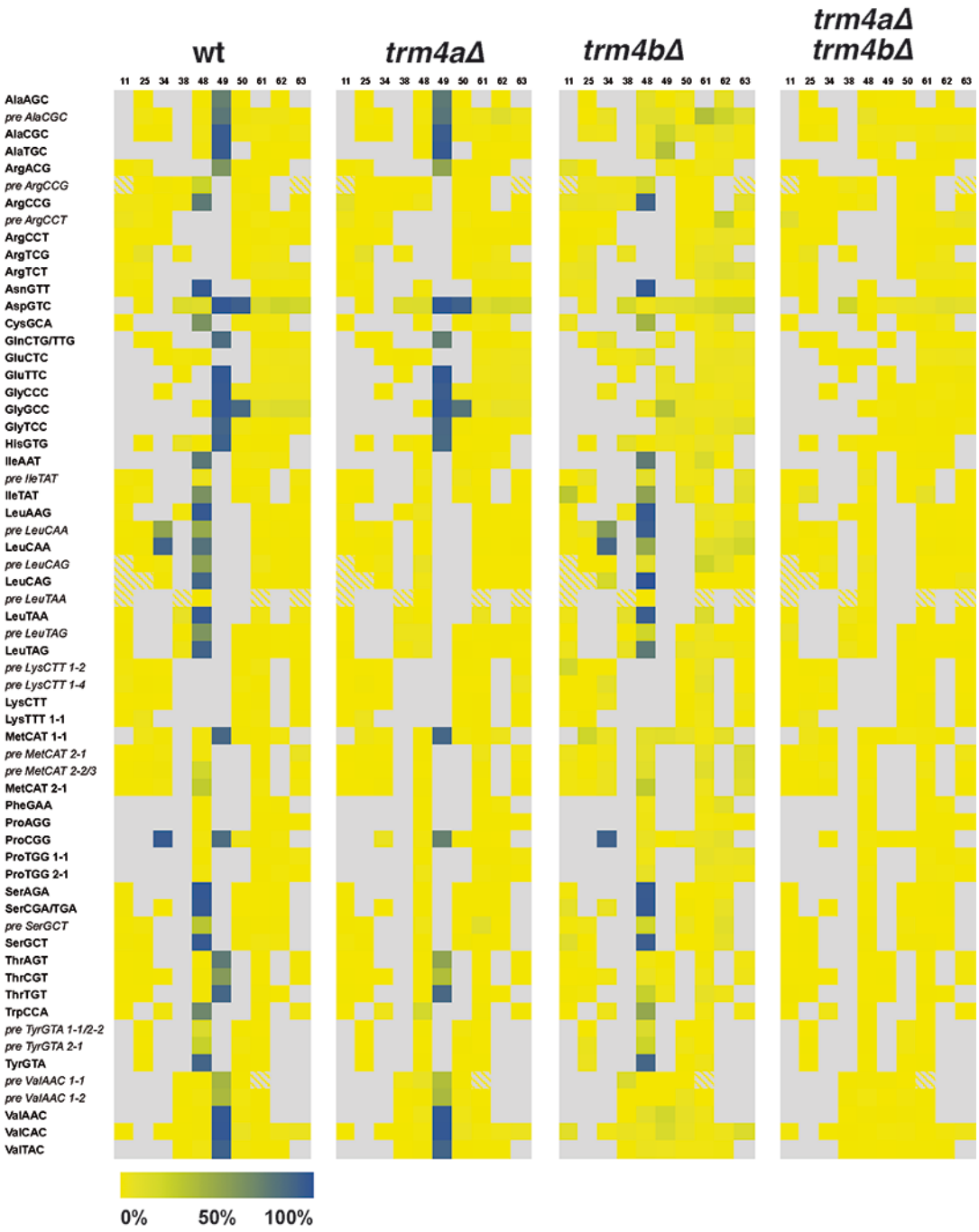
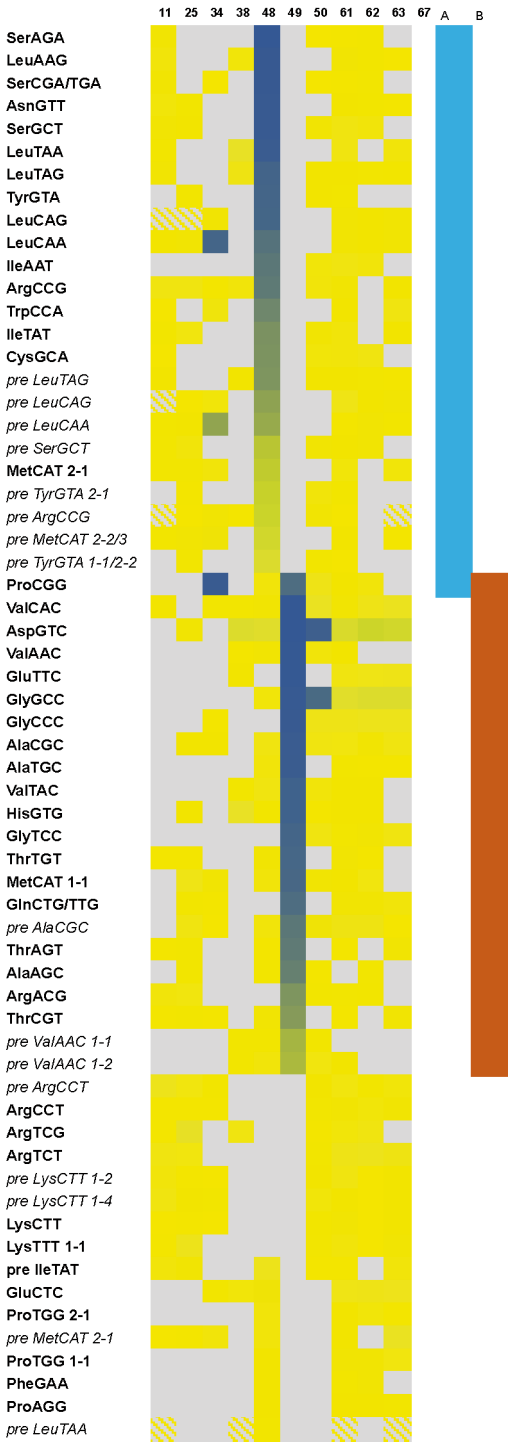
Supplementary materials

