

RESEARCH PAPER



Mouse *Trmt2B* protein is a dual specific mitochondrial methyltransferase responsible for m⁵U formation in both tRNA and rRNA

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ABSTRACT

RNA molecules of all species contain modified nucleotides and particularly m⁵U residues. The vertebrate mitochondrial small subunit rRNA contains m⁵U nucleotide in a unique site. In this work we found an enzyme, TRMT2B, responsible for the formation of this nucleotide and m⁵U residues in a number of mitochondrial tRNA species. Inactivation of the *Trmt2B* gene leads to a reduction of the activity of respiratory chain complexes I, III and IV, containing the subunits synthesized by the mitochondrial protein synthesis apparatus. Comparative sequence analysis of m⁵U-specific RNA methyltransferases revealed an unusual evolutionary pathway of TRMT2B formation which includes consecutive substrate specificity switches from the large subunit rRNA to tRNA and then to the small subunit rRNA.

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Introduction

RNA molecules of all living organisms contain nearly two hundred kinds of modified nucleotides, the majority being methylated [1]. An emerging field of epitranscriptomics [2–4] addresses the significance of RNA modifications in normal and pathological [5–7] organism functioning. Mapping of modified residues within the predominant types of functional RNA molecules of the model species has been essentially completed [8,9], see [10–13] for reviews. The major quest to match existing modification sites with possible RNA modification enzyme candidates has already been finished for *E. coli* [14] and yeast [10] rRNA methyltransferases. At the same time, the annotation of mammalian RNA modification machinery is still underway [10,11].

Methylated nucleotides within the ribosomal RNA molecules are clustered in the ribosomal functional centres [11,15,16], mammalian ribosomes being no exception. In the course of evolution, some modification sites were preserved most likely from the last universal common ancestor, while other sites are lineage specific. Multiple events of convergent and divergent evolution were proposed for the enzymes involved in methylation of ribosomal RNA and other RNA types involved in translation, such as transfer RNA (see [11] for discussion). In a number of cases RNA methyltransferases were proposed to acquire a new RNA target, e.g. bacterial RlmN was shown to modify both 23S rRNA [17] and tRNA [18]. Mammalian methyltransferase METTL16, which is a

likely homolog of bacterial 23S rRNA methyltransferase RlmF [19] modifies U6 snRNA and pre-mRNAs [20]. Dual specificity towards mitochondrial 16S rRNA [21] and tRNA [22] arose in the evolution of the TRMT61B enzyme.

Vertebrate mitochondrial ribosomal RNA molecules possess a number of distinct methylated residues [4,13,23–25] largely reminiscent of those found in other types of ribosomes [11]. Only two residues, m¹A947 [26] of 16S rRNA and m⁵U429 [24] of 12S rRNA (human numbering) could only be found in the ribosomes of vertebrate mitochondria. An enzyme responsible for the modification of the former, TRMT61B [22], is a likely product of ancestral tRNA-specific methyltransferase gene duplication. An enzyme responsible for the formation of the m⁵U429 nucleotide of 12S rRNA was unknown, while TRMT2B was recently suggested to be responsible for the formation of m⁵U54 in human mitochondrial tRNAs [27].

In this work we pursued an idea that an enzyme capable of methylating 12S rRNA nucleotide U429 evolved from an ancestor methyltransferase responsible for the generation of m⁵U residue in other types of RNA. This idea led us to the characterization of mouse protein TRMT2B as a dual specificity RNA methyltransferase responsible for the generation of m⁵U425 (mouse numbering, equivalent to human m⁵U429) residue of the mitochondrial 12S rRNA (Fig. 1A) and m⁵U54 residue in a number of mitochondrial tRNAs (Fig. 2A).

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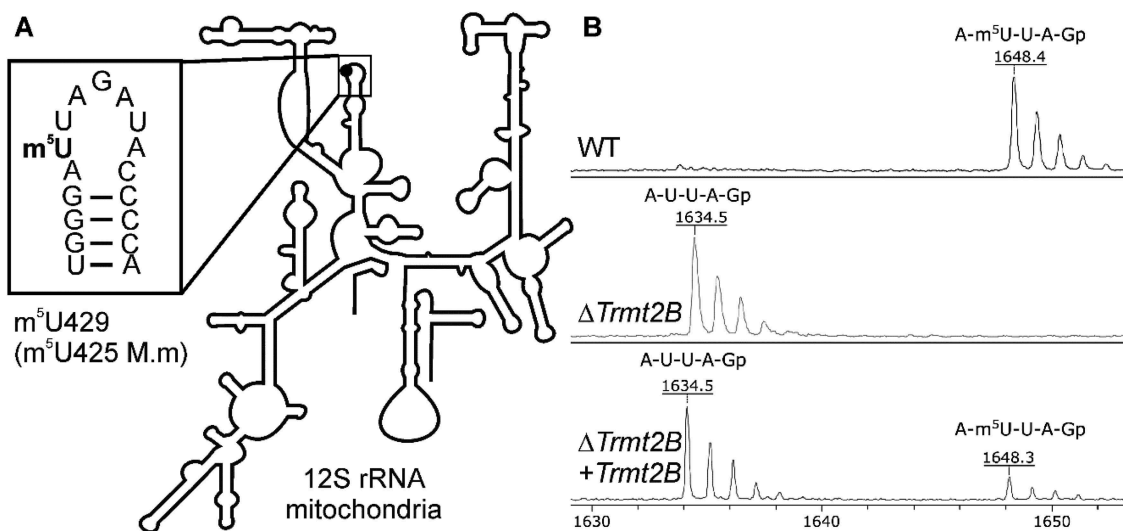


