The tRNA methyltransferase Trm1 promotes eukaryotic pre-tRNA maturation through catalytic and non-catalytic activities

Jennifer Porat[1], Ana Vakiloroayaei[1], Taylor Cargill[1], and Mark A. Bayfield[1]*
1. Department of Biology, York University, Toronto, Canada
*To whom correspondence should be addressed: bayfield@yorku.ca (M.A.B.)

Abstract
tRNAs undergo an extensive maturation process involving post-transcriptional modifications often associated with tRNA structural stability and promoting the native fold. Impaired post-transcriptional modification has been linked to human disease, likely through defects in translation, mitochondrial function, and increased susceptibility to degradation by various tRNA decay pathways. More recently, evidence has emerged that bacterial tRNA modification enzymes can act as tRNA chaperones to guide tRNA folding in a manner independent from catalytic activity. Here, we provide evidence that the fission yeast tRNA methyltransferase Trm1, which dimethylates nuclear- and mitochondrial-encoded tRNAs at G26, can also promote tRNA functionality in the absence of catalysis. We show that wild type and catalytic-dead Trm1 are active in an *in vivo* tRNA-mediated suppression assay and possess *in vitro* RNA folding activity, suggesting an alternate function as a tRNA chaperone. Further, we demonstrate crosstalk between Trm1 and the RNA chaperone La, with La binding to the 3' end and body of nascent pre-tRNA inhibiting tRNA dimethylation *in vivo* and *in vitro*. Collectively, these results support the hypothesis for multi-functional tRNA modification enzymes that combine catalytic and non-catalytic activities to shape tRNA structure and function.

Introduction
Owing to their critical role in translation, tRNAs are subject to numerous processing and quality control steps to ensure their structural stability and functionality. In eukaryotes, this involves removal of a 5' leader and 3' trailer sequence; where applicable, removal of an intron; CCA addition and aminoacylation of the mature 3' end; as well as the acquisition of post-transcriptional modifications (reviewed in (1, 2)). While most post-transcriptional modifications are non-essential, especially in yeast, the combination of modifications on a given tRNA is an important determinant for structural stability and functionality. Modifications to the anticodon loop affect translational fidelity by maintaining the correct open reading frame and influencing codon-anticodon base-pairing, whereas modifications to the tRNA body primarily affect structure and folding (3). Importantly, studies have found that pre-tRNAs lacking certain post-transcriptional modifications are prone to misfolding and subsequently targeted for decay by the nuclear surveillance machinery (4, 5), while hypomodified mature tRNAs are degraded in the cytoplasm by the rapid tRNA decay pathway (6).

Links between pre-tRNA structure and escape from decay are especially relevant in the context of the function of the eukaryotic RNA chaperone La. The La protein interacts with the 3' uridylate trailer of RNA Polymerase III transcripts, including nascent pre-tRNAs, through a binding pocket formed by the eponymous La motif and RNA recognition motif 1 (RRM1) (7–10). As such, a major role of the La protein is to protect the 3' end of pre-tRNAs from exonucleolytic degradation and direct the order of pre-tRNA end processing (reviewed in (11)). La binding to the 3' trailer results in 5' leader processing by RNase P followed by 3' trailer cleavage, the latter

of which enables La dissociation and recycling onto a new pre-tRNA substrate (12, 13). 3' end binding by La is particularly important for structurally defective pre-tRNAs, which rely on La for protection from decay by the nuclear exosome (14). However, 3' end protection alone is insufficient to rescue increasingly defective pre-tRNAs from nuclear surveillance, necessitating a second activity mapping to the canonical RNA binding surface of the RRM1 (13, 14). Further insight into this alternate activity revealed that the RRM1 interacts with the pre-tRNA body, where it can act as an RNA chaperone to assist pre-tRNA folding (13, 15, 16). Coupling of La's two distinct binding modes—3' uridylate binding and contacts to the tRNA body—enables high-affinity engagement of pre-tRNAs, resulting in their stabilization and proper folding (13).

As tRNAs can also achieve their native, functional conformation through post-transcriptional modifications, the La protein has been proposed to function redundantly with tRNA modification enzymes. In agreement with this, deletion of La is synthetically lethal with the deletion of several tRNA modification enzymes in budding and fission yeast grown at elevated temperatures, where tRNA misfolding can occur (16, 17). Rescue of synthetic lethality by wild type La, but not RRM1 mutants defective for RNA chaperone activity, further supports the idea that La and tRNA modification enzymes have roles in promoting correct tRNA folding (16). Among the modification enzymes that function redundantly with La, N2, N2-dimethylation at G26 by the tRNA methyltransferase Trm1 (18), has been demonstrated to stabilize pre-tRNA *in vitro* and *in vivo* (16, 19). G26 dimethylation has been linked to structural stability at the junction between the anticodon and variable arm, or hinge region; accordingly, the absence of Trm1 in fission yeast is associated with charging defects for certain tRNA species, implying a role in promoting correct folding (16). While studies on the structure stabilizing effect of Trm1-catalyzed methylation have largely been limited to pre-tRNAs in the nucleus, a mitochondrial Trm1 isoform containing an N-terminal mitochondrial targeting sequence has also been described, leading to the modification of select G26-containing mitochondrial-encoded tRNAs (20, 21). The human mitochondrial isoform, which has been linked to promoting protein synthesis, cellular proliferation, and redox homeostasis through modification of mt-tRNA Ile^{UAU}, mt-tRNA Ala^{UGC}, and mt-tRNA Arg^{UCG}, arises from alternative splice isoforms that include or exclude the mitochondrial targeting sequence (21). In contrast, budding yeast Trm1 isoforms arise from alternate transcription start sites that yield proteins differing in the presence of the mitochondrial targeting sequence (20).

The structural and functional importance of post-transcriptional modifications on both nuclear- and mitochondrial-encoded tRNAs has been well-established (reviewed in (22)), but recent evidence points to the idea that post-transcriptional modification enzymes may have alternate functions related to tRNA folding (2). Notably, the bacterial tRNA pseudouridine synthase TruB and methyltransferase TrmA promote tRNA folding even in the absence of catalysis, leading to their characterization as tRNA chaperones (23, 24). Although a catalytically inactive mutant of the eukaryotic TrmA homolog Trm2 can rescue a growth defect associated with a mutant allele of tRNA Ser^{CGA}, suggesting that the dual function of tRNA modification enzymes is evolutionarily conserved (25), to date there has been no mechanistic insight into how eukaryotic tRNA modification enzymes promote tRNA folding independent from catalytic activity.

Here, we use the tRNA methyltransferase Trm1 from *Schizosaccharomyces pombe* to examine how catalytic and catalytic-independent activities shape eukaryotic pre-tRNA maturation and processing. We demonstrate that mutation of a key catalytic residue results in a complete loss of dimethylation on nuclear- and mitochondrial-encoded tRNAs *in vitro* and *in vivo*, yet still promotes pre-tRNA maturation and function in a tRNA-mediated suppression assay. We also uncover the timing of Trm1-mediated dimethylation with respect to other pre-tRNA processing activities and unexpectedly show that the *S. pombe* La protein Sla1 opposes tRNA modification by Trm1. Finally, we provide evidence that both wild-type and catalytically inactive Trm1 are functional in an RNA folding assay *in vitro*, suggesting that it also acts as a tRNA chaperone. These data are thus consistent with the idea that tRNA modification enzymes have retained modification and folding activities throughout evolution and use a combination of these activities to ensure proper tRNA structure and function.

Materials and Methods

Yeast strains and constructs

Standard laboratory techniques were used to culture *S. pombe* cells at 32°C. Tag integration and knockouts were generated with a previously described PCR-based strategy and verified by PCR and western blotting (26) (strains are provided in supplementary table 1). Plasmids were introduced with lithium acetate and selected on minimal media lacking supplements (EMM-ura) (27) (supplementary table 2). Mutations were introduced by site-directed mutagenesis and verified by sequencing. tRNA-mediated suppression growth assays were performed as described (28).

RNA and protein extractions and northern and western blotting

S. pombe cells were grown at 32°C and harvested at mid-log phase. Total RNA was isolated with hot acid phenol and northern analysis was performed as described using 15% TBE-urea polyacrylamide gels (28). Band intensities were quantified with ImageQuant TL software. For PHA26 northerns (19), relative modification index values represent the intensity of the TUC signal divided by the intensity of the PHA26 signal and normalized to a wild type strain or empty vector. For tRNA-mediated suppression northerns, blots were probed with the indicated ^{32}P-labeled probes and an equimolar amount of unlabeled competitor probe to prevent hybridization of the labeled probe with the endogenous tRNA Ser^{UGA} (probe sequences are provided in supplementary table 3). Total protein was extracted according to (28) and western blots were probed with a custom anti-Trm1 antibody (gift from Dr. Richard Maraia, NIH) at 1:2000 and for β-actin at 1:1250.

cDNA synthesis and qRT-PCR

1 μg Turbo DNase-treated RNA was reverse transcribed with the iScript cDNA synthesis kit (BioRad, 1708890), treated with 0.5 μL RNase cocktail (Invitrogen, AM2286), and diluted 1:10 before quantification using the SensiFAST SYBR No-Rox kit (Bioline, BIO-98005). qRT-PCR was performed with 1 μM of each primer (a common reverse primer for both Trm1 isoforms and isoform-specific forward primers, probes provided in supplementary table 3) with the following qRT-PCR settings: 95˚C for 10 minutes and 40 cycles consisting of 10 seconds at 95˚C, 20 seconds at 60˚C, and 20 seconds at 72˚C. Trm1 levels were normalized to *act1* mRNA and

normalized M1 Trm1 signal was subtracted from normalized M24 Trm1 signal to calculate relative M24 Trm1 levels.

