

SUPPLEMENTARY DATA

Guanosine 10 methylation by TRMT11–TRMT112 optimizes human mitochondrial tRNAs for efficient translation

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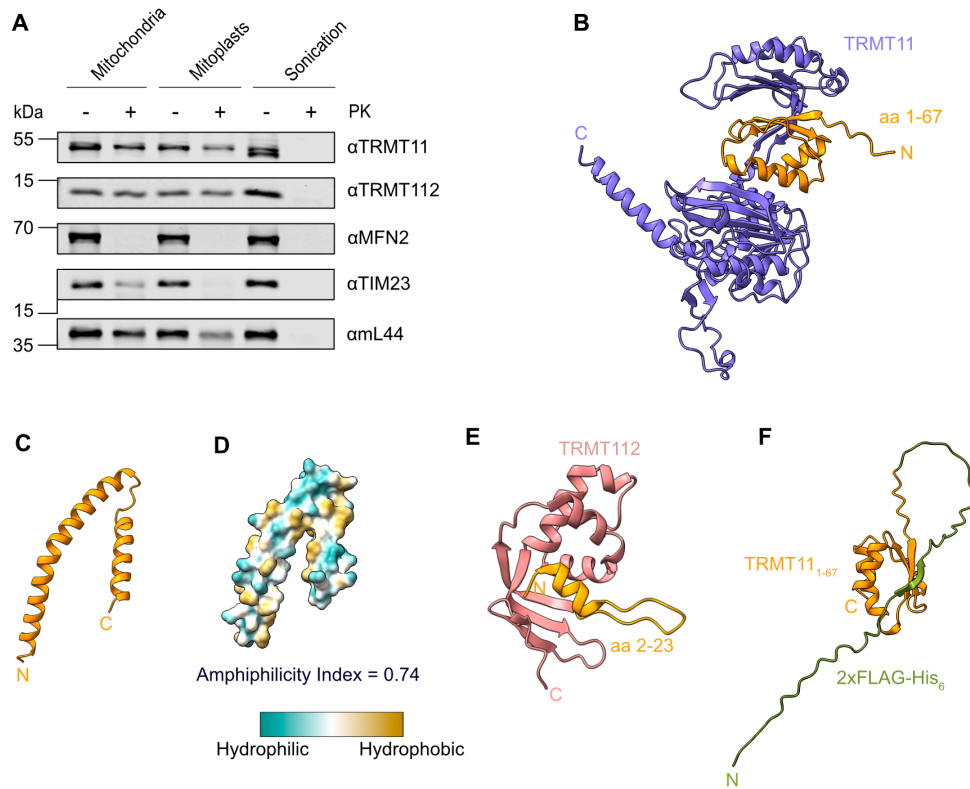
SUPPLEMENTARY DATA – see associated .xlsx files

Supplementary Data 1. TRMT11-His-FLAG CRAC datasets.

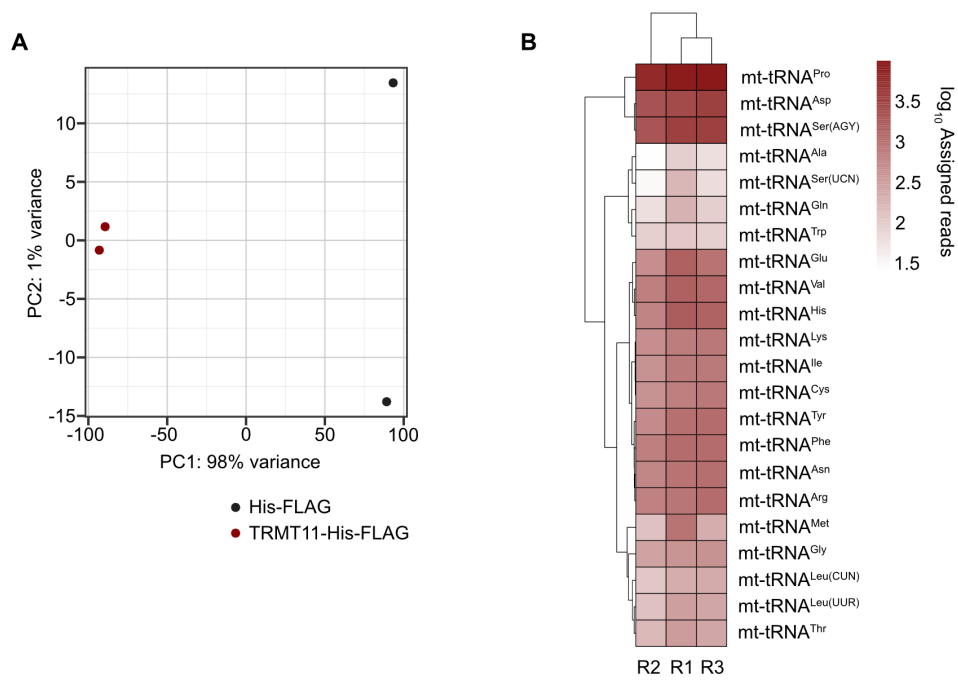
Supplementary Data 2. Small-RNA-seq analysis of mt-tRNA expression levels.

Supplementary Data 3. HPLC-MS analysis of modified nucleotides in mitochondrial RNA from WT and TRMT11^{KO} cells.

