^{Arg}, tRNA^{Gly}, and tRNA^{Phe} were as follows: oligodeoxynucleosides specific for tRNAs were used:

5'-TGGTATAGATAGCGAGAATCGAAC-3' (tRNA^{Gly});

5'-TTGGTGCCCTTAATGAGAATCGAACT-3' (tRNA^{Phe});

5'-TGGTACTCTCTCCATGATTTGAAC-3' (tRNA^{Arg});

(GenBank accession no: AJ011856).³¹ DIG-labeled oligodeoxynucleosides were generated by using the DIG oligonucleoside Tailing kit (Roche). The hybridization was performed as detailed elsewhere. ^{21,22} Quantification of density in each band was made as detailed elsewhere.^{21,22}

Mitochondrial tRNA aminoacylation analysis

Total mitochondrial RNAs were isolated as above but under acid condition.⁵² 2 µg of total mitochondrial RNA were electrophoresed at 4°C through an acid 10% polyacrylamide/7 M urea gel in a 0.1M sodium acetate (pH 5.0) to separate the charged and uncharged tRNA, as detailed elsewhere.⁵² Then RNAs were electro-blotted onto a positively charged membrane (Roche) and hybridized sequentially with the specific tRNA probes as above.

Mitochondrial mRNA analysis

Total cellular RNA was obtained using a Totally RNATM kit (Ambion) from midlog phase yeast cultures (2.0×10^7 cells) according to the manufacturer's instructions. Equal amounts (20 µg) of total RNA were fractionated by electrophoresis through a 1.8% agarose-formaldehyde gel,

transferred onto a positively charged membrane (Roche Applied Science), and initially hybridized with a probe specific for *COX1* RNA. The probe was synthesized on the corresponding restriction enzyme-linearized plasmid using a DIG RNA labeling kit (Roche Applied Science). RNA blots were then stripped and rehybridized with DIG-labeled *CYTB*, 15S rRNA and 21S rRNA probes, respectively. The plasmids used for *CYTB*, *COX1*, 15S rRNA and 21S rRNA probes were as described previously.¹²

Analysis of mitochondrial protein synthesis

Yeast strains were pulse-labeled for 2.5 min with [³⁵S]methionine in methionine-free medium in the presence of cycloheximide to inhibit cytoplasmic protein synthesis, as described elsewhere.⁵³ The radiolabeled proteins were separated on SDS-exponential polyacrylamide gradient gels.^{12,53} The gels were treated with Me₂SO/2,5-diphenyloxazole, dried, and exposed for fluorography.

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References

1. Hopper AK, Phizicky EM. tRNA transfers to the limelight. *Genes Dev* 2003;17:162–180. [PubMed: 12533506]
2. Agris PF, Vendeix FA, Graham WD. tRNA's wobble decoding of the genome: 40 years of modification. *J Mol Biol* 2007;366:1–13. [PubMed: 17187822]
3. Czerwoniec A, Dunin-Horkawicz S, Purta E, Kaminska KH, Kasprzak JM, Bujnicki JM, Grosjean H, Rother K. MODOMICS: a database of RNA modification pathways. 2008 update. *Nucleic Acids Res* 2008;37:D118–121. [PubMed: 18854352]
4. Björk, GR. Stable RNA modification. In: Neidhardt, FC.; Curtiss, R., III; Ingraham, JL.; Lin, ECC.; Low, BK.; Magasanik, B.; Reznikoff, WS.; Riley, M.; Schaechter, M.; Umberger, HE., editors. *Escherichia coli and Salmonella: Cellular and Molecular Biology*. American Society for Microbiology; Washington, D. C: 1996. p. 861-886.
5. Yan Q, Guan MX. Identification and characterization of mouse *TRMU* gene encoding the mitochondrial 5-methylaminomethyl-2-thiouridylate-methyltransferase. *Biochim Biophys Acta* 2004;1676:119–126. [PubMed: 14746906]
6. Ishitani R, Yokoyama S, Nureki O. Structure, dynamics, and function of RNA modification enzymes. *Curr Opin Struct Biol* 2008;18:330–339. [PubMed: 18539024]
7. Brégeon D, Colot V, Miroslav M, Radman M, Taddei F. Translational misreading: a tRNA modification counteracts a +2 ribosomal frameshift. *Genes Dev* 2001;15:2295–2306. [PubMed: 11544186]
8. Moukadir I, Prado S, Piera J, Velazquez-Campoy A, Bjork GR, Armengod M-E. Evolutionarily conserved proteins MnmE and GidA catalyze the formation of two methyluridine derivatives at tRNA wobble positions. *Nucleic Acids Res* 2009;0:gkp762v1–gkp762.
9. Li X, Guan MX. A human mitochondrial GTP binding protein related to RNA modification may modulate the phenotypic expression of the deafness-associated mitochondrial 12S rRNA mutation. *Mol Cell Biol* 2002;22:7701–7711. [PubMed: 12370316]
10. Li R, Li X, Yan Q, Mo JQ, Guan MX. Isolation and characterization of mouse *MTO1* related to mitochondrial tRNA modification. *Biochim Biophys Acta* 2003;1629:53–59. [PubMed: 14522080]
11. Kambampati R, Lauhon CT. MnmA and IscS are required for in vitro 2-thiouridine biosynthesis in *Escherichia coli*. *Biochemistry* 2003;42:1109–1117. [PubMed: 12549933]
12. Yan Q, Li X, Faye G, Guan MX. Mutations in *MTO2* related to tRNA modification impair mitochondrial gene expression and protein synthesis in the presence of a paromomycin resistance mutation in mitochondrial 15S rRNA. *J Biol Chem* 2005;280:29151–29157. [PubMed: 15944150]