Pulse labeling of mitochondrial protein synthesis

Pulse labeling was performed according to (29). Briefly, *S. pombe* cells were initially grown in EMM-ura + 2% glucose, then inoculated into fresh EMM-ura with 0.1% glucose and 2% galactose and grown at 32˚C for 6 generations to a final OD_{600} less than 1 x 10^7 cells/mL. Cell pellets corresponding to ~2.5 x 10^7 cells were washed with 500 μL reaction buffer (40 mM potassium phosphate pH 6.0, 2% galactose, 0.1% glucose), pelleted, resuspended in 500 μL reaction buffer with 10 mg/mL cycloheximide, and incubated at room temperature for 15 minutes. Cycloheximide was omitted to evaluate cytoplasmic translation. 5 μL [^{35}S]-methionine was directly added to the cell suspension, mixed thoroughly, and incubated for 30 minutes at room temperature. Cells were pelleted and the pellet was resuspended in 75 μL solubilization buffer (1.8 M NaOH, 1 M β-mercaptoethanol, 0.01 mM PMSF) and mixed. 500 μL water was added and proteins were TCA-precipitated, followed by separation on a 17.5% SDS gel, transfer to a nitrocellulose membrane, and exposed to a Phosphor screen overnight.

Recombinant protein purification and electrophoretic mobility shift assay (EMSA)

Recombinant His-tagged protein expression was induced in *Escherichia coli* with 1 mM IPTG at 16˚C for 18 hours and purified over a Ni^{2+} column (His-TRAP, GE-Amersham), followed by a second round of purification over a heparin column (Hi-TRAP, GE-Amersham). Proteins were concentrated into 1X EMSA buffer (20 mM Tris HCl pH 7.6, 100 mM KCl, 0.2 mM EDTA pH 8.0, 1 mM DTT) and quantified by SDS-PAGE. EMSAs were performed as described (16). Briefly, 3000 cpm PAGE purified, T7-transcribed ^{32}P α-ATP-labeled pre-tRNAs (sequences provided in supplementary table 4) were heated to 95°C and slow-cooled to room temperature before addition to a 20 uL reaction containing 1X EMSA buffer. Increasing amounts of recombinant Trm1 were added to the reaction mix, followed by incubation at 32°C for 20 minutes. Reactions were cooled on ice for 2 minutes and complexes were resolved on 8% non-denaturing polyacrylamide gels run at 4°C and 100V. The proportion of bound tRNA was quantified with ImageQuant TL software and binding curves were fit to a non-linear specific binding curve (Hill slope) with GraphPad Prism 6.0.

In vitro methylation and primer extension

5 μM T7-transcribed pre-tRNA Ser^{UGA} was methylated for 3 hours at 32˚C in a 25 μL reaction containing 100 mM Tris HCl pH 7.5, 0.1 mM EDTA pH 8.0, 10 mM $MgCl_2$, 40 mM NH_4Cl_2, 1 mM DTT, 100 mM SAM (NEB, B9003S), and 5 μM recombinant Trm1. For competition between hLa and Trm1, 5 μM pre-tRNA Ser^{UGA} was pre-incubated alone or with 5 μM recombinant hLa in a 50 μL reaction for 20 minutes at 32˚C in methylation reaction buffer without $MgCl_2$ and SAM. Reactions were snap chilled on ice to preserve complexes, followed by the addition of recombinant Trm1, 2 mM $MgCl_2$, and 100 mM SAM. Reactions were incubated at 32˚C and samples were removed at indicated time points and immediately purified by phenol: chloroform: isoamyl alcohol (25:24:1) extraction. Primer extensions were performed with SuperScript III (Invitrogen, 18080093) following standard methods.

FRET assays

FRET assays were performed as described (15, 30). Briefly, unlabeled and Cy5- and Cy3-labeled RNA substrates were synthesized by IDT (sequences provided in supplementary table 4) and used at a final concentration of 25 nM. Where applicable, recombinant proteins were added to 400 μL reactions at a final concentration of 100 nM immediately prior to taking measurements. Fluorescence emission at 590 and 680 nm was recorded on a Cary Eclipse fluorimeter with readings taken in half-second time-points over a period of 180 seconds. Strand dissociation measurements were initiated immediately following strand annealing with the addition of 1 μM unlabeled competitor RNA. Strand annealing and dissociation rate constants were determined by calculating a FRET index (emission at 680 nm divided by emission at 590 nm) over time and normalizing values between 0 and 1. Curves were fitted to an equation for one-phase association (strand annealing) or one-phase decay (strand dissociation) in Graphpad Prism 6.0.

Results

Alternate transcriptional start sites yield nuclear- and mitochondrially-targeted Trm1 in *S. pombe*

As in Trm1 from budding yeast, fission yeast Trm1 can exist as a nuclear- or mitochondrial-targeted isoform produced from alternate transcription start sites (Figure 1A). Two alternate transcription start sites have been mapped for fission yeast Trm1: one producing a ~250 nucleotide 5' UTR ahead of the first ATG (this isoform will henceforth be referred to as M1, for beginning at the first methionine), and a second with a ~10 nucleotide 5'UTR ahead of a downstream ATG (M24, beginning at the 24th amino acid) (Figure 1A) (31). While both isoforms have been shown to target mitochondrially-encoded tRNAs in budding yeast, the N-terminal extension in the longer isoform enhances the efficiency of mitochondrial targeting, resulting in a predominantly mitochondrial-localized protein compared to the nuclear-localized shorter isoform (20). Quantification of the relative abundance of both transcripts in wild type cells grown in rich media revealed that the nuclear-targeted transcript is approximately four times as abundant as the mitochondrially-targeted transcript at steady state levels (Figure 1B). Consistent with the idea that modifications to the tRNA body do not dramatically alter bulk translation, pulse labeling revealed no defects in mitochondrial or cytoplasmic translation upon Trm1 deletion (Figure S1).

D201 is a key catalytic residue for N2, N2-dimethylation of nuclear- and mitochondrially-encoded tRNAs

To investigate potential modification-independent functions of Trm1, we aligned an AlphaFold (32) prediction of *S. pombe* Trm1 to the structure of an archaeal Trm1 homolog bound to SAH (33), mutated an aspartic acid in the conserved catalytic site to alanine (D201A) (Figure 1C) and monitored Trm1-catalyzed modification of nuclear- and mitochondrially-encoded tRNAs with a northern blotting assay, positive hybridization in the absence of modification at G26 (PHA26) (19, 34). This assay uses a probe complementary to the region overlapping G26, such that dimethylation at G26 impairs probe hybridization, resulting in a decrease in signal intensity. An additional probe targeting the TUC loop, which is free of modifications that interfere with probe hybridization, serves as an internal normalization for tRNA abundance (Figure 1D). The D201A

mutant showed a comparable decrease in modification as *trm1Δ* cells transformed with an empty vector, in the context of the nuclear- and mitochondrially-targeted isoforms, despite similar levels of protein accumulation as the wild type isoforms (Figure 1D, E). Moreover, these northern blots demonstrate subcellular targeting of the Trm1 isoforms: M24 Trm1 robustly modifies nuclear- and mitochondrial-encoded tRNAs, consistent with its proposed localization to the nucleus and mitochondria, while M1 Trm1 only modifies mitochondrially-encoded tRNAs, suggesting that it is solely present in the mitochondria (Figure 1D). These results also confirmed the previously demonstrated substrate specificity of Trm1, where G26-containing tRNAs exhibit different levels of Trm1-catalyzed modification (19). Certain nuclear-encoded tRNAs, including tRNA Ser^{UGA} and tRNA Leu^{AAG} are robustly modified by endogenous Trm1 (Figure 1D, "wild type") and overexpressed M24 Trm1, while the PHA26 probe anneals to the G26-containing tRNA Phe^{GAA} in a manner that remains unchanged based on the presence of Trm1, consistent with a lack of modification.

To rule out defects in tRNA binding contributing to a lack of modification by D201A, we purified recombinant wild type and D201A M24 Trm1 and measured *in vitro* binding affinity to pre-tRNA Ser^{UGA} using electrophoretic mobility shift assays (EMSA). Wild type and D201A exhibited comparable binding affinity, suggesting that disruption of the putative catalytic site does not impair tRNA binding (Figure 2A, B). We also set up *in vitro* methylation reactions with pre-tRNA Ser^{UGA}, recombinant Trm1, and SAM, and measured modification efficiency by primer extension, as dimethylation on the Watson-Crick face is sufficient to cause a reverse transcriptase (RT) stop (16, 35). The lack of modification by D201A *in vitro* (Figure 2C), evident by the lack of RT stop, and by PHA26 northern blotting *in vivo* (Figure 1D) confirm that D201A is indeed catalytically inactive, thus enabling further studies into potential catalytic-independent functions of Trm1.

Trm1 promotes tRNA-mediated suppression through catalytic and catalytic-independent activities

The tRNA-mediated suppression assay has been used to test various aspects of pre-tRNA processing including 3' processing, pre-tRNA folding, and modification (reviewed in (28, 36)). The assay relies on a mutation to the anticodon of tRNA Ser^{UCA}, allowing stop codon readthrough of a nonsense mutation in the AIR carboxylase gene which, when fully functional, prevents the accumulation of a red metabolic intermediate (36, 37). The G35C mutation that enables nonsense decoding also impairs anticodon-intron base pairing in the suppressor pre-tRNA, resulting in a misfold that increases susceptibility of the pre-tRNA to exosome-mediated decay (14). Suppressor pre-tRNA misfolding can be rescued by overexpression of pre-tRNA processing factors, thus enabling identification and insight into the mode of action of pre-tRNA binding proteins, including the RNA chaperone La (13, 14). We and others have previously demonstrated that Trm1 is active in promoting tRNA-mediated suppression (16, 19), although this could be due to a stabilizing effect from the modification, a separate pre-tRNA processing activity that functions independently from modification, or a combination of these.