Figure 1. TRMT2B forms methylated m^5U425 residue of mouse mitochondrial 12S rRNA. (A) Secondary structure of mouse mitochondrial 12S rRNA [65]. Inset the closeup of the loop of the rRNA helix 24 containing the methylated residue. (B) Mass spectra of the 12S rRNA fragment with U425 nucleotide digested by RNase T1. The upper panel corresponds to wild type cells, the middle panel corresponds to Trmt2B knockout cells, while the lower panel corresponds to the complementation of Trmt2B knockout cells by a reintroduced Trmt2B gene. Mass of 1648 Da corresponds to fully methylated fragment, 1634 Da – fragment without methyl group.

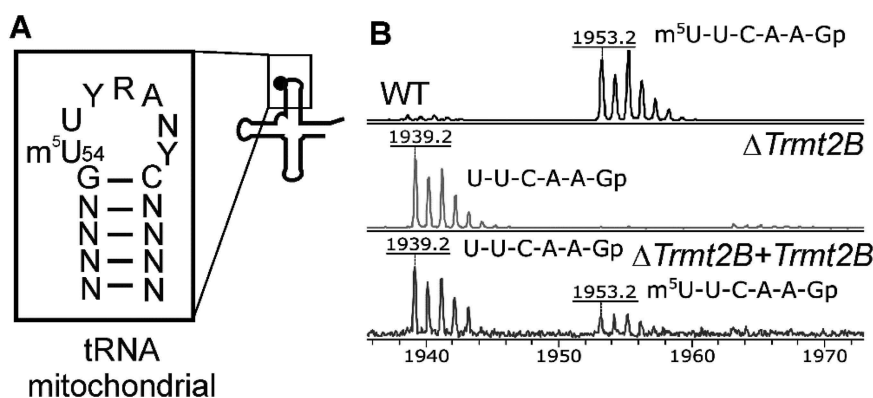


Figure 2. TRMT2B forms methylated m^5U54 residue of mouse mitochondrial $tRNA^{le}$. (A) Secondary structure of mouse mitochondrial $tRNA^{le}$ [41]. Inset the closeup of the T-loop consensus of mouse mitochondrial tRNA species containing the methylated residue m^5U54 . (B) Mass spectra of mouse mitochondrial $tRNA^{le}$ fragment with U54 nucleotide digested by RNase T1. The upper panel corresponds to wild type cells, the middle panel corresponds to Trmt2B knockout cells, while the lower panel corresponds to the complementation of Trmt2B knockout cells by a reintroduced Trmt2B gene. Mass of 1953 Da corresponds to a fully methylated fragment, 1939 Da – a fragment without a methyl group.

Results

Searching for possible mammalian m^5U RNA methyltransferases

Two possible mechanisms are documented for m^5U formation in RNA. The major pathway involves Rossman fold S-adenosyl-L-methionine (SAM) dependent methyltransferases [28], while an alternative reaction makes use of N^5,N^{10} -methylene-tetrahydrofolate (MTHF) as a cofactor to introduce an m^5U modification into tRNA of Gram-positive bacteria [29,30] or 23S rRNA of mycoplasma [31]. A homology search [32] for MTHF dependent RNA modification enzymes among mice or human proteins revealed only homologs of yeast MTO1 [33] involved in the formation of a hypermodified τm^5s^2U residue in the anticodon of mitochondrial tRNAs [34] and thus unlikely to be involved in formation of m^5U residues due to the significant difference in the chemical reaction mechanisms.

A search for possible Rossman fold m^5U RNA methyltransferases in the set of mice or human proteins revealed two possible candidates for making methylated residue in the mitochondrial 12S rRNA, TRMT2A and TRMT2B. The former, TRMT2A, was recently described as a protein with nuclear localization likely to be responsible for the formation of m^5U54 residue within cytoplasmic tRNAs [35]. In contrast to TRMT2A, TRMT2B is likely to be localized in mitochondria [36,37]. Thus, we considered TRMT2B protein as a primary candidate for the putative methyltransferase forming m^5U425 12S rRNA residue in the mouse mitochondrial ribosome.

TRMT2B methylates mitochondrial 12S rRNA

A model cell line for analysis of the *Trmt2B* gene's function was chosen on the basis of *Trmt2B* gene expression among a

number of mouse cell lines, such as NIH 3T3, NS0, Hepa1-6 and Aml12 using RT qPCR (SI Appendix, Fig. S1). An NS0 mouse myeloma cell line was used for further experimental analysis due to the highest *Trmt2B* gene expression and the ease of producing large amounts of biomass.

To investigate if TRMT2B methylates 12S rRNA we inactivated the *Trmt2b* gene in the NS0 mouse myeloma cell line using CRISPR/Cas9 system [38] to introduce double strand break in the first coding exon and selected clones with frameshift mutations generated due to the non-homologous end joining (NHEJ). As a result we obtained two independent cell lines with different sets of frameshift mutations in the *Trmt2b* gene (Fig. S2). To be sure that the phenotype of the knockout cell lines was not a result of the unlikely event of an off-target genome cleavage, we also checked 5 off-target sites (SI Appendix, Table S1) with the highest score according to Benchling CRISPR guide RNA designing tool (<https://benchling.com>) and found out there were no differences between WT and KO cell lines in possible off-target cleavage sites (Fig. S3).

