

# Unexpected expansion of tRNA substrate recognition by the yeast m<sup>1</sup>G<sub>9</sub> methyltransferase Trm10

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## ABSTRACT

*N*-1 Methylation of the nearly invariant purine residue found at position 9 of tRNA is a nucleotide modification found in multiple tRNA species throughout Eukarya and Archaea. First discovered in *Saccharomyces cerevisiae*, the tRNA methyltransferase Trm10 is a highly conserved protein both necessary and sufficient to catalyze all known instances of m<sup>1</sup>G<sub>9</sub> modification in yeast. Although there are 19 unique tRNA species that contain a G at position 9 in yeast, and whose fully modified sequence is known, only 9 of these tRNA species are modified with m<sup>1</sup>G<sub>9</sub> in wild-type cells. The elements that allow Trm10 to distinguish between structurally similar tRNA species are not known, and sequences that are shared between all substrate or all nonsubstrate tRNAs have not been identified. Here, we demonstrate that the *in vitro* methylation activity of yeast Trm10 is not sufficient to explain the observed pattern of modification *in vivo*, as additional tRNA species are substrates for Trm10 m<sup>1</sup>G<sub>9</sub> methyltransferase activity. Similarly, overexpression of Trm10 in yeast yields m<sup>1</sup>G<sub>9</sub> containing tRNA species that are ordinarily unmodified *in vivo*. Thus, yeast Trm10 has a significantly broader tRNA substrate specificity than is suggested by the observed pattern of modification in wild-type yeast. These results may shed light onto the suggested involvement of Trm10 in other pathways in other organisms, particularly in higher eukaryotes that contain up to three different genes with sequence similarity to the single *TRM10* gene in yeast, and where these other enzymes have been implicated in pathways beyond tRNA processing.

**Keywords:** tRNA methyltransferase; Trm10; m<sup>1</sup>G; substrate specificity

## INTRODUCTION

tRNA species from all three domains of life contain numerous post-transcriptional modifications to nucleotide bases and ribose sugars (Grosjean et al. 1995; Grosjean and Benne 1998; Czerwonec et al. 2009; Jühling et al. 2009; Phizicky and Hopper 2010; Jackman and Alfonzo 2013; Machnicka et al. 2013). Nucleotide modifications found in tRNAs range in complexity from single methyl groups to large chemical groups, such as wybutosine. Many tRNA modifications, and the enzymes that catalyze them, are highly conserved throughout multiple domains of life, suggesting that modified tRNA nucleotides are biologically important. Consistent with this hypothesis, modified nucleotides in the anticodon stem and loop are thought to function in maintaining fidelity and/or efficiency of translation, and deletion of the genes that encode these modification enzymes often causes severe growth defects (Ericson and Björk 1986; Gerber and Keller 1999; Björk et al. 2001; Brégeon et al. 2001; Urbonavicius et al. 2001; Pintard et al. 2002; Gustilo et al. 2008; Phillips

and de Crécy-Lagard 2011). For the majority of modifications found throughout the rest of the tRNA, however, deletion of the corresponding modification enzyme does not cause measurable growth defects. Therefore, the biological function of modified tRNA nucleotides outside of the anticodon stem and loop is largely unknown. However, strong synthetic lethal phenotypes have been associated with different pairwise deletions of yeast modification enzymes, consistent with the idea that a network of modifications with some degree of built-in redundancy is required to support the optimal function of tRNA *in vivo* (Alexandrov et al. 2006; Chernyakov et al. 2008; Kotelawala et al. 2008; Phizicky and Alfonzo 2010; Whipple et al. 2011).

The yeast tRNA m<sup>1</sup>G<sub>9</sub> methyltransferase (Trm10) is one such tRNA modification enzyme, which is nonessential for viability in *S. cerevisiae* but is associated with various phenotypes, including 5-fluorouracil hypersensitivity and temperature sensitivity when deleted in combination with the m<sup>7</sup>G<sub>46</sub> methyltransferase *TRM8/TRM82* (Jackman et al. 2003; Alexandrov et al. 2006; Gustavsson and Ronne 2008). Purified yeast Trm10 catalyzes S-adenosyl methionine (SAM)-dependent methylation of the *N*-1 position of guanosine residues. Although m<sup>1</sup>G is observed at positions 9 and 37 in yeast tRNAs, Trm10 only modifies G<sub>9</sub>, whereas m<sup>1</sup>G<sub>37</sub>

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modification is catalyzed by an unrelated methyltransferase, Trm5 (Bjork et al. 2001). The complete modification status has been determined for 28 yeast tRNA species with unique anticodon sequences; of these, 9 tRNAs contain the m<sup>1</sup>G modification at position 9 (Jühling et al. 2009; Machnicka et al. 2013). Deletion of *TRM10* in yeast leads to loss of m<sup>1</sup>G<sub>9</sub> modification on all tRNAs for which the modification can be detected, suggesting that the single enzyme is responsible for modifying all substrate tRNAs in vivo (Jackman et al. 2003).

tRNA substrate recognition by modification enzymes is accomplished by numerous mechanisms. For enzymes that only act on a single tRNA species, recognition of the correct tRNA substrate is readily accomplished by distinct sequence elements, such as the tRNA<sup>His</sup> anticodon that is the critical element recognized by the tRNA<sup>His</sup>-specific guanylyltransferase, Thg1 (Jackman and Phizicky 2006). However, enzymes that modify multiple tRNA substrates face a more challenging task: How are specific substrates recognized from among a pool of highly similar tRNA species? These enzymes can be further divided into two general classes based on the minimum molecular requirements that define cognate tRNAs. The first class includes enzymes that require only the presence of the correct nucleotide at the position to be modified, such as the tRNA adenosine deaminase (Tad2/Tad3 in yeast) that catalyzes I<sub>34</sub> formation (Gerber and Keller 1999). The adenosine deaminase modifies all A<sub>34</sub>-containing tRNAs in the cell (8/8 sequenced tRNAs in yeast), and identity of the tRNA plays a minor role in substrate selection (Auxilien et al. 1996). The second class includes enzymes for which the presence of the correct nucleotide alone is not sufficient to specify modification and therefore whose substrate specificity must be determined by additional parameters. Trm10 is a representative of this second class, since in yeast, 19 sequenced tRNAs contain a G residue at position 9, but only about half of these G<sub>9</sub>-containing tRNAs are modified to m<sup>1</sup>G<sub>9</sub> by the action of Trm10 in vivo. The features that cause Trm10 to distinguish which G<sub>9</sub>-containing tRNAs to modify are not obvious from sequence comparisons and led us to investigate the determinants for Trm10-tRNA recognition.