Wild type and catalytically inactive nuclear-targeted Trm1 promote suppression of the tRNA Ser^{UCA} allele in a *sla1* null background, suggesting that modification is not strictly required

for suppression activity (Figure 3A). Still, the addition of the U47:6C mutation to the suppressor tRNA, which is associated with further misfolding and a reliance on the RNA chaperone activity of La (15, 38), resulted in partial suppression by wild type Trm1 but not D201A (Figure S2A). This is suggestive of two distinct activities for Trm1: its established methyltransferase activity, which has been previously shown to stabilize tRNA structure (16), and a modification-independent activity that is dependent on tRNA binding. tRNA binding alone is sufficient for suppression activity in the context of the tRNA Ser^{UCA} allele, whereas binding and modification are required for the more defective suppressor tRNA allele.

It has also been reported that deletion of the RNA Polymerase III repressor Maf1 causes anti-suppression, which is counter-intuitive with the increase in tRNA transcription observed upon Maf1 deletion (19). However, the anti-suppression phenotype can be explained by hypomodification of the suppressor tRNA by Trm1, as Trm1—and presumably other pre-tRNA binding proteins— become limiting with the resultant increase in the pre-tRNA pool (19). Complete and near-complete suppression of tRNA Ser^{UCA} is achieved by wild type and catalytically inactive Trm1, respectively, in the *maf1Δ* strain (Figure 3A). In the model whereby Trm1 becomes limiting in the absence of Maf1, suppression by D201A suggests that it is not only the Trm1-catalyzed modification that becomes limiting, but also tRNA binding by Trm1, further supporting the notion of a catalytic-independent function for Trm1.

To decipher the mechanism by which Trm1 promotes tRNA-mediated suppression, we quantified the abundance of the suppressor tRNA at the precursor and mature level (Figure 3B, S2B-D). Probes against the tRNA Ser^{UCA} intron were used to detect the precursor, which migrates as 2 distinct bands in the absence of the yeast La protein Sla1, representing the leader-containing, trailer-processed and leader- and trailer-processed species, while a probe overlapping the exon junctions was used to detect the mature suppressor tRNA. We did not observe an increase in mature suppressor tRNA levels upon expression of wild type or catalytically inactive Trm1, suggesting that suppression by Trm1 is not the result of increased suppressor tRNA levels, as has previously been reported for suppression observed upon suppressor tRNA gene duplication (Figure 3B, S2D) (38). Similarly, tRNA-mediated suppression of tRNA Ser^{UCA} by human or *S. pombe* La is largely due to 3' trailer binding and protection of the suppressor pre-tRNA from exosome-mediated decay, which is characterized by the stabilization of a 3' trailer-containing suppressor pre-tRNA species relative to an empty vector (13, 14). We can rule out suppression due to 3' end protection by Trm1, as evident by the lack of stabilization of the 3' trailer-containing species relative to the empty vector (Figure 3B, pre-tRNA Ser^{UCA} and endogenous pre-tRNA Lys^{CUU}, compare to stabilization of the nascent pre-tRNA with hLa in Figure 4C). It is worth noting that stabilization of the 5' and 3' processed pre-tRNA species occurs upon overexpression of D201A, which supports the idea that in the absence of catalysis, Trm1 remains bound to and stabilizes the pre-tRNA (Figure 3B, top panel).

We also performed the PHA26 assay to investigate the role of Trm1 modification in tRNA-mediated suppression (Figure 3B, third panel) The suppressor tRNA-containing strain possesses endogenous Trm1, so the lack of complete modification (represented by a decrease in signal intensity with the PHA26 probe) of the suppressor tRNA with an empty vector agrees with

previous reports that Trm1 is normally the limiting factor in tRNA modification (19). The suppressor tRNA only becomes fully modified upon wild type Trm1 overexpression, with overexpression of D201A resulting in comparable modification levels to an empty vector (Figure 3B). The PHA26 assay also provides information on the timing of Trm1 modification with respect to pre-tRNA processing activities. We observed no change in PHA signal intensity upon Trm1 expression in the trailer-processed, leader-containing pre-tRNA (Figure 3B, top band in top panel), suggesting that it is not a substrate for modification, but noted a decrease in signal intensity for the leader- and trailer-processed pre-tRNA and mature tRNA, supporting a previously proposed model that Trm1 modification occurs after Sla1 binding and 3' end processing (16) (Figure 3C-E).

Trm1 competes with the RNA chaperone La for pre-tRNA binding

While previous reports indicate that tRNAs become hypomodified at G26 upon Maf1 deletion (19), we did not observe any modification differences with and without endogenous Maf1 (Figure 3C-E). Since those previous measurements were performed in a *sla1+* strain and our data are from a *sla1Δ* strain, we examined the relationship between Sla1 and Trm1-dependent modification. Strikingly, we detected an increase in G26 modification upon Sla1 deletion for leucine and serine tRNAs, suggesting a possible competition between Sla1 and Trm1 for pre-tRNA binding (Figure 4A). As the La protein has been reported to make multiple contacts to pre-tRNAs—engagement of the uridylate trailer and pre-tRNA body (13, 14, 39)—we took advantage of previously characterized mutants of the human La protein (hLa) to map competition to La's various domains and RNA binding modes (Figure 4B). We measured tRNA-mediated suppression activity, steady-state precursor and mature tRNA levels, and G26 modification status for the suppressor tRNA Ser^{UCA} in a *sla1Δ* strain transformed with wild type or mutant hLa (Figure 4B, C). As has been previously reported, mutations to uridylate-binding residues in the La motif (Q20A Y24A D33I) led to decreased suppression activity. In contrast, mutations to the RRM1, which result in impaired RNA chaperone activity but unchanged uridylate binding (13), exhibited no defects in the tRNA-mediated suppression assay, in agreement with the idea that tRNA Ser^{UCA} predominantly requires 3' end protection, but not RNA chaperone activity, for complete suppression (14) (Figure 4B). This is evident from stabilization of the top suppressor tRNA band on the northern blots, corresponding to the nascent, 5' leader- and 3' trailer-containing pre-tRNA species, for wild type and RRM1 hLa mutants (Figure 4C).

Like overexpressed Trm1 (Figure 3B), endogenous Trm1 displays the same modification preferences for modifying end-processed pre-tRNA (Figure 4C). Further, we observed a decrease in G26 modification of the suppressor tRNA in strains transformed with wild type hLa and the RRM1 mutant Y114A F155A, which displays decreased RNA chaperone activity but no pre-tRNA binding defects (14), a complete rescue of modification similar to the empty vector by the uridylate-binding mutant, and partial rescue by the RRM1 loop-3 R142A R143A K148A K151A mutant, which is defective in both RNA chaperone activity and pre-tRNA binding (13, 15) (Figure 4C). These results suggest that competition between La and Trm1 is linked to La's pre-tRNA affinity, with mutations that disrupt uridylate or tRNA body binding, which contribute additively to pre-tRNA affinity (13), leading to increased modification at G26. Notably, these

results also reveal that the apparent hLa-dependent increases in mature suppressor tRNA levels, which has been used as an indicator of the degree of tRNA-mediated suppression activity, can arise from a combination of changes in mature tRNA levels and differences in G26 modification. Changes in mature suppressor tRNA levels in the presence or absence of wild type or mutant hLa are less pronounced when using a probe that does not overlap with G26 (Figure 4C, compare relative mature tRNA Ser^{UCA} to apparent relative mature tRNA Ser^{UCA}). Thus, mature suppressor tRNA levels are not the sole determinant that can be used to predict or assign meaning to tRNA-mediated suppression activity, providing additional evidence for the hypothesis that an increase in specific tRNA activity due to modification or folding is linked to tRNA-mediated suppression activity.

To determine whether La binding to pre-tRNA acts as a shield to prevent Trm1 binding and modification of nuclear-encoded pre-tRNAs, we pre-incubated pre-tRNA with and without recombinant hLa and set up an *in vitro* methylation time course to examine changes in G26 dimethylation. We observed decreased methylation in the presence of hLa, particularly at earlier time points in the reaction (Figure 4D, E), consistent with our *in vivo* data demonstrating that La inhibits Trm1 modification. As the methylation reaction occurs in the absence of any cellular factors that promote tRNA processing, including other modification enzymes and end-processing endo and exonucleases, this is suggestive of La itself acting as a barrier to Trm1-mediated methylation, rather than decreasing dimethylation indirectly, through its roles in facilitating pre-tRNA processing.

Trm1 folds tRNA *in vitro*

As our data point towards Trm1 possessing a catalytic-independent function that promotes tRNA functionality *in vivo*, we considered the possibility that Trm1 might function as a tRNA chaperone, much like the bacterial tRNA modifying enzymes TruB and TrmA (23, 24). We employed an *in vitro* FRET-based RNA annealing and dissociation assay that has been used to characterize RNA chaperone activity for La and La Related Proteins (15, 30). This assay uses complementary RNA oligos 5' end labeled with Cy3 or Cy5, such that annealing of the oligos will result in FRET between the two fluorophores, from which we can calculate the rate of annealing (k_{ann}), followed by the addition of an unlabeled competitor to assess strand dissociation activity (k_{SD}), which is accompanied by a decrease in FRET between the two fluorophores (Figure 5A). In the absence of strand dissociation, as measured by an increase in FRET following competitor addition, the rate constant calculated in the second phase is considered k_{ann2}. Consistent with previous data (15, 30), strand annealing and strand dissociation were enhanced by the addition of recombinant human La (Figure 5B, C). We also observed strand annealing and dissociation for wild type and catalytically inactive Trm1, suggesting that they are capable of folding RNA *in vitro* (Figure 5B, C). Our *in vivo* data demonstrating that Trm1 is active in a tRNA-mediated suppression assay independent of catalysis, coupled with our *in vitro* data supporting an RNA chaperone-like activity, point towards Trm1 acting as a tRNA chaperone, similar to prokaryotic tRNA modification enzymes.