To check whether mitochondrial 12S rRNA remained methylated at the U425 site upon *Trmt2b* inactivation, we prepared the total RNA from the wild type and KO cells and used it for affinity purification of the 12S rRNA fragment. To this end, we designed a 5'-biotinylated oligodeoxyribonucleotide complementary to region 423–460 nucleotides of the 12S rRNA (SI Appendix, Table S2), hybridized it with the total RNA sample and cleaved the excess unprotected RNA with RNase T1. The biotinylated DNA/RNA duplex was bound to streptavidin sepharose and later denatured to release the 12S rRNA fragment containing the site of modification. The fragment was cleaved by a guanilate-specific RNase T1 and the resulting short oligonucleotides were analysed by MALDI mass spectrometry (Fig. 1B). The mass peak corresponding to the fragment containing U425 from the Δ *Trmt2B* cell line (Fig. 1B, middle panel, 1634 Da peak) appeared to be 14 Da lighter than the one from WT cell line (Fig. 1B, upper panel, 1648 Da peak), which indicates a lack of methylation due to *Trmt2B* gene inactivation.

To complement the inactivation of the *Trmt2B* gene in the knockout cell line, we cloned *Trmt2B* cDNA to the vector [39] capable of *Sleeping beauty* transposase [40]-driven incorporation into the genome. Complementation of the *Trmt2B* gene knockout cells resulted in a partial restoration of the 12S rRNA residue m⁵U425 modification (Fig. 1B, lower panel). Incompleteness of the complementation in our view might be explained either by suboptimal functionality of the TRMT2B protein produced from the artificial construct or by expression of the construct in only a subset of cells in a population.

TRMT2B methylates several mitochondrial tRNAs

Due to structural similarity of TRMT2B with tRNA specific methyltransferases of the TrmA/Trm2 family we hypothesized that TRMT2B may be capable to methylate the T-loop of mitochondrial tRNAs (Fig. 2A). To test this hypothesis we isolated mitochondrial tRNA^{Ile} from the wild type, *Trmt2B* knockout cell lines and the cell line where *Trmt2B* knockout was partially complemented by reintroduction of *Trmt2B*

gene (Fig. 2B). As a result we saw that methylation of m⁵U54 of the mitochondrial tRNA^{Ile} observed in the wild type cells (Fig. 2B, upper panel, 1953 Da peak) disappeared upon *Trmt2B* gene inactivation (Fig. 2B, middle panel, 1939 Da peak). Complementation of the *Trmt2B* gene knockout cells resulted in a partial restoration of the tRNA^{Ile} modification (Fig. 2B, lower panel).

Next, we addressed the question whether TRMT2B is responsible for modification of other mitochondrial tRNA T-loops. Sequences of mitochondrial tRNAs are less conserved than those of cytoplasmic or bacterial tRNAs [41]. Only four of them, namely tRNA^{Ile}, tRNA^{Gln}, tRNA^{Leu}_(tm⁵UAA) and tRNA^{Ser}_(UGA) have a 'canonical' UUC patch at the 5'-side of the T-loop, while other tRNAs diverge from the T-loop consensus in both the length and composition up to a complete lack of the U54 nucleotide. We managed to check for the m⁵U54 modification in 10 out of 12 mitochondrial tRNAs that contain the U54 nucleotide (Table 1, Fig. S4). We also analysed possible T-loop methylation of tRNA^{Gly} that lacks U54 to make sure that TRMT2B does not methylate other nucleotides of the T-loop. Other mitochondrial tRNAs either lacked U54 or were not amenable to the mass-spectrometry based analysis method we used.

As a result we demonstrated that most of the mitochondrial tRNAs whose T-loop starts with UU are methylated by TRMT2B, while those whose T-loop starts with UC or lack U54 are not (Table 1, Fig. S4). Exceptions are serine specific mitochondrial tRNAs. Partial methylation was observed for tRNA^{Ser}_(UGA), while tRNA^{Ser}_(GCU) was shown to lack m⁵U54 methylation. As a control, we also analysed m⁵U methylation of cytoplasmic tRNA^{Sec} and tRNA^{Gly} and found that TRMT2B is not responsible for methylation of those tRNAs.