13. Elseviers D, Petrullo LA, Gallagher P. Novel *E. coli* mutants deficient in biosynthesis of 5-methylaminomethyl-2-thiouridine. *Nucleic Acids Res* 1984;12:3521–3534. [PubMed: 6427754]
14. Meyer S, Scrima A, Versées W, Wittinghofer A. Crystal structures of the conserved tRNA-modifying enzyme GidA: implications for its interaction with MnmE and substrate. *J Mol Biol* 2008;380:532–547. [PubMed: 18565343]
15. Scrima A, Vetter IR, Armengod MF, Wittinghofer A. The structure of the TrmE GTP-binding protein and its implications for tRNA modification. *EMBO J* 2005;24:23–33. [PubMed: 15616586]
16. Ikeuchi Y, Shigi N, Kato J, Nishimura A, Suzuki T. Mechanistic insights into sulfur relay by multiple sulfur mediators involved in thiouridine biosynthesis at tRNA wobble positions. *Mol Cell* 2006;21:97–108. [PubMed: 16387657]
17. Numata T, Ikeuchi Y, Fukai S, Suzuki T, Nureki O. Snapshots of tRNA sulphuration via an adenylated intermediate. *Nature* 2006;442:419–424. [PubMed: 16871210]
18. Colby G, Wu M, Tzagoloff A. *MTOI* codes for a mitochondrial protein required for respiration in paromomycin-resistant mutants of *Saccharomyces cerevisiae*. *J Biol Chem* 1998;273:27945–27952. [PubMed: 9774408]
19. Decoster E, Vassal A, Faye G. *MSSI*, a nuclear-encoded mitochondrial GTPase involved in the expression of COX1 subunit of cytochrome *c* oxidase. *J Mol Biol* 1993;232:79–88. [PubMed: 8392589]
20. Umeda N, Suzuki T, Yukawa M, Ohya Y, Shindo H, Watanabe K, Suzuki T. Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in mitochondrial tRNAs: implications for the molecular pathogenesis of human mitochondrial diseases. *J Biol Chem* 2004;280:1613–1624. [PubMed: 15509579]
21. Wang X, Yan Q, Guan MX. Deletion of the *MTO2* gene related to tRNA modification causes a failure in mitochondrial RNA metabolism in the yeast *Saccharomyces cerevisiae*. *FEBS Lett* 2007;581:4228–4234. [PubMed: 17706197]
22. Wang X, Yan Q, Guan MX. Mutation in *MTOI* involved in tRNA modification impairs mitochondrial RNA metabolism in the yeast *Saccharomyces cerevisiae*. *Mitochondrion* 2009;9:180–185. [PubMed: 19460296]
23. Zhao H, Li R, Wang Q, Yan Q, Deng JH, Han D, Bai Y, Young WY, Guan MX. Maternally inherited aminoglycoside-induced and non-syndromic deafness is associated with the novel C1494T mutation in the mitochondrial 12S rRNA gene in a large Chinese family. *Am J Hum Genet* 2004;74:139–152. [PubMed: 14681830]
24. Guan MX, Yan Q, Li X, Bykhovskaya Y, Gallo-Teran J, Hajek P, Umeda N, Zhao H, Garrido G, Mengesha E, Suzuki T, del Castillo IL, Peters J, Li R, Qian Y, Wang X, Ballana E, Shohat M, Lu J, Estivill X, Watanabe K, Fischel-Ghodsian N. Mutation in *TRMU* related to transfer RNA modification modulates the phenotypic expression of the deafness-associated mitochondrial 12S ribosomal RNA mutations. *Am J Hum Genet* 2006;79:291–302. [PubMed: 16826519]
25. Clark-Walker GD, Miklos GLG. Complementation in cytoplasmic petite mutants of yeast to form respiratory competent cells. *Proc Natl Acad Sci USA* 1975;72:372–375. [PubMed: 1090936]
26. Moriya J, Yokogawa T, Wakita K, Ueda T, Nishikawa K, Crain PF, Hashizume T, Pomerantz SC, McCloskey JA, Kawai G, Hayashi N, Yokoyama S, Watanabe K. A novel modified nucleoside found at the first position of the anticodon of methionine tRNA from bovine liver mitochondria. *Biochemistry* 1994;33:2234–2239. [PubMed: 7509636]
27. Shigi N, Suzuki T, Tamakoshi M, Oshima T, Watanabe K. Conserved bases in the TΨC Loop of tRNA are determinants for thermophile-specific 2-thiouridylation at position 54. *J Biol Chem* 2002;277:39128–39135. [PubMed: 12177072]
28. Suzuki T, Suzuki T, Wada T, Saigo K, Watanabe K. Taurine as a constituent of mitochondrial tRNA: new insights into the functions of taurine and human mitochondrial diseases. *EMBO J* 2002;21:6581–6589. [PubMed: 12456664]
29. Krüger MK, Sørensen MA. Aminoacylation of hypomodified tRNA^{Glu} *in vivo*. *J Mol Biol* 1998;284:609–620. [PubMed: 9826502]
30. Sylvers LA, Rogers KC, Shimizu M, Ohtsuka E, Soll D. A 2-thiouridine derivative in tRNA^{Glu} is a positive determinant for aminoacylation by *Escherichia coli* glutamyl-tRNA synthetase. *Biochemistry* 1993;32:3836–3841. [PubMed: 8385989]

