S. cerevisiae Trm140 has two recognition modes for 3-methylcytidine modification of the anticodon loop of tRNA substrates

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ABSTRACT

The 3-methylcytidine (m^3C) modification is ubiquitous in eukaryotic tRNA, widely found at C_{32} in the anticodon loop of $tRNA^{Thr}$, $tRNA^{Ser}$, and some $tRNA^{Arg}$ species, as well as in the variable loop (V-loop) of certain $tRNA^{Ser}$ species. In the yeast *Saccharomyces cerevisiae*, formation of m^3C_{32} requires Trm140 for six tRNA substrates, including three $tRNA^{Thr}$ species and three $tRNA^{Ser}$ species, whereas in *Schizosaccharomyces pombe*, two Trm140 homologs are used, one for $tRNA^{Thr}$ and one for $tRNA^{Ser}$. The occurrence of a single Trm140 homolog is conserved broadly among Ascomycota, whereas multiple Trm140-related homologs are found in metazoans and other fungi. We investigate here how *S. cerevisiae* Trm140 protein recognizes its six tRNA substrates. We show that Trm140 has two modes of tRNA substrate recognition. Trm140 recognizes G_{35}-U_{36}-t^6A_{37} of the anticodon loop of $tRNA^{Thr}$ substrates, and this sequence is an identity element because it can be used to direct m^3C modification of $tRNA^{Phe}$. However, Trm140 recognition of $tRNA^{Ser}$ substrates is different, since their anticodons do not share G_{35}–U_{36} and do not have any nucleotides in common. Rather, specificity of Trm140 for $tRNA^{Ser}$ is achieved by seryl-tRNA synthetase and the distinctive $tRNA^{Ser}$ V-loop, as well as by t^6A_{37} and i^6A_{37}. We provide evidence that all of these components are important in vivo and that seryl-tRNA synthetase greatly stimulates m^3C modification of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ in vitro. In addition, our results show that Trm140 binding is a significant driving force for tRNA modification and suggest separate contributions from each recognition element for the modification.

Keywords: 3-methylcytidine; methyltransferase; modification; tRNA; specificity; anticodon loop

INTRODUCTION

tRNA undergoes extensive post-transcriptional modifications in all domains of life to ensure the efficiency and accuracy of translation. In the yeast *Saccharomyces cerevisiae*, each cytoplasmic tRNA has an average of 12.6 modifications, with ∼10 modifications in the main body of the tRNA and ∼2.6 modifications in the anticodon loop region, comprising residues N_{32}–N_{38} of the anticodon loop and the neighboring N_{31}–N_{39} base pair of the anticodon stem (Juhling et al. 2009). Modifications within the tRNA body often contribute to folding or stability (Helm et al. 1999; Kadaba et al. 2004; Alexandrov et al. 2006; Whipple et al. 2011). In contrast, modifications in the anticodon (primarily at the wobble nucleotide N_{34}) or at N_{37} often contribute to accurate decoding and reading frame maintenance (Gerber and Keller 1999; Bjork et al. 2001, 2007; Urbonavicius et al. 2001; Murphy and Ramakrishnan 2004; Esberg et al. 2006; Agris et al. 2007; Waas et al. 2007; Weixlbaumer et al. 2007; Johansson et al. 2008; Chen et al. 2011; El Yacoubi et al. 2011).

Modifications occurring at other residues within the anticodon loop region also have important roles in tRNA function. For example, a yeast *pus3*Δ mutant, which lacks pseudouridine at U_{38} or U_{39}, is temperature sensitive, primarily due to reduced $tRNA^{Gln(UUG)}$ function (Han et al. 2015); and a yeast *trm7*Δ mutant, which lacks 2′-*O*-methylation at C_{32} (Cm) as well as Gm_{34}, grows poorly due to reduced translation and reduced $tRNA^{Phe}$ function (Pintard et al. 2002; Guy et al. 2012). N_{32} and N_{38} are at the borders of the anticodon loop, often form a noncanonical base pair (Auffinger and Westhof 1999), and have been shown to be critical for ribosome binding and decoding (Lustig et al. 1993; Olejniczak et al. 2005; Olejniczak and Uhlenbeck 2006; Ledoux et al. 2009).