Discussion

In this work, we provide evidence of both catalytic and catalytic-independent roles for the fission yeast tRNA methyltransferase Trm1. That these dual functions appear to be conserved across prokaryotes and eukaryotes speaks to both the propensity for RNA to adopt misfolded or alternate, non-functional conformations (40), and the importance of resolving tRNA misfolds to ensure optimal function in translation. While we have shown that wild type and catalytically inactive Trm1 promotes the proper processing and folding of the Ser^{UCA} suppressor tRNA, it remains to be found how Trm1 chaperones endogenous misfolded pre-tRNAs. We anticipate that Trm1 uses its tRNA folding activity to guide pre-tRNA folding in the nucleus, while Trm1-catalyzed dimethylation is particularly important for additional stabilization to resolve more severe misfolds. This is especially evident when comparing tRNA-mediated suppression of various suppressor tRNA mutants. The G35C suppressor tRNA mutation (Ser^{UCA}) only results in a misfold at the pre-tRNA level, due to altered base-pairing with the intron which is later removed and thus the mature suppressor tRNA possesses the same fold as endogenous tRNA Ser^{UGA}. In contrast, adding the U47:6C mutation leads to a misfold that persists in the mature tRNA in the cytoplasm, compounded by the fact that the degree of misfold in U47:C is also greater than that of tRNA Ser^{UCA}. Therefore, while the catalytic-independent activity alone is sufficient for Trm1 associated tRNA Ser^{UCA} stabilization and folding in the nucleus, tRNA Ser^{UCA} U47:6C requires both Trm1-mediated pre-tRNA stabilization in the nucleus and the Trm1-catalyzed modification that remains on the mature tRNA in the cytoplasm for suppression activity (Figure 3, Figure S2).

The finding that the catalytically inactive D201A mutant promotes tRNA-mediated suppression in a *sla1Δ* strain, but only partial suppression in a *sla1Δ maf1Δ* strain provides additional evidence supporting the importance of both catalytic and non-catalytic functions to ensure proper tRNA structure and function (Figure 3). The increase in tRNA transcription resulting from the deletion of the RNA Polymerase III repressor Maf1 may in turn lead to an increase in the persistence of defective pre-tRNAs as tRNA processing factors, including Trm1, become limiting. Thus, the ability to benefit from Trm1's modification and folding activity could be more effective at resolving misfolds. Alternately, since tRNA modification can lead to product release from the enzyme, as has been demonstrated for the tRNA pseudouridine synthase TruB (41, 42), the lack of catalysis observed with D201A might be expected to lead to pre-tRNA retention by Trm1, thus compounding the effect of Trm1 becoming limited if it is unable to effectively recycle onto new substrates. Another unexpected result concerns the nature of the correlation between mature suppressor tRNA levels and the degree of tRNA-mediated suppression activity, as has been posited to partially explain why the La protein promotes tRNA-mediated suppression (14). We found that the apparent increase in mature suppressor tRNA levels can at least partially be attributed to a decrease in modification at G26, supporting the idea of exercising caution when designing northern blotting probes to avoid sites of bulky modifications on the Watson-Crick face (34). Rather, our results point towards tRNA-mediated suppression activity as a function of the combination of suppressor tRNA levels and specific activity per suppressor tRNA molecule (Figure 3, Figure 4). For Trm1, which promotes tRNA-mediated suppression in the absence of increasing mature tRNA levels, this may be due in part to stabilization imparted by the modification, or Trm1-mediated tRNA folding leading to increased stability or accommodation by the ribosome. We can also infer differences in the

mechanism of tRNA-mediated suppression for tRNA Ser^{UCA} by hLa and Trm1: hLa primarily acts by stabilizing the 3' end of the nascent suppressor pre-tRNA to protect it from degradation by the nuclear surveillance pathway, while Trm1 appears to increase suppressor tRNA functionality without affecting tRNA levels. Moreover, La and Trm1 act on different pre-tRNA species—La targeting nascent, trailer-containing pre-tRNA and Trm1 modifying intron-containing, end-processed pre-tRNA—suggesting that pre-tRNAs have multiple chances to fold correctly at different stages in the pre-tRNA processing pathway.

Additionally, while this work serves to reinforce the importance of La-mediated 3' end protection and tRNA chaperone activity for tRNA-mediated suppression, it also revealed a surprising link between the La protein and Trm1 modification (Figure 4). Previously, Trm1 and La were hypothesized to function redundantly in the tRNA biogenesis pathway (16, 17), with La-mediated pre-tRNA stabilization thought to increase the window in which Trm1 could modify pre-tRNAs in the nucleus (16). While it is indeed likely that Trm1 and La have redundant functions — both promote pre-tRNA stability and folding— it is interesting that they oppose each other with respect pre-tRNA occupancy, in that the interaction between La and pre-tRNA prevents Trm1 from accessing and modifying pre-tRNAs, presumably through a shielding effect (13, 39). It remains to be found whether a relationship between La and Trm1 exists in humans, where La proteins harbor an additional RNA recognition motif (RRM2 or xRRM) within a more divergent C-terminal domain that has been implicated in La's RNA chaperone activity (43–45). The extent to which the C-terminal domain influences tRNA binding *in vivo* remains unknown, although its propensity for sequence-independent binding to structured, hairpin-containing RNA (44, 46) may affect how La engages the pre-tRNA body. Further, exploring how La may influence the installation of other modifications occurring at the pre-tRNA stage will likely be an active area of future research.

Finally, our work identifying nuclear and mitochondrial functions for Trm1 in *S. pombe* adds to the growing body of literature proposing coordination of the nuclear and mitochondrial genomes through tRNA modifications (47). In budding and fission yeast, this may be achieved by altering the balance of transcription of the nuclear- and mitochondrially-targeted Trm1 isoforms, which will in turn influence the degree of G26 dimethylation of nuclear- and mitochondrially-encoded tRNAs. Still, how Trm1-catalyzed tRNA modifications may alter cytoplasmic and mitochondrial translation remains unknown. Although we showed that there are no defects in bulk translation upon deletion of Trm1, we do not exclude the possibility that dimethylation at G26 alters the dynamics of codon recognition, as has been described for methylation of human mitochondrial Serine and Threonine tRNAs at position 32 by METTL8 (48). Additionally, studying the interplay between Trm1 and other nuclear-encoded enzymes that similarly modify mitochondrial tRNAs will continue to inform our understanding of the control and potential co-regulation of the nuclear and mitochondrial genomes.

Our work underscores the complexity of tRNA maturation in a model eukaryote by highlighting the multi-functional nature of a tRNA modification enzyme. The data presented here support the growing idea that tRNA modification enzymes can have roles beyond catalysis, and that this holds true in prokaryotes and eukaryotes. The extent to which this applies to other tRNA

modification enzymes, and RNA modification enzymes more generally, will be an exciting area of future research that will continue to inform our understanding of the mechanisms underlying RNA fold and function.

Data Availability

All data pertaining to this manuscript is included in the main manuscript and supplementary files.

Author Contributions

Jennifer Porat: Conceptualization, Formal analysis, Methodology, Validation, Writing—original draft, Writing—review & editing. Ana Vakiloroayaei: Formal analysis, Methodology, Writing—review & editing. Taylor Cargill: Validation, Writing—review & editing. Mark A. Bayfield: Conceptualization, Writing—review & editing.

Acknowledgements

We thank Dr. Richard Maraia for the Trm1 antibody and *maf1Δ* yeast strain.

Funding

J.P. is supported by a Canada Graduate Scholarship (Doctoral) from the National Sciences and Engineering Research Council of Canada. M.A.B. is supported by a Discovery Grant from NSERC ("Impact of chemical modification of noncoding RNAs on gene expression in *S. pombe*").

References

1. Phizicky,E.M. and Hopper,A.K. (2010) tRNA biology charges to the front. *Genes Dev.*, **24**, 1832–1860.
2. Porat,J., Kothe,U. and Bayfield,M.A. (2021) Revisiting tRNA chaperones: New players in an ancient game. *RNA*, **27**, 543–559.
3. Hopper,A.K. (2013) Transfer RNA post-transcriptional processing, turnover, and subcellular dynamics in the yeast Saccharomyces cerevisiae. *Genetics*, 10.1534/genetics.112.147470.
4. Kadaba,S., Wang,X. and Anderson,J.T. (2006) Nuclear RNA surveillance in Saccharomyces cerevisiae: Trf4p-dependent polyadenylation of nascent hypomethylated tRNA and an aberrant form of 5S rRNA. *RNA*, 10.1261/rna.2305406.
5. Kadaba,S., Krueger,A., Trice,T., Krecic,A.M., Hinnebusch,A.G. and Anderson,J. (2004) Nuclear surveillance and degradation of hypomodified initiator tRNA Met in S. cerevisiae. *Genes Dev.*, **18**, 1227–1240.
6. Alexandrov,A., Chernyakov,I., Gu,W., Hiley,S.L., Hughes,T.R., Grayhack,E.J. and Phizicky,E.M. (2006) Rapid tRNA decay can result from lack of nonessential modifications. *Mol. Cell*, 10.1016/j.molcel.2005.10.036.
7. Stefano,J.E. (1984) Purified lupus antigen la recognizes an oligouridylate stretch common to the 3' termini of RNA polymerase III transcripts. *Cell*, **36**, 145–154.
8. Rinke,J. and Steitz,J.A. (1982) Precursor molecules of both human 5S ribosomal RNA and transfer RNAs are bound by a cellular protein reactive with anti-La Lupus antibodies. *Cell*, 10.1016/0092-8674(82)90099-X.
9. Teplova,M., Yuan,Y.R., Phan,A.T., Malinina,L., Ilin,S., Teplov,A. and Patel,D.J. (2006) Structural