Lack of TRMT2B influences activity of ETC complexes I, III and IV

To analyse an influence of *Trmt2B* gene inactivation on the function of mitochondria we performed a number of assays. The amount of mitochondrial DNA in a cell is a widely used proxy for the mitochondrial content [42]. The number of mitochondria in the wild type cells and cells with inactivated *Trmt2b* gene was compared by assessment of the amount of

Table 1. m5U methylation of mito-tRNA T-loop by TRMT2B protein.

	tRNA amino acid	T-loop	m5U methylation by TRMT2B
T-loop starts with UUC	Ile	UUCAAGC	+
	Gln	UUCAAUU	+
	Leu(tm ⁵ UAA)	UUCAAAU	+
	Ser(UGA)	UUCGAUU	partial
T-loop starts with UU, but the third nucleotide is not C	Tyr	UUUAAAU	+
	Asn	UUUAAUU	+
	Met	UUUAAAU	+
	Ser(GCU)	UUUAAAAA	-
	Glu	UUGAAUG	not analysed
T-loop starts with U, but the second nucleotide is not U	Thr	UCUUC	-
	Asp	UCAAUAA	-
	Arg	UGAUGU	not analysed
	Leu(UAG)	UGCAAAU	not analysed
T-loop doesn't start with U	Gly	AUAAAC	-
	Ala, Cys, His, Lys, Phe, Pro, Trp, Val		not analysed

mitochondrial DNA with the help of qPCR (Fig. S5A) and found to be the same. Likewise to check whether inactivation of the *Trmt2B* gene would result in the decrease in the 12S rRNA abundance we used RT qPCR (Fig. S5B) and found no significant difference.

The role of the TRMT2B methyltransferase in the efficiency of mitochondrial protein synthesis was assessed via [³⁵S]methionine incorporation into the proteins synthesized by the parental NS0 cells and *Trmt2b* knockout cells, while protein synthesis by the cytoplasmic ribosomes is inhibited by cycloheximide. No significant difference in the radioactive methionine inclusion into proteins synthesized by a mitochondrial protein synthesis apparatus was revealed (Fig. S5C).

While the yield of proteins made with the help of mitochondrial translation machinery was not affected by inactivation of the *Trmt2B* gene, the activity of the synthesized proteins might be compromised, e.g. if the fidelity of protein synthesis or the folding of nascent proteins is affected. The mammalian mitochondrial genome encodes 13 proteins, all of them being subunits of electron transfer chain (ETC) complexes. To this end we examined ETC complexes' activity by measuring oxygen consumption in permeabilized cells while being sequentially supplied with appropriate substrates and, later, inhibitors of specific respiratory chain complexes. This way we revealed a small, but statistically significant decrease in the activity of ETC complexes I, III and IV, the ones that contain subunits synthesized in mitochondria. At the same time, the activity of complex II, which is composed entirely of subunits encoded by a nuclear genome was found not to depend on the *Trmt2B* gene's inactivation (Fig. 3).

Discussion

The mitochondrial ribosome most likely evolved from the ribosome of a bacterial endosymbiont of the predecessor of

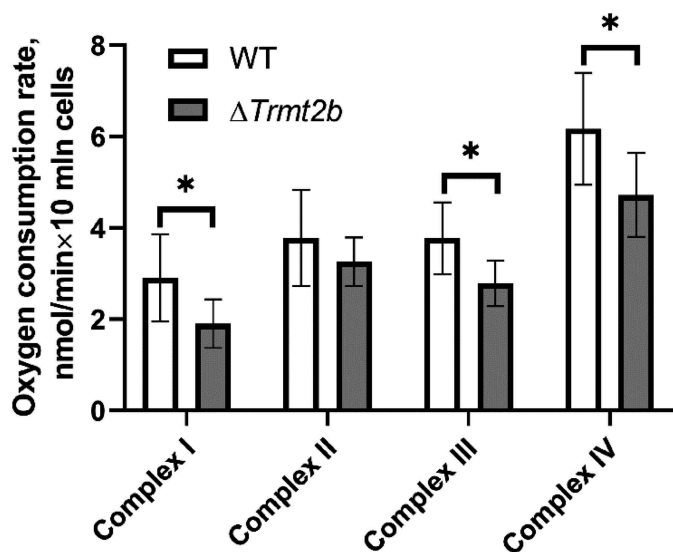


Figure 3. TRMT2B inactivation leads to statistically significant reduction of the respiratory chain complexes I, III and IV activity. Shown are oxygen consumption rates of the permeabilized wild type (open bars) and *Trmt2B* knockout (grey bars) cells. Substrates and inhibitors of the complexes I-IV were added sequentially, see materials and methods.

eukaryotic cells [43]. In the process of evolution, the mitochondrial ribosome underwent a significant reduction of its RNA components concomitant with a swelling of ribosomal proteins [44,45]. Likely, because of that, a set of modified nucleotides of the mitochondrial ribosome, particularly mammalian mitoribosome [4,11,13,23,24], resembles a subset of those in a bacterial ribosome (Table 2). Unique for the mitochondrial rRNA are modified nucleotides m¹A947 [26] of the 16S rRNA and m⁵U429 [24] of the 12S rRNA (human numbering), both modified with dual-specific rRNA/tRNA methyltransferases. Curiously, in our analysis of tRNA T-loop modification we observed a lack of m¹A58 modification in mouse mitochondrial tRNA^{Leu}_(tm5UAA), which was previously reported to be methylated by TRMT61B in human cells [21]. This apparent discrepancy is explained by the lack of *Trmt61B* gene functionality in mice [46] which is remarkably accompanied by the substitution of its target nucleotide m¹A947 of the mitochondrial 16S rRNA to uridine.