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The 3-methylcytidine (m^3C) modification is also found at C_{32} in the anticodon loop of tRNAs and likely has an important role. m^3C is found at C_{32} of all four characterized eukaryotic cytoplasmic $tRNA^{Thr}$ species, 18 of the 20 characterized cytoplasmic $tRNA^{Ser}$ species, and two of five characterized eukaryotic $tRNA^{Arg}$ species that have an encoded C_{32}, as well as at residue e2 of the $tRNA^{Ser}$ variable loop (V-loop) in animals (Weissenbach et al. 1977; Juhling et al. 2009; Machnicka et al. 2013; Arimbasseri et al. 2016). The m^3C modification is formed by members of the Trm140 m^3C methyltransferase family. In *S. cerevisiae*, *TRM140* is required for m^3C_{32} modification of six tRNA species, including all three $tRNA^{Thr}$ species, with anticodons IGU, CGU, and UGU, and the three $tRNA^{Ser}$ species with anticodons CGA, UGA, and GCU (D'Silva et al. 2011; Noma et al. 2011), while $tRNA^{Ser(IGA)}$ does not have C_{32}. In contrast, in *Schizosaccharomyces pombe* there are two *TRM140* homologs; *trm140*$^+$ is required for modification of all three $tRNA^{Thr}$ species, whereas *trm141*$^+$ is required for modification of all four $tRNA^{Ser}$ species (Arimbasseri et al. 2016). Although *S. cerevisiae trm140*Δ mutants and *S. pombe* mutants lacking *TRM140* and/or *TRM141* have no growth defect in a variety of media (D'Silva et al. 2011; Arimbasseri et al. 2016), the importance of m^3C_{32} is underscored by the growth defect of *S. cerevisiae trm140*Δ *trm1*Δ mutants in the presence of cycloheximide (D'Silva et al. 2011), and by the broad conservation of the *TRM140* family and the m^3C modification in eukaryotes.

One important question about *S. cerevisiae* Trm140 is how it recognizes its specific tRNA substrates. For enzymes such as Pus3 that modify the same residues in every tRNA, recognition typically exploits a common structural feature of tRNAs (Hur and Stroud 2007). For enzymes such as the $tRNA^{His}$ guanylyltransferase Thg1, which only modifies $tRNA^{His}$, recognition of the unique GUG anticodon drives modification (Jackman and Phizicky 2006; Nakamura et al. 2013). However, several modifying enzymes recognize a specific subset of tRNAs without obvious recognition elements; examples include the m^1G_9 modification catalyzed by Trm10 for 13 of 24 species with G_9 (Swinehart et al. 2013; Swinehart and Jackman 2015) and the Cm_{32} and Gm_{34} modification catalyzed by Trm7 on three tRNA species (Pintard et al. 2002), as well as the m^3C_{32} modification catalyzed by *S. cerevisiae* Trm140 on its six substrates (D'Silva et al. 2011; Noma et al. 2011). Indeed, simple sequence inspection suggests no common theme that would direct Trm140 modification of its three $tRNA^{Thr}$ substrates and its three $tRNA^{Ser}$ substrates that distinguishes these tRNAs from the 17 other *S. cerevisiae* tRNAs with C_{32}.

Here we define the specificity of *S. cerevisiae* Trm140 by both in vivo and in vitro approaches. We show that there are two distinct modes of Trm140 recognition of tRNA substrates for m^3C_{32} modification. For $tRNA^{Thr}$ species, Trm140 reads the anticodon nucleotides and the t^6A modification, whereas for $tRNA^{Ser}$ species, recognition is achieved through seryl-tRNA synthetase (SerRS, encoded by *SES1*) and the V-loop region, as well as i^6A_{37} or t^6A_{37}.