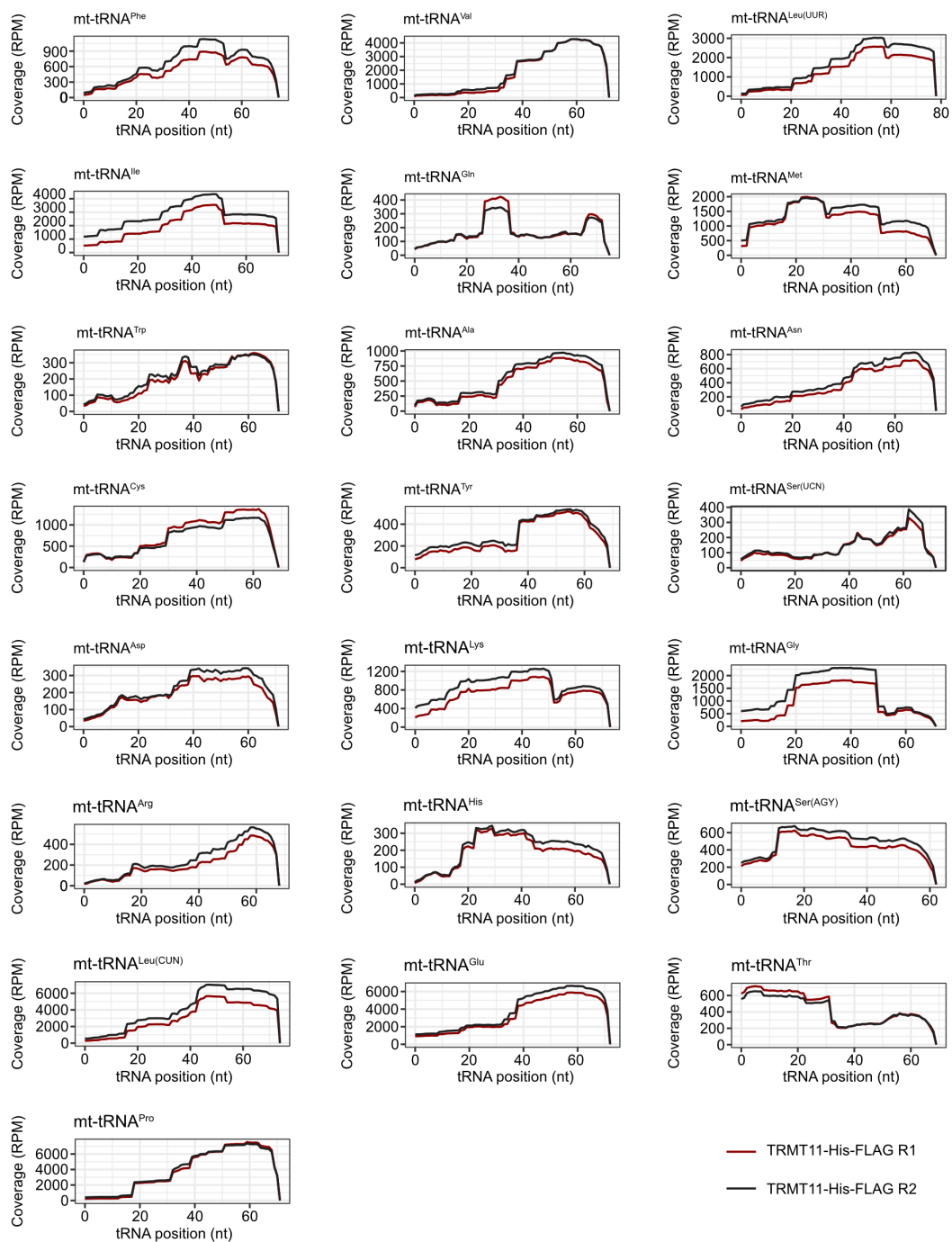
SUPPLEMENTARY FIGURES AND LEGENDS



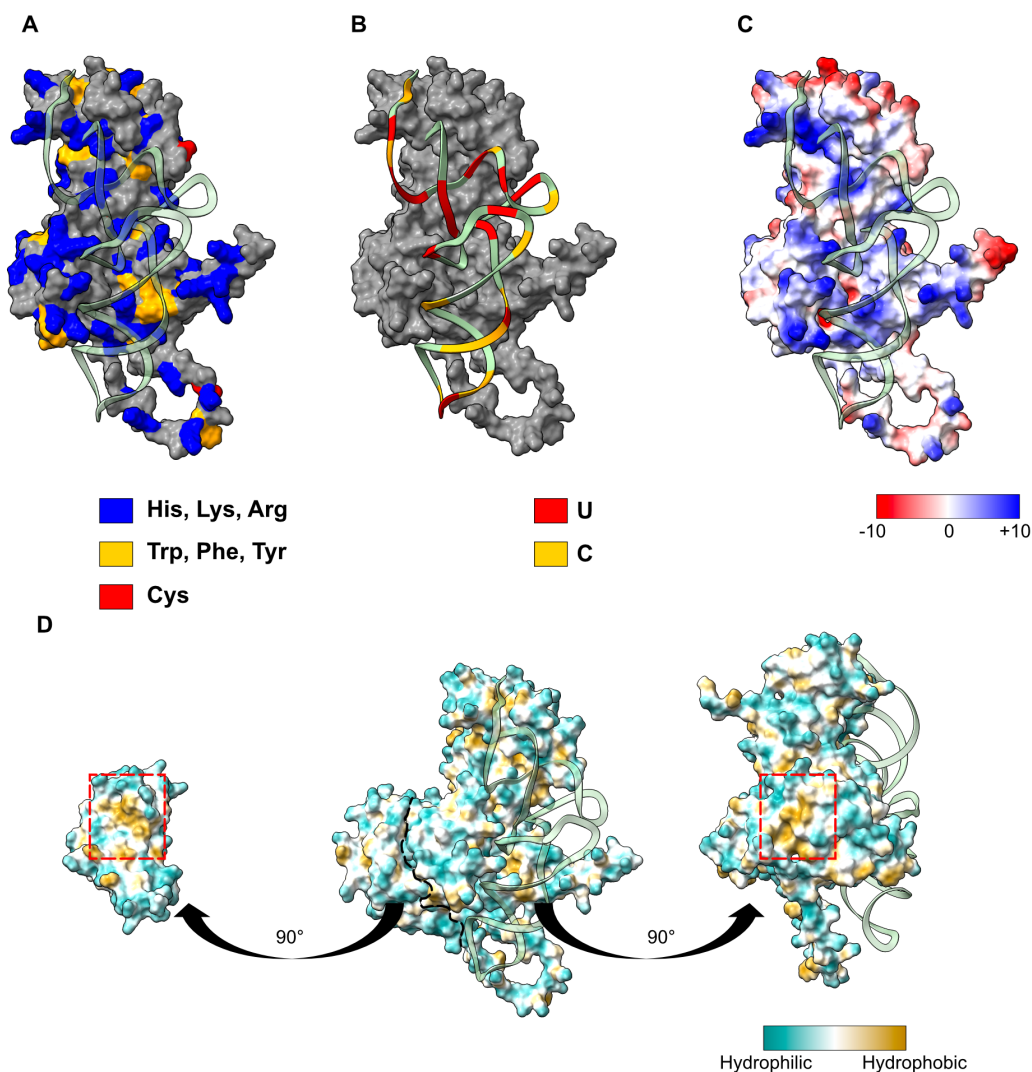
Supplementary Figure S1. Mitochondrial localization and targeting of TRMT11 and TRMT112. **(A)** Mitochondria, mitoplasts or sonicated mitochondria isolated from HCT116 cells were left untreated (-) or treated with (+) proteinase K (PK), and proteins were analyzed by western blotting using the indicated antibodies. Representative data from n=3 independent experiments are shown. **(B)** AlphaFold model of TRMT11 (UniProt ID: Q7Z4G4) with the N-terminal 67 amino acids (aa) shown in orange and the remaining amino acids in purple. **(C)** AlphaFold prediction of the structure of the N-terminal 67 amino acids of TRMT11 alone. **(D)** Model shown in (d) colored according to amphiphilicity. Amphiphilicity index according to MF scale (<https://dbaasp.org/>) (1) **(E)** Crystal structure of TRMT112 (PDB:6H2U) (2) with the N-terminal 25 amino acids (aa) shown in orange and the remaining amino acids in salmon. **(F)** AlphaFold model of a N-terminally 2xFLAG-His₆ tagged version of the first 67 amino acids of TRMT11.