Supplementary Figure 1: Phylogenetic tree of Trm4/ NSun2 homologs. The HOG000205147 group of Trm4/ NSun2 sequences from the HOGENOM database [1] was taken, which comprises 116 Trm4 protein sequences. The alignment and tree as generated on http://doua.prabi.fr using Clustalomega and Phyml, respectively, are displayed using iTOL [2]. Members from organisms of interest are shown in red.



Supplementary Figure 2: tRNA methylome of wt, *trm4aΔ, trm4bΔ* and *trm4aΔ trm4bΔ* as determined by genome-wide high-throughput bisulfite sequencing (as in Fig. 1A).



Supplementary Figure 3: Trm4a is responsible for *in vivo* methylation of C34 and C48 methylation, whereas Trm4b methylates C49 and C50. Genome-wide high-throughput bisulfite sequencing data from wt (same data as in Fig. 1A and Suppl. Fig. 1) was sorted by the level of methylation at C48, followed by the level of C49 methylation. Blue bar, Trm4a targets; red bar, Trm4b targets. tRNAProCGG is the only tRNA methylated by both Trm4a and Trm4b.

Supplementary Table 1: Experimental conditions that have no effect on *trm4aΔ, trmb4Δ and trm4aΔ trm4bΔ*

Condition	Remark
High temperature (37º C)	-
DMSO	Solvent
Ethanol	Solvent
Caffeine	Checkpoint inhibitor
H_2O_2	Oxidative stress
Hygromycin B	Translation inhibitor
Cycloheximide	Translation inhibitor
Hydroxyurea	Translation inhibitor
Paromomycin*	Translation inhibitor
Anisomycin	Translation inhibitor

* Paromomycin sensitivity was not tested in *trm4aΔ trm4bΔ,* because the presence of *trm4bΔ::kanMX* in this strain causes paromomycin resistance due to the *kanMX* cassette and thus is unrelated to Trm4b function.