31. Foury F, Roganti T, Lecrenier N, Purnelle B. The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. FEBS Lett 1998;440:325–331. [PubMed: 9872396]
32. Villarroya M, Prado S, Esteve JM, Soriano MA, Aguado C, Pérez-Martínez D, Martínez-Ferrandis JI, Yim L, Victor VM, Cebolla E, Montaner A, Knecht E, Armengod ME. Characterization of human GTPBP3, a GTP-binding protein involved in mitochondrial tRNA modification. Mol Cell Biol 2008;28:7514–7531. [PubMed: 18852288]
33. Sundaram M, Durant PC, Davis DR. Hypermodified nucleosides in the anticodon of tRNA^(Lys) stabilize a canonical U-turn structure. Biochemistry 2000;39:12575–12584. [PubMed: 11027137]
34. Hagervall TG, Pomerantz SC, McCloskey JA. Reduced misreading of asparagine codons by *Escherichia coli* tRNA^{Lys} with hypomodified derivatives of 5-methylaminomethyl-2-thiouridine in the wobble position. J Mol Biol 1998;284:33–42. [PubMed: 9811540]
35. Alexandrov A, Chernyakov I, Gu W, Hiley SL, Hughes TR, Grayhack EJ, Phizicky EM. Rapid tRNA decay can result from lack of nonessential modifications. Mol Cell 2006;21:87–96. [PubMed: 16387656]
36. Chernyakov I, Whipple JM, Kotelawala L, Grayhack EJ, Phizicky EM. Degradation of several hypomodified mature tRNA species in *Saccharomyces cerevisiae* is mediated by Met22 and the 5′-3′ exonucleases *Rat1* and *Xrn1*. Genes Dev 2008;22:1369–1380. [PubMed: 18443146]
37. Ashraf SS, Sochacka E, Cain R, Guenther R, Malkiewicz A, Agris PF. Single atom modification (O→S) of tRNA confers ribosome binding. RNA 1999;5:188–194. [PubMed: 10024171]
38. Hagervall TG, Björk GR. Undermodification in the first position of the anticodon of supG-tRNA reduces translational efficiency. Mol Gen Genet 1984;196:194–200. [PubMed: 6387394]
39. Urbonavicius U, Qian Q, Durand JM, Hagervall TG, Björk GR. Improvement of reading frame maintenance is a common function for several tRNA modifications. EMBO J 2001;20:4863–4873. [PubMed: 11532950]
40. Yarian C, Townsend H, Czeszkowski W, Sochacka E, Malkiewicz AJ, Guenther R, Miskiewicz A, Agris PF. Modified nucleoside dependent Watson–Crick and wobble codon binding by tRNA^{Lys}_{UUU} species. Biochemistry 2000;39:13390–13395. [PubMed: 11063576]
41. Yasukawa T, Suzuki T, Ishii N, Ohta S, Watanabe K. Wobble modification defect in tRNA disturbs codon–anticodon interaction in a mitochondrial disease. EMBO J 2001;20:4794–4802. [PubMed: 11532943]
42. Costanzo MC, Fox TD. Control of mitochondrial gene expression in *Saccharomyces cerevisiae*. Annu Rev Genet 1990;24:91–108. [PubMed: 2088182]
43. Islas-Osuna MA, Ellis TP, Marnell LL, Mittelmeier TM, Dieckmann CL. Cbp1 Is Required for Translation of the Mitochondrial Cytochrome b mRNA of *Saccharomyces cerevisiae*. J Biol Chem 2002;277:37987–37990. [PubMed: 12149267]
44. Myers AM, Pape LK, Tzagoloff A. Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae*. EMBO J 1985;4:2087–2092. [PubMed: 3905388]
45. Barrientos A, Korr D, Barwell KJ, Sjulsen C, Gajewski CD, Manfredi G, Ackerman S, Tzagoloff A. MTG1 codes for a conserved protein required for mitochondrial translation. Mol Biol Cell 2003;14:2292–2302. [PubMed: 12808030]
46. Williams EH, Butler CA, Bonnefoy N, Fox TD. Translation initiation in *Saccharomyces cerevisiae* mitochondria: functional interactions among mitochondrial ribosomal protein Rsm28p, initiation factor 2, methionyl-tRNA-formyltransferase and novel protein Rmd9p. Genetics 2007;175:1117–1126. [PubMed: 17194786]
47. Hensgens LA, Bonen L, de Haan M, van der Horst G, Grivell LA. Two intron sequences in yeast mitochondrial COX1 gene: homology among URF-containing introns and strain-dependent variation in flanking exons. Cell 1983;32:379–389. [PubMed: 6297792]
48. Van Dyck E, Clayton DA. Transcription-dependent DNA transactions in the mitochondrial genome of a yeast hypersuppressive petite mutant. Mol Cell Biol 1998;18:2976–2985. [PubMed: 9566917]
49. Burnett KG, Scheffler IE. Integrity of mitochondria in a mammalian cell mutant defective in mitochondrial protein synthesis. J Cell Biol 1981;90:108–115. [PubMed: 6265473]