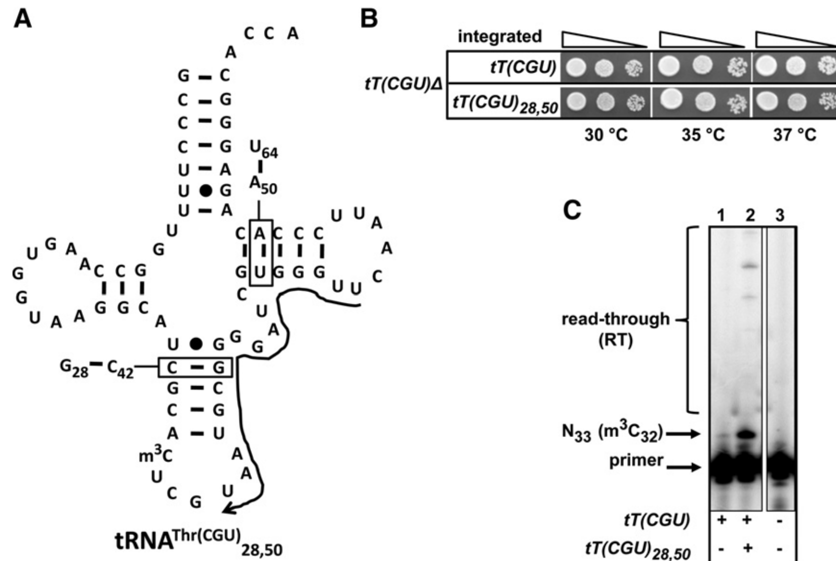


FIGURE 1. The $tRNA^{Thr(CGU)}{}_{28,50}$ scaffold used for analysis of $tRNA^{Thr}$ variants is fully functional and efficiently modified to m^3C. (*A*) Schematic of $tRNA^{Thr(CGU)}{}_{28,50}$. The secondary structure of $tRNA^{Thr(CGU)}$ is shown. The base pairs of residues 28:42 and 50:64 are switched as indicated in the boxes. The primer complementary to residues 55–36 is indicated with a 3′ arrow. (*B*) $tRNA^{Thr(CGU)}{}_{28,50}$ is functional in vivo. *tT(CGU)*Δ cells containing the integrated WT *tT (CGU)* or *tT(CGU)$_{28,50}$* as indicated were grown overnight in YPD medium, serially diluted and spotted onto YPD medium, and plates were incubated at indicated temperatures for 3 d. (*C*) $tRNA^{Thr(CGU)}{}_{28,50}$ is efficiently modified to m^3C_{32}. Bulk RNA from cells containing the integrated WT *tT(CGU)* or *tT(CGU)$_{28,50}$* as indicated were analyzed by primer extension assay, as described in Materials and Methods, using the primer shown in *A*. The vast majority of $tRNA^{Thr(CGU)}{}_{28,50}$ has m^3C_{32} based on the primer extension stop at U_{33}, compared to the amount of read-through (RT).

RESULTS

An XGU anticodon and t^6A_{37} are necessary and sufficient for m^3C modification of $tRNA^{Thr}$ species

To begin to elucidate the specificity of Trm140, we designed a scaffold based on $tRNA^{Thr(CGU)}$ [gene name: *tT(CGU)*] that would enable analysis of m^3C in variants with this target scaffold independent of other tRNAs present in the cell. We altered the identity of base pairs 28:42 and 50:64 to allow us to analyze this tRNA species ($tRNA^{Thr(CGU)}{}_{28,50}$) with oligonucleotides spanning these residues (Fig. 1A). This variant was fully functional because an otherwise lethal *tT(CGU)*Δ strain was completely rescued upon integration of *tT(CGU)$_{28,50}$*, with

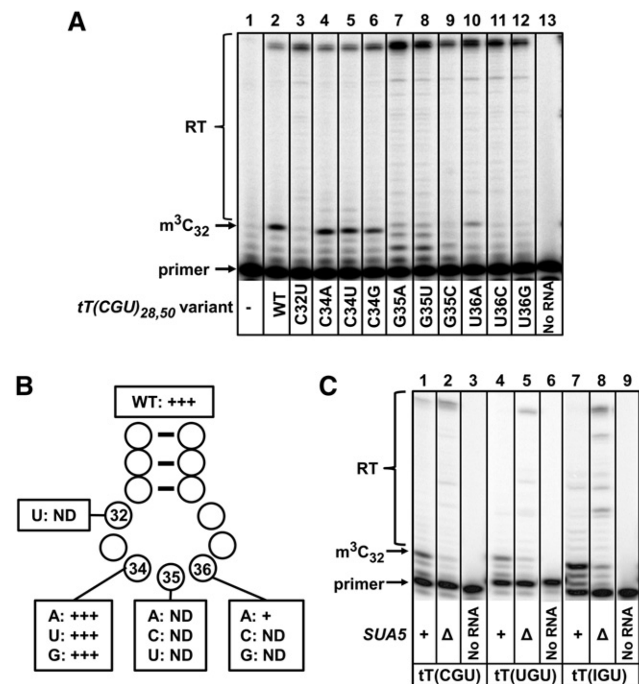


FIGURE 2. An XGU anticodon and t^6A_{37} are important for m^3C modification of $tRNA^{Thr(CGU)}$. (*A*) G_{35} and U_{36} of $tRNA^{Thr(CGU)}$ are required for m^3C formation. Bulk RNA from strains containing integrated *tT (CGU)*$_{28,50}$ variants with mutations as indicated were analyzed for m^3C by primer extension, using a primer complementary to residues 51–37 of $tRNA^{Thr(CGU)}{}_{28,50}$. The major read-through band on the *top* of the gel corresponds to the full-length tRNA. Note that for variants with G_{35} and U_{36}, the primer extension is slightly displaced, presumably due to a different primer extension sequence. (*B*) Summary of primer extension results of m^3C modification in *A*. +++, WT levels of m^3C; +, low but detectable m^3C; ND, m^3C not detected. (*C*) t^6A_{37} is important for m^3C formation of $tRNA^{Thr}$ species. Bulk RNA from *SUA5*$^+$ and *sua5*Δ cells was analyzed by primer extension with primers as indicated, to evaluate the importance of t^6A for m^3C modification of $tRNA^{Thr(CGU)}$, $tRNA^{Thr(UGU)}$, and $tRNA^{Thr(IGU)}$.