Suppl. Table 2: Oligonucleotides used in this study*

ProCGG1	CTCGAGTTTTTCAGCAAGATTTGTAATACGACTCACTATAGCGGG CCT
ProCGG2	GAACCAGGGATCACCTCTACCCGAAAGAGGTATGTTAACCACTAC ACTATTAGGCCCGCTATAGTGAGTC
ProCGG2 C34A	GAACCAGGGATCACCTCTACCCTAAAGAGGTATGTTAACCACTAC ACTATTAGGCCCGCTATAGTGAGTC
ProCGG2 C49A	GAACCAGTGATCACCTCTACCCGAAAGAGGTATGTTAACCACTAC ACTATTAGGCCCGCTATAGTGAGTC
ProCGG2intron	CTCAGCATACAAGTGGGGATTACCCGAAAGAGGTATGTTAACCAC TACACTATTAGGCCCGCTATAGTGAGTC
ProCGG2intron C34A	CTCAGCATAAAAGTGGGGATTACCCTAAAGAGGTATGTTAACCAC TACACTATTAGGCCCGCTATAGTGAGTC
ProCGG3-b	CGGGTAGAGGTGATCCCTGGTTCGAATCCTGGTTAGGCCCCCAT GG
ProCGG3-b C34A	AGGGTAGAGGTGATCCCTGGTTCGAATCCTGGTTAGGCCCCCAT GG
ProCGG3-b C49A	CGGGTAGAGGTGATCACTGGTTCGAATCCTGGTTAGGCCCCCAT GG
ProCGG3-bintron	CGGGTAATCCCCACTTGTATGCTGAGTTTAGAGGTGATCCCTGGT TCGAATCCTGGTTAGGCCCCCATGG
ProCGG3-bintron C34A	AGGGTAATCCCCACTTTTATGCTGAGTTTAGAGGTGATCCCTGGT TCGAATCCTGGTTAGGCCCCCATGG
ProCGG4	CCATGGGGGCCTAACC
Pro CGG Intron C49A fw	GAGGTGATCACTGGTTCGAATCCTGGTTAG
Pro CGG Intron C49A rev	CGAACCAG**T**GATCACCTCTAAACTCAGCATAC
Pro CGG Intron G65T 2 fw	GAATCCTGTTTAGGCCC CCATGGAAAATCGATGTTCTT
Pro CGG Intron G65T 2 rev	GGGCCTAA**A**CAGGATTCGAACCAG**T**GATCACCTC

Mini ProCGG.fw	TCGAGTTTTTCAGCAAGATTTGTAATACGACTCACTATAGCGACCT CTTTCGGGTAATCCCCACTTGTATGCTGAGTTTAGAGGCCAC
Mini ProCGG.rev	CATGGTGGCCTCTAAACTCAGCATACAAGTGGGGATTACCCGAAA GAGGTCGCTATAGTGAGTCGTATTACAAATCTTGCTGAAAAAC
Mini ProCGGC34A.fw	TCGAGTTTTTCAGCAAGATTTGTAATACGACTCACTATAGCGACCT CTTTAGGGTAATCCCCACTTTTATGCTGAGTTTAGAGGCCAC
Mini ProCGGC34A.rev	CATGGTGGCCTCTAAACTCAGCATAAAAGTGGGGATTACCCTAAA GAGGTCGCTATAGTGAGTCGTATTACAAATCTTGCTGAAAAAC
proRTprimer_Sp	CTCAACTGGATTGGCTNNNNNGATAAATCCAGTTGAGTGGAAACC TAACC
Sp_pro_bisulfit.fw	TTTAATAGTGTAGTGGTTAATATA
Stemloop.rev	CTCAACTGGATTGGCT
BclI-Trm4a.fw	CGATCATGATCAGATGGGTCGTAAGCATTATTCTAG
BclI-Trm4a.rev	CGATCATGATCATCAAGTGTTATGTTTGTGTATTG
XhoI-Trm4b.fw	CGATCACTCGAGATGGGGAAAAGAAATAAAAAGG
BamHI-Trm4b.rev	CGATCAGGATCCTTACACGTCCATTCCAAATAATTCC

* N indicates any of A, T, C or G.

Supplementary references

1. Penel S, Arigon AM, Dufayard JF, Sertier AS, Daubin V, Duret L, et al. Databases of homologous gene families for comparative genomics. BMC bioinformatics 2009; 10 Suppl 6:S3.
2. Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res 2016; 44:W242-5.