Now, when the search for mammalian mitochondrial rRNA methyltransferases has been completed with this work and the work of Powell and Minczuk [49] and identification of METTL15 as m⁴C-specific 12S rRNA methyltransferase (REF [50] and Laptev et al., under revision) we can compare the essentiality of all the enzymes from the list (Table 2). On the average, the more conserved mitochondrial rRNA methyltransferases are the more severe is the phenotype of their knockout. The universally conserved TFB1M [51] and MRM2 [52,53] proteins which belong to the families of bacterial KsgA [54] and RlmE [55,56] methyltransferases are important for mitochondrial ribosome assembly and translation [53,57]. The MRM3 protein responsible for the modification of nucleotide Gm1370 of the mitochondrial 16S rRNA A-loop, next to the Um1369 nucleotide modified by MRM2, is also important, since its deficiency results in a marked decrease of mitochondrial translation [52]. Modification of the 16S rRNA P-loop by MRM1 [52] appears to be less crucial [58], and likewise modification of the bacterial P-loop by its ortholog, RlmB [59]. Similarity in the significance of bacterial rRNA methyltransferases and their mitochondrial counterparts does not always hold true. E.g. a lack of bacterial RsmF protein responsible for the formation of the m⁵C1407 nucleotide of 16S rRNA does not have momentous consequences beyond moderate growth

Table 2. Enzymes responsible for the modification of mammalian mitochondrial rRNA.

12S rRNA	Enzyme/reference	Bacterial homolog
m ⁵ U429(m ⁵ U425)	TRMT2B (this study)	no
m ⁴ C839	METTL15[50] and Laptev et al, in revision	RsmH[47]
m ⁵ C841	NSUN4[60]	RsmF**[48]
m ₂ ⁶ A936, m ₂ ⁶ A937	TFB1M[51]	KsgA[54]
16S rRNA		
m ¹ A947	TRMT61B[22]	no
Gm1145	MRM1[52]	RlmB[59]
Um1369	MRM2[52]	RlmE[55,56]
Gm1370	MRM3[52]	no

*Human numbering, mouse numbering is provided in parenthesis.

***E.coli* RsmF modifies the nucleotide m⁵C1407 of the 16S rRNA [48], which is proximal to C1404, equivalent to m⁵C841.

retardation, while inactivation of its mitochondrial ortholog NSUN4 is embryonically lethal due to mitochondrial ribosome instability and ETC complexes' assembly failure [60]. In addition to its function as a modification enzyme, NSUN4 is known to form a stable complex with the mitochondrial ribosome assembly factor MTERF4, thus playing a role in both ribosome modification and assembly [61,62]. Not all mitochondrial rRNA methyltransferase knockouts resulted in spectacular phenotypes. E.g. a deficiency of TRMT61B [22] is likely to manifest itself in some specific and yet to be established conditions. Inactivation of TRMT2B, as we demonstrated in this work, resulted in statistically significant reduction in the activity of ETC complexes containing mitochondrially encoded subunits. However, the consequences of TRMT2B knockout are much milder than those of TFB1M or NSUN4 inactivation (see above). While, apparently, the mitochondrial protein synthesis yield was unreduced upon *Trmt2b* gene inactivation (Fig. S5C) in total agreement with the results of Minczuk group [49], the reduction in the activity of ETC complexes containing mitochondrially encoded subunits might hypothetically be explained by a reduction of protein synthesis fidelity. Unfortunately, an *in vitro* translation system is unavailable for mitochondria making us unable to directly test this possibility.

We also have to admit that we could not definitely state whether the observed phenotype is explained by the lack of 12S rRNA or tRNA modification. Methylation of the tRNA T-loop might be important for its stability, at least when combined with secondary tRNA mutations, decreasing its propensity to fold into a correct structure [63]. Curiously, bovine mitochondrial tRNAs lack U54 methylation [64]. Although it is not known whether bovine mitochondrial 12S rRNA carries m⁵U residue, the genome of *Bos taurus* contains a seemingly functional *Trmt2B* gene, hypothetically responsible only for 12S rRNA modification. Among human mitochondrial tRNAs, whose modification patterns are known only tRNA^{Leu}_(tm UAA)⁵ and tRNA^{Pro}_(UGG), contain m⁵U54 residue [1]. These two species are the only among the known set containing a 7 nucleotide T-loop starting with UU, thus the rules determining whether tRNA would be a substrate for TRMT2B are likely to be the same for mouse and human mitochondria.

The dual specificity of the TRMT2B methyltransferase might be explained by a remarkable similarity between the structures of the 12S rRNA loop 24 (Fig. 1A) and the T-loop of tRNA (Fig. 2A). However, a possibility that modification of the 12S rRNA by TRMT2B is simply a coincidence explained by the structural similarity of the loops that are modified, in our view is unlikely. While the 16S nucleotide m¹A947 modified by another dual specific rRNA/tRNA methyltransferase is not conserved, nucleotide m⁵U425 of the 12S rRNA is universally conserved and located in the highly conserved [65] loop 24, contacting mRNA [66] and forming a ridge between the P and E-site bound tRNA anticodon loops [67]. While the methyl group of the nucleotide m⁵U425 is not in direct contact with the ligands of the ribosome it is likely that this modification could stabilize the structure of the RNA loop 24 it belongs to. In line with this idea, cytoplasmic ribosomes contain pseudouridine [68], known to stabilize stacking [69], at the position equivalent to that of nucleotide m⁵U425 of mitochondrial 12S rRNA.