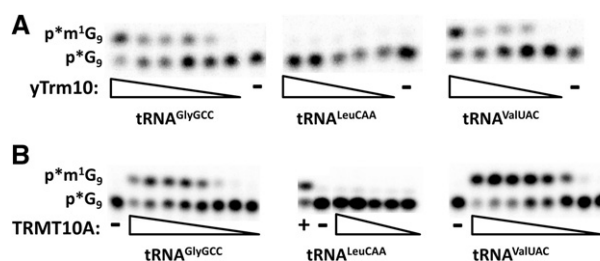
In this work, we tested whether purified Trm10 exhibits in vitro substrate selectivity consistent with the observed pattern of modification in vivo in yeast. Surprisingly, we observed robust methylation of multiple tRNAs in vitro that are not normally modified in vivo in wild-type yeast. The discrepancy between the observed in vitro methylation activities and the lack of modification in vivo cannot be fully explained by substantial differences in catalytic efficiency or by contributions of other modified nucleotides on the tRNA, although these factors may contribute to tRNA recognition. These data support a model wherein Trm10 exhibits generally broader intrinsic substrate selectivity than is indicated by the identity of tRNA species that are modified within the cell. These results have implications for alternative biological functions associated with Trm10 family enzymes in higher eukaryotes.

## RESULTS

### Trm10 catalyzes unexpected methylation of tRNAs that lack m<sup>1</sup>G<sub>9</sub> in yeast

To investigate the molecular basis for Trm10 tRNA recognition, we selected three yeast tRNAs to test for in vitro m<sup>1</sup>G<sub>9</sub> modification by Trm10, including one known in vivo substrate (tRNA<sup>GlyGCC</sup>) and two G<sub>9</sub> containing tRNAs that are not methylated in vivo in yeast (tRNA<sup>ValUAC</sup> and tRNA<sup>LeuCAA</sup>). In vitro methylation assays were performed using tRNA substrates uniquely labeled with <sup>32</sup>P at the G<sub>9</sub> 5'-phosphate, created using a variation of the previously described protocol for generating Trm10 tRNA substrates labeled at the phosphate located 3' to G<sub>9</sub> (Jackman et al. 2003). Labeled substrates were used in in vitro activity assays with purified yeast Trm10 (yTrm10) in the presence of SAM. After digestion with nuclease P1, reactions yielded either p\*G for unreacted substrate, or p\*m<sup>1</sup>G<sub>9</sub> for the Trm10-methylated tRNA, which are resolved from one another by thin-layer chromatography (TLC) (Fig. 1A).

Consistent with the previously observed modification of tRNA<sup>GlyGCC</sup> both in vitro and in vivo, the assay demonstrated strong m<sup>1</sup>G<sub>9</sub> modification of this tRNA by yTrm10 (Fig. 1A). For the tRNA<sup>LeuCAA</sup> substrate, also consistent with the lack of m<sup>1</sup>G<sub>9</sub> on this tRNA in vivo, little or no modification by yTrm10 was detected in the in vitro assays. However, m<sup>1</sup>G<sub>9</sub> methylation was observed with the tRNA<sup>ValUAC</sup> substrate at similar levels to those observed with the bona fide substrate tRNA<sup>GlyGCC</sup>. Since the closest human ortholog to yTrm10 (TRMT10A) exhibited an apparently more restrictive tRNA substrate specificity than yTrm10 with some previously tested tRNA substrates (Vilardo et al. 2012), we tested the ability of purified human TRMT10A to methylate the same three yeast tRNAs (Fig. 1B). Nonetheless, TRMT10A exhibits the same



**FIGURE 1.** Trm10 methylates the noncognate substrate tRNA<sup>ValUAC</sup> in vitro. Activity assays were performed with (A) yeast Trm10 (10-fold dilutions starting with 1.8 mg/mL purified protein in highest concentration reactions) or (B) human TRMT10A (fivefold dilutions starting with 5.0 mg/mL purified protein in highest concentration reactions). Substrate tRNAs (as indicated) were uniquely labeled at the 5'-phosphate to G<sub>9</sub>. After 2 h, tRNAs were digested to nucleotide 5'-monophosphates with nuclease P1, and the p\*m<sup>1</sup>G reaction products were resolved from the remaining unreacted substrate (p\*G) using cellulose TLC. Lanes indicated with (–) are no enzyme control reactions; lane indicated with (+) is positive control with substrate to show the position of migration of m<sup>1</sup>G<sub>9</sub> for tRNA<sup>Leu</sup> assay.

**TABLE 1.** Steady-state kinetic parameters for m<sup>1</sup>G<sub>9</sub> methylation by purified Trm10

Enzyme	tRNA	k <sub>cat</sub> (min <sup>-1</sup> )	K <sub>M,tRNA</sub> (nM)	k <sub>cat</sub> /K <sub>M</sub> (M <sup>-1</sup> s <sup>-1</sup> )
yTrm10	tRNA <sup>GlyGCC</sup>	0.54 ± 0.11	2400 ± 1200	0.36 ± 0.11 × 10 <sup>4</sup>
yTrm10	tRNA <sup>ValUAC</sup>	0.31 ± 0.09	1860 ± 1310	0.28 ± 0.13 × 10 <sup>4</sup>
TRMT10A	tRNA <sup>GlyGCC</sup>	0.14 ± 0.032	160 ± 80	1.4 ± 0.5 × 10 <sup>4</sup>
TRMT10A	tRNA <sup>ValUAC</sup>	0.72 ± 0.08	233 ± 97	5.2 ± 1.6 × 10 <sup>4</sup>

pattern of methylation as yTrm10, acting on tRNA<sup>GlyGCC</sup> and tRNA<sup>ValUAC</sup> but not tRNA<sup>LeuCAA</sup>, suggesting that at least for these three substrates, the enzymes may be relying on similar features for substrate discrimination.

### Modification of the noncognate tRNA<sup>ValUAC</sup> is efficient in vitro

We tested whether Trm10 methylates tRNA<sup>GlyGCC</sup> more efficiently than tRNA<sup>ValUAC</sup>, despite similar levels of product formation observed in the endpoint assays above. Measurement of steady-state kinetic parameters for yTrm10 and TRMT10A activity revealed similar efficiency of methylation (in terms of k<sub>cat</sub>/K<sub>M</sub>) for both tRNA<sup>GlyGCC</sup> and tRNA<sup>ValUAC</sup> (Supplemental Fig. S1; Table 1). Precise determination of the K<sub>M,tRNA</sub> for each tRNA substrate was difficult because of significant product inhibition observed in the time courses, likely due to inhibition by the S-adenosylhomocysteine (SAH) product of the methyltransferase reaction, as is frequently observed with other tRNA methyltransferases (Shugart 1978; Hjalmarsson et al. 1983; Ochi et al. 2010). Nonetheless, by restricting measurements of initial rate to <5% of product formation, where the product inhibition was not as evident, we were able to measure observed rates that agreed well (within threefold), even when measured on different days. Thus, purified Trm10 (from either yeast or humans) does not exhibit a sufficiently strong biochemical preference for methylation of the tRNA<sup>GlyGCC</sup> substrate that would explain the complete absence of detectably modified tRNA<sup>ValUAC</sup> in yeast.