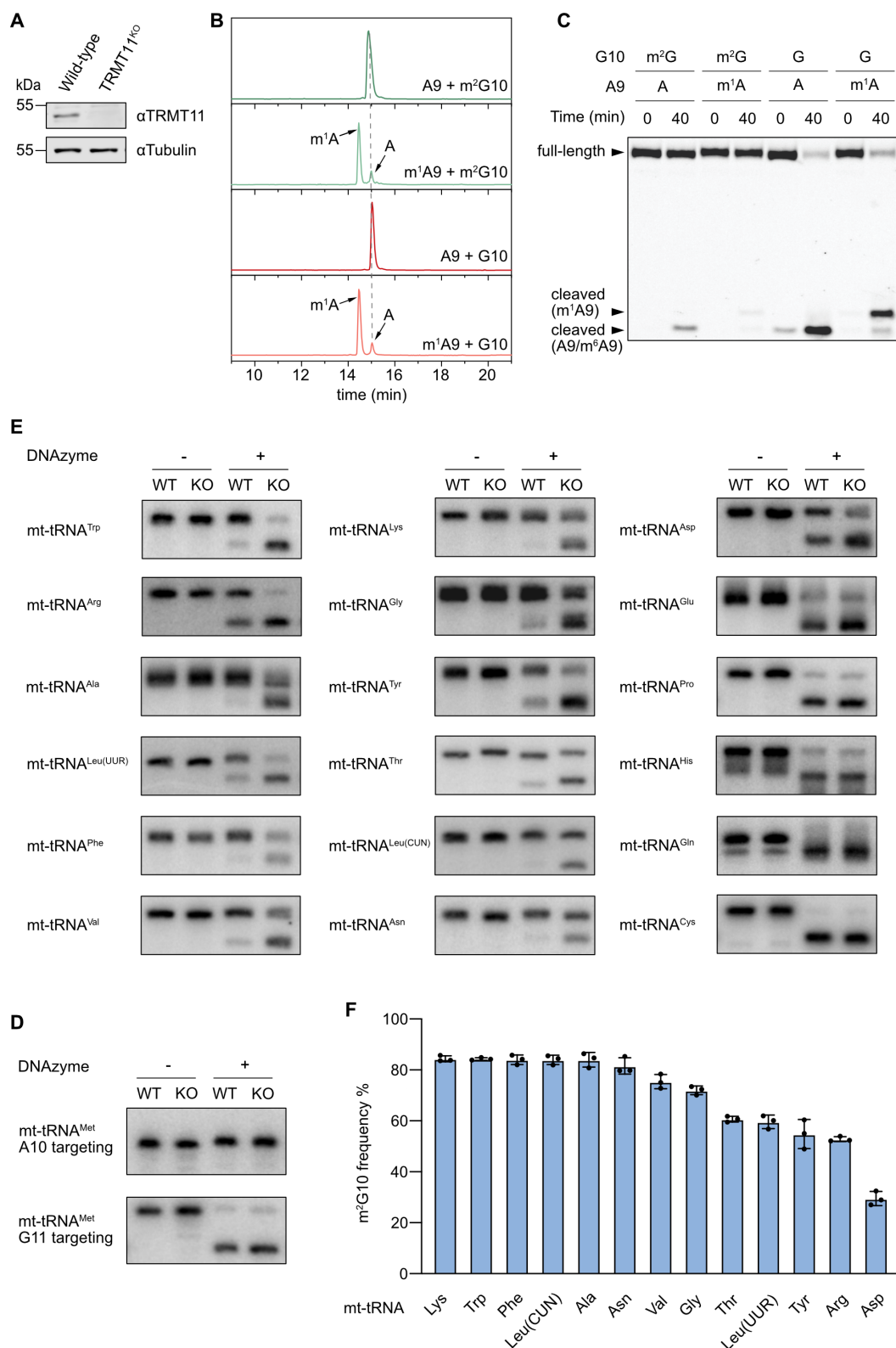
Supplementary Figure S2. CRAC analysis of TRMT11 and expression levels of mt-tRNAs. **(A)** Principal component analysis of His-FLAG (negative control) and TRMT11-His-FLAG CRAC datasets (n=2). **(B)** Heatmap showing relative expression levels of mt-tRNAs in wild-type HEK293 cells as determined by small RNA-seq in n=3 independent experiments (R1-R3). Mt-tRNAs are hierarchically clustered according to enrichment.



Supplementary Figure S3. Profiles of TRMT11 crosslinking on mt-tRNAs. Numbers of sequencing reads in significant peaks in the TRMT11-His-FLAG datasets compared to the control mapping to each nucleotide of each mt-tRNA are shown.

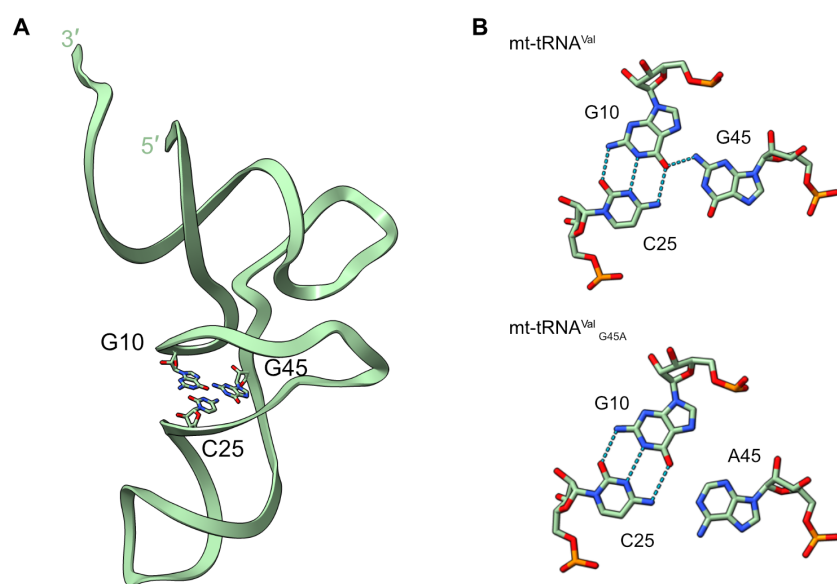


Supplementary Figure S4. Models of TRMT11–TRMT112–mt-tRNA^{Trp} complex. (A) Model of TRMT11 and mt-tRNA^{Trp} with UV-reactive amino acids on the surface highlighted. (B) As in (A), but with UV-crosslinkable nucleotides in mt-tRNA^{Trp} indicated. (C) Model from (A) presented with the indicated color scale depicting electrostatic potential. (D) Model from (A) presented with the indicated color scale depicting hydrophobicity.

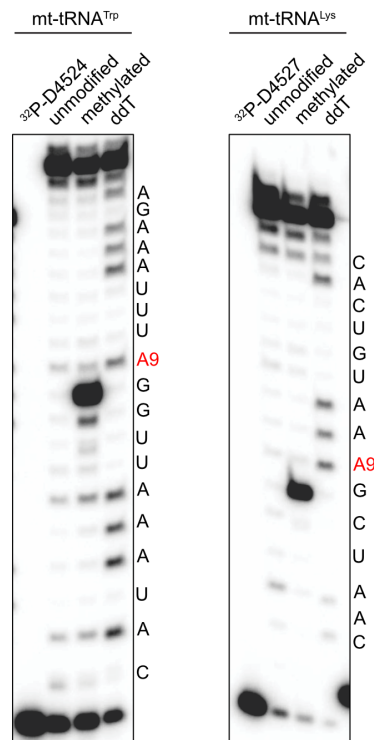


Supplementary Figure S5. DNAzyme-mediated cleavage of mt-tRNAs. (A) Proteins extracted from wild-type HCT116 cells and those containing a single nucleotide insertion in *TRMT11* exon 11 leading to a frameshift and premature stop codon (p.Tyr377Leufs*6; *TRMT11*^{KO}) (3) were analyzed by western blotting using antibodies against TRMT11 and, as a loading control, Tubulin. n=3 independent experiments were performed and representative