In an enzymatic system of m⁵U formation one could find examples of both convergent and divergent evolution (see [11] for discussion) including switches between Rossmann fold SAM-dependent RNA methyltransferases and MTHF dependent enzymes [29–31]. Various blends of single and dual specificity enzymes acting on rRNA and tRNA are present even among the Rossmann fold m⁵U forming RNA methyltransferases (Fig. 4A, Fig. S6). The ancestral m⁵U RNA methyltransferase is likely to be bacterial RlmD [70], an enzyme which modifies nucleotide m⁵U1939 of the 23S rRNA. In a number of bacterial lineages, RlmD-like enzymes acquired an ability to modify both m⁵U1939 and m⁵U747 of the 23S rRNA [71]. This new function is likely to require an insertion of two loops into the structure of the central domain of the parental RlmD-like protein [72] (Fig. 4B, Fig. S6). In proteobacteria, such as *E. coli*, most likely due to gene duplication and the following specialization, RlmC and TrmA enzymes have been evolved in addition to RlmD (Fig. 4). RlmC, an enzyme that lacks the TRAM domain of RlmD and RlmCD, but retains insertions in the central domain, is responsible for the modification of m⁵U747 of the 23S rRNA [73]. TrmA is the m⁵U54 tRNA methyltransferase [74] that lacks RlmC and RlmCD-specific insertions, but has a new loop in the Rossmann fold methyltransferase domain (Fig. 4B, Fig. S6) [75]. Surprisingly, fungal Trm2 proteins responsible for m⁵U54 modification in tRNA [76] form a separate lineage close to bacterial rRNA-specific RlmCD methyltransferases rather than to bacterial TrmA tRNA-specific methyltransferases (Fig. 4). Enzymes of higher eukaryotes responsible for m⁵U formation are also likely to originate from the predecessor rRNA-specific methyltransferase (Fig. 4). They lack a TrmA-specific extension in the Rossmann fold methyltransferase domain (Fig. 4, Fig. S6), but have insertions in the central domain reminiscent of bacterial RlmCD/RlmC. Unlike fungal Trm2 and bacterial RlmD and RlmCD, the ancestor of m⁵U specific methyltransferases of higher eukaryotes acquired an RRM domain instead of a TRAM domain (Fig. 4). It is likely that this enzyme was tRNA-specific since tRNAs are the common targets for all enzymes of this clade. Finally, in the evolution of vertebrates, TRMT2B methyltransferase, as we demonstrated in this work, became again a dual specificity RNA methyltransferase, this time capable of modifying not only mitochondrial tRNA, but the mitochondrial small subunit ribosomal 12S RNA, thus being the only group in the entire family acting on the small ribosomal subunit. Enzymes responsible for m⁵U modification switched the substrate several times from rRNA to tRNA and later to an unrelated site of another rRNA molecule.

Materials and methods

Cell culture

Mouse NS0 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS, GlutaMAX and penicillin and streptomycin at 37°C, 5% CO₂. Large cell culture volumes were grown in Thomson Optimum Growth 1.6 L flasks in a shaker-incubator at 110 rpm. NIH3T3, Hepa1-6 and Aml12 were cultured in tissue treated flasks in DMEM/F12 medium

