



RESEARCH PAPER



Division of labour: tRNA methylation by the NSun2 tRNA methyltransferases Trm4a and Trm4b in fission yeast

Martin Müller^a, Anke Samel-Pommerencke^a, Carine Legrand^b, Francesca Tuorto ^b, Frank Lyko^b, and Ann E. Ehrenhofer-Murray ^a

^aInstitut für Biologie, Molekulare Zellbiologie, Humboldt-Universität zu Berlin, Berlin, Germany;; ^bDivision of Epigenetics, DKFZ-ZMBH Alliance, German Cancer Research Center, Heidelberg, Germany

ABSTRACT

Enzymes of the cytosine-5 RNA methyltransferase Trm4/NSun2 family methylate tRNAs at C48 and C49 in multiple tRNAs, as well as C34 and C40 in selected tRNAs. In contrast to most other organisms, fission yeast *Schizosaccharomyces pombe* carries two Trm4/NSun2 homologs, Trm4a (SPAC17D4.04) and Trm4b (SPAC23C4.17). Here, we have employed tRNA methylome analysis to determine the dependence of cytosine-5 methylation (m⁵C) tRNA methylation *in vivo* on the two enzymes. Remarkably, Trm4a is responsible for all C48 methylation, which lies in the tRNA variable loop, as well as for C34 in tRNA^{Leu}_{CAA} and tRNA^{Pro}_{CGG}, which are at the anticodon wobble position. Conversely, Trm4b methylates C49 and C50, which both lie in the TΨC-stem. Thus, *S. pombe* show an unusual separation of activities of the NSun2/Trm4 enzymes that are united in a single enzyme in other eukaryotes like humans, mice and *Saccharomyces cerevisiae*. Furthermore, *in vitro* activity assays showed that Trm4a displays intron-dependent methylation of C34, whereas Trm4b activity is independent of the intron. The absence of Trm4a, but not Trm4b, causes a mild resistance of *S. pombe* to calcium chloride.

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Introduction

The Trm4 family of methyltransferases catalyses the methylation of position 5 on cytosine to create 5-methyl-cytosine (m⁵C) in several tRNAs. Trm4 (also termed Ncl1) from *S. cerevisiae* generates m⁵C at four different positions in yeast tRNA [1]: C34, C40, C48 and C49. Position 48 lies at the junction between the variable loop and the TΨC-stem of tRNAs, and C49 is at the first position of the TΨC-stem. Either C48 or C49 alone, but not both simultaneously, are frequently methylated (m⁵C48 in 13 of 15 cases, m⁵C49 in 12 of 13 cases in *S. cerevisiae*) [2]. Furthermore, tRNA^{Phe}_{GAA} carries Trm4-dependent m⁵C40 in *S. cerevisiae*, which lies in the anticodon stem-loop [1]. Finally, tRNA^{Leu}_{CAA} is methylated at the wobble position C34 by Trm4. Interestingly, both respective tRNA^{Phe} and tRNA^{Leu} genes carry an intron, and Trm4 requires the presence of the intron to carry out C34 and C40 methylation, whereas its activity on C48 and C49 is independent of the intron [1].

Functionally, m⁵C34 wobble base modification affects the efficiency of tRNA^{Leu} to act as an amber suppressor tRNA *in vivo* [3], whereas m⁵C40 is important for the spatial organization of the anticodon stem-loop and for formation of the Mg²⁺ binding pocket (reviewed in [4]). The absence of Trm4 in *S. cerevisiae* causes sensitivity towards the translational inhibitor paromomycin and synthetic growth defects in combination with the absence of other tRNA modifications, which is due to the fact that undermodified tRNAs are subject to rapid tRNA decay [5–7]. Also, the level of m⁵C34 methylation in tRNA^{Leu} is increased when


S. cerevisiae cells are under oxidative stress, and this results in a codon-biased translation of proteins required for the response to the stress condition [8].

The Trm4 homolog from higher eukaryotes, NSun2 (NOP2/Sun domain family, member 2; MYC-induced SUN domain-containing protein, Misu) also is a tRNA methyltransferase, but NSun2 has also been reported to methylate non-tRNA substrates, including rRNA and other non-coding and coding RNAs [9,10]. For instance, NSun2 has been shown to methylate vault ncRNAs, which affects their processing into small regulatory RNAs [10,11]. The absence of NSun2 furthermore increases cleavage of tRNAs by angiogenin and causes higher accumulation of 5' tRNA fragments [9], which affects translation and cellular stress response. These effects of NSun2 on RNA processing are hypothesized to contribute to disease states in humans, where homozygous mutation of the NSUN2 gene leads to the Dubowitz-like syndrome [12].

Intriguingly, widespread m⁵C in mRNAs has been reported in human [13,14] and plant cells [15,16], where hundreds of mRNA methylation sites were reported, and their methylation in *Arabidopsis* has been shown to depend on the TRM4B methyltransferase. However, this view of pervasive mRNA methylation has been challenged, since many methylation sites as determined by high-throughput bisulfite sequencing do not withstand stringent statistical filtering [17].

Most organisms, including human, mouse, *Drosophila* and *S. cerevisiae*, contain a single Trm4/NSun2 homolog. In contrast,

CONTACT Ann E. Ehrenhofer-Murray  ann.ehrenhofer-murray@hu-berlin.de  Humboldt-Universität zu Berlin, Institut für Biologie, Unter den Linden 6, Sitz: Philippstr. 13, Rhoda-Erdmann-Haus, 10099 Berlin, Germany

 Supplemental data for this article can be accessed [here](#).

the fission yeast *Schizosaccharomyces pombe* [18] as well as *Arabidopsis thaliana* and some other plants carry two homologs that are termed Trm4a and Trm4b [15] (Suppl. Figure 1). This raises the question how the two homologs affect (t)RNA methylation, and whether they have additional substrates. In an earlier study of tRNA methylation in *S. pombe*, we observed that m⁵C34 is present not only on tRNA^{Leu}, but also on a novel site, tRNA^{Pro} (CGG) [19], though the dependence of Trm4a or Trm4b remained to be determined.