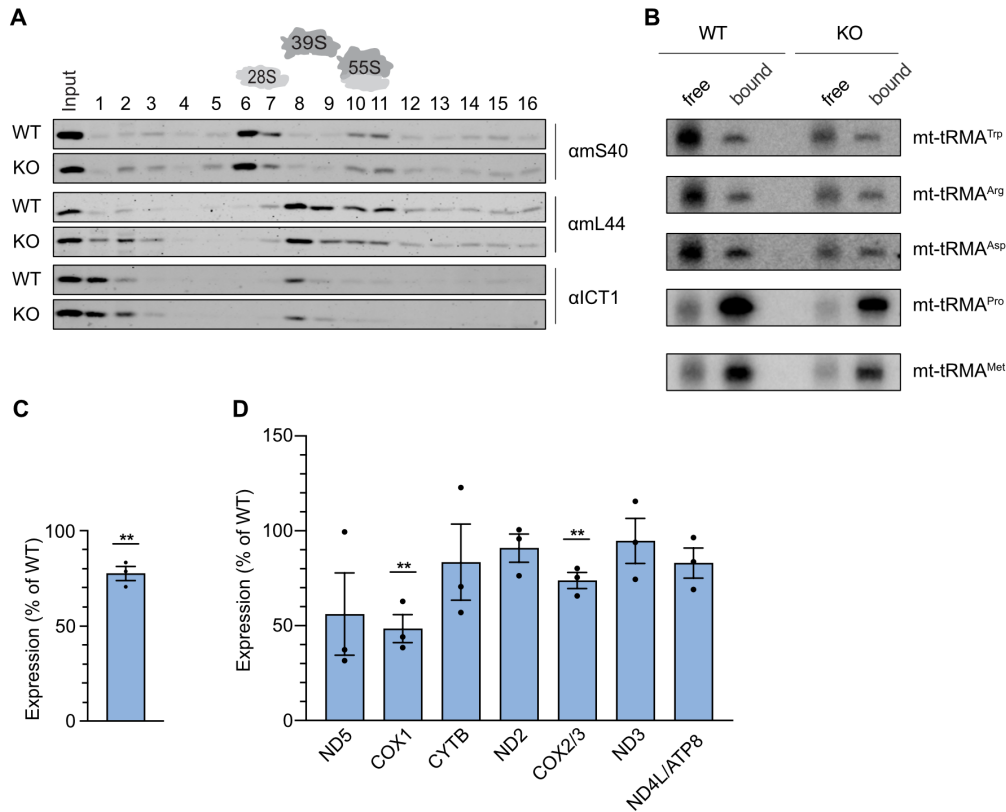
data are shown. **(B)** Anion-exchange HPLC analysis of the indicated mt tRNA^{Trp} 5' fragments, prepared by solid-phase synthesis with m²G phosphoramidite and ribozyme-mediated installation of m¹A. **(C)** Synthetic RNA oligonucleotides corresponding to the first 20 nucleotides of mt-tRNA^{Trp} with A9 or m¹A9 combined with G10 or m²G10 were incubated with an m²G-sensitive DNAzyme. Samples were separated by denaturing gel electrophoresis and RNAs were detected by SYBRGold staining. Note that i) m¹A9-containing RNAs migrate more slowly than A9-containing RNAs due to the positive charge of m¹A, and ii) during sample analysis, due to Dimroth rearrangement, a small fraction of m¹A may be converted to m⁶A; migration of m⁶A9-containing RNAs is indistinguishable from those containing A9. n=2 independent experiments were performed and representative data are shown (see also Fig. 3C for time course of cleavage by DNAzymes). **(D)** Small RNAs from wild-type (WT) and TRMT11^{KO} (KO) cells were left untreated (-) or subjected to DNAzyme-mediated cleavage (+) using DNAzymes designed to place A10 or G11 of mt-tRNA^{Met} in the active sites. mt-tRNA^{Met} was detected by northern blotting. n=3 independent experiments were performed and representative data are shown. **(E)** DNAzyme cleavage assays were performed as in (D) and Fig. 3E using DNAzymes targeting (m²)G10 of the indicated mt-tRNAs, and the corresponding mt-tRNAs were detected by northern blotting. n=3 independent experiments were performed and representative data are shown here; see Fig. 3F for quantification. **(F)** m²G10 stoichiometry of mt-tRNAs determined by DNAzyme-mediated cleavage of RNAs from TRMT11^{KO} vs. wild-type cells using the formula $(\text{KO}_{\text{cleavage}} - \text{WT}_{\text{cleavage}}) / \text{KO}_{\text{cleavage}} \times 100$.



Supplementary Figure S6. Interactions between G10, C25 and G45 of mt-tRNA^{Val}. (A) Three-dimensional model (AlphaFold) of mt-tRNA^{Val} highlighting the spatial arrangement of nucleotides G10, C25 and G45. (B) Magnified views of the interactions of G10 and C25 with G45 (upper) and A45 (lower). Dashed lines indicate hydrogen bonds.



Supplementary Figure S7. Ribozyme-mediated installation of m¹A9 in *in vitro* transcribed mt-tRNAs. Reverse transcription stop assays using unmodified and m¹A9-containing *in vitro* transcribed mt-tRNA^{Trp} (left) or mt-tRNA^{Lys} (right) as substrates. m¹A stalls reverse transcriptase progression leading to accumulation of cDNA fragments terminating one nucleotide before the modification site. The non-extended [³²P]-labeled primer was analyzed alongside as well as a sequencing ladder generated with chain terminating dideoxyTTP (ddT).



Supplementary Fig. S8. Mitochondrial translation in wild-type and TRMT11^{KO} cells. **(A)** Mitochondrial extracts from wild-type (WT) and TRMT11^{KO} cells were separated by sucrose density gradient centrifugation and proteins in each fraction were analyzed by western blotting using the antibodies to detect the indicated proteins. **(B)** Fractions containing non-mitoribosome-associated mt-tRNAs (1-2; free) and 55S monosomes (11-12; bound), respectively, were pooled and the indicated mt-tRNAs were analyzed by northern blotting. **(C, D)** Nascent mitochondrial proteins in mitochondria isolated from WT and TRMT11^{KO} cells grown in media containing glucose as a carbon source were labeled with [³⁵S]methionine, separated by denaturing PAGE and detected using a phosphorimager. The expression levels (collectively (C) and individually (D)) of *de novo* synthesized proteins in n=3 experiments were quantified and are shown as mean ± standard error in TRMT11^{KO} relative to WT. Statistical significance was calculated using an unpaired, two-sided Student's t-test; ***p* < 0.01.

SUPPLEMENTARY TABLES

Supplementary Table S1. Oligonucleotides used for molecular cloning.

Name	Sequence (5'-3')	Application
10456 pcDNA5-FLAG-TRMT11.fw	AGGGGCCCCTGCATCACCACCAT CACCATGGATCCGGTGGCGGTGG ATCGGGCGGTGGCGGATCGATG GCGCTGTCGTGTACCCT	Cloning into pcDNA5 plasmid
10457 pcDNA5-FLAG-TRMT11.rv	CGAGGCTGATCAGCGGGTTTAAA CGGGCCCTCTAGACTCGAGTCAT TCCTGGTGGATTTTCTTCCTTGG CAATTC	Cloning into pcDNA5 plasmid
10458 pcDNA5-TRMT11-FLAG.fw	GACTCTAGCGTTTAACTTAAGCT TGGTACCGCCACCATGGCGCTGT CGTGTACCCTTAACAG	Cloning into pcDNA5 plasmid
11200 pcDNA5-TRMT11-FLAG.rv	CGTGATGGTGTATGATGGTGGCTA GCTTCCTGGGTGGATTTTCTTCC TTGGC	Cloning into pcDNA5 plasmid
11248 pcDNA5-TRMT11-D244A-FLAG.fw	GCATATGTGTATGGGACAGCCAT AGACTACAACACAGTTC	Site-directed mutagenesis
11249 pcDNA5-TRMT11-D244A-FLAG.rv	GAAGTGTGTTGTAGTCTATGGCTG TCCCATAACACATATGC	Site-directed mutagenesis
11250 pcDNA5-TRMT11-D285R-FLAG.fw	GTTTAGAGAAGTATTACCTTCGTG TCCTGGTTTCAGATGCATC	Site-directed mutagenesis
11251 pcDNA5-TRMT11-D285R-FLAG.rv	GATGCATCTGAAACCAGGACACG AAGGTAATACTTCTCTAAAC	Site-directed mutagenesis
12535 pcDNA5-TRMT11-Δ2-67-FLAG.fw	CTCTAGCGTTTAACTTAAGCTTG GTACCGCCACCATGGTGTGTGCC AAGTCTATATTTG	Cloning into pcDNA5 plasmid
11697 pcDNA5-TRMT112-FLAG.fw	GTTTAACTTAAGCTTGGTACCGC CACCATGAACTGCTTACCCACAA TCTGCTGAG	Cloning into pcDNA5 plasmid
11698 pcDNA5-TRMT112-FLAG.rv	GTGATGGTGTATGATGGTGGCTAG CACTCTCAGTTTCTTCACTCA GCAG	Cloning into pcDNA5 plasmid
11742 pcDNA5-TRMT112-Δ2-23-FLAG.fw	GCGTTTAACTTAAGCTTGGTACC GCCACCATGCGCCTCCAGGCCAC CGAGGTCCGTATC	Cloning into pcDNA5 plasmid
12883 pcDNA5-COX8-1-30-Venus-N.fw1	GTGTTTAACTGGTTCTGCTCGTC GTTTACCTGTTCTCGTGCTAAAA TTCATTCTTTACCTGGGGCGGA GGTTCGGGGATGGTGAGCAAGG GCGAGGAGC	Cloning into pcDNA5 plasmid
12884 pcDNA5-COX8-1-30-Venus-N.rv	CTAGTGATCCGTGATGGTGTATGAT GGTGGCTAGCCTACTGCTTGTCTG GCGGTGATATAGACG	Cloning into pcDNA5 plasmid
12885 pcDNA5-COX8-1-30-Venus-N.fw2	GCCTCCGGACTCTAGCGTTTAAA CTTAAGCTTGGTACCGCCACCAT GTCTGTTTTAACTCCTTTATTATTA CGTGGTTTAACTGGTTCTGCTC	Cloning into pcDNA5 plasmid
11246 pClpuro-FLAG-TRMT11.fw	CTATAGGCTAGCCCTCGAGATGG ACTACAAAGACGATG	Cloning into pClpuro plasmid
11247 pClpuro-FLAG-TRMT11.rv	CTAAAGGGAAGCGGCCGAATTC ATTCCTGGGTGGATTTTTC	Cloning into pClpuro plasmid
11244 pClpuro-TRMT11-FLAG.fw	CTCACTATAGGCTAGCCCTCGAG AATTCGCCACCATGGCGCTGTCTG	Cloning into pClpuro plasmid
11245 pClpuro-TRMT11-FLAG.rv	CACTAAAGGGAAGCGGCCGAATT CTCGAGCTAACCTTTATCATCGTC	Cloning into pClpuro plasmid