basis for recognition and sequestration of UUUOH 3' temini of nascent RNA polymerase III transcripts by La, a rheumatic disease autoantigen. *Mol. Cell*, **21**, 75–85.
10. Kotik-Kogan,O., Valentine,E.R., Sanfelice,D., Conte,M.R. and Curry,S. (2008) Structural Analysis Reveals Conformational Plasticity in the Recognition of RNA 3' Ends by the Human La Protein. *Structure*, **16**, 852–862.
11. Bayfield,M.A., Vinayak,J., Kerkhofs,K. and Mansouri-Noori,F. (2019) La proteins couple use of sequence-specific and non-specific binding modes to engage RNA substrates. *RNA Biol.*, **18**, 168–177.
12. Yoo,C.J. and Wolin,S.L. (1997) The yeast La protein is required for the 3' endonucleolytic cleavage that matures tRNA precursors. *Cell*, 10.1016/S0092-8674(00)80220-2.
13. Bayfield,M.A. and Maraia,R.J. (2009) Precursor-product discrimination by la protein during tRNA metabolism. *Nat. Struct. Mol. Biol.*, **16**, 430–437.
14. Huang,Y., Bayfield,M.A., Intine,R. V. and Maraia,R.J. (2006) Separate RNA-binding surfaces on the multifunctional la protein mediate distinguishable activities in tRNA maturation. *Nat. Struct. Mol. Biol.*, **13**, 611–618.
15. Naeeni,A.R., Conte,M.R. and Bayfield,M.A. (2012) RNA chaperone activity of human La protein is mediated by variant RNA recognition motif. *J. Biol. Chem.*, **287**, 5472–5482.
16. Vakiloroayaei,A., Shah,N.S., Oeffinger,M. and Bayfield,M.A. (2017) The RNA chaperone La promotes pre-tRNA maturation via indiscriminate binding of both native and misfolded targets. *Nucleic Acids Res.*, **45**, 11341–11355.
17. Copela,L.A. Chakshusmathi,G., Sherrer,R.L. and Wolin,S.L. (2006) The La protein functions redundantly with tRNA modification enzymes to ensure tRNA structural stability. *RNA*, **12**, 644–654.
18. Ellis,S.R., Morales,M.J., Li,J.M., Hopper,A.K. and Martin,N.C. (1986) Isolation and characterization of the TRM1 locus, a gene essential for the N2,N2-dimethylguanosine modification of both mitochondrial and cytoplasmic tRNA in Saccharomyces cerevisiae. *J. Biol. Chem.*, **261**, 9703–9709.
19. Arimbasseri,A.G., Blewett,N.H., Iben,J.R., Lamichhane,T.N., Cherkasova,V., Hafner,M. and Maraia,R.J. (2015) RNA Polymerase III Output Is Functionally Linked to tRNA Dimethyl-G26 Modification. *PLoS Genet.*, **11**, e1005671.
20. Ellis,S.R., Hopper,A.K. and Martin,N.C. (1987) Amino-terminal extension generated from an upstream AUG codon is not required for mitochondrial import of yeast N2,N2-dimethylguanosine-specific tRNA methyltransferase. *Proc. Natl. Acad. Sci. U. S. A.*, **84**, 5172–5176.
21. Dewe,J.M., Fuller,B.L., Lentini,J.M., Kellner,S.M. and Fu,D. (2017) TRMT1-catalyzed tRNA modifications are required for redox homeostasis to ensure proper cellular proliferation and oxidative stress survival. *Mol. Cell. Biol.*, **37**, MCB.00214-17.
22. Suzuki,T. (2021) The expanding world of tRNA modifications and their disease relevance. *Nat. Rev. Mol. Cell Biol.*, **22**, 375–392.
23. Keffer-Wilkes,L.C., Soon,E.F. and Kothe,U. (2020) The methyltransferase TrmA facilitates tRNA folding through interaction with its RNA-binding domain. *Nucleic Acids Res.*, **48**, 7981–7990.
24. Keffer-Wilkes,L.C., Veerareddygari,G.R. and Kothe,U. (2016) RNA modification enzyme TruB is a tRNA chaperone. *Proc. Natl. Acad. Sci. U. S. A.*, **113**, 14306–14311.

25. Johansson,M.J.O. and Byström,A.S. (2002) Dual function of the tRNA(m5U54)methyltransferase in tRNA maturation. *RNA*, **8**, 324–335.
26. Bähler,J., Wu,J.Q., Longtine,M.S., Shah,N.G., McKenzie,A., Steever,A.B., Wach,A., Philippsen,P. and Pringle,J.R. (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast*, 10.1002/(SICI)1097-0061(199807)14:10<943::AID-YEA292>3.0.CO;2-Y.
27. Kanter-Smoler,G., Dahlkvist,A. and Sunnerhagen,P. (1994) Improved method for rapid transformation of intact Schizosaccharomyces pombe cells. *Biotechniques*.
28. Porat,J. and Bayfield,M.A. (2020) Use of tRNA-Mediated Suppression to Assess RNA Chaperone Function. In Heise,T. (ed), *RNA Chaperones: Methods and Protocols*. Springer US, New York, NY, pp. 107–120.
29. Gouget,K., Verde,F. and Barrientos,A. (2008) In vivo labeling and analysis of mitochondrial translation products in budding and in fission yeasts. *Methods Mol. Biol.*, **457**, 113–124.
30. Hussain,R.H., Zawawi,M. and Bayfield,M.A. (2013) Conservation of RNA chaperone activity of the human La-related proteins 4, 6 and 7. *Nucleic Acids Res.*, 10.1093/nar/gkt649.
31. Thodberg,M., Thieffry,A., Bornholdt,J., Boyd,M., Holmberg,C., Azad,A., Workman,C.T., Chen,Y., Ekwall,K., Nielsen,O., *et al.* (2019) Comprehensive profiling of the fission yeast transcription start site activity during stress and media response. *Nucleic Acids Res.*, **47**, 1671–1691.
32. Jumper,J., Evans,R., Pritzel,A., Green,T., Figurnov,M., Ronneberger,O., Tunyasuvunakool,K., Bates,R., Žídek,A., Potapenko,A., *et al.* (2021) Highly accurate protein structure prediction with AlphaFold. *Nature*, **596**, 583–589.
33. Awai,T., Ochi,A., Ihsanawati, Sengoku,T., Hirata,A., Bessho,Y., Yokoyama,S. and Hori,H. (2011) Substrate tRNA recognition mechanism of a multisite-specific tRNA methyltransferase, Aquifex aeolicus Trm1, based on the X-ray crystal structure. *J. Biol. Chem.*, **286**, 35236–35246.
34. Khalique,A., Mattijssen,S. and Maraia,R.J. (2022) A versatile tRNA modification-sensitive northern blot method with enhanced performance. *RNA*, **28**, 418–432.
35. Motorin,Y., Muller,S., Behm-Ansmant,I. and Branlant,C. Identification of modified residues in RNAs by reverse transcription-based methods. *Methods Enzymol.*, **425**, 21–53.
36. Rijal,K., Maraia,R.J. and Arimbasseri,A.G. (2015) A methods review on use of nonsense suppression to study 3’ end formation and other aspects of tRNA biogenesis. *Gene*, **556**, 35–50.
37. Huang,Y., Intine,R. V., Mozlin,A., Hasson,S. and Maraia,R.J. (2005) Mutations in the RNA Polymerase III Subunit Rpc11p That Decrease RNA 3’ Cleavage Activity Increase 3’-Terminal Oligo(U) Length and La-Dependent tRNA Processing. *Mol. Cell. Biol.*, **25**, 621–636.
38. Iben,J.R., Epstein,J.A., Bayfield,M.A., Bruinsma,M.W., Hasson,S., Bacikova,D., Ahmad,D., Rockwell,D., Kittler,E.L.W., Zapp,M.L., *et al.* (2011) Comparative whole genome sequencing reveals phenotypic tRNA gene duplication in spontaneous Schizosaccharomyces pombe la mutants. *Nucleic Acids Res.*, **39**, 4728–4742.
39. Gogakos,T., Brown,M., Garzia,A., Meyer,C., Hafner,M. and Tuschl,T. (2017) Characterizing Expression and Processing of Precursor and Mature Human tRNAs by Hydro-tRNAseq and PAR-CLIP. *Cell Rep.*, **20**, 1463–1475.
40. Herschlag,D. (1995) RNA chaperones and the RNA folding problem. *J. Biol. Chem.*, **270**,