### Identification of additional Trm10 substrates in vivo in *S. cerevisiae*

Since our data suggested that the pattern of substrate recognition for Trm10 was more complicated than is indicated by the known in vivo modifications, we sought to obtain a more complete picture of how many tRNA species in yeast are normally modified with m<sup>1</sup>G<sub>9</sub>. In yeast, there are 27 unique tRNA species that contain a G residue at the 9 position (Table 2), but only 19 of these have been previously sequenced at the RNA level so that their m<sup>1</sup>G<sub>9</sub> modification status is known (Table 2; Jühling et al. 2009). We used a primer extension assay to assess methylation of tRNAs isolated from *TRM10* and *trm10Δ* strains. Each assay contained a labeled primer that binds to sequences of the anticodon

stem/D-loop of a specific tRNA (Fig. 2A). If G<sub>9</sub> is not methylated, extension of the primer by reverse transcriptase results in a stop corresponding to the full-length tRNA; but if m<sup>1</sup>G<sub>9</sub> is present, the hydrogen-bonding proton from N-1 of G<sub>9</sub> is removed, and the primer extension is blocked at position 9 due to interference with Watson-Crick base-pairing.

Trm10-catalyzed methylation is thus indicated by the presence of a primer extension stop in the wild-type RNA that is not observed in the RNA isolated from the *trm10Δ* strain.

Using the primer extension assay, we probed the methylation status of the eight remaining unsequenced yeast tRNAs. Three tRNAs (Arg<sup>CCU</sup>, Ser<sup>GCU</sup>, and Ser<sup>CGA</sup>) were

**TABLE 2.** Modification at G<sub>9</sub> of *S. cerevisiae* tRNA

tRNA species (anticodon)	tRNA type <sup>a</sup>	G <sub>9</sub> status-wild-type yeast	Percentage modified with m <sup>1</sup> G <sub>9</sub> upon Trm10 overexpression <sup>b</sup>
Previously sequenced tRNA <sup>c</sup>			
Ala(AGC)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Arg(ACG)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Arg(UCU)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Gly(GCC)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Ile(AAU)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Ini(CAU)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Pro(UGG)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Trp(CCA)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Val(AAC)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Lys(CUU)	I	m <sup>2</sup> G <sub>9</sub>	80
Asn(GUU)	I	G <sub>9</sub>	ND <sup>e</sup>
Cys(GCA)	I	G <sub>9</sub>	75
Thr(AGU)	I	G <sub>9</sub>	60
Val(UAC)	I	G <sub>9</sub>	38
Leu(UAG)	II	G <sub>9</sub>	
Leu(CAA)	II	G <sub>9</sub>	2
Leu(UAA)	II	G <sub>9</sub>	
Ser(AGA)	II	G <sub>9</sub>	
Ser(UGA)	II	G <sub>9</sub>	
G <sub>9</sub> status investigated in this work			
Ala(UGC)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Pro(AGG)	I	m <sup>1</sup> G <sub>9</sub>	NA <sup>d</sup>
Gly(CCC)	I	m <sup>1</sup> G <sub>9</sub> (partial)	62
Thr(CGU)	I	m <sup>1</sup> G <sub>9</sub> (partial)	60
Leu(GAG)	II	G <sub>9</sub>	2
Ser(GCU)	II	ND <sup>e</sup>	
Ser(CGA)	II	ND <sup>e</sup>	
Arg(CCU)	I	ND <sup>e</sup>	

<sup>a</sup>Type I tRNA with standard 4–6 nucleotide variable loop or Type II with extended variable loop sequence, as indicated.

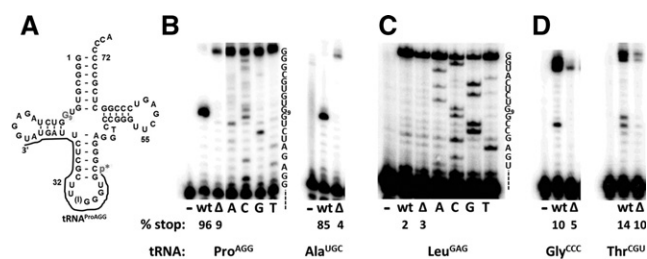
<sup>b</sup>Measured by primer extension assay (Fig. 4); percentage modification determined by intensity of primer extension stop before G<sub>9</sub> compared to intensity of total primer extension products for each tRNA.

<sup>c</sup>From references Czerwoniec et al. (2009) and Jühling et al. (2009).

<sup>d</sup>(NA) not applicable; tRNA already fully modified with m<sup>1</sup>G<sub>9</sub>.

<sup>e</sup>(ND) tRNA species not detectable using primer extension assay; G<sub>9</sub>-status remains unknown.





**FIGURE 2.** Determining  $m^1G_9$  status for previously unsequenced tRNAs in wild-type yeast. (A) Primer extension assays were performed using a 5'-end labeled primer complementary to sequences of the tRNA anticodon stem and D-loop as indicated by the solid line for the tRNA<sup>ProAGG</sup> example indicated in the diagram. (B) tRNA<sup>ProAGG</sup> and tRNA<sup>AlaUGC</sup> are quantitatively modified with  $m^1G_9$  in wild-type yeast. Primer extension assays with primer complementary to the indicated tRNAs using RNA isolated from either *TRM10* (wt) or *trm10Δ* ( $\Delta$ ) yeast strains. Representative sequencing lanes for tRNA<sup>ProAGG</sup> are shown, where the identity of the dideoxy-NTP included in each lane is indicated (lanes A,C,G,T). The 5'-end sequence of tRNA<sup>ProAGG</sup> is indicated next to the sequencing lanes. (% stop) Corresponds to the approximate proportion of the indicated tRNA that is modified with  $m^1G_9$  and is indicated below the lanes for wild-type and *trm10Δ* RNA; (–) these lanes correspond to primer only control reactions. (C) tRNA<sup>LeuGAG</sup> is not modified with  $m^1G_9$  to detectable levels in wild-type yeast. Samples were assayed by primer extension as described in B with a primer corresponding to the tRNA<sup>LeuGAG</sup> sequence. (D) tRNA<sup>GlyCCC</sup> and tRNA<sup>ThrCGU</sup> are partially modified with  $m^1G_9$  in wild-type yeast. Samples were assayed by primer extension as described in B with primers corresponding to the indicated tRNAs.

not detected using primer extension, presumably due to low levels of these minor tRNAs in cells. Two of the eight tRNAs (Pro<sup>AGG</sup> and Ala<sup>UGC</sup>) are quantitatively  $m^1G_9$ -modified in a Trm10-dependent manner (Fig. 2B; Table 2). One tRNA (Leu<sup>GAG</sup>), like the other tRNA<sup>Leu</sup> species investigated previously, is fully unmodified in vivo as evidenced by the absence of a detectable primer extension stop above background at the position expected for  $m^1G_9$  modification in both strains (Fig. 2C). Interestingly, for the remaining two tRNA species (Gly<sup>CCC</sup> and Thr<sup>CGU</sup>), the primer extension data indicate that these exist as incompletely modified species in vivo, with 10%–20% modification of each tRNA observed, as judged by the percent of the  $m^1G_9$  stop relative to the stop corresponding to full-length tRNA in the primer extension assay (Fig. 2D; Table 2). Thus, of the 24 tRNAs whose modification status is now known, 13 of these are modified with  $m^1G_9$  (either quantitatively or partially) in the wild-type strain (Table 2).