12881 pClpuro-TRMT11-Venus-C.fw	GGAAGAAAAATCCACCCAGGAAG GGGGCGGAGGTTTCGGGGGGCGG AGGTTTCGAAGAACGGCATCAAGG CCAAC	Cloning into pClpuro plasmid
12882 pClpuro-TRMT11-Venus-C.rv	CATTAACCCTCACTAAAGGGAAGC GGCCGAATTCTCGAGCTACTTGTA CAGCTCGTCCATGCCG	Cloning into pClpuro plasmid
12880 pClpuro-TRMT11-Venus-C.rv2	GTTGGCCTTGATGCCGTTCTTCGA ACCTCCGCCCCCGAACCTCCGC CCCCTTCTGCGGTGGATTTTCTT CC	Cloning into pClpuro plasmid
12930 pClpuro-TRMT112-Venus-C.fw	GCTGAGTGAAGAGGAACTGAGA GTGGGGGCGGAGGTTTCGGGGGG CGGAGGTTTCGAAGAACGGCATCA AGGCCAAC	Cloning into pClpuro plasmid
12931 pClpuro-TRMT112-Venus-C.fw2	GACTCACTATAGGCTAGCCCTCG AGAATTCGCCACCATGAACTGCT TACCCACAATCTG	Cloning into pClpuro plasmid
12932 pClpuro-TRMT112-Venus-C.rv	GTTGGCCTTGATGCCGTTCTTCGA ACCTCCGCCCCCGAACCTCCGC CCCCACTCTCAGTTTCTTCTTCA TCAGC	Cloning into pClpuro plasmid
oMG731	GTATGGGACAGCCATAGACTACA ACACAGTTCATGG	Site-directed mutagenesis
oMG732	GTAGTCTATGGCTGTCCCATACAC ATATGCACC	Site-directed mutagenesis

Supplementary Table S2. Plasmids used in this study.

Name	Application
pMB2188 pcDNA5-TRMT11-His6-2xFLAG	Stable cell line generation
pMB2189 pcDNA5-TRMT11-D244A-His6-2xFLAG	Stable cell line generation
pMB2190 pcDNA5-TRMT11-D285R-His6-2xFLAG	Stable cell line generation
pMB2435 pcDNA5-TRMT11-Δ2-67-His6-2xFLAG	Stable cell line generation
pMB2283 pcDNA5-TRMT112-His6-2xFLAG	Stable cell line generation
pMB2284 pcDNA5-TRMT112-Δ2-23-His6-2xFLAG	Stable cell line generation
pMB2436 pcDNA5-COX8A ₁₋₃₀ -Venus-N ₁₋₁₅₈	Stable cell line generation
pMB2192 pClpuro-FLAG-TRMT11	Transient transfection
pMB2193 pClpuro-TRMT11-FLAG	Transient transfection
pMB2194 pClpuro-TRMT11-D244A-FLAG	Transient transfection
pMB2195 pClpuro-TRMT11-D285R-FLAG	Transient transfection
pMB2237 pClpuro-TRMT11-Venus-C ₁₅₉₋₂₃₉	Transient transfection
pMB2439 pClpuro-TRMT112-Venus-C ₁₅₉₋₂₃₉	Transient transfection
pFF6 pACYCDuet-1-HsTRMT112	Expression of recombinant proteins in <i>E. coli</i>
pMG715 pnEA-vH-HsTRMT11-His6 WT	Expression of recombinant proteins in <i>E. coli</i>
pMG1254 pnEA-vH-HsTRMT11-His6 D244A	Expression of recombinant proteins in <i>E. coli</i>

Supplementary Table S3. Antibodies used in this study

Name	Source	Dilution
Anti-FLAG M2 Mouse	Sigma (F3165)	1:10000
Anti-αTubulin Rabbit	Sigma (T6199)	1:3000
Anti-TRMT11 Rabbit	ProteinTech (17555-1-AP)	1:500
Anti-TRMT112 Mouse	Santa Cruz (sc-398481)	1:100
Anti-MFN2 Rabbit	ProteinTech (121861-1-AP)	1:600
Anti-mL44 Rabbit	ProteinTech (16394-1-AP)	1:5000
Anti-uL23m Rabbit	P. Rehling (4)	1:10000
Anti-TIM23 Rabbit	ProteinTech (11123-1-AP)	1:2500

Anti-TIM44 Rabbit	ProteinTech (13859-1-AP)	1:2000
Anti-mL62 (ICT1) Rabbit	ProteinTech (10403-1-AP)	1:750
IRDye 680LT Donkey anti-Mouse	LI-COR Biosciences (P/N: 926-68022)	1:10000
IRDye 800CW Donkey anti-Rabbit	LI-COR Biosciences (P/N: 925-32213)	1:10000
IRDye 680LT Goat anti-Mouse IG2a	LI-COR Biosciences (P/N: 926-68051)	1:10000

Supplementary Table S4. Oligonucleotides used as northern blot probes.

Detected RNA	Sequence (5'-3')
mt-tRNA ^{Trp}	TAAGTATTGCAACTTACTGAG
mt-tRNA ^{Arg}	AATATGATTATCATAATTTA
mt-tRNA ^{Ala}	CTGCAAAACCCCACTCTGCATCAA
mt-tRNA ^{Leu(UUR)}	AAGAGGAATTGAACCTCTGACTGT
mt-tRNA ^{Phe}	GGGGTGATGTGAGCCCGTCTAA
mt-tRNA ^{Val}	GGTCAAGTTAAGTTGAAATCTC
mt-tRNA ^{Lys}	TAAAGAGGTGTTGGTTCTCTTAATCTT
mt-tRNA ^{Gly}	TTTTTGAATGTTGTCAAACACTAGT
mt-tRNA ^{Tyr}	AAGAGGCCTAACCCCTGTCTTT
mt-tRNA ^{Thr}	GGAAAAAGGTTTTTCATCTC
mt-tRNA ^{Leu(CUN)}	ATTTGGAGTTGCACCAAATTTT
mt-tRNA ^{Asn}	CAATGGGACTTAAACCCACAAACACT
mt-tRNA ^{Asp}	ATATAGGATTTAGCCTATAATTTA
mt-tRNA ^{Glu}	GCACGGACTACAACCACGACCAA
mt-tRNA ^{Pro}	GAAAAAGTCTTTAACTCCACCATT
mt-tRNA ^{His}	TAAGGGGTCGTAAGCCTCTGTTGTC
mt-tRNA ^{Gln}	CTATGAGAATCGAACCCATCCCTG
mt-tRNA ^{Cys}	GGCAGGTTTGAAGCTGCTTCTT
mt-tRNA ^{Met(A10)}	GGGAAGGGTATAACCAACATTTTC
mt-tRNA ^{Met(G11)}	GGGAAGGGTATAACCAACATTTTC
Nu-tRNA ^{Met}	GGATGGTTTCGATCCATCGACCTC
5S rRNA	CCGAGATCAGACGAGATCGGGCGCGTTCAGGGTGGTATGG

Supplementary Table S5. Sequences of DNAzymes and disruptor oligonucleotides. Red indicates the m²G-sensitive catalytic sequence.