In this study, we have used tRNA methylome analysis to determine the specificity of Trm4a and Trm4b from *S. pombe*. Interestingly, we found a clear division of labour in that Trm4a conducted all C48 methylation as well as C34 methylation on both tRNA^{Leu} and tRNA^{Pro}_{CGG}, whereas Trm4b methylated all C49 sites on tRNAs. *In vitro*, Trm4a was only able to methylate C34 of tRNA^{Pro}_{CGG} in the presence of the intron of the tRNA. Trm4b methylated C49 of tRNA^{Pro}_{CGG} *in vitro* independently of the intron, and it showed *in vitro* activity on C34. The absence of Trm4a causes *S. pombe* cells to be mildly resistant to calcium chloride, an indicator of mitochondrial function, whereas no obvious defects were observed in the absence of Trm4b. Altogether, our results highlight a strict distribution of tRNA substrates between the two NSUN2 homologs, which suggests distinct functions for methylation at two adjacent tRNA sites, positions C48 and C49.

Results

tRNA methylome analysis shows specialization in *S. pombe* of Trm4a for C34 and C48 methylation and Trm4b for C49

Trm4/NSun2-dependent tRNA methylation is known on C34, C40, C48 and C49 [1]. Since *S. pombe* carries two homologs, Trm4a and Trm4b, we asked which m⁵C sites on tRNAs depended on which of the two enzymes. For this purpose, we determined the complete tRNA methylome of *S. pombe* wt, *trm4aΔ*, *trm4bΔ* and *trm4aΔ trm4bΔ* strains by RNA bisulfite treatment and next-generation sequencing of tRNAs. The resulting reads were mapped to the known *S. pombe* tRNAs [20] and analyzed for cytosine methylation. Comparison of tRNA methylation patterns in the individual strains allowed us to assign methylation sites to Trm4a and Trm4b (Figure 1(a), Suppl. Figure 1).

Interestingly, C34 methylation depended on Trm4a in both tRNA^{Leu}_{CAA} and tRNA^{Pro}_{CGG} (Figure 1(a), middle), since methylation levels at this site were at background levels in the *trm4aΔ* strain (Suppl. Figure 2). To further confirm this for tRNA^{Pro}_{CGG}, we performed high-throughput tRNA bisulfite sequencing on tRNA^{Pro}_{CGG} (Figure 1(b)). m⁵C34 and m⁵C49 levels were determined to be 89 and 83%, respectively, in a wild-type strain. In *trm4aΔ*, the C34 methylation level dropped to background levels (8%), whereas C49 methylation remained at 82%. Conversely, m⁵C34 was at 94% in *trm4bΔ*, and m⁵C49 dropped to 9%, thus confirming that Trm4a is responsible for m⁵C34 and Trm4b for m⁵C49 on tRNA^{Pro}_{CGG} *in vivo*.

For the methylation of C48 and C49, we observed a surprising selectivity of the Trm4 enzymes. Namely, Trm4a was responsible for all methylation of C48 (Figure 1

(a), middle), and conversely, methylation of all C49 sites depended only on Trm4b (Figure 1(a), right). This was also apparent when the tRNA methylome data was not sorted alphabetically by the respective amino acid they carry (Figure 1(a)), but by the degree of methylation at C48 and C49 (Suppl. Figure 3). Also, as has been noted earlier [1], C48 and C49 are never methylated in the same tRNA. Thus, a picture emerged where C48 is carried out exclusively by Trm4a and C49 methylation by Trm4b. tRNA^{Pro}_(CGG) is the only tRNA that is methylated both by Trm4a (C34) and Trm4b (C49).

In vitro activity of Trm4b on C49 and Trm4a on C34 of intron-containing tRNA^{Pro}

To verify the specificity of Trm4a and Trm4b, we investigated their *in vitro* methylation activity on *in vitro*-transcribed tRNAs (Figure 2(a)). Both proteins were heterologously expressed and purified from *E. coli* and used in *in vitro* tRNA methylation assays using tRNA^{Pro}_{CGG} as a substrate, which is methylated *in vivo* on C34 by Trm4a and on C49 by Trm4b. We also generated mutant versions of the tRNA in which either C34 or C49 was mutated to adenine (C34A, C49A).

Significantly, Trm4b showed robust *in vitro* activity on wild-type tRNA^{Pro}_{CGG}. However, it was inactive on the C49A mutant version under the reaction conditions used here while maintaining activity on the C34A mutant version (Figure 2(b)). To further evaluate this, a time course of methylation was conducted with Trm4b and wild-type tRNA^{Pro}_{CGG} (wt) or the C34A and C49A versions. This showed that Trm4b was active on the wt tRNA; its activity was slightly enhanced on C34A and completely abrogated on C49A (Figure 2(c)), indicating that Trm4b has *in vitro* activity on tRNA^{Pro}_{CGG} and is specific for C49, which was in agreement with *in vivo* methylation data.