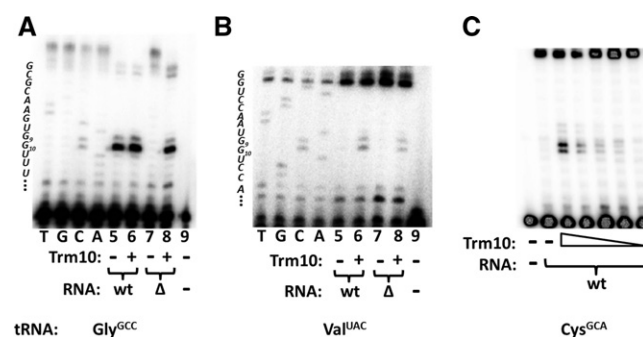
### Presence of native modifications does not prevent methylation of noncognate tRNAs in vitro

We considered the possibility that similar levels of  $m^1G_9$  methylation of tRNA<sup>GlyCCC</sup> and tRNA<sup>ValUAC</sup> substrates may reflect the use of otherwise unmodified transcripts for the in vitro assays. Therefore, we tested the ability of yTrm10 to methylate tRNA derived from the *trm10Δ* yeast strain, which specifically lacks  $m^1G_9$  methylation on tRNA<sup>Gly</sup>, yet

otherwise contains the normal complement of modified nucleotides on the tRNA (Jackman et al. 2003). We isolated bulk tRNA from *TRM10* and *trm10Δ* yeast strains using the hot phenol method, and then used the bulk tRNA as the substrate for in vitro methylation in a reaction containing purified yTrm10 and SAM, under the same conditions used for the assays with labeled tRNAs above. To assess methylation of the resulting RNAs, we used the primer extension assay described above, with labeled primers that bind to similar sequences of the tRNA anticodon stem/D-loop for each tRNA (see Fig. 2A).

As expected, tRNA<sup>GlyCCC</sup> isolated from the *trm10Δ* strain was a substrate for in vitro methylation by yeast Trm10, indicated by the strong primer extension stop before G<sub>9</sub> induced upon treatment of *trm10Δ* RNA with purified Trm10 (Fig. 3A, cf. lanes 7,8). For tRNA<sup>ValUAC</sup>,  $m^1G_9$  was not present at detectable levels in RNA isolated from either *trm10Δ* or *TRM10* strains, consistent with the reported lack of modification of this tRNA in vivo (Fig. 3B, lanes 5,7). However, a block to primer extension was observed for tRNA<sup>ValUAC</sup> after treatment of RNA isolated from both strains with purified Trm10, indicating that the in vivo isolated tRNA is a substrate for  $m^1G_9$  modification (Fig. 3B, lanes 6,8).

We noted that the extent of  $m^1G_9$  modification of the non-substrate tRNA<sup>ValUAC</sup> (~20% of the total tRNA as judged by the intensity of the primer extension stop in Fig. 3B) is less than that observed with the in vivo substrate tRNA<sup>GlyCCC</sup> (>90%  $m^1G_9$ ). Therefore, we tested the generality of the results by performing the same assay with another tRNA that lacks  $m^1G_9$  modification in vivo (tRNA<sup>CysGCA</sup>). As with tRNA<sup>ValUAC</sup>, tRNA<sup>CysGCA</sup> isolated from yeast is a substrate



**FIGURE 3.** Yeast Trm10 methylates modified tRNA species derived from yeast. RNAs derived from *TRM10* (wt) or *trm10Δ* ( $\Delta$ ) strains were incubated under in vitro reaction conditions with or without purified Trm10 (as indicated by + or –) and then assayed by primer extension to determine levels of  $m^1G_9$  modification of the resulting tRNA. Primer extension assays were performed using a 5'-end labeled primer complementary to tRNA<sup>GlyCCC</sup> (A) or tRNA<sup>ValUAC</sup> (B). Sequencing reactions contain each of the four ddNTPs (A,C,G,T) as indicated in the first four lanes of each panel. Lane 9 is the primer extension result from an assay containing no RNA and no added Trm10. (C) Primer extension to assess  $m^1G_9$  methylation of tRNA<sup>CysGCA</sup> formed by incubating tRNA from wild-type yeast (that does not normally contain  $m^1G_9$  on tRNA<sup>CysGCA</sup>, as observed in lane 2) with decreasing concentrations of purified Trm10.

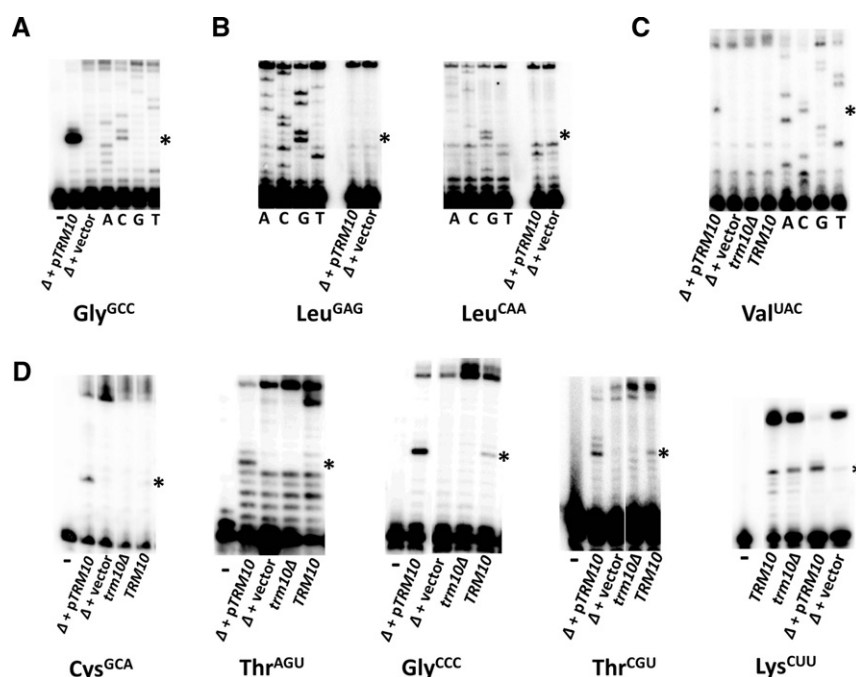
for methylation by Trm10 *in vitro*, and the level of modification is dependent on the amount of Trm10 included in the assays, with detectable modification occurring even at 13 nM Trm10 (Fig. 3C). Again, the tRNA is not completely modified by Trm10 (only 38% methylation was observed at the highest concentration of Trm10 in the assay), suggesting that the efficiency of methylation of the modified tRNA is lower for these noncognate substrates than for the *in vivo* substrate tRNA<sup>GlyGCC</sup>. Thus, other modifications appear to play some role in determining specificity *in vivo*. Nonetheless, as with the *in vitro* transcripts, the ability of Trm10 to methylate these tRNAs at any level is at odds with the complete absence of modification observed in the wild-type yeast and suggests that other mechanisms must also exist to restrict Trm10 from acting on these tRNA substrates *in vivo*.