Target RNA	DNAzyme sequence (5'-3')	Disruptor sequence (5'-3')
mt-tRNA ^{Trp}	GGCTCTTGGTCTGTATTTAACTGTCAGCGACTCG AAAAATTTCT	TAAGTATTGCAACTTACTGA G
mt-tRNA ^{Arg}	AATCATTCGTTTTGTTTAAATGTCAGCGACTCGAA ATATACCA	AATATGATTATCATAATTTA
mt-tRNA ^{Ala}	AATCAGCCACTTTAATTAAGTGTGTCAGCGACTCGA AAAGCCCTT	CTGCAAAACCCCACTCTGC ATCAA
mt-tRNA ^{Leu(UUR)}	AGTTTTATGCGATTACCGGGCTCTGTGTGTCAGCGA CTCGAAATCTTAAC	AAGAGGAATTGAACCTCTGA CTGT
mt-tRNA ^{Phe}	AGTGTATTGCTTTGAGGAGGTAAGTGTGTCAGCGAC TCGAACATAAAC	GGGGTGATGTGAGCCCGTCT TAA
mt-tRNA ^{Val}	AGTTGGGTGCTTTGTGTTAAGTGTGTCAGCGACTCG AAACACTCTG	GGTCAAGTTAAGTTGAAATC TC
mt-tRNA ^{Lys}	AGGTTAATGCTAAGTTAGTGTGTCAGCGACTCGAAT TACAGTG	TAAAGAGGTGTTGGTTCTCT TAATCTT
mt-tRNA ^{Gly}	AGTTAACGGTACTATTTATATGTGTCAGCGACTCGA AAAAAGAGT	TTTTTGAATGTTGTCAAAC TAGT
mt-tRNA ^{Tyr}	AGTCCAATGCTTCACTCAGTGTGTCAGCGACTCGAA ATTTTACC	AAGAGGCCTAACCCCTGTCT TTT
mt-tRNA ^{Thr}	AGACTGGTGTATTAGTTTATATGTGTCAGCGACTCG AAACAAGGAC	GGAAAAAGGTTTTTCATCTC

mt-tRNA ^{Leu(CUN)}	AGACCAATGGATAGCTGTTATCTGTCAGCGACTC GAATTTAAAGT	ATTTGGAGTTGCACCAAAAT TTT
mt-tRNA ^{Asn}	AGCTAAGCACCCTAATCAACTGGTGTGTCAGCGACT CGAATCAATCTA	CAATGGGACTTAAACCCACA AACACT
mt-tRNA ^{Asp}	AAAGTTATGAAATGGTTTTTGTGTCAGCGACTCGA AAATACCTT	ATATAGGATTTAGCCTATAA TTTA
mt-tRNA ^{Glu}	AAACCATCGTTGTATTTCAATGTGTCAGCGACTCGA ACAAGAAC	GCACGGACTACAACCACGA CCAA
mt-tRNA ^{Pro}	AAGCTAAGATTCTAATTTAAATGTGTCAGCGACTCG AAATTCTCTG	GAAAAAGTCTTTAACTCCAC CATT
mt-tRNA ^{His}	AATCTGATGTTTTGGTTAAATGTGTCAGCGACTCGA AATATTTACC	TAAGGGGTCGTAAGCCTCT GTTGTC
mt-tRNA ^{Gln}	ATTCTCCGTGCCACCTATCACATGTGTCAGCGACTC GAACCATCCTA	CTATGAGAATCGAACCCATC CCTG
mt-tRNA ^{Cys}	AATTCATATGAAAATCATGTGTCAGCGACTCGAAT CGGAGCT	GGCAGGTTTGAAGCTGCTT CTT
mt-tRNA ^{Met} _A10	GGCCCGATAGCTTATTTAGCTGTGTCAGCGACTCGA AACCTTACT	GGGAAGGGTATAACCAACA TTTTC
mt-tRNA ^{Met} _G11	GGCCCGATAGCTTATTTAGTGTGTCAGCGACTCGAA GACCTTAC	GGGAAGGGTATAACCAACA TTTTC
nu-tRNA ^{iMet}	GGCCAGCACGCTTCCGCTGCGTGTGTCAGCGACT CGAAACTCTGCT	GGATGGTTTCGATCCATCG ACCTC

Supplementary Table S6. Oligonucleotides used for *in vitro* transcription template generation.

Name	Sequence (5'-3')	Application
mt-tRNA ^{Trp} _wt_ivt.sense	CTGTAATACGACTCACTATAGGAGAA ATTTAGGTTAAATACAGACCAAGAGC CTTCAAAGCCCTCAGTAAGTTGCAAT ACTTAATTTCTGcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp}
mt-tRNA ^{Trp} _wt_ivt.antisense	ggCAGAAATTAAGTATTGCAACTTACT GAGGGCTTTGAAGGCTCTTGGTCTG TATTTAACCTAAATTTCTCCTATAGTG AGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp}
mt-tRNA ^{Trp} _G10A_ivt.sense	CTGTAATACGACTCACTATAGGAGAA ATTTAAGTTAAATACAGACCAAGAGC CTTCAAAGCCCTCAGTAAGTTGCAAT ACTTAATTTCTGcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{G10A}
mt-tRNA ^{Trp} _G10A_ivt.antisense	tggCAGAAATTAAGTATTGCAACTTAC TGAGGGCTTTGAAGGCTCTTGGTCT GTATTTAACTTAAATTTCTCCTATAGT GAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{G10A}
mt-tRNA ^{Trp} _pre_ivt.sense	CTGTAATACGACTCACTATAGGcttatA GAAATTTAGGTTAAATACAGACCAAG AGCCTTCAAAGCCCTCAGTAAGTTGC AATACTTAATTTCTGtaaca	Template generation for <i>in vitro</i> transcription of pre-mt-tRNA ^{Trp}
mt-tRNA ^{Trp} _pre_ivt.antisense	tggttaCAGAAATTAAGTATTGCAACTTA CTGAGGGCTTTGAAGGCTCTTGGTC TGTATTTAACCTAAATTTCTataagCCT ATAGTGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of pre-mt-tRNA ^{Trp}
mt-tRNA ^{Trp} _-CCA_ivt.sense	CTGTAATACGACTCACTATAGGAGAA ATTTAGGTTAAATACAGACCAAGAGC CTTCAAAGCCCTCAGTAAGTTGCAAT ACTTAATTTCTG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{-CCA}
mt-tRNA ^{Trp} _-CCA_ivt.antisense	CAGAAATTAAGTATTGCAACTTACTG AGGGCTTTGAAGGCTCTTGGTCTGTA	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{-CCA}