50. Guan MX. Cytoplasmic tyrosyl-tRNA synthetase rescues the defect in mitochondrial genome maintenance caused by the nuclear mutation *mgm104-1* in the yeast *Saccharomyces cerevisiae*. *Mol Gen Genet* 1997;255:525–532. [PubMed: 9294037]
51. Sherman, F.; Fink, GR.; Hicks, JB. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 1983.
52. Enríquez JA, Attardi G. Analysis of aminoacylation of human mitochondrial tRNAs. *Methods Enzymol* 1996;264:183–196. [PubMed: 8965692]
53. Barrientos A, Korr D, Tzagoloff A. Shy1p is necessary for full expression of mitochondrial COX1 in the yeast model of Leigh's syndrome. *EMBO J* 2002;21:43–52. [PubMed: 11782424]

Lack of modifications causes mitochondrial dysfunction

FIG. 1. Growth properties of yeast wild-type (WT) and mutant (MT) strains

Series dilutions of the wild-type strain W303-1B, *mtol* and *mtol2* null and *mss1-18* strains, *mtol/mtol2*, *mtol/mss1-18*, and *mtol2/mss1-18* double mutant strains and *mtolmtol2/mss1* strain were spotted on glucose and glycerol plates. The plate was then incubated at 30°C in glucose medium for 3 days and in glycerol medium for 5 days.