### Overexpression of Trm10 causes m<sup>1</sup>G<sub>9</sub> modification of additional tRNA species *in vivo*

Deletion of *trm10* in yeast leads to complete loss of m<sup>1</sup>G<sub>9</sub> without any detectable growth defect of the resulting *trm10Δ* strain in standard media (Jackman et al. 2003). A plasmid containing yeast *TRM10* under control of a galactose-inducible promoter (*CEN LEU2 P<sub>GAL</sub>-TRM10*) was transformed into the *trm10Δ* strain and tRNA was isolated from the strain grown under inducing conditions (in the presence of 2% galactose). The primer extension assay was then used to assess the extent of m<sup>1</sup>G<sub>9</sub> modification of individual tRNA species in the plasmid-complemented strain. We demonstrated that normal levels (92%) of m<sup>1</sup>G<sub>9</sub> modification were restored to tRNA<sup>GlyGCC</sup> when the wild-type copy of *TRM10* was expressed in *trm10Δ* cells, thus confirming that the plasmid-expressed Trm10 is functional (Fig. 4A). Lack of an antibody against Trm10 prevents quantitation of the precise level of Trm10 overexpression in the galactose-controlled strain. However, expression levels from a Trm10 construct expressed under the control of the same promoter (created as part of a proteomic yeast collection) were quantified by Western blotting using an antibody targeting an affinity purification tag fused to Trm10 (Gelperin et al. 2005). In this collection, Trm10 was classified as a high-expressing protein (≥1 mg/mL culture), suggesting that the expression levels achieved upon galactose-induction are substantial.

Consistent with the *in vitro* methylation assays with purified Trm10, there was no detectable methylation of tRNA<sup>LeuGAG</sup> or tRNA<sup>LeuCAA</sup> even when Trm10 expression is induced by growth in the presence of galactose (Fig. 4B). Interestingly, however, in the same cells we observed m<sup>1</sup>G<sub>9</sub> methylation of tRNA<sup>ValUAC</sup> at levels significantly above the levels observed in wild-type yeast (38% modified as judged by the intensity of the primer extension stop before m<sup>1</sup>G<sub>9</sub>) (Fig. 4C). We tested additional tRNAs that are not usually efficient substrates for modification by Trm10 *in vivo* and found that all of these also accumulate m<sup>1</sup>G<sub>9</sub> methylation upon Trm10 overexpression (Fig. 4D). Methylation of tRNA<sup>CysGCA</sup> and tRNA<sup>ThrAGU</sup>, neither of which was detectably modified in wild-type yeast, increased to 75% and 60%, respectively, as judged by the intensity of the new primer extension block in this strain. Moreover, partially modified tRNA<sup>GlyCCC</sup> and tRNA<sup>ThrCGU</sup> also exhibited a substantial increase in modification up to 62% and 60%, respectively.

Analysis of tRNA<sup>LysCUU</sup> revealed a more complex pattern of modification. tRNA<sup>LysCUU</sup> is not modified with m<sup>1</sup>G<sub>9</sub> but instead is reported to contain m<sup>2</sup>G at the 9 position, catalyzed by an unknown enzyme (Jühling et al. 2009). The m<sup>2</sup>G modification does not normally cause a block to



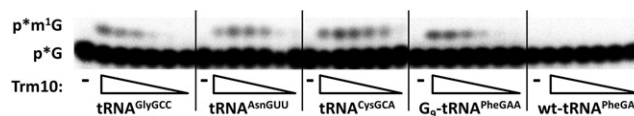
**FIGURE 4.** Additional yeast tRNAs accumulate m<sup>1</sup>G<sub>9</sub> *in vivo* under conditions of Trm10 overexpression. Primer extension assays were performed using tRNA isolated from the *trm10Δ* strain containing either a plasmid for overexpression of *TRM10* (Δ + pTRM10) or empty vector control (Δ + vector) using primers complementary to the indicated tRNA species in each panel. The position of the stop at G<sub>10</sub> (which would correspond to m<sup>1</sup>G<sub>9</sub> modification, if present) is indicated by a star to the right of each panel. Sequencing lanes are shown for RNA derived from *trm10Δ* cells, with the ddNTP included in each reaction (A,C,G,T) indicated below each lane; (–) primer only control lanes. Panels A and B reflect analysis of RNA derived from *trm10Δ* cells in the presence and absence of Trm10 overexpression. Panels C and D also include primer extension results for wild-type (*TRM10*) or *trm10Δ* strains so that the level of modification upon Trm10 overexpression can be readily compared to that observed in wild-type cells.

primer extension, consistent with the retention of base-pairing ability by the remaining N2 proton after methylation (for example, see Fig. 4C; tRNA<sup>ValUAC</sup> contains m<sup>2</sup>G<sub>10</sub>, but no primer extension stop is observed before G<sub>10</sub>). Yet, tRNA<sup>LysCUU</sup> derived from wild-type cells clearly exhibits a primer extension stop at the G<sub>10</sub> position (Fig. 4D). Importantly, however, the observed stop is not Trm10-dependent (the same % stop is observed in tRNA<sup>LysCUU</sup> from *trm10Δ* cells), so the cause of the primer extension block in the absence of Trm10, and whether it is related to the presence of m<sup>2</sup>G<sub>9</sub> on this tRNA, is not known. Nonetheless, upon Trm10 overexpression, there is a pronounced increase in the primer extension block at G<sub>10</sub> (<80% of the total primer extension products), suggesting that this tRNA, which is reportedly m<sup>2</sup>G<sub>9</sub>-modified, can also become a substrate for m<sup>1</sup>G<sub>9</sub> modification by Trm10 (Fig. 4D). The precise modification status of tRNA<sup>LysCUU</sup> at G<sub>9</sub> under these conditions remains to be fully determined but appears likely to be an unusual instance of dual methylation of G at both the N1 and N2 positions on the same nucleotide.

We note that these data further support the observation that the presence of other tRNA modifications is not sufficient to completely prevent m<sup>1</sup>G<sub>9</sub> modification in yeast, since for the assays shown in Figure 4, methylation occurs in vivo prior to isolation of the tRNA and visualization using the primer extension assay. The increased level of methylation observed in Figures 1 and 2 is thus not an artifact of the in vitro methylation conditions. Furthermore, these assays reveal that the additional reactivity of Trm10 toward tRNA<sup>ValUAC</sup> is not an isolated example of mismodification of a single tRNA but instead represents an overall expansion of potential tRNA substrates for Trm10 in yeast that can be affected by the expression level of Trm10 in cells.