In contrast to Trm4b, Trm4a was inactive on tRNA^{Pro} and its mutant versions (Figure 2(b)). Since C34 methylation in tRNA^{Leu} by Trm4 from *S. cerevisiae* has previously been shown to depend on the presence of an intron in this tRNA [1], we asked whether Trm4a from *S. pombe* also depended on intronic sequences to be active. Therefore, we tested the activity of Trm4a on the intron-containing tRNA^{Pro}_{CGG} (pre-tRNA^{Pro}, Figure 2(a)) and a mini-tRNA^{Pro}, which consists of the anticodon loop that is extended by the intron (Figure 2(a)). Significantly, Trm4a activity was readily detected on the wild-type pre-tRNA^{Pro}_{CGG} and mini-tRNA^{Pro}_{CGG} substrates, but not in the C34A mutant versions (Figure 2(d,e)), indicating that Trm4a is a C34 methyltransferase for the intron-containing tRNA^{Pro}_{CGG} precursor.

To further characterize the activities of Trm4a as well as Trm4b on pre-tRNA^{Pro}, time courses of methylation on the wt as well as the mutant versions were conducted. Trm4a was active on wt pre-tRNA^{Pro} and pre-tRNA^{Pro} with C49A, but was inactive when C34 or C34 and C49 were mutated (Figure 2(f)). This supported the notion that Trm4a is only active on the intron-containing pre-tRNA^{Pro} to methylate C34.

The activity of Trm4b on pre-tRNA^{Pro} was more complex (Figure 2(g)). It showed robust activity on wt pre-tRNA^{Pro}. Surprisingly, Trm4b retained activity on C49-mutated pre-tRNA^{Pro}, which was in disagreement with our observation that all m⁵C49 *in vivo* depends on Trm4b and suggested