20871–20874.
41. Wright,J.R., Keffer-Wilkes,L.C., Dobing,S.R. and Kothe,U. (2011) Pre-steady-state kinetic analysis of the three Escherichia coli pseudouridine synthases TruB, TruA, and RluA reveals uniformly slow catalysis. *RNA*, 10.1261/rna.2905811.
42. Ramamurthy,V., Swann,S.L., Paulson,J.L., Spedaliere,C.J. and Mueller,E.G. (1999) Critical aspartic acid residues in pseudouridine synthases. *J. Biol. Chem.*, **274**, 22225–22230.
43. Kuehnert,J., Sommer,G., Zierk,A.W., Fedarovich,A., Brock,A., Fedarovich,D. and Heise,T. (2015) Novel RNA chaperone domain of RNA-binding protein la is regulated by AKT phosphorylation. *Nucleic Acids Res.*, 10.1093/nar/gku1309.
44. Brown,K.A., Sharifi,S., Hussain,R., Donaldson,L., Bayfield,M.A. and Wilson,D.J. (2016) Distinct Dynamic Modes Enable the Engagement of Dissimilar Ligands in a Promiscuous Atypical RNA Recognition Motif. *Biochemistry*, **55**, 7141–7150.
45. Bayfield,M.A., Yang,R. and Maraia,R.J. (2010) Conserved and divergent features of the structure and function of La and La-related proteins (LARPs). *Biochim. Biophys. Acta - Gene Regul. Mech.*, **1799**, 365–378.
46. Martino,L., Pennell,S., Kelly,G., Bui,T.T.T., Kotik-Kogan,O., Smerdon,S.J., Drake,A.F., Curry,S. and Conte,M.R. (2012) Analysis of the interaction with the hepatitis C virus mRNA reveals an alternative mode of RNA recognition by the human la protein. *Nucleic Acids Res.*, **40**, 1381–1394.
47. Bohnsack,M.T. and Sloan,K.E. (2018) The mitochondrial epitranscriptome: the roles of RNA modifications in mitochondrial translation and human disease. *Cell. Mol. Life Sci.*, **75**, 241–260.
48. Schöller,E., Marks,J., Marchand,V., Bruckmann,A., Powell,C.A., Reichold,M., Mutti,C.D., Dettmer,K., Feederle,R., Hüttelmaier,S., *et al.* (2021) Balancing of mitochondrial translation through METTL8-mediated m3C modification of mitochondrial tRNAs. *Mol. Cell*, **81**, 4810-4825.E12.
49. Ihsanawati, Nishimoto,M., Higashijima,K., Shirouzu,M., Grosjean,H., Bessho,Y. and Yokoyama,S. (2008) Crystal Structure of tRNA N-2, N-2-Guanosine Dimethyltransferase Trm1 from Pyrococcus horikoshii. *J. Mol. Biol.*, **383**, 871–884.

Figure captions

Figure 1: *S. pombe* Trm1 modifies nuclear- and mitochondrial-encoded tRNAs at G26

A) Schematic of alternate transcription start sites giving rise to nuclear- and mitochondrial-targeted Trm1 isoforms.

B) qRT-PCR of *trm1* mRNA isoforms normalized to *act1* mRNA (mean± standard error, two-tailed unpaired t-test * at $p<0.05$) (n= 5 biological replicates).

C) AlphaFold (32) structure prediction of Trm1 aligned to SAH-bound Trm1 from *Pyrococcus horikoshii* (PDB 2EJU) (49). Inset: Hydrogen bonding between SAH (yellow) and D201 (red).

D) PHA26 northern blot of nuclear- and mitochondrial-encoded tRNA. Northern blots were stripped and re-probed for U5 as a loading control. Relative modification index represents the TUC signal divided by the PHA26 signal and normalized to a wild type strain (mean±SEM, n= 3 biological replicates). Left: schematic depicting binding sites of the PHA26 and TUC probes (red). G26 is represented by a blue circle.

E) Western blot of Trm1 and β-actin in a *trm1Δ* strain transformed with the indicated plasmids.

Figure 2: D201A supports *in vitro* tRNA binding, but not methylation
A) EMSAs of Trm1 M24 and M24 D201A with radiolabeled pre-tRNA Ser^{UGA}.
B) Binding curves of A) (mean±SEM, n= 3 technical replicates).
C) Primer extension of *in vitro* methylated tRNA Ser^{UGA}. The sequences flanking G26 are indicated.

Figure 3: Trm1 promotes tRNA-mediated suppression independent of catalytic activity
A) tRNA-mediated suppression with wild type and catalytically inactive Trm1 in a *sla1Δ* and *sla1Δ maf1Δ* background.
B) Northern blot of tRNA Ser^{UCA} processing intermediates and G26 modification status and 3' end protection of pre-tRNA Lys^{CUU}.
C-E) Quantification of relative modification at G26, relative to the *sla1Δ* strain expressing an empty vector. Relative modification was calculated by normalizing the PHA26 signal (overlapping G26) to probes targeting the intron-containing precursor (C and D) and mature suppressor (E).

Figure 4: La and Trm1 influence pre-tRNA end-processing and G26 dimethylation
A) PHA26 northern blots of nuclear-encoded tRNA in wild type, *maf1Δ, sla1Δ,* and *sla1Δ maf1Δ* cells. Northern blots were stripped and reprobed for U5 as a loading control. Relative modification index represents the TUC signal divided by the PHA26 signal and normalized to a wild type strain (mean±SEM, n= 3 biological replicates)
B) Schematic of human La (hLa) domains with indicated mutations and tRNA-mediated suppression of wild type and mutant hLa constructs in a *sla1Δ* background.
C) Northern blot of tRNA Ser^{UCA} processing intermediates and G26 modification status and 3' end protection of pre-tRNA Lys^{CUU}. Apparent relative abundance refers to intensity of the PHA26 probe normalized to U5.
D) Primer extension of *in vitro* methylated tRNA Ser^{UGA} over time
E) Quantification of *in vitro* methylation of tRNA Ser^{UGA} over time, calculated as the ratio of the G26 RT stop to full length tRNA (mean±SEM, n= 4 independent replicates).

Figure 5: Trm1 folds RNA *in vitro*
A) RNA strand annealing (phase I) and dissociation (phase II), as indicated by changes in FRET index (emission at 680 nm/emission at 590 nm) between the Cy3- and Cy5-labeled substrates. Phase II was initiated with the addition of an unlabeled competitor RNA at t=180 seconds. Representative traces are shown.
B-C) Strand annealing (B) and dissociation (C) rate constants calculated with RNA alone, recombinant hLa, or wild type or catalytically inactive M24 Trm1 (mean± standard error, two-tailed unpaired t-test * at $p<0.05$, ** at $p<0.01$, *** at $p<0.001$) (n= 7 independent replicates).

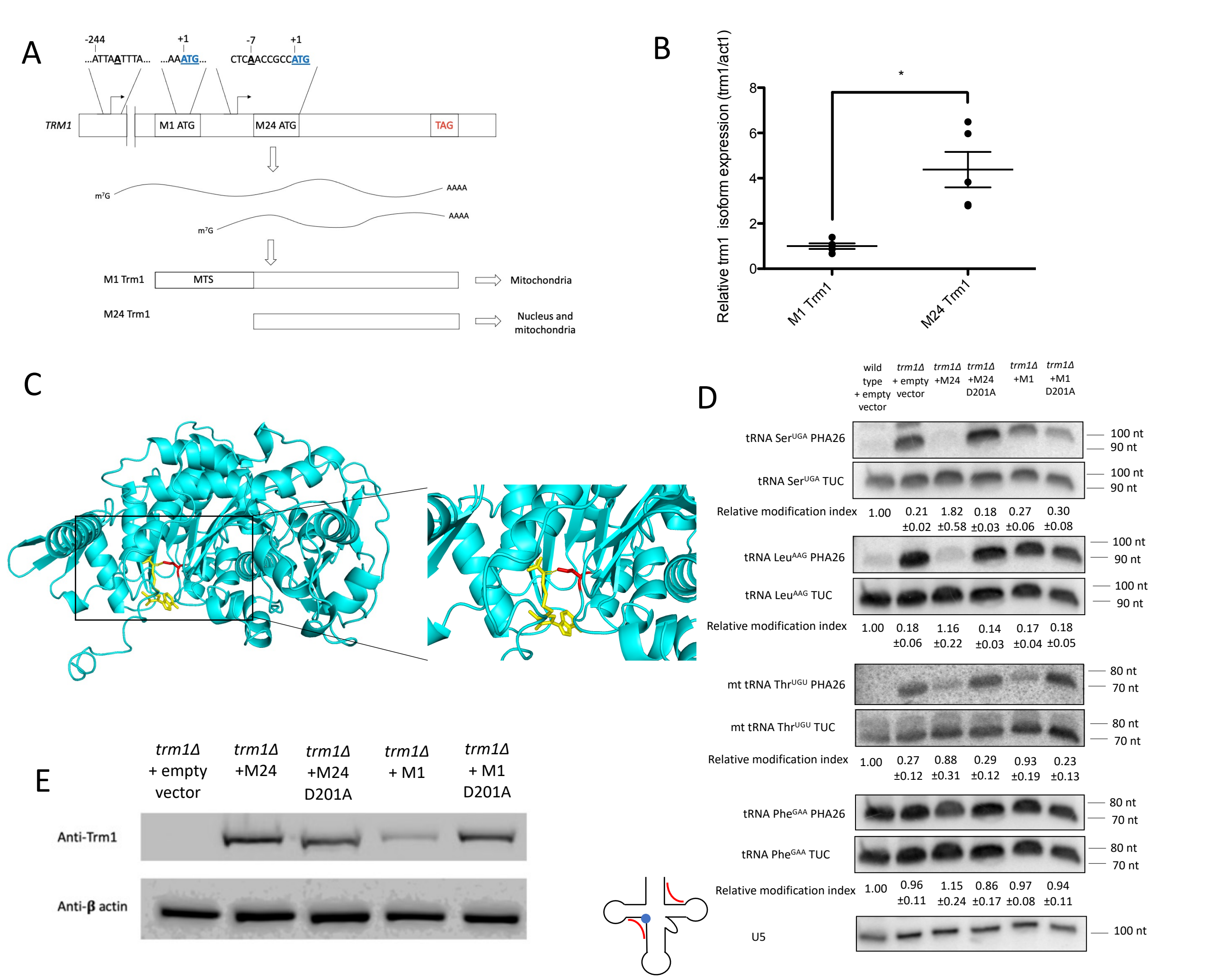

Figure 1: *S. pombe* Trm1 modifies nuclear- and mitochondrial-encoded tRNAs at G26

A) Schematic of alternate transcription start sites giving rise to nuclear- and mitochondrial-targeted Trm1 isoforms.