	TTTAACCTAAATTTCTCCTATAGTGAG TCGTATTACAG	
mt-tRNA ^{trp} _C25U_ivt.antisense	tggCAGAAATTAAGTATTGCAACTTAC TGAGGGCTTTGAAGGCTCTTAGTCTG TATTTAACCTAAATTTCTCCTATAGTG AGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{C25U}
mt-tRNA ^{trp} _C32U_ivt.sense	CTGTAATACGACTCACTATAGGAGAA ATTTAGGTAAATACAGACCAAGAGC TTTCAAAGCCCTCAGTAAGTTGCAAT ACTTAATTTCTGcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{C32U}
mt-tRNA ^{trp} _C32U_ivt.antisense	tggCAGAAATTAAGTATTGCAACTTAC TGAGGGCTTTGAAAGCTCTTGGTCTG TATTTAACCTAAATTTCTCCTATAGTG AGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{C32U}
mt-tRNA ^{trp} _C32U,A38U_ivt.sense	CTGTAATACGACTCACTATAGGAGAA ATTTAGGTAAATACAGACCAAGAGC TTTCAATGCCCTCAGTAAGTTGCAAT ACTTAATTTCTGcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{C32U,A38U}
mt-tRNA ^{trp} _C32U,A38U_ ivt.antisense	tggCAGAAATTAAGTATTGCAACTTAC TGAGGGCATTGAAAGCTCTTGGTCT GTATTTAACCTAAATTTCTCCTATAGT GAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{C32U,A38U}
mt-tRNA ^{trp} _G45A_ivt.sense	CTGTAATACGACTCACTATAGGAGAA ATTTAGGTAAATACAGACCAAGAGC CTTCAAAGCCCTCAATAAGTTGCAAT ACTTAATTTCTGcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{G45A}
mt-tRNA ^{trp} _G45A_ivt.antisense	tggCAGAAATTAAGTATTGCAACTTATT GAGGGCTTTGAAGGCTCTTGGTCTG TATTTAACCTAAATTTCTCCTATAGTG AGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Trp} _{G45A}
mt-tRNA ^{val} _wt_ivt.sense	CTGTAATACGACTCACTATAGGCAGA GTGTAGCTTAACACAAAGCACCCAAC TTACACTTAGGAGATTTCAACTTAACT TGACCGCTCTGAcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val}
mt-tRNA ^{val} _wt_ivt.antisense	tggTCAGAGCGGTCAAGTTAAGTTGAA ATCTCCTAAGTGTAAGTTGGGTGCTT TGTGTTAAGCTACACTCTGCCTATAG TGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val}
mt-tRNA ^{val} _G10A_ivt.sense	CTGTAATACGACTCACTATAGGCAGA GTGTAAGTTAACACAAAGCACCCAAC TTACACTTAGGAGATTTCAACTTAACT TGACCGCTCTGAcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{G10A}
mt-tRNA ^{val} _G10A_ivt.antisense	tggTCAGAGCGGTCAAGTTAAGTTGAA ATCTCCTAAGTGTAAGTTGGGTGCTT TGTGTTAAGTTACACTCTGCCTATAG TGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{G10A}
mt-tRNA ^{trp} _pre_ivt.sense	CTGTAATACGACTCACTATCAGGacga acCAGAGTGTAGCTTAACACAAAGCA CCCAACTTACACTTAGGAGATTTCAA CTTAACCTTGACCGCTCTGAgctaaa	Template generation for <i>in vitro</i> transcription of pre-mt-tRNA ^{Val}
mt-tRNA ^{trp} _pre_ivt.antisense	ttagcTCAGAGCGGTCAAGTTAAGTTG AAATCTCCTAAGTGTAAGTTGGGTGC TTTGTGTTAAGCTACACTCTGgtcgtCC TATAGTGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of pre-mt-tRNA ^{Val}
mt-tRNA ^{val} _CCA_ivt.sense	CTGTAATACGACTCACTATAGGCAGA GTGTAGCTTAACACAAAGCACCCAAC TTACACTTAGGAGATTTCAACTTAACT TGACCGCTCTGA	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{CCA}

mt-tRNA ^{Val} _CCA_ivt.antisense	TCAGAGCGGTCAAGTTAAGTTGAAAT CTCCTAAGTGTAAGTTGGGTGCTTTG TGTTAAGCTACACTCTGCCTATAGTG AGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} -CCA
mt-tRNA ^{Val} _C25U_ivt.sense	CTGTAATACGACTCACTATAGGCAGA GTGTAGCTTAACACAAAGTACCCAAC TTACACTTAGGAGATTTCAACTTAACT TGACCGCTCTGAcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{C25U}
mt-tRNA ^{Val} _C25U_ivt.antisense	tggTCAGAGCGGTCAAGTTAAGTTGAA ATCTCCTAAGTGTAAGTTGGGTACTT TGTGTTAAGCTACACTCTGCCTATAG TGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{C25U}
mt-tRNA ^{Val} _C38U_ivt.sense	CTGTAATACGACTCACTATAGGCAGA GTGTAGCTTAACACAAAGCACCCAAC TTACATTTAGGAGATTTCAACTTAACT TGACCGCTCTGAcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{C38U}
mt-tRNA ^{Val} _C38U_ivt.antisense	tggTCAGAGCGGTCAAGTTAAGTTGAA ATCTCCTAAATGTAAGTTGGGTGCTT TGTGTTAAGCTACACTCTGCCTATAG TGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{C38U}
mt-tRNA ^{Val} _C32A,C38U_ivt.sense	CTGTAATACGACTCACTATAGGCAGA GTGTAGCTTAACACAAAGCACCCAAT TTACAATTAGGAGATTTCAACTTAACT TGACCGCTCTGAcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{C32A,C38U}
mt-tRNA ^{Val} _C32A,C38U_ ivt.antisense	tggTCAGAGCGGTCAAGTTAAGTTGAA ATCTCCTAATTGTAAGTTGGGTGCTT TGTGTTAAGCTACACTCTGCCTATAG TGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{C32A,C38U}
mt-tRNA ^{Val} _G5A_ivt.sense	CTGTAATACGACTCACTATAGGCAGA ATGTAGCTTAACACAAAGCACCCAAC TTACACTTAGGAGATTTCAACTTAACT TGACCGCTCTGAcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{G5A}
mt-tRNA ^{Val} _G5A_ivt.antisense	tggTCAGAGCGGTCAAGTTAAGTTGAA ATCTCCTAAGTGTAAGTTGGGTGCTT TGTGTTAAGCTACATTCTGCCTATAG TGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{G5A}
mt-tRNA ^{Val} _A31G_ivt.sense	CTGTAATACGACTCACTATAGGCAGA GTGTAGCTTAACACAAAGCACCCAG CTTACACTTAGGAGATTTCAACTTAA CTTGACCGCTCTGAcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{A31G}
mt-tRNA ^{Val} _A31G_ivt.antisense	tggTCAGAGCGGTCAAGTTAAGTTGAA ATCTCCTAAGTGTAAGCTGGGTGCTT TGTGTTAAGCTACACTCTGCCTATAG TGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{A31G}
mt-tRNA ^{Val} _G45A_ivt.sense	CTGTAATACGACTCACTATAGGCAGA GTGTAGCTTAACACAAAGCACCCAAC TTACACTTAGGAAATTTCAACTTAACT TGACCGCTCTGAcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{G45A}
mt-tRNA ^{Val} _G45A_ivt.antisense	tggTCAGAGCGGTCAAGTTAAGTTGAA ATTCCTAAGTGTAAGTTGGGTGCTT TGTGTTAAGCTACACTCTGCCTATAG TGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Val} _{G45A}
mt-tRNA ^{Pro} _wt_ivt.sense	CTGTAATACGACTCACTATAGGCAGA GAATAGTTTAAATTAGAATCTTAGCTT TGGGTGCTAATGGTGGAGTTAAAGA CTTTTCTCTGAcca	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Pro}
mt-tRNA ^{Pro} _wt_ivt.antisense	tggTCAGAGAAAAAGTCTTTAACTCCA CCATTAGCACCCAAAGCTAAGATTCT AATTTAACTATTCTCTGCCTATAGTG AGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Pro}