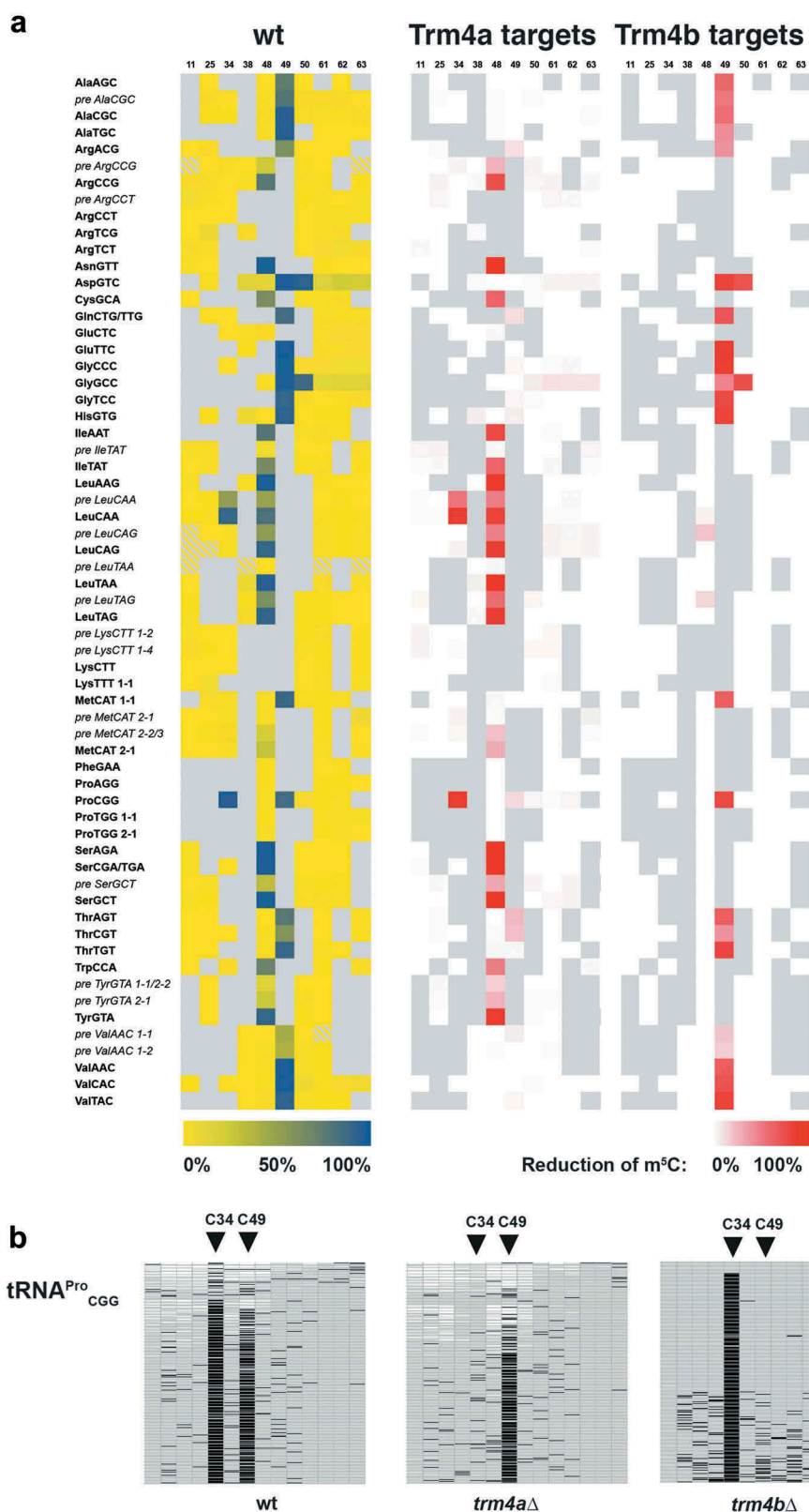


Figure 1. Genome-wide high-throughput tRNA bisulfite sequencing in *Schizosaccharomyces pombe* reveals selectivity of Trm4a for C34 and C48 methylation and Trm4b for C49. (a) Left, cytosine methylation levels are shown for individual tRNA species in wt cells. The top row indicates the position of the cytosine using standard tRNA numbering. tRNAs are sorted alphabetically by the respective amino acids they encode. The prefix 'pre' designates the unspliced version of selected tRNAs. Blue, strong methylation; yellow, no methylation. Grey fields indicate the absence of a C at that position. Yellow/grey cross-hatched fields indicate positions that constitute a C, but were not covered or had too low coverage in the high-throughput sequencing data. Middle, difference in tRNA methylation between wt and *trm4aΔ* cells. Red colours indicate a strong loss of methylation in *trm4aΔ* compared to wt. Right, difference in tRNA methylation between wt and *trm4bΔ* cells. Representation as in the middle panel. (b) Methylation of tRNA^{Pro}_{CGG} at C34 depended on Trm4a, and C49 methylation on Trm4b. Methylation levels were determined by targeted sequencing of bisulfite-treated tRNA^{Pro}_{CGG}.