B) qRT-PCR of *trm1* mRNA isoforms normalized to *act1* mRNA (mean± standard error, two-tailed unpaired t-test * at $p<0.05$) (n= 5 biological replicates).

C) AlphaFold (32) structure prediction of Trm1 aligned to SAH-bound Trm1 from *Pyrococcus horikoshii* (PDB 2EJU) (49). Inset: Hydrogen bonding between SAH (yellow) and D201 (red).

D) PHA26 northern blot of nuclear- and mitochondrial-encoded tRNA. Northern blots were stripped and re-probed for U5 as a loading control. Relative modification index represents the TUC signal divided by the PHA26 signal and normalized to a wild type strain (mean±SEM, n= 3 biological replicates). Left: schematic depicting binding sites of the PHA26 and TUC probes (red). G26 is represented by a blue circle.

E) Western blot of Trm1 and β-actin in a *trm1Δ* strain transformed with the indicated plasmids.

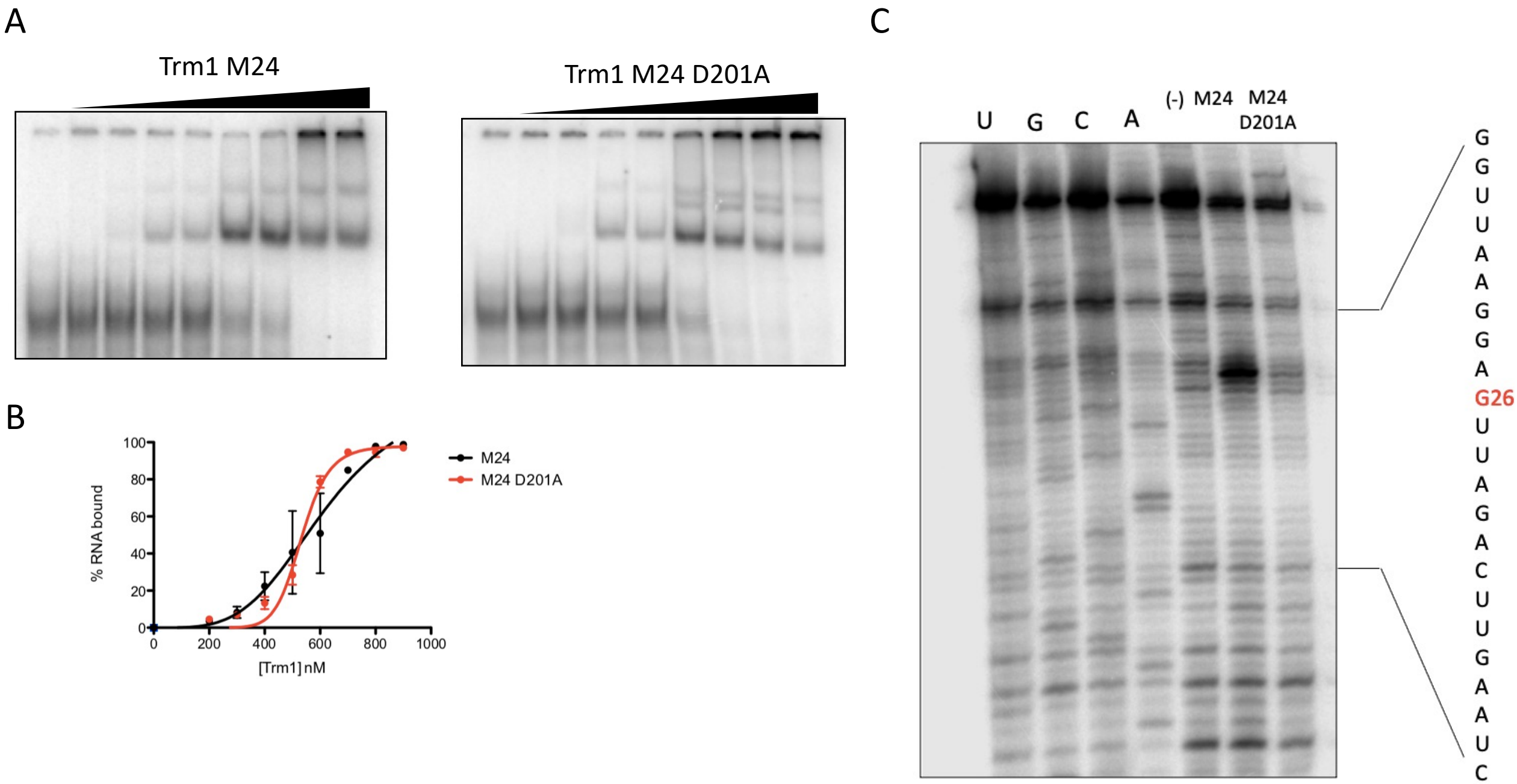

Figure 2: D201A supports *in vitro* tRNA binding, but not methylation
A) EMSAs of Trm1 M24 and M24 D201A with radiolabeled pre-tRNA Ser^{UGA}.
B) Binding curves of A) (mean±SEM, n= 3 technical replicates).
C) Primer extension of *in vitro* methylated tRNA Ser^{UGA}. The sequences flanking G26 are indicated.

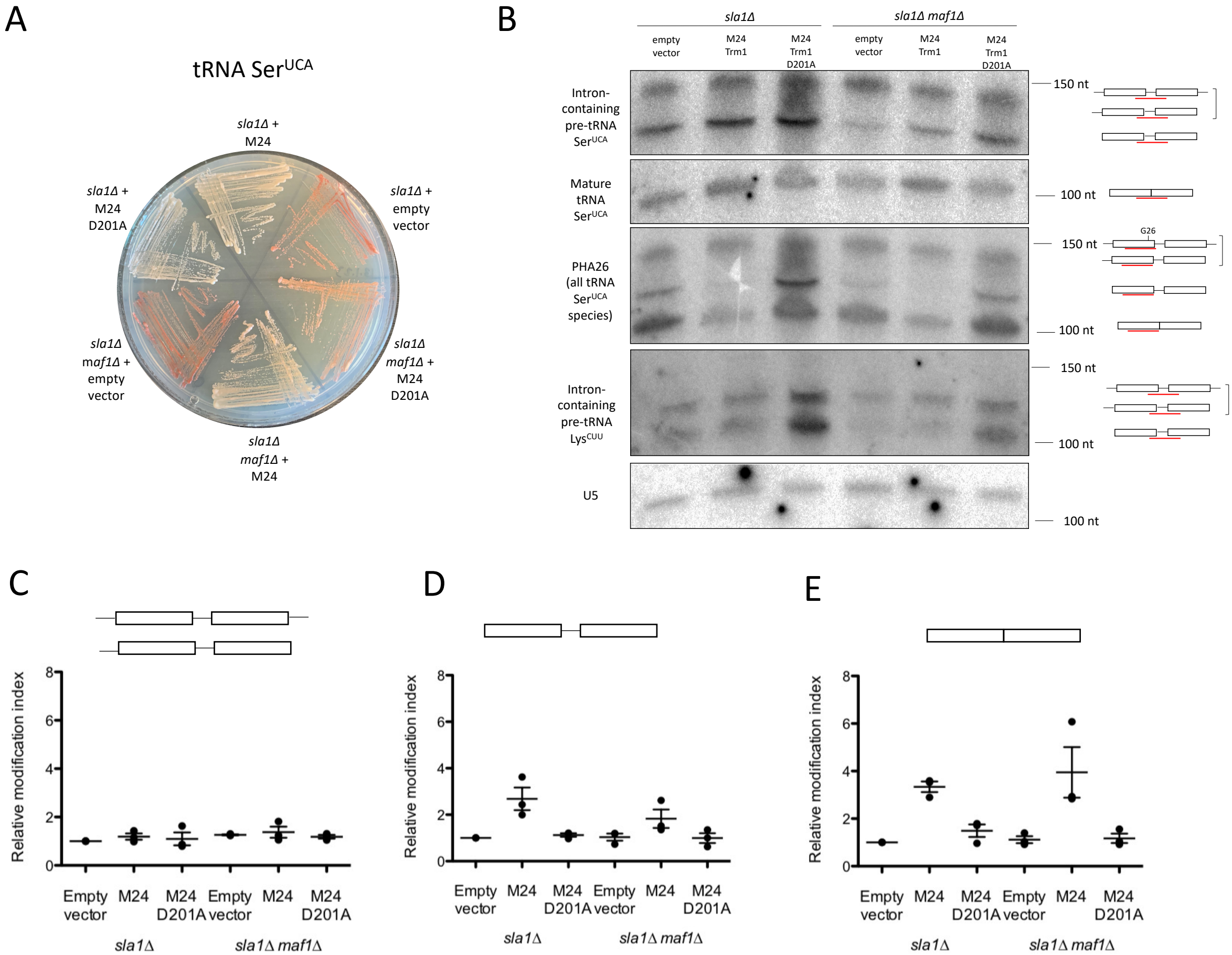

Figure 3: Trm1 promotes tRNA-mediated suppression independent of catalytic activity

A) tRNA-mediated suppression with wild type and catalytically inactive Trm1 in a *sla1Δ* and *sla1Δ maf1Δ* background.

B) Northern blot of tRNA Ser^{UCA} processing intermediates and G26 modification status and 3' end protection of pre-tRNA Lys^{CUU}.