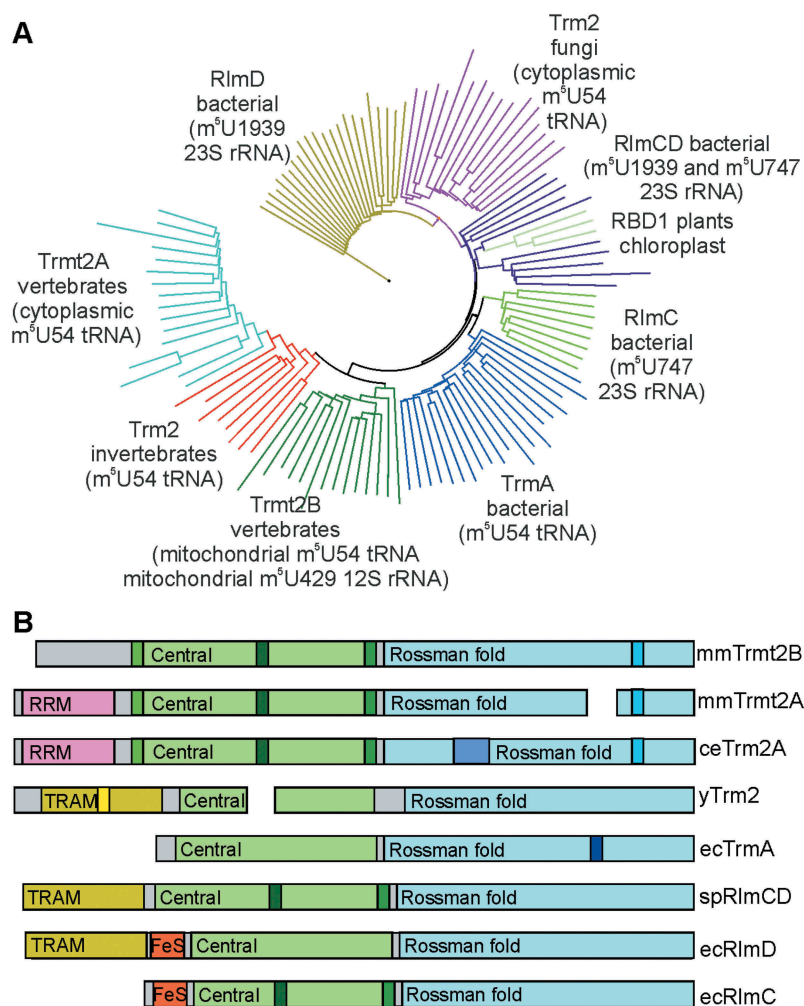


Figure 4. Evolution of m^5U RNA methyltransferases. (A) Evolutionary tree of m^5U RNA methyltransferases. Different clades are marked by different colours and labelled with the name of enzyme, group of organisms and substrate. (B) A scheme of domain structures and group-specific insertions. Enzyme names preceded by organism designations are shown on the right side of the scheme: *Mus musculus* (mm), *Caenorhabditis elegans* (ce), yeast *Saccharomyces cerevisiae* (y), *Escherichia coli* (ec) and *Streptococcus pneumoniae* (sp). Different colours correspond to common protein domains labelled accordingly, such as Central domain, Rossman fold domain, TRAM domain, RRM domain and iron-sulphur cluster (FeS). Group specific insertions are marked with different hues.

(Gibco) supplemented with 10% FBS, GlutaMAX and penicillin and streptomycin at 37°C, 5% CO₂.

Trmt2b gene inactivation

The sequence of gRNA (5'-GTGCTTCTTAGCTATCACGC-3') for Cas9 targeting the first coding exon of Trmt2b was generated using the Benchling CRISPR guide RNA designing tool (<https://benchling.com>). Two complementary oligodeoxyribonucleotides containing the gRNA sequence and BbsI ligation adapters were annealed and ligated into BbsI-digested pX458 vector [38] (Addgene #48138). NS0 cells were transfected with the pX458 plasmid containing the gRNA sequence using a Lipofectamine 3000 reagent (Thermo Scientific). 24 hours after transfection GFP-positive cells were sorted using BD FACS Aria III in 96-well plates containing 0.2 mL of RPMI medium per well. Individual clones were analysed by PCR amplification of ~250 bp Trmt2b fragment (5'-TCAAGAGTCCTAAATGCACAACC-3' and 5'-CCAGGAGTCATCTCTACAATGC-3') and sequencing of the amplicon. For off-target analysis we chose 5 off-targets with the highest score according to the Benchling

CRISPR guide RNA designing tool (<https://benchling.com>). Each off-target was analysed by PCR amplification of ~250 bp. Sequences of possible off-targets and primers for them are shown in the Table S1.

Ectopic expression of the Trmt2b gene

Total RNA was isolated from the NS0 cell line using a PureLink RNA Mini Kit (Thermo Scientific). TRMT2B cDNA was synthesized with reverse transcription by Maxima Reverse Transcriptase (Thermo Scientific) and a specific primer (5'-TGTCTCCTATGGCAACCTGG-3') followed by PCR with Q5 High-Fidelity DNA Polymerase (NEB) using primers (5'-GGCCTCTGAGGCCATGCACAACCC-AAGACTATTC-3' and 5'-GGCCTGACAGGCCTTACCGAGTGAAGAGGAG AAC-3'). PCR product was ligated into a SmaI-digested and dephosphorylated pUC19 vector. TRMT2B ORF was then inserted into pSBtet-Neo [39] (Addgene #60509) by restriction free cloning [77] using the following primers for the first PCR: forward – 5'-TCGAAAGGCCTCTGAGGCCATG-3', reverse – 5'-GCTT-GGCCTGACAGGCCTTAC-3'.

The Trmt2b KO cell line cell line was electroporated with plasmids coding for the TRMT2B and SB100x transposase [40] (pCMV(CAT)T7-SB100, Addgene #34879) in a mass ratio of 19:1 using a Neon Transfection System (Thermo Scientific) according to the manufacturer's instructions. 24 hours after transfection, electroporation cells were subjected to G418 (1 mg/mL, Thermo Scientific). Selection was carried out for 2 weeks and then cells were cultivated in the medium without G418.

12S rRNA and mt-tRNAs isolation

Total RNA was isolated using an ExtractRNA reagent (Evrogen). The 12S rRNA fragment and tRNAs was isolated using 5'-biotinylated oligodeoxyribonucleotides (Table S2) complementary to the region containing the nucleotide to be analysed. Total RNA (2 mg/mL, 6 mg) and the oligonucleotide (200 pmol/mL) were heated in a 6x SSC buffer for 5 min at 95°C and left in the dry block to anneal. In case of rRNA, after cooling to ~37°C, RNase T1 (Thermo Scientific) was added to the mixture to a final concentration of 1 unit/ul and incubated for 1 h at 37°C. Then the solution was mixed with Streptavidin Sepharose High performance (GE Healthcare) and incubated for 20 min at room temperature with rotation. The resin was washed 3 times with 3x SSC, 4 times with 1x SSC and 4 times with 0.1x SSC. The 12S rRNA fragment was eluted from the resin by adding elution buffer (0.1x SSC, 6M urea) and incubating for 5 min at 75°C. RNA from the eluate was precipitated with isopropanol overnight at -20°C. The precipitate was dissolved in 1x RNA Loading Dye (Thermo Scientific) and loaded on to 12% denaturing polyacrylamide gel. The gel was stained by ethidium bromide and the desired band was excised. The RNA band was fragmented and washed twice with a washing buffer (25 mM ammonium citrate, 50% acetonitrile) at room temperature for 10 min and dried with 100% acetonitrile.