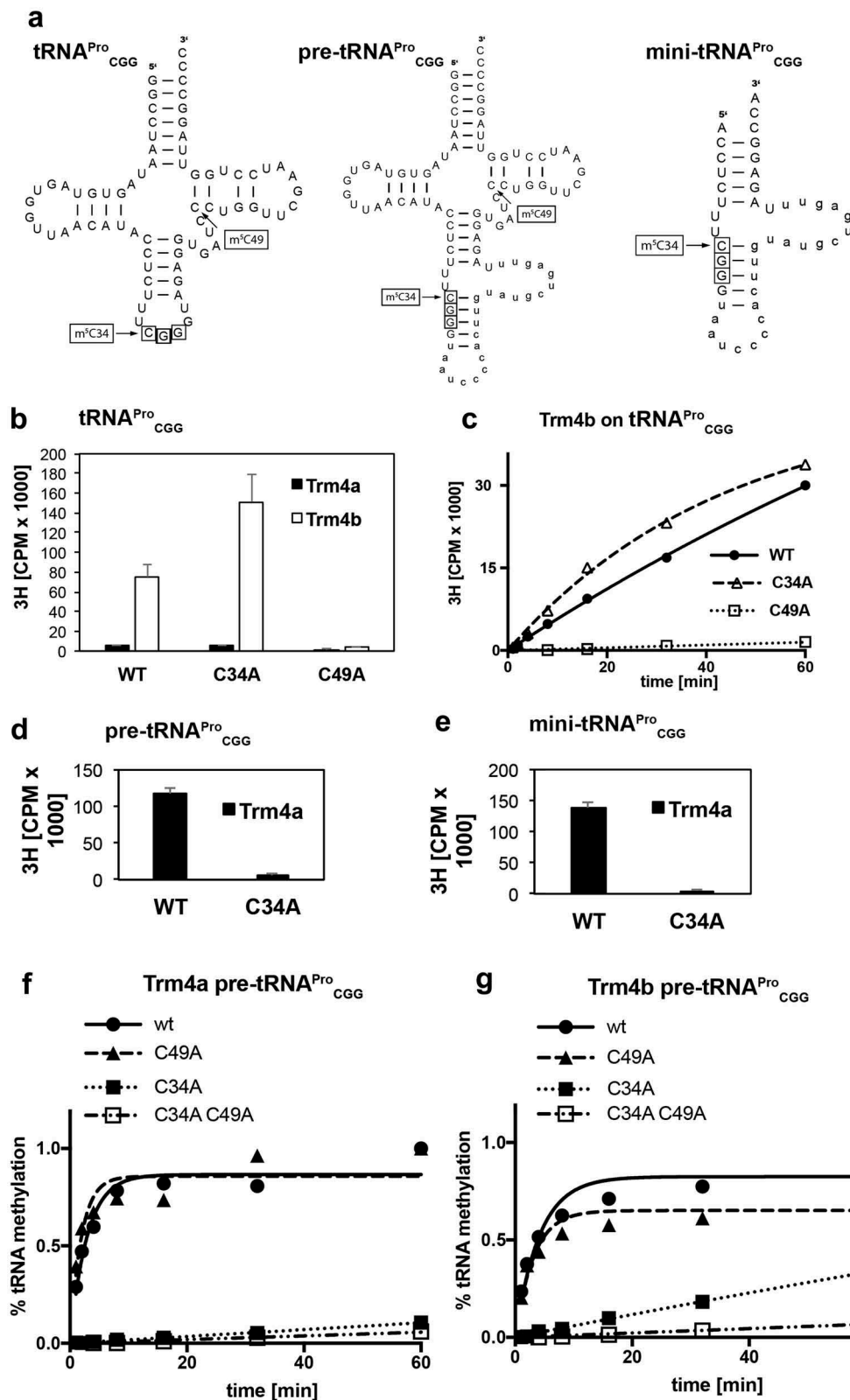


Figure 2. *In vitro* methylation activity of Trm4a and Trm4b shows that Trm4a catalyses intron-dependent C34 methylation in pre-tRNA^{Pro}_{CGG}. (a) tRNA sequences of tRNA^{Pro}_{CGG} (left), the intron-containing tRNA^{Pro}_{CGG} (middle, intron sequence in lower case) and a mini-tRNA^{Pro}_{CGG} version comprising the anticodon stem-loop and the intron. Anticodon nucleotides are boxed, and the position of C34 and C49 are indicated. (b) *In vitro* methylation of tRNA^{Pro}_{CGG} by Trm4a and Trm4b as determined by incorporation of ³H-labeled methyl groups into *in vitro*-transcribed tRNA substrates. The activity of Trm4b is abrogated by mutation of C49 to adenine (C49A), and Trm4a is inactive on tRNA^{Pro}_{CGG}. Error bars show the standard deviation (n = 3; reaction time 60 minutes). (c) Time course of tRNA^{Pro}_{CGG} methylation by Trm4b. Trm4b is inactive on the tRNA when C49 is mutated. (d, e) *In vitro* activity of Trm4a on pre-tRNA^{Pro}_{CGG} (d) and mini tRNA^{Pro}_{CGG} (e) is abrogated by mutation of C34 to adenine (C34A). The experiment was performed as in (b). (f) Time course of methylation of the intron-containing pre-tRNA^{Pro}_{CGG} by Trm4a. Trm4a activity is abrogated on pre-tRNA^{Pro}_{CGG} - C34A and - C34A C49A. (g) Time course of methylation of the intron-containing pre-tRNA^{Pro}_{CGG} by Trm4b. Trm4b activity was reduced on pre-tRNA^{Pro}_{CGG}-C34A and abrogated on - C34A C49A.

that Trm4b methylated another cytosine *in vitro* on pre-tRNA^{Pro}. Furthermore, Trm4b showed reduced activity on the pre-tRNA^{Pro} version with C34A, which may indicate that full Trm4b activity requires methylation at C34. *In vitro* activity of Trm4b was only abrogated when both C34 and C49 were mutated, which is consistent with the notion that Trm4b methylates both C34 and C49 *in vitro*, even though it does not methylate C34 *in vivo*.

Altogether, these data suggest a scenario in which Trm4a first methylates C34 on the intron-containing tRNA, which subsequently is spliced. Trm4b then methylates C49, and its activity is stimulated by prior Trm4a-mediated methylation on C34. Alternatively, Trm4b activity could be stimulated by other modifications on mature tRNA^{Pro}.

Absence of Trm4, but not Trm4b, causes mild resistance to CaCl₂

We next investigated whether the absence of Trm4a or Trm4b had phenotypic consequences in *S. pombe*. To this end, we determined how their absence affected the growth characteristics of *S. pombe* cells as well as their drug sensitivity. None of the strains showed a phenotype under a variety of conditions (Suppl. Table 1). In particular, the strains did not display sensitivity to paromomycin or oxidative stress, though the absence of *TRM4* in *S. cerevisiae* has been shown to cause sensitivity to these agents [8,21]. Thus, the Trm4 homologs in *S. pombe* have a different function than their equivalent in *S. cerevisiae*.

Among the growth conditions we tested was cellular survival on increased levels of CaCl₂, which is an indicator of mitochondrial function in *S. cerevisiae* [22]. Indeed, we observed that *trm4aΔ* showed a slightly increased resistance to CaCl₂ (Figure 3(a)), and no effect was detected for *trm4bΔ*. Accordingly, *trm4aΔ trm4bΔ* cells showed a similar CaCl₂ resistance as *trm4aΔ* alone.

Since the CaCl₂ resistance of *trm4aΔ* was rather mild, we sought to verify whether it could be complemented with *trm4a*⁺. For this purpose, the *trm4aΔ trm4bΔ* strain was transformed with a *trm4a*⁺, a *trm4b*⁺, or with both plasmids and tested for growth on CaCl₂. The strain grew less well on CaCl₂ when it carried the *trm4a*⁺ plasmid compared to the vector control, and this was irrespective of the presence of the *trm4b*⁺ plasmid (Figure 3(b)). This confirmed that the CaCl₂ resistance was due to absence of Trm4a.

The CaCl₂ resistance phenotype indicates that Trm4a may affect mitochondrial function in *S. pombe*, though how this occurs is unclear. The *S. pombe* mitochondrial genome encodes 25 tRNAs [23], necessitating the import of tRNAs into the

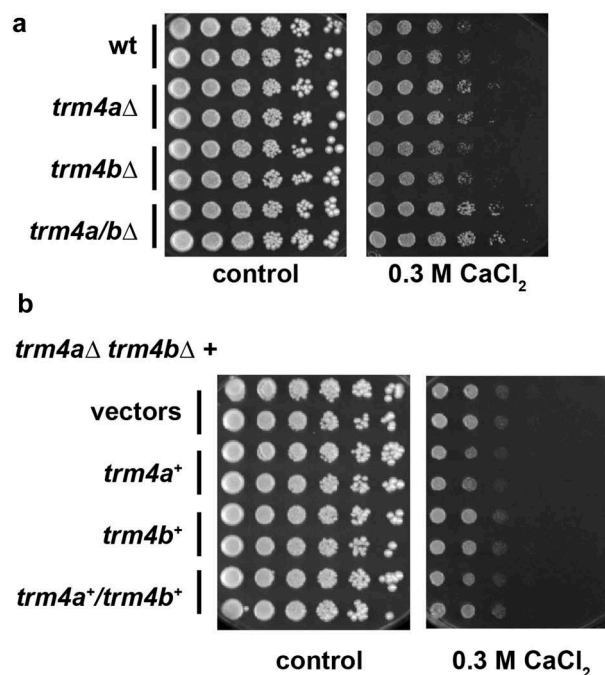


Figure 3. *trm4aΔ* causes mild resistance to calcium chloride. (a) The indicated strains (AEP1, AEP499, AEP391, AEP501) were serially diluted and spotted on full medium with or without 0.3M CaCl₂. Plates were incubated for four days at 30°C. (b) The CaCl₂ resistance of *trm4aΔ trm4bΔ* (AEP501) was complemented by plasmid-borne *trm4a*⁺, but not *trm4b*⁺.

mitochondria that are modified by Trm4a and Trm4b. However, both enzymes are localized to the nucleus (*S. pombe* Postgenome Database, <http://www.riken.jp/SPD>), and the coordination of methylation, splicing and subcellular localization of the tRNAs remains to be determined. An alternative possibility is that tRNA methylation by Trm4a affects translation of a protein involved in CaCl₂ uptake or metabolism.