C-E) Quantification of relative modification at G26, relative to the *sla1Δ* strain expressing an empty vector. Relative modification was calculated by normalizing the PHA26 signal (overlapping G26) to probes targeting the intron-containing precursor (C and D) and mature suppressor (E).

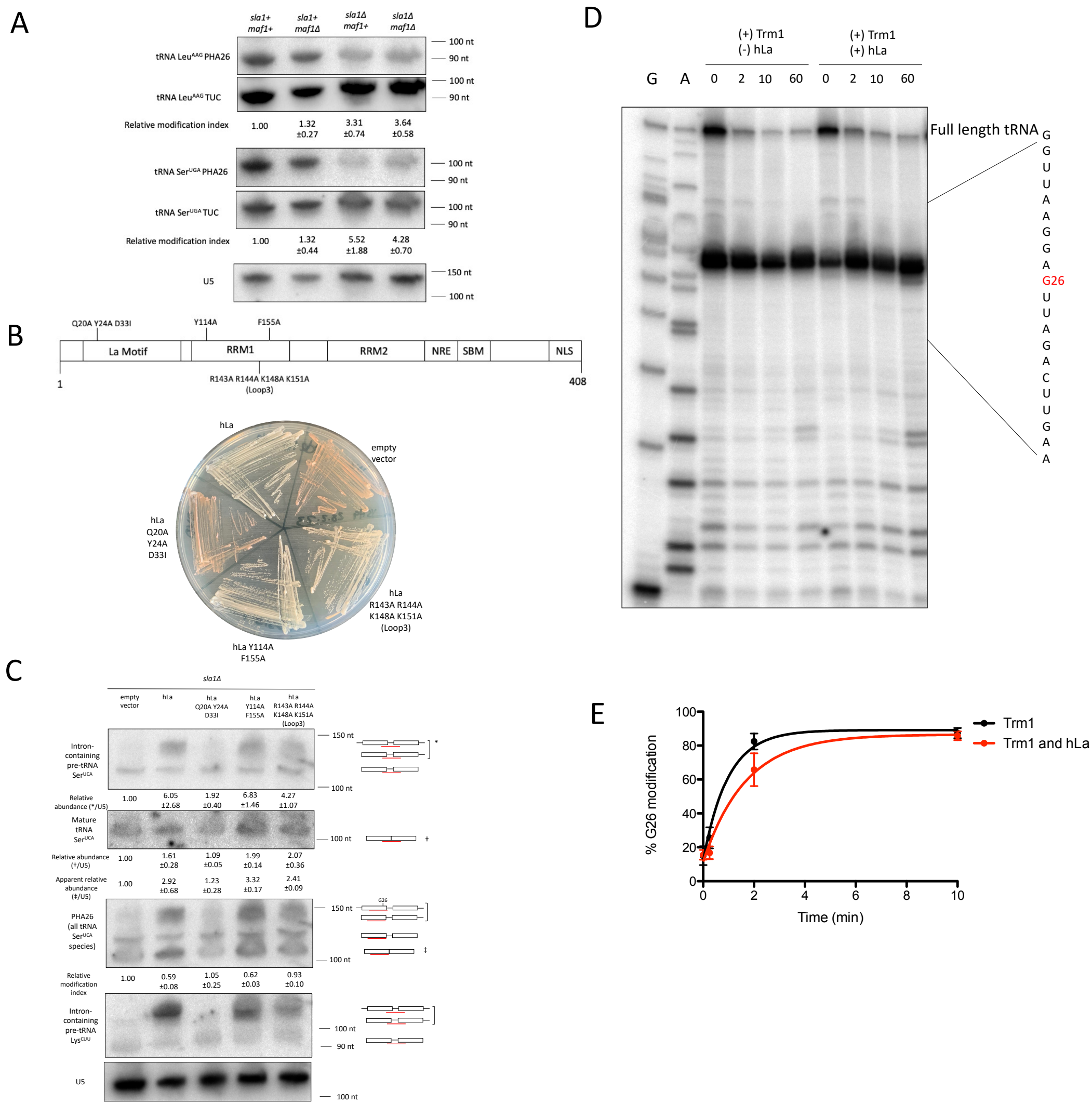

Figure 4: La and Trm1 influence pre-tRNA end-processing and G26 dimethylation

A) PHA26 northern blots of nuclear-encoded tRNA in wild type, *maf1Δ, sla1Δ,* and *sla1Δ maf1Δ* cells. Northern blots were stripped and reprobed for U5 as a loading control. Relative modification index represents the TUC signal divided by the PHA26 signal and normalized to a wild type strain (mean±SEM, n= 3 biological replicates)

B) Schematic of human La (hLa) domains with indicated mutations and tRNA-mediated suppression of wild type and mutant hLa constructs in a *sla1Δ* background.

C) Northern blot of tRNA Ser^{UCA} processing intermediates and G26 modification status and 3' end protection of pre-tRNA Lys^{CUU}. Apparent relative abundance refers to intensity of the PHA26 probe normalized to U5.

D) Primer extension of *in vitro* methylated tRNA Ser^{UGA} over time

E) Quantification of *in vitro* methylation of tRNA Ser^{UGA} over time, calculated as the ratio of the G26 RT stop to full length tRNA (mean±SEM, n= 4 independent replicates).

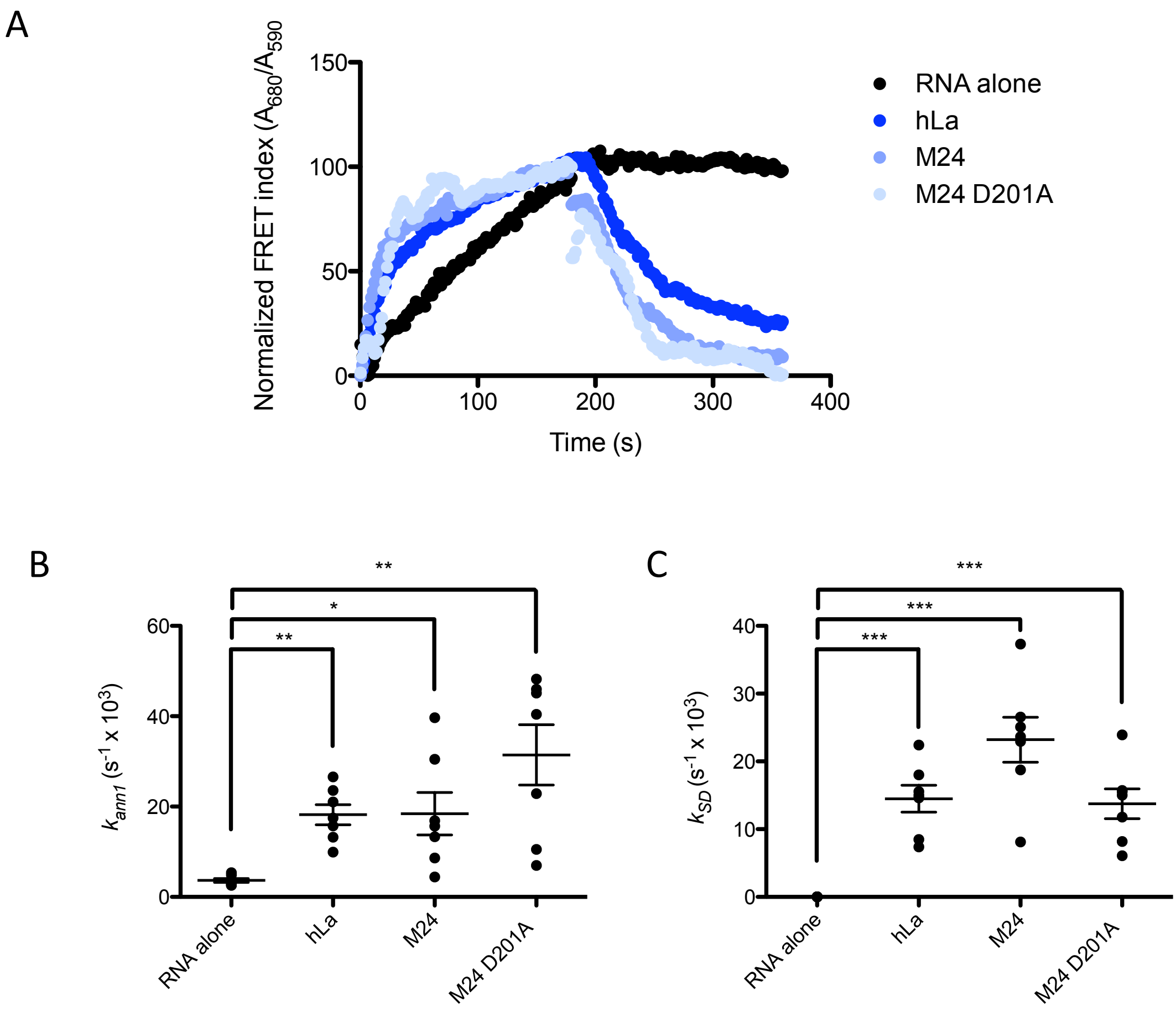

Figure 5: Trm1 folds RNA *in vitro*

A) RNA strand annealing (phase I) and dissociation (phase II), as indicated by changes in FRET index (emission at 680 nm/emission at 590 nm) between the Cy3- and Cy5-labeled substrates. Phase II was initiated with the addition of an unlabeled competitor RNA at t=180 seconds. Representative traces are shown.

B-C) Strand annealing (B) and dissociation (C) rate constants calculated with RNA alone,

recombinant hLa, or wild type or catalytically inactive M24 Trm1 (mean± standard error, two-tailed unpaired t-test * at $p<0.05$, ** at $p<0.01$, *** at $p<0.001$) (n= 7 independent replicates).