MALDI MS analysis

For MALDI MS a fragmented gel band was dried from acetonitrile in air at room temperature and RNA was digested with 1 unit/ul RNase T1 or 20 ug/mL RNase A in 50 mM ammonium citrate for 3 hours at 37°C. A digestion mixture (0.5 ul) was mixed with 50 mg/mL 2,5-dihydroxybenzoic acid in 0.5% trifluoroacetic acid and 30% acetonitrile (1.5 ul) and was left to air dry at room temperature. MALDI mass spectrometry was performed in a reflector mode on an Ultraflex III BRUKER equipped with a UV laser (Nd, 335 nm) detecting positive ions.

qPCR

To compare the amount of different rRNAs in WT and KO cell lines, total RNA was isolated using PureLink RNA Mini Kit (Thermo Scientific). Total RNA was treated with DNase I (Thermo Scientific) followed by reverse transcription using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). SYBR Green PCR Master Mix (Thermo Scientific) was used to carry out real-time PCR reactions using a Biorad CFX96 Touch Real-Time PCR Detection System. Each sample was run in triplicate and

samples from 4 different experiments were analysed in parallel. The primer sequences for analysis are the following:

12S rRNA – 5'-CTCAAAGGACTTGGCGGTAC-3', 5'-GT TTGCTGAAGATGGCGGTA-3'; 16S rRNA – 5'-TGAAATT TCGGTTGGGGTGA-3', 5'-TCCCTAGGGTAACTTGGTCC-3'; 18S rRNA – 5'-GTAACCCGTTGAACCCCAT-3', 5'-GG CCTCACTAAACCATCCAAT-3'; Gapdh – 5'-GGTCCCAG CTTAGGTTTCATCAG-3', 5'-GTCGTTGATGGCAACAATC TCCAC-3'.

Relative changes in transcript levels were calculated using the Δ Ct method. The mean expression level of *Gapdh* was used for normalization.

For comparison of mtDNA copy number, total DNA was isolated by a phenol-chloroform extraction method and precipitated by ethanol. qPCR reactions were performed as described before, except that the reverse transcription step was omitted. Each sample was run in triplicate and samples from 4 different experiments were analysed in parallel. Primers for 12S rRNA and 16S rRNA were the same as for the analysis of the rRNA amount. Relative changes in DNA levels were calculated using the Δ Ct method. The concentration of a single copy locus of the nuclear DNA (primers – 5'-CTTCTTCTCTTACCTGCACGCC-3', 5'-GGTTACCAATG TCAGCGACGAGG-3') was used for normalization.

Mitochondrial protein synthesis

Mitochondrial protein biosynthesis was analysed as described in [78] with minor modifications. Briefly, $\sim 1 \cdot 10^6$ cells, cultured at 37°C and in a 5% CO₂ atmosphere in the appropriate medium, were harvested by centrifugation at 200 × g for 5 min, rinsed with 1 ml of PBS and resuspended in 1 ml of methionine-free medium supplemented with 200 ug/ml cycloheximide. After 5 minute incubation at 37°C in 5% CO₂, 5–10 uCi of EasyTag™ L-[³⁵S]-labelled methionine (Perkin Elmer) was added, and incubation continued for 45 minutes. The labelling was stopped by addition of non-radioactive methionine (final concentration 80 mM) and puromycin (final concentration 4 ug/ml). Cells were harvested, washed with PBS twice, resuspended in 100–150 ul of PBS and lysed with ultrasound. Protein concentrations were measured by Bradford, equal amounts of total protein from different samples (30–50 ug) were applied to 15% – 20% gradient denaturing polyacrilamide gel. After electrophoresis at 25 mA for 5–6 hours, gels were stained with Coomassie G-250, dried and analysed by autoradiography with a Storm 865 scanner (GE Healthcare). Gels images were analysed using ImageJ software (NCBI)

Activity of respiratory complexes

NS0 cells were harvested at $\sim 10^6$ cells/ml, 10^7 cells were washed with PBS, resuspended in respiration medium (0.137 M NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄, 25 mM Tris-HCl solution with pH 7.4), incubated with 20 ug/mL digitonin 5 min at 37°C and were placed in an Oxytherm (Hansatech, UK) chamber heated to 37°C. Stepwise control of the ETC-CI linked respiration was

assessed using the protocol described in [79]. Briefly, substrates or their precursors and then inhibitors of ETC complexes I (glutamate, malate – rotenone), II (succinate – malonate), III (glycerol-3-phosphate – antimycin A) and IV (TMPD, ascorbate – KCN) were added to the chamber sequentially. The next substance was added to the chamber when the oxygen consumption rate was stable for 2 min. Activity of ETC complexes were calculated as the difference in oxygen consumption rates after addition of the substrate and inhibitor.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author contributions

PS designed the project and performed bioinformatic analysis of putative RNA methyltransferases, IL and ES performed major part of experimental work except the parts listed below, SL performed mitochondrial translation assays, MS performed mass spectrometry analysis, MR performed cell sorting to create cell lines; AB, IL, PS, PK, and OD contributed to manuscript writing.

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