Discussion

Unlike most model organisms, *S. pombe* carries two paralogs of the Trm4/NSun2 family of RNA methyltransferases, which are termed Trm4a and Trm4b. Here, we report that the two enzymes have distinct specificities in that Trm4a is responsible for methylation of all C48 sites, whereas Trm4b methylates all C49 sites in tRNA. Furthermore, Trm4a, but not Trm4b, carries out C34 methylation *in vivo*. C34 methylation is found in tRNA^{Leu}, and we have reported a second C34 methylation site in tRNA^{Pro}_{CGG} in *S. pombe* whose modification also depends on Trm4a. *In vitro*, recombinant Trm4a showed activity on C34, though it was only active on an intron-containing version of the tRNA. Thus, with respect to its methylation activity, Trm4a most closely reflects the characteristics of Trm4/NSun2 homologs from other organisms that also depend on the presence of the intron for activity [1], whereas Trm4b methylates its tRNA substrates at C49 regardless of the presence or absence of the intron. Surprisingly, Trm4b *in vitro* was active on C34, although m⁵C34 *in vivo* was independent of Trm4b. At the cellular level, the absence of C34 and C48 in *trm4aΔ*, but not C49 methylation in *trm4bΔ*, causes a mild increase in resistance to CaCl₂, which

Table 1. *S. pombe* strains used in this study.

Designation	Genotype	Source
AEP1	h ⁻ <i>leu1-32 ura4-D18 his3-D3</i>	YGRC
AEP102	h ⁺ <i>leu1-32 ade6-216 ura4-D18 trm4aΔ::kanMX</i>	Bioneer
AEP162	h ⁺ <i>leu1-32 ade6-216 ura4-D18 trm4aΔ::kanMX trm4b::NatMX</i>	[18]
AEP391	h ⁻ <i>leu1-32 ura4-D18 his3-D3 trm4bΔ::NatMX</i>	This study
AEP499	h ⁻ <i>leu1-32 ura4-D18 his3-D3 trm4aΔ::NatMX</i>	This study
AEP501	h ⁻ <i>leu1-32 ura4-D18 his3-D3 trm4aΔ::kanMX trm4bΔ::NatMX</i>	This study

may indicate an effect of C34 and C48 methylation in mitochondria and/or calcium homeostasis.

The specificity of Trm4b on C49/C50 is in apparent conflict with our earlier work, where we reported that tRNA^{ASP} methylation at C49, C50 and C61 – C63 depended on both Trm4a and Trm4b [18]. However, re-analysis of the *trm4bΔ* strain employed in that study revealed that it does not contain the *trm4b*⁺ deletion.

This apparent specialization of the two Trm4 homologs for C48 or C49 methylation raises the question of the evolutionary origin of these paralogs. Interestingly, the phylogenetic analysis (Suppl. Figure 1) shows that they derive from a relatively recent duplication event that occurred independently of the duplication in plants (e.g. *Arabidopsis*, *Oryza sativa*, *Zea mays*) and is not shared by other closely related fungi from the *Candida* or *Aspergillus* families. It remains to be seen what the relevance of this duplication in *S. pombe* is, especially since the absence of the two proteins causes only mild phenotypes.

A further question is what distinguishes the tRNA groups that are methylated at C48 or C49 from each other. Notably, those tRNAs that carried a methylated C48 at the edge of the variable loop did not carry a cytosine at position C49. Thus, it can be hypothesized that Trm4a is specific for the cytosine that is within this variable loop, adjacent to the TΨC-stem. If a tRNA carries both C48 and C49 (e.g. tRNA^{Thr}, tRNA^{Val}, tRNA^{Gln}), then this tRNA is not a substrate for C48 methylation by Trm4a, but is methylated at C49 by Trm4b (Figure 1(a)).

In contrast to C48, C49 lies within the TΨC-stem and forms the first base-pair of the stem loop. We hypothesize that methylation by Trm4b is specific for this structure, since it methylates only C49 even in those tRNAs that also possess C48 (e.g. tRNA^{Thr}, tRNA^{Val}, tRNA^{Gln}). For those tRNAs with C49 only, but no C48 (e.g. tRNA^{Arg}_{ACG}, tRNA^{Glu}_{TTC}, tRNA^{Gly}_{TCC}), this position invariably is methylated by Trm4b.

Interestingly, while most tRNAs are methylated at either C48 or C49, but not both, we observed two tRNAs, tRNA^{ASP} and tRNA^{Gly}_{GCC}, that are methylated at two adjacent cytosines, the positions C49 and C50. These two sites lie within the TΨC-stem. There exist other tRNAs with C49 and C50 that are only methylated at C49, but not C50 (e.g. tRNA^{Ala}_{AGC}, tRNA^{Arg}_{ACG}). Why some, but not other such C50-carrying tRNAs are targets for Trm4b methylation remains to be determined.

There also are some tRNAs that possess a C48, but are not subject to C48 methylation by Trm4a (e.g. tRNA^{Glu}_{CTC}, tRNA^{Met}_{CAT2-1, 2-2, 2-3}; tRNA^{Pro}_{TGG}). Again, it is unclear what distinguished these tRNAs from the Trm4a targets. The 3' base seems not to play a role, since both targets and non-targets can carry G or A at the position following C48.