mt-tRNA ^{Pro} _{U25C} _ivt.sense	CTGTAATACGACTCACTATAGGCAGAGAATAGTTTAAATTAGAACCTTAGCTTTGGGTGCTAATGGTGGAGTTAAAGACTTTTTCTCTGA _{Acca}	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Pro} _{U25C}
mt-tRNA ^{Pro} _{U25C} _ivt.antisense	tggTCAGAGAAAAAGTCTTTAACTCCA CCATTAGCACCCAAAGCTAAGGTTCTAATTTAACTATTCTCTGCCTATAGTGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Pro} _{U25C}
mt-tRNA ^{Pro} _{U32C,U38A} _ivt.sense	CTGTAATACGACTCACTATAGGCAGAGAATAGTTTAAATTAGAACTTAGCCTTGGGAGCTAATGGTGGAGTTAAAGACTTTTTCTCTGA _{Acca}	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Pro} _{U32C,U38A}
mt-tRNA ^{Pro} _{U32C,U38A} _ivt.antisense	tggTCAGAGAAAAAGTCTTTAACTCCA CCATTAGCTCCCAAGGCTAAGATTCTAATTTAACTATTCTCTGCCTATAGTGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Pro} _{U32C,U38A}
mt-tRNA ^{Pro} _{U25C,U32C,U38A} _ivt.sense	CTGTAATACGACTCACTATAGGCAGAGAATAGTTTAAATTAGAACCTTAGCCTTGGGAGCTAATGGTGGAGTTAAAGACTTTTTCTCTGA _{Acca}	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Pro} _{U25CU32C,U38A}
mt-tRNA ^{Pro} _{U25C,U32C,U38A} _ivt.antisense	tggTCAGAGAAAAAGTCTTTAACTCCA CCATTAGCTCCCAAGGCTAAGGTTCTAATTTAACTATTCTCTGCCTATAGTGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of mt-tRNA ^{Pro} _{U25CU32C,U38A}
D2162 mt-tRNA-Lys _{rv} primer	TGGTCACTGTAAAGAGGTGTTGGTTCCTTAATCTTTAACTTAAAAG	Template generation for <i>in vitro</i> transcription of (m ¹)A9-containing mt-tRNA ^{Lys}
D2161 mt-tRNA-Lys _{fw} primer	CTGTAATACGACTCACTATAGGCACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAAAGATT	Template generation for <i>in vitro</i> transcription of (m ¹)A9-containing mt-tRNA ^{Lys}
D4520 mt-tRNA-Trp _{rv} primer	TGGCAGAAATTAAGTATTGCAACTTACTGAGGGCTTTGAAGGCTCTTGGTCT	Template generation for <i>in vitro</i> transcription of (m ¹)A9-containing mt-tRNA ^{Trp}
D4521 mt-tRNA ^{Trp} _{fw} primer	CTGTAATACGACTCACTATAGGAGAAATTTAGGTAAATACAGACCAAGAGCCTTCAAA	Template generation for <i>in vitro</i> transcription of (m ¹)A9-containing mt-tRNA ^{Trp}
D4522 MTR1_mt-tRNA ^{Trp} A9	AGAAATTTTATGTCTAGTTGTCCCCGAGCGAACTCGGGGGGTGCGTCAGGTAAATACCTATAGTGAGTCGTATTACAG	Template generation for <i>in vitro</i> transcription of MTR1 targeting A9 of mt-tRNA ^{Trp}
D4534 MTR1_mtRNA ^{Lys} A9 _{fw} primer	CTGTAATACGACTCACTATAGGAAGTAGCTGACCGACCCCCCGAGTTCGC	Template generation for <i>in vitro</i> transcription of MTR1 targeting A9 of mt-tRNA ^{Lys}
D4535 MTR1_mtRNA ^{Lys} A9 _{rv} primer	CACTGTAATATGTCTAGTTGTCCCCGAGCGAACTCGGGGGGTGCGTCAGCTAACTT	Template generation for <i>in vitro</i> transcription of MTR1 targeting A9 of mt-tRNA ^{Lys}

SUPPLEMENTARY METHODS

Small RNA-seq-based analysis of mt-tRNA levels

Total RNA extracted from HEK293 Flp-In cells using TRI reagent (Sigma Aldrich) was treated with Turbo DNase (Thermo Fisher Scientific), and after re-isolation using the RNA Clean & Concentrator-5 kit (Zymo Research) following the manufacturer's instructions, small RNA-seq library preparation was performed using the Qiagen miRNA Library Kit according to the manufacturer's instructions. Library concentrations were determined using a Qubit fluorometer (Thermo Fisher Scientific), and Illumina next-generation sequencing was carried out at the NGS Integrative Genomics Core Unit (University Medical Center Göttingen) on a NovaSeq6000. Fastq files were quality checked using FastQC (v0.11.0) before merging of paired-end reads using PEAR (v0.9.6) with the settings: -p 0.05 -n 4 -v 6. Assembled reads were adapter- and quality-trimmed using Flexbar (v3.5.0) with the settings: i1.8 -qt 13 -u 0 -m 15 -n 8 -at RIGHT -z GZ -o -ao 2 -ae 0.1 -as AACTGTAGGCACCATCAAT. Filtered reads were aligned to the human genome supplemented with a custom pre-rRNA reference using STAR (v2.7.11a) with following settings --outFilterMultimapNmax 450, --winAnchorMultimapNmax 450 --outSAMmultNmax -1 --scoreDelOpen -2 --scoreDelBase -2 -outFilterMismatchNoverReadLmax 0.04 --outSAMtype BAM SortedByCoordinate --outMultimapperOrder Random --outFilterType BySJout --alignSJoverhangMin 8 --alignSJDBoverhangMin 1--outSJfilterOverhangMin -1 -1 -1 -1 --outSJfilterCountTotalMin -1 -1 -1 -1 --outSAMattributes NH HI AS MD nM --alignEndsType Local --seedSearchStartLmaxOverLread 0.5 --outFilterScoreMinOverLread 0 --outFilterMatchNminOverLread 0.5. Bam files were indexed and distributions of reads across different RNA species was determined by featureCounts (v2.0.0) with the settings: -s 1 -t exon -g gene_type -M --largestOverlap --fracOverlap 0.8 --fraction. Counting was performed using the comprehensive GENCODE gene annotation v47, supplemented with the corresponding GENCODE tRNA annotation in GTF format. The two annotation files were combined, and the feature type of tRNA entries was annotated as exon to ensure compatibility with featureCounts and to minimize ambiguous assignment to intronic regions. Assigned reads were analyzed and visualized in R.

Reverse transcription stop assays

For analysis of tRNA methylation state by reverse transcription stop assay, the respective primers were first 5'-end labelled with ³²P by mixing 150-200 pmol of DNA oligonucleotides (mt-tRNA^{Trp} 5'-TTTGAAGGCTCTTGGTCT-3'; mt-tRNA^{Lys} 5'-TTAAAAGGTTAATGCTAA-3') with 5 µCi γ-[³²P]-ATP and 5 U of T4 PNK in 10 µL of 1x PNK buffer (70 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM DTT). The reaction mixture was

incubated for 1-1.5 h at 37 °C followed by two rounds of ethanol precipitation to remove excess of γ -[³²P]-ATP.

4 pmol of the methylated or unmodified, purified tRNA and 100 IPS (impulses per second; approx. 4 pmol) of the respective 5'-[³²P]-labeled primer were annealed (3 min at 95 °C, 10 min at 25 °C) in 5 μ L 1x annealing buffer (5 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). 5 mM DTT, 0.5 mM of each dNTP and 0.25 μ L MMLV RT (0.5 mg/mL) (5) were added to give a total volume of 10 μ L reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂). After 15 min incubation at 42 °C, 1 μ L 2 N NaOH was added, followed by 5 min incubation at 95 °C to degrade the RNA. Sequencing ladders were generated analogously using unmodified tRNA together with dNTP/ddNTP mixtures containing 0.5 mM ddTTP, 0.05 mM dTTP, 0.5 mM each of the other three dNTPs. The reaction products were recovered by ethanol precipitation, dissolved in loading dye, resolved by denaturing PAGE and visualized by digital autoradiography using a Typhoon Phosphorimager (Cytiva).

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