### Additional tRNA substrates are methylated by purified Trm10 in vitro

To directly test the ability of Trm10 to methylate some of the normally unmodified tRNA species described above, as well as the tRNA<sup>AsnGUU</sup> that could not be detected with the primer extension assay, we cloned tRNA genes for tRNA<sup>CysGCA</sup> and tRNA<sup>AsnGUU</sup> and generated uniformly labeled versions of each tRNA by in vitro transcription in the presence of [ $\alpha^{32}$ P]-GTP. Since unlike the single-labeled substrates used in Figure 1, all of the G-residues in these tRNA transcripts are labeled, the maximum amount of observable methylation due specifically to modification at G<sub>9</sub> is significantly decreased. For example, for tRNA<sup>CysGCA</sup>, even if 100% m<sup>1</sup>G modification occurs at position 9, the maximum level of methylated product that would be observed in the assay is 1/23 total G residues, or ~4.3%. We readily detected m<sup>1</sup>G<sub>9</sub> modification of the tested tRNA substrates, with maximal amounts of product formation consistent with that predicted for each tRNA (Fig. 5). The overall efficiency of methylation of each substrate based on these endpoint assay measure-



**FIGURE 5.** Additional yeast tRNAs are substrates for efficient m<sup>1</sup>G<sub>9</sub> methylation by yeast Trm10 in vitro. Activity assays were performed with yeast Trm10 (fivefold dilutions starting with 1.1 mg/mL purified protein in highest concentration reactions) and tRNA substrates (as indicated) uniformly labeled at the 5'-phosphate of every G-residue in the tRNA. After reaction with Trm10, tRNAs were digested to nucleotide 5'-monophosphates with nuclease P1, and the p\*m<sup>1</sup>G reaction products were resolved from the remaining unreacted substrate (p\*G) using cellulose TLC. (–) These lanes are no enzyme control reactions. For each tRNA, the maximum amount of observed m<sup>1</sup>G product was quantified and compared to the maximum amount of m<sup>1</sup>G if all G<sub>9</sub> nucleotides were methylated. For tRNA<sup>AsnGUU</sup>, the maximum m<sup>1</sup>G product was 4.4% compared to 4.5% expected if all G<sub>9</sub> nucleotides were methylated. For tRNA<sup>CysGCA</sup>, the maximum m<sup>1</sup>G observed/maximum m<sup>1</sup>G expected was 5.3%/4.3%; and for the G<sub>9</sub>-containing tRNA<sup>PheGAA</sup>, the maximum m<sup>1</sup>G observed/maximum m<sup>1</sup>G expected was 3.5%/4.2%.

ments appears similar to that observed for tRNA<sup>ValUAC</sup> above (Fig. 1).

To probe the apparent generality of Trm10-tRNA recognition further, we tested whether a tRNA that normally contains A<sub>9</sub>, which is not methylated to m<sup>1</sup>A<sub>9</sub> by yTrm10, could become a substrate for methylation by introducing G<sub>9</sub> into the tRNA. Wild-type (A<sub>9</sub>-containing) tRNA<sup>PheGAA</sup> was uniformly labeled with [ $\alpha^{32}$ P]-GTP, and as expected, contained no detectable m<sup>1</sup>G after incubation with purified Trm10 (Fig. 5, last panel). However, when A<sub>9</sub> was replaced with G<sub>9</sub>, purified yTrm10 catalyzed m<sup>1</sup>G formation with an observed efficiency similar to that with the cognate tRNA<sup>GlyGCC</sup> substrate. These data further confirm that Trm10 is capable of acting on a wider set of tRNA substrates than the pattern of modification in wild-type yeast would suggest, a result that may have implications for Trm10 function in other species that contain alternative patterns of tRNA m<sup>1</sup>G<sub>9</sub> modification.

### DISCUSSION

m<sup>1</sup>G methylation at position 9 of tRNA is a highly conserved modification found in Eukaryotes and Archaea and is catalyzed by the SAM-dependent methyltransferase, Trm10. In wild-type yeast strains, approximately half of all tRNA species that contain a G-nucleotide at position 9 are modified by Trm10 (Fig. 2; Table 2), but the molecular basis for selection of this subset of tRNA species for modification is not obvious from simple sequence comparisons. Here, we investigated the tRNA substrate specificity of yeast Trm10, both in vitro and in vivo. We demonstrated that purified yeast Trm10 catalyzes m<sup>1</sup>G<sub>9</sub> formation in vitro with a number of yeast tRNA transcripts that are not normally modified in wild-type yeast (Figs. 1, 5). Comparison of steady-state kinetic parameters (for methylation of tRNA<sup>ValUAC</sup>) and endpoint enzyme titration assays (for methylation of tRNA<sup>ValUAC</sup>, tRNA<sup>CysGCA</sup>, and tRNA<sup>AsnGUU</sup>) revealed that the apparent catalytic efficiency



of methylation of these substrates is similar to that observed with physiological tRNA substrates, such as tRNA<sup>GlyGCC</sup> (Figs. 1, 5; Table 1). In the presence of other modified nucleotides on these additional species, the overall efficiency of m<sup>1</sup>G<sub>9</sub> modification is somewhat reduced, suggesting that other modifications play some role in tRNA recognition by Trm10 (Fig. 3). However, the presence of other nucleotide modifications on the tRNA does not completely prevent methylation of these additional species, either in vitro or in vivo, and thus cannot fully explain the complete lack of modification of the majority of these species in vivo in yeast (Figs. 3, 4; Table 2). Taken together, these data suggest that Trm10 exhibits a broader pattern of substrate recognition than would be expected based on the limited pattern of in vivo m<sup>1</sup>G<sub>9</sub> modification in wild-type yeast.

If additional tRNA species can become substrates for Trm10 methylation, what is the basis for the observed modification of only a subset of the potential substrate tRNAs in yeast? We noted that type II tRNA species such as tRNA<sup>LeuCAA</sup>, which contain extended variable loop sequences, remain consistently unmodified in our assays, even under conditions of Trm10 over-expression that lead to modification of other typically unmodified tRNAs in yeast (Fig. 4; Table 2). In fact, of the 90 different type II tRNAs that have been sequenced, 71 of these contain a G<sub>9</sub>, and none of these are observed to be m<sup>1</sup>G<sub>9</sub> modified in any organism. Moreover, only two tRNA<sup>Leu</sup> species are known to contain m<sup>1</sup>G<sub>9</sub>, and both are atypical tRNAs found in mitochondria of cow and humans that do not contain the extended variable loop feature. Therefore, it appears likely that the presence of a long variable loop may prevent recognition by Trm10, perhaps by restricting the tRNA to a certain structural conformation that prevents accessibility of Trm10 to the G<sub>9</sub> residue at the core of the L-shaped tRNA structure. Further structural and biochemical studies are needed to understand the role of the variable loop in tRNA recognition by Trm10.