Of note, Trm4 has previously been reported to methylate C40 in tRNA^{Phe}. tRNA^{Phe} in *S. pombe* possesses a cytosine at position 40, which our data indicate to be unmethylated, though the coverage of this site was very low in our tRNA methylome data, such that this has to be interpreted with caution (data not shown).

Next to determining the effects of both Trm4a and Trm4b on the full *S. pombe* tRNA methylome, we also investigated the phenotypic consequences of the absence of the two enzymes. Surprisingly, neither *trm4aΔ* nor *trm4bΔ* or both deletions combined caused strong phenotypes, even though *trm4Δ* in

S. cerevisiae is known to cause sensitivity to the translation inhibitor paromomycin [21] as well as to oxidative stress [8]. Also, several genome-wide studies have reported phenotypes for *trm4Δ* in *S. cerevisiae*, including sensitivity to benomyl [24], hydroxyurea [25], caffeine [26] and cycloheximide [25]. *S. cerevisiae trm4Δ* furthermore shows a temperature-sensitive growth defect in combination with *trm8Δ* [5]. *TRM8* encodes the methyltransferase for m⁷G46, a position that lies close to C48 and C49 in the variable loop of the tRNA. The temperature sensitivity arises due to the rapid tRNA decay (RTD) of tRNA^{Val}_{AAC} [5]. Also, *trm4Δ* in *S. cerevisiae* is temperature-sensitive in the absence of Trm1, which generates N2, N2-dimethylguanosine at G26. Both *trm4Δ trm1Δ* and *trm4Δ trm8Δ* are suppressed by overexpression of the translation elongation factor EF-1A, which thus counteracts tRNA decay by the RTD pathway [7]. Whether *trm4aΔ* and *trm4bΔ* of *S. pombe* show similar synthetic genetic interactions with deletions in genes encoding other tRNA-modifying enzymes remains to be determined. In mice, the deletion of NSun2 is synthetically lethal with the deletion of Dnmt2, the m⁵C methyltransferase for C38 of tRNA^{ASP} [27]. However, *S. pombe* cells lacking both Trm4 homologs as well as the Dnmt2 homolog Pmt1 are viable and have no obvious phenotype [18].

It will also be interesting to see whether Trm4a and Trm4b methylate mRNAs or other small RNAs in *S. pombe*, as has been reported for NSun2/TRM4B in mouse and plants [9–11,15,16], especially since the presence of m⁵C in mRNA has been widely discussed, with some reports arguing for widespread mRNA methylation [13], while stringent statistical filtering points towards only few m⁵C-modified mRNAs [17].

Materials and methods

S. pombe strains and growth conditions

The *S. pombe* strains used in this study are shown in Table 1. They were cultured in standard full medium (YES, 5 g/l yeast extract, 30 g/l glucose, 250 mg/l adenine, 250 mg/l histidine, 250 mg/l leucine, 250 mg/l uracil, 250 mg/l lysine).

To test CaCl₂ sensitivity, serial dilutions of *S. pombe* strains were spotted on YES agar plates with or without CaCl₂ (0.3 M), starting with OD₆₀₀ = 2 and a dilution factor of 1:6 between consecutive spots.

Knockout of *SPAC23C4.17 (trm4b⁺)* in *S. pombe* was obtained by homologous recombination, and correct knockout was verified by polymerase chain reaction (PCR) analysis [28].

Plasmid constructions

For the construction of *S. pombe* vectors carrying *trm4a⁺* and *trm4b⁺*, fragments with 500 bp upstream and downstream of the open reading frame were amplified from genomic DNA and cloned into PstI/SacI of pREP3x or pREP4x (Table 2).

tRNA methylome analysis

The methylome of *S. pombe* tRNAs was analyzed as described previously [19]. tRNAs from *S. pombe* were obtained by separating total RNA on a denaturing polyacrylamide gel

Table 2. Plasmids used in this study.

Designation	Description	Source
pAE1688	pJET1-tRNA ^{ASP} (<i>S.pombe</i>)	[18]
pAE2394	pET15b- <i>trm4a</i> ⁺	This study
pAE2396	pET15b- <i>trm4b</i> ⁺	This study
pAE2644	pJET1-tRNA ^{Pro} _{CGG} (<i>S.pombe</i>)	This study
pAE2645	pJET1-tRNA ^{Pro} _{CGG} (C34A) (<i>S.pombe</i>)	This study
pAE2646	pJET1-tRNA ^{Pro} _{CGG} (C49A) (<i>S.pombe</i>)	This study
pAE2720	pJET1-mini tRNA ^{Pro} _{CGG} (<i>S.pombe</i>)	This study
pAE2721	pJET1-mini tRNA ^{Pro} _{CGG} (C34A) (<i>S.pombe</i>)	This study
pAE2725	pJET1-pre-tRNA ^{Pro} _{CGG}	This study
pAE2726	pJET1-pre-tRNA ^{Pro} _{CGG} (C34A)	This study
pAE2931	pREP3x- <i>trm4a</i> ⁺ (<i>LEU2</i>)	This study
pAE2933	pREP4x- <i>trm4b</i> ⁺ (<i>ura4</i> ⁺)	This study
pAE2939	pJET1-pre-tRNA ^{Pro} _{CGG} (C49A)	This study
pAE2949	pJET1-pre-tRNA ^{Pro} _{CGG} (C34A C49A)	This study

and size-selection for tRNAs. Extracted RNAs were subjected to bisulfite conversion using the EZ RNA Methylation kit (Zymo). 3' dephosphorylation and 5' phosphorylation were performed using T4 polynucleotide kinase (TaKaRa). Library preparation for deep sequencing was done using the NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs). A total of 200 ng of tRNAs per library were used as starting material and ligations done with undiluted adaptors. Adaptor-ligated cDNA was amplified with 12 cycles of PCR reaction and purified using the QIAQuick PCR Purification kit (Qiagen). Libraries were size selected with a 6% Novex tris-borate-EDTA (TBE) polyacrylamide gel (Life Technologies), extracted and ethanol precipitated according to NEBNext instruction manual and resuspended in EB buffer (Qiagen).