FIG. 2. Southern blot analysis of mitochondrial genomes

Equal amounts (10 μ g) of genomic DNA were digested with restriction enzyme *Cfo*I, fractionated by electrophoresis through a 1.8% agarose gel, transferred onto a positively charged membrane, and hybridized with DIG-labeled probes specific for *CYT*B, *COX*1, *COX*2, *COX*3, *ATP*6, *ATP*9, and 21S rRNA, respectively. As an internal control, DNA blots were stripped and rehybridized with DIG-labeled nuclear 25S rRNA probe.

FIG. 3. APM gel electrophoresis combined with Northern blotting of yeast mitochondrial tRNAs Equal amounts (2 μ g) of total mitochondrial RNA were separated by polyacrylamide gel electrophoresis that contains 0.05 mg/ml (N-acryloylamino)phenylmercuric chloride (APM) and electroblotted onto positively charged membrane, and hybridized with DIG-labeled oligonucleotide probes specific for the mitochondrial tRNA^{Lys}. The blots were then stripped and re-hybridized with DIG-labeled probes for tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Met}, and tRNA^{His}, respectively. The retarded bands of 2-thiolated tRNAs and non-retarded bands of tRNA without thiolation are marked by an arrow.

FIG. 4. Northern-blot analysis of mitochondrial tRNA

Equal amounts (2 μ g) of total mtRNA samples from the wild type and mutant yeast strains were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with DIG-labeled oligonucleotide probes specific for the mitochondrial tRNA^{Lys}. The blots were then stripped and re-hybridized with DIG-labeled probes for tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Gly}, tRNA^{Met}, tRNA^{Arg}, tRNA^{Phe}, and tRNA^{His}, respectively.

FIG. 5. *In vivo* aminoacylation assays for mitochondrial tRNA

Equal amounts (2 μg) of total mitochondrial RNA purified from various yeast strains under acid conditions were treated with electrophoresis at 4°C through an acid (pH 5.1) 10% polyacrylamide/7M urea gel, electroblotted onto a positively charged nylon membrane, and hybridized with DIG-labeled oligonucleotide probes specific for mitochondrial tRNA^{Lys}. The blots were then stripped and rehybridized with DIG-labeled probes for tRNA^{Leu}, tRNA^{Arg} and tRNA^{His}, respectively.

FIG. 6. Northern-blot analysis of mitochondrial mRNAs

Equal amounts (20 μ g) of total cellular RNA from various mutant and control strains were electrophoresed through a 1.8% agarose-formaldehyde gel, transferred onto a positively charged membrane and hybridized first with a DIG-labeled *COXI* specific RNA probe. After stripping, the blots were rehybridized with DIG-labeled mtRNA probes *CYTB*, 15S rRNA and 21S rRNA probes, respectively.

FIG. 7. Mitochondrial protein labeling analysis

Electrophoretic patterns of the mitochondrial translation products of wild type and mutant cells labeled for 2.5 min with [35 S]methionine in the presence of 150 μ g of cycloheximide/ml. Samples containing equal amounts of total cellular protein (50 μ g) were run in SDS/polyacrylamide gradient gels. Var1 (a ribosomal protein), Cox1, -2, and -3 (subunits I, II, and III of cytochrome *c* oxidase), Atp6 (subunits 6 of the H⁺-ATPase), and Cytb (apocytochrome *b*) are shown.

Table 1

Genotype and sources of yeast strains

Strains	Genotype	Source
M12	a, <i>ilv5</i> , <i>trp2</i>	Ref. 19.
W1021ΔMTO2/ <i>mss1</i> -18	a <i>his3</i> -1, <i>ura3</i> -1, <i>mss1</i> -18, <i>mto2</i> :: <i>HIS3</i>	Ref. 12
W303ΔMTO1	α <i>ade2</i> -1, <i>his3</i> -1, <i>leu2</i> -3, <i>trp1</i> -1, <i>ura3</i> -1, <i>mto1</i> :: <i>URA3</i>	Ref. 22
ΔMTO1	α <i>his3</i> -1, <i>ura3</i> -1, <i>trp1</i> -1, <i>mto1</i> :: <i>URA3</i>	This study
ΔMTO2	a <i>ade2</i> -1, <i>his3</i> -1, <i>ura3</i> -1, <i>leu2</i> -3, <i>mto2</i> :: <i>HIS3</i>	This study
<i>mss1</i> -18	α <i>his3</i> -1, <i>ura3</i> -1, <i>mss1</i> -18	This study
ΔMTO1/MTO2	a <i>his3</i> -1, <i>ura3</i> -1, <i>trp1</i> -1, <i>mto1</i> :: <i>URA3</i> , <i>mto2</i> :: <i>HIS3</i>	This study
Δ MTO1/ <i>mss1</i> -18	a <i>his3</i> -1, <i>ura3</i> -1, <i>trp1</i> -1, <i>mto1</i> :: <i>URA3</i> , <i>mss1</i> -18	This study
Δ MTO2/ <i>mss1</i> -18	α <i>his3</i> -1, <i>ura3</i> -1, <i>mss1</i> -18, <i>mto2</i> :: <i>HIS3</i>	This study
ΔMTO1/MTO2/ <i>mss1</i> -18	a <i>ade2</i> -1, <i>his3</i> -1, <i>ura3</i> -1, <i>mto1</i> :: <i>URA3</i> , <i>mss1</i> -18, <i>mto2</i> :: <i>HIS3</i>	This study