For the remaining type I tRNA species (containing the typical short 4–6 nucleotide variable loop sequence), the basis for tRNA recognition is still not clear. Differences in the catalytic efficiency of methylation of nonsubstrates versus substrates are not sufficient to explain the absolute lack of any detectable m<sup>1</sup>G<sub>9</sub> modification observed with nonsubstrates such as tRNA<sup>ValUAC</sup> in vivo. The presence of other modifications appears to exert a somewhat restrictive effect on the efficiency of methylation with noncognate tRNAs (Fig. 3); but similar to the minimal differences in kinetic efficiency, these effects on their own cannot rationalize the observed pattern of m<sup>1</sup>G<sub>9</sub>-modified tRNAs in vivo. Taken together, these results suggest that selection of substrates for m<sup>1</sup>G<sub>9</sub> modification in yeast may be controlled by a variety of factors. These factors may include reliance on additional protein or RNA components to provide an additional level of recognition, and contributions of tRNA dynamics and stability may also serve to restrict the in vivo activity of γTrm10 to only a subset of G<sub>9</sub>-containing tRNAs.

The relative lack of complete tRNA sequences, including modifications, for organisms other than *S. cerevisiae* makes it difficult to fully assess how Trm10 substrate recognition might be accomplished in other species. The observation that the human Trm10 ortholog most closely related to γTrm10 (TRMT10A) catalyzes similarly efficient in vitro methylation of the yeast tRNA<sup>GlyGCC</sup> and tRNA<sup>ValUAC</sup> substrates (Fig. 1) is intriguing, since the sequence of human tRNA<sup>GlyGCC</sup> has been determined; and in humans, tRNA<sup>GlyGCC</sup> contains an unmodified G<sub>9</sub>. The human and yeast tRNA<sup>GlyGCC</sup> species share >70% overall sequence identity; and although specific sequences may be responsible for the apparent discrimination between the two tRNAs, these are again not obvious from simple sequence comparison to known Trm10 substrates. Further investigation of TRMT10A methyltransferase activity with bona fide human tRNA substrates is required, but these data suggest that similar patterns may be observed in human cells, where cytosolic Trm10 acts on a more limited set of tRNA species in vivo than the in vitro substrate specificity would suggest. It is interesting that the same tRNA species are not similarly modified in different organisms, such as the tRNA<sup>GlyGCC</sup> described above (modified with m<sup>1</sup>G<sub>9</sub> in yeast but not in humans), or tRNA<sup>AsnGUU</sup> and tRNA<sup>ThrAGU</sup>, which contain unmodified G<sub>9</sub> in yeast but are both modified with m<sup>1</sup>G<sub>9</sub> in bovine tRNA. This observed lack of consensus in terms of substrates for methylation is consistent with a scenario in which Trm10 enzymes generally exhibit the ability to methylate a wide variety of substrate tRNAs, and the actual selection of specific substrates to be modified in vivo is controlled by organism-specific factors.

The observation that Trm10 methyltransferase acts on additional substrates beyond those with a demonstrated connection to methylation in vivo may be relevant to the observed connection between a Trm10 ortholog in humans and the unusual proteinaceous ribonuclease P enzyme in human mitochondria (Holzmann et al. 2008; Vilardo et al. 2012). The mitochondrial enzyme, unlike all other ribonucleoprotein versions of RNase P found to date, is a patchwork of three previously unrelated proteins, including one of the three human paralogs of Trm10 (originally RG9MTD1, now also known as TRMT10C). As postulated by the discoverers of this complex, TRMT10C appears to participate in tRNA recognition during 5'-end maturation, since of the three mitochondrial RNase P components, TRMT10C is the only one to have any previously annotated relationship with tRNA. However, the methylation activity of TRMT10C is not strictly required for cleavage of pre-tRNA by the mitochondrial RNase P holoenzyme, since a mutant form of TRMT10C with impaired methylation activity still supports formation of a functional 5'-end cleavage enzyme (Vilardo et al. 2012). Interestingly, although yeast Trm10 substrate specificity is more flexible than may have been previously expected, the mitochondrial TRMT10C ortholog appears to exhibit even broader tRNA substrate selectivity than yeast

Trm10, human TRMT10A, or TRMT10B, since TRMT10C modifies several mt-tRNA species that appear to be less efficiently modified by any of the other cytosolic enzymes (Vilardo et al. 2012). Since interaction with a wide variety of tRNA substrates is predictably necessary for a Trm10-type enzyme to function as the recognition factor for efficient tRNA processing by the mt-RNase P enzyme, the observation of even more extensive substrate recognition capabilities of TRMT10C is perhaps not surprising. Nonetheless, the relationship between the further expanded tRNA substrate selectivity of mitochondrial TRMT10C and the varying tRNA substrate specificities exhibited by the cytosolic enzymes will be interesting to investigate in the future.

## MATERIALS AND METHODS

### Purification of Trm10

N-terminally His<sub>6</sub>-tagged yeast Trm10 or Human TRMT10A was expressed in *E. coli* as previously described (Jackman et al. 2003) and purified using immobilized metal ion affinity chromatography (IMAC); all steps were performed at 4°C. Harvested cells (from 0.5 L culture) were washed and resuspended in buffer A (20 mM HEPES, 4 mM MgCl<sub>2</sub>, 10% glycerol, 5 mM β-mercaptoethanol [BME], pH 7.5) containing 1 M NaCl and protease inhibitors (1 μg/mL each pepstatin and leupeptin and 1 mM phenylmethylsulfonyl fluoride). The resuspended cells were lysed by French press, and cellular debris was removed by centrifugation. The NaCl concentration in the soluble crude extract was adjusted to 0.5 M by the addition of an equal volume of buffer A (containing no added NaCl), and the resulting crude extract was incubated with 1 mL TALON resin (Clontech), pre-equilibrated in buffer B (20 mM HEPES, 0.5 M NaCl, 4 mM MgCl<sub>2</sub>, 10% glycerol, 5 mM β-mercaptoethanol [BME], pH 7.5) with gentle mixing. The bound resin was washed with at least 20 column volumes of buffer B, followed by at least 40 column volumes of buffer B containing 10 mM imidazole. Trm10 was eluted from the column by the addition of 10 mL buffer B containing 250 mM imidazole; 1-mL fractions were collected and assessed for protein content by visual inspection using BioRad protein assay. Fractions that contained the highest protein concentration were combined and dialyzed against buffer containing 25 mM sodium phosphate, 0.055 M NaCl, 1 mM MgCl<sub>2</sub>, 1 μM EDTA, 1 mM dithiothreitol, 50% glycerol, pH 7.5) to remove imidazole and concentrate protein samples; purified protein was stored at −20°C. SDS-PAGE analysis and activity assays were used to assess the purity and stability of purified protein.