The libraries were multiplexed in equimolar ratios and sequenced on one lane of the Illumina HiSeq 2000 platform using paired-end 100 bp sequencing. Sequenced reads were aligned using BSMAP [29] with reference tRNA sequences downloaded from the genomic tRNA database <http://gtrnadb.ucsc.edu/> and from Pombase <https://www.pombase.org/>. Sequences were summarized so as to remove sequences that would be identical if all Cs are converted to Us using a custom bash script. Additionally, close sequences were identified using Clustal Omega <https://www.ebi.ac.uk/Tools/msa/clustalo/> and further pruned manually, so as to obtain a subset of sequences both representative and unique as viewed from the aligner. Aligned reads were quality-controlled, filtered for non-conversion artefacts and ambiguous alignment. Non-conversion ratio was calculated as the ratio of non-converted reads to all reads covering a specific position.

High-throughput bisulfite sequencing of individual tRNAs

Total RNA was isolated from 50 ml yeast cultures (OD₆₀₀ = 1) using TriFast (PeqLab) according to the manufacturers instructions. Bisulfite sequencing of tRNAs was performed as described previously [19]. Primer sequences are listed in Suppl. Table 2. Processing included trimming of PCR primers, selection of high quality reads and sorting of the reads based on the sequence in the degenerate region of the RT-primer. Processed reads were analyzed for bisulfite conversion using BiQ Analyzer HT [30].

Purification of recombinant Trm4a and Trm4b

Total RNA extracted from *wt S. pombe* was used to generate cDNA with TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher). The cDNAs of *trm4a*⁺ and *trm4b*⁺ were subsequently amplified using gene specific primers and cloned into the pET15b vector using restriction sites introduced by the primers (*BclI* for *trm4a*⁺; *XhoI* and *BamHI* for *trm4b*⁺). His₆-Trm4a and His₆-Trm4b were expressed in *Escherichia coli* (DE3) Rosetta cells, and protein production was induced by auto-induction [31]. Purification of the recombinant protein was carried out using Profinity IMAC resin (Bio-Rad) and 200 mM imidazole (elution buffer: 30 mM potassium phosphate, 300 mM KCl, 10% glycerol, 0.1 mM DTT, 200 mM imidazole). The protein was then dialysed against dialysis buffer I (30 mM potassium phosphate pH 7, 200 mM KCl, 20% glycerol, 0.1 mM EDTA, 1 mM DTT) and dialysis buffer II (30 mM potassium phosphate pH 7, 100 mM KCl, 50% glycerol, 0.1 mM EDTA, 1 mM DTT).

RNA substrates for in vitro methylation

S. pombe tRNA substrates were obtained as previously described [18]. Briefly, tRNA sequences were either obtained from recursive PCR using four oligonucleotides (full-length tRNA) or from annealing of two complementary oligonucleotides (mini-tRNAs) and subsequently cloned into the pJET1 vector (Suppl. Table 2). *In vitro* transcription was performed using the TranscriptAid T7 High Yield Transcription Kit (ThermoFisher) according to the manufacturers instructions. 2 µg of the template vector, linearized with *NcoI*, was incubated for 4 hours at 37°C with nucleotides and T7 RNA polymerase in reaction buffer. After DNase I treatment, tRNAs were purified from the reaction using phenol/chloroform extraction followed by gel filtration with Sephadex G50 (GE Healthcare).

In vitro methylation assay

In vitro methylation activity of recombinant Trm4a and Trm4b was analyzed by tritium incorporation as previously described [32]. 20 µl reactions were set up by first diluting 2 µg total RNA or 250 ng tRNA in water and heating for 5 min at 65°C. After addition of methylation buffer and DTT (final concentration: 5 mM Tris-HCl pH 7.5, 5 mM NaCl, 0.5 mM MgCl₂, 0.1 mM DTT) the mixture was allowed to cool to room temperature for 15 min, in order to allow for proper refolding of RNA substrates. Afterwards, 1.25 nM [methyl-³H]-AdoMet (Hartmann Analytic) and 1 µM of Trm4a or Trm4b was added to start the reaction (reaction temperature 30°C). Assays were run for 60 min (Figure 2(b-e)) at 30°C and then spotted onto DE81 filters (Whatman). (t)RNA was precipitated by putting the filter in ice-cold 5% TCA. Filters were washed twice in 5% TCA and once in ethanol for 10 min each. Filters were dried and tritium incorporation was determined by liquid scintillation counting for 10 min per sample. Background was determined from reactions lacking an enzyme and later subtracted from obtained values. For the time courses of tRNA methylation, 500 ng of *in vitro*-transcribed tRNA in a 40 µl reaction was incubated with 1 µM of

enzyme for the indicated times. Samples were processed as described above. Data were fitted to a single exponential reaction progress curve as described [33].

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Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Francesca Tuorto  <http://orcid.org/0000-0003-1625-1181>

Ann E. Ehrenhofer-Murray  <http://orcid.org/0000-0001-8709-1942>

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