Table 2

Quantification of the levels of mitochondrial tRNAs.

	WT	ΔMTO2	ΔMTO1	mss <i>I</i> -18	ΔMTO2/ <i>mss I</i> -18	ΔMTO1/MTO2	ΔMTO1/ <i>mss I</i> -18	ΔMTO1/MTO2/ <i>mss I</i> -18
tRNA ^{Lys}	100 ± 7	52 ± 5	70 ± 7	66 ± 8	0	0	91 ± 8	0
tRNA ^{Glu}	100 ± 8	38 ± 4	66 ± 8	104 ± 9	0	0	110 ± 12	0
tRNA ^{Gln}	100 ± 7	22 ± 5	70 ± 16	105 ± 13	0	0	100 ± 14	0
tRNA ^{Leu}	100 ± 6	55 ± 9	61 ± 11	63 ± 6	0	0	137 ± 10	0
tRNA ^{Gly}	100 ± 9	46 ± 7	66 ± 7	110 ± 12	0	0	176 ± 16	0
tRNA ^{Arg}	100 ± 5	55 ± 8	43 ± 6	104 ± 9	0	0	119 ± 13	0
tRNA ^{Met}	100 ± 11	58 ± 7	102 ± 9	114 ± 11	0	0	149 ± 14	0
tRNA ^{Phe}	100 ± 7	44 ± 6	74 ± 6	94 ± 7	0	0	130 ± 17	0
tRNA ^{His}	100 ± 11	122 ± 9	141 ± 17	176 ± 16	0	0	193 ± 19	0

Average relative each tRNA content per cell was normalized to average content per cell of 21S rRNA.
The values were expressed as percentages of the average values for the wild type (WT) strain.
Calculations were based on samples from three independent cultures of each tRNA in each strain.

Table 3

Quantification of the levels of aminoacylated mitochondrial tRNA.

	WT	ΔMTO2	ΔMTO1	<i>mssL</i> -18	ΔMTO2/ <i>mssL</i> -18	ΔMTO1/MTO2	ΔMTO1/ <i>mssL</i> -18	ΔMTO1/MTO2/ <i>mssL</i> -18
tRNA ^{Lys}	46 ± 4	29 ± 2	28 ± 2	43 ± 5	0	0	38 ± 7	0
tRNA ^{Arg}	40 ± 3	21 ± 2	22 ± 3	35 ± 4	0	0	36 ± 3	0
tRNA ^{His}	44 ± 7	43 ± 5	41 ± 6	47 ± 8	0	0	44 ± 5	0

Quantification of the levels of aminoacylated tRNA^{Lys}, tRNA^{Arg} and tRNA^{His} were based on samples from three independent cultures of each tRNA in each strain. The values were expressed as percentages of the average values for the aminoacylated mitochondrial tRNA.

Table 4
Summary of relationship between nucleotide modifications at U34 of tRNA^{Lys} and genotypes of yeast *mtol2*, *mtol1* and *mss1* mutants

	WT	Δ MTO2	Δ MTO1	<i>mss1</i> -18	Δ MTO2/ <i>mss1</i> -18	Δ MTO1/ <i>MTO2</i>	Δ MTO1/ <i>mss1</i> -18	Δ MTO1/ <i>MTO2</i> / <i>mss1</i> -18
U ₃₄	+	+	+	+	+	+	+	+
S ² U ₃₄	+	-	+	+	-	-	+	-
cmnm ⁵ U ₃₄	+	+	-	-	-	-	-	-
cmnm ^{5,2} U ₃₄	+	-	-	-	-	-	-	-