### In vitro assay for m<sup>1</sup>G<sub>9</sub> methylation

tRNA substrates for methyltransferase assays were uniquely labeled at the 5'-phosphate of G<sub>9</sub> using a variation of the previously described protocol (Jackman et al. 2003). To generate these substrates, the first eight nucleotides were removed from the coding sequence of each of the three yeast tRNA genes in plasmids designed for in vitro transcription with T7 RNA polymerase. The resulting plasmids were used as templates to produce in vitro transcripts initiating at G<sub>9</sub> and the 5'-truncated transcripts were 5'-<sup>32</sup>P-labeled by treatment

with phosphatase, followed by T4 polynucleotide kinase in the presence of [γ-<sup>32</sup>P]-ATP according to Jackman et al. (2003). A synthetic RNA oligonucleotide comprising nucleotides 1–8 was ligated onto the labeled transcript using T4 DNA ligase and a bridging DNA splint to regenerate a full-length tRNA with the unique 5'-<sup>32</sup>P-G<sub>9</sub> label (Jackman et al. 2003). Ligated full-length tRNAs were excised from denaturing (Urea) polyacrylamide gels, purified by phenol-extraction and ethanol purification, and evaluated by nuclease digestion to confirm the correct ligation site junction for each labeled tRNA product. For assays in Figure 5, uniformly labeled tRNAs were produced by in vitro transcription of full-length tRNAs and tRNA variants (cloned into appropriate plasmids for transcription by T7 RNA Polymerase) in the presence of [α-<sup>32</sup>P]-GTP; labeled transcripts were purified by gel electrophoresis followed by phenol extraction and ethanol precipitation for use in methyltransferase assays.

Activity assays were performed at 30°C with purified yeast Trm10 and labeled tRNA substrate (either uniquely or uniformly labeled, as indicated) in assay buffer containing 50 mM Tris pH 8.0 and 1.5 mM MgCl<sub>2</sub> in the presence of 0.5 mM SAM. At the desired time points, 10-μL aliquots of each reaction were quenched by phenol extraction and concentrated by ethanol precipitation. The purified RNA was digested to mononucleotides by resuspension of the RNA pellet from ethanol precipitation in 4 μL of a solution containing 1 unit nuclease P1 in 20 mM sodium acetate, pH 5.2, 1 mM ZnCl<sub>2</sub> buffer. Digestion reactions were incubated for 30 min at 30°C, and then 2-μL aliquots of each nuclease-digested reaction were spotted to cellulose TLC plates (EM Science). Trm10 reaction products (p<sup>m</sup>G) were resolved from unreacted substrate (p<sup>\*</sup>G) using a 66:33:1 (v:v:v) isobutyric acid:H<sub>2</sub>O:NH<sub>4</sub>OH solvent system; reactions were visualized and quantified using a Typhoon trio imager and ImageQuant (GE Healthsciences).

### Determination of steady-state kinetic parameters for Trm10 activity with in vitro tRNA substrates

Steady-state kinetic parameters for the in vitro tRNA substrates, tRNA<sup>GlyGCC</sup>, and tRNA<sup>ValUAC</sup> were determined using standard in vitro assay conditions as described above. Linear initial rates were measured under saturating conditions for SAM (25 μM for yeast Trm10 and 500 μM for TRMT10A) with varying concentration of tRNA substrate (by inclusion of defined concentrations of unlabeled tRNA transcript along with the labeled tRNA substrate). Assays were conducted using 100–8000 nM tRNA and 5–50 nM enzyme for assays with yeast Trm10, and 50–3000 nM tRNA and 5–150 nM enzyme in assays with human TRMT10A. Assays typically contained at least 10-fold excess tRNA over enzyme except for assays with the lowest concentration of tRNA (50–100 nM), where slightly higher concentrations of enzyme were required to visualize product formation within the time courses, and the excess of substrate over enzyme was about fivefold. Time courses were chosen to yield <5% product formation during the time of the assay to reduce the effects of product inhibition on the observed rates. Linear initial velocities (*v*<sub>o</sub>) were normalized for enzyme concentration and measured in three independent assays for each substrate/enzyme combination and at tRNA substrate concentrations in the range of about 0.3 to 3 times the apparent *K*<sub>M,tRNA</sub>. For each substrate, *v*<sub>o</sub>/[E] was plotted as a function of [tRNA], and fit to the Michaelis-Menten equation using nonlinear least-squares regression analysis using Kaleidagraph



(Synergy software) to yield kinetic parameters  $k_{\text{cat}}$ ,  $K_{\text{M,tRNA}}$ , and  $k_{\text{cat}}/K_{\text{M,tRNA}}$ .

## Primer extension analysis of m<sup>1</sup>G<sub>9</sub> methylation

Yeast strains were grown at 30°C to an OD<sub>600</sub> of 1, and low molecular weight RNAs were extracted using the hot phenol method (Jackman et al. 2003). For Trm10 treatment of in vivo isolated RNAs (Fig. 2), 10 µg of total RNA was incubated with 12 µg purified Trm10 in 50 mM Tris pH 8.0, 1.5 mM MgCl<sub>2</sub>, 0.5 mM SAM for 2 hr at 30°C. Primers were 5'-end labeled and gel-purified prior to their use in the primer extension reactions. Reactions contained 1 pmol labeled primer and 10 µg total RNA (either directly after isolation, or after Trm10-treatment as described above) and were performed as previously described (Jackman et al. 2003). Where indicated, the percent of m<sup>1</sup>G<sub>9</sub> in each tRNA was quantified by measuring the intensity of the band corresponding to the m<sup>1</sup>G<sub>9</sub> primer extension block divided by the sum of the primer extension stop due to m<sup>1</sup>G<sub>9</sub> and the primer extension stop corresponding to full-length tRNA (total primer extension products in each reaction). Sequencing reactions were performed using the same primer extension conditions in the presence of 0.2 mM of each ddNTP, as indicated. Primer sequences used for each tRNA (listed 5'-3') were AlaUCG: CTTGCAAGGGAAGCGCGCTA; ArgCCU: TTAGGA GGGAGACGCGTTAC; GlyCCC: TTGGGAAGCATAAATTCTAA C; ProAGC: CACCCTAAGCGAGAATCATA; LeuGAG: ACCTC GACCTGACGCCTTAGA; ThrCGU: TTACGAGTGCGATGCC TTAC; SerGCU: TTAGCAGGGCATCGCCTTAA; SerCGA: TTT CGAGTCTCTCGCCTTAA; GlyGCC: TGGCAACGTTGGATTTT ACC; ValUAC: GTGTAAGGCGACGTCTTGA; LeuCAA: GCTT GAATCAGGCGCCTTAG; LysCUU: ATTAAGAGTCATACGCGC TA; ThrAGU: ACTAGTGTGGCGCCTTACCA; AsnGUU: TTAAC AGTCGCACGCCTTAACC; and CysGCA: TTTGCAATCTGGTG CGCTACC.

## SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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## Unexpected expansion of tRNA substrate recognition by the yeast m<sup>1</sup>G<sub>9</sub> methyltransferase Trm10

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