no discernable growth defect in the range of 30°C to 37°C, compared to the corresponding strain bearing wild-type *tT (CGU)* at the same locus (Fig. 1B). The $tRNA^{Thr(CGU)}{}_{28,50}$ variant was easily analyzed by primer extension independent of the wild-type $tRNA^{Thr(CGU)}$ (Fig. 1C), and was efficiently modified to m^3C; the vast majority of this tRNA has m^3C_{32}, based on the prominent primer extension block at N_{33}, compared to the relatively minor read-through signal (Fig. 1C), similar to the amount of m^3C modification observed for wild-type $tRNA^{Thr(CGU)}$ (Fig. 2).

Since the $tRNA^{Thr}$ species bearing m^3C_{32} all have similar anticodons (CGU, UGU, and IGU), we made and analyzed a set of yeast strains, each with an integrated $tRNA^{Thr(CGU)}{}_{28,50}$ variant bearing a single mutation in the anticodon loop (Fig. 2A,B). Primer extension analysis showed that alteration of C_{34} to other nucleotides resulted in retention of the major primer extension block at N_{33}, indicative of m^3C_{32}. In contrast, substitution of residue G_{35} or U_{36} with each of the other nucleotides resulted in almost complete loss of m^3C_{32}. These results suggest that the conserved G_{35} and U_{36} residues in the anticodons of $tRNA^{Thr}$ species are required for m^3C formation. As expected, substitution of C_{32} to U_{32} resulted in no primer extension stop.

Since U_{36} was important for m^3C modification activity, and U_{36} is always followed by t^6A_{37} (Machnicka et al. 2013) or, as frequently occurs in yeast and some other organisms, a cyclized derivative of t^6A called ct^6A (Miyauchi et al. 2013), it was possible that t^6A_{37} or ct^6A_{37} (collectively referred to as t^6A for simplicity) also had a role in m^3C formation. To examine the contribution of t^6A to m^3C modification, we analyzed m^3C levels in wild-type $tRNA^{Thr}$ species from an *sua5*Δ strain, which lacks t^6A_{37} (El Yacoubi et al. 2009). The m^3C levels in each of the three $tRNA^{Thr}$ species from the *sua5*Δ strain were substantially reduced compared to those from the corresponding wild-type strain (Fig. 2C). This result suggested that t^6A_{37} was important, but not absolutely required, for m^3C modification of the three $tRNA^{Thr}$ species in vivo, along with the required G_{35}–U_{36} anticodon sequence.

To probe the connection between Trm140 tRNA modification specificity and Trm140 binding, we developed a pull-down assay. In a strain in which Trm140 was overproduced with a C-terminal tandem affinity purification tag (PT), we observed highly specific copurification of tRNA substrates. Trm140 was purified using IgG Sepharose in buffer conditions predicted to maintain native protein–RNA interactions, and then bound protein was washed once and eluted with protease to release Trm140 and retained RNAs. Northern analysis of the eluted tRNAs (Fig. 3A) showed highly efficient retention of Trm140 substrate tRNAs, including $tRNA^{Thr(IGU)}$, $tRNA^{Thr(UGU)}$, $tRNA^{Thr(CGU)}$, relative to that in a vector control, whereas several nonsubstrate tRNAs examined did not copurify with Trm140, including $tRNA^{Leu(UAA)}$, $tRNA^{Phe(GAA)}$, and $tRNA^{Trp(CCA)}$. Similar copurification results were obtained upon overproduction of ORF240 (Fig. 3B), the C-terminal domain of Trm140 (comprising residues 277–628), which is necessary and sufficient for the methyltransferase activity in vivo (D'Silva et al. 2011); with either Trm140 or ORF240, we observed efficient retention of $tRNA^{Thr(IGU)}$, $tRNA^{Ser(GCU)}$, and $tRNA^{Ser(UGA)/(CGA)}$ (which were not distinguished by the hybridization probe we used), compared to the vector control. Because ORF240 was expressed at higher levels, had slightly stronger tRNA binding signals than Trm140, and also lacked the unnecessary N-terminal domain of Trm140, we continued binding experiments with ORF240. To quantify the specificity of ORF240 tRNA binding, we analyzed biological triplicate samples for ORF240 binding of a number of different tRNAs, using two washes before the release of bound protein by proteolytic cleavage. Under these conditions, $tRNA^{Thr(CGU)}$ bound very efficiently and reproducibly, whereas four nonsubstrate tRNAs did not, including $tRNA^{Phe(GAA)}$, $tRNA^{Lys(CUU)}$, $tRNA^{Met_i(CAU)}$, and $tRNA^{Ala(UGC)}$ (Fig. 3C). For these and subsequent experiments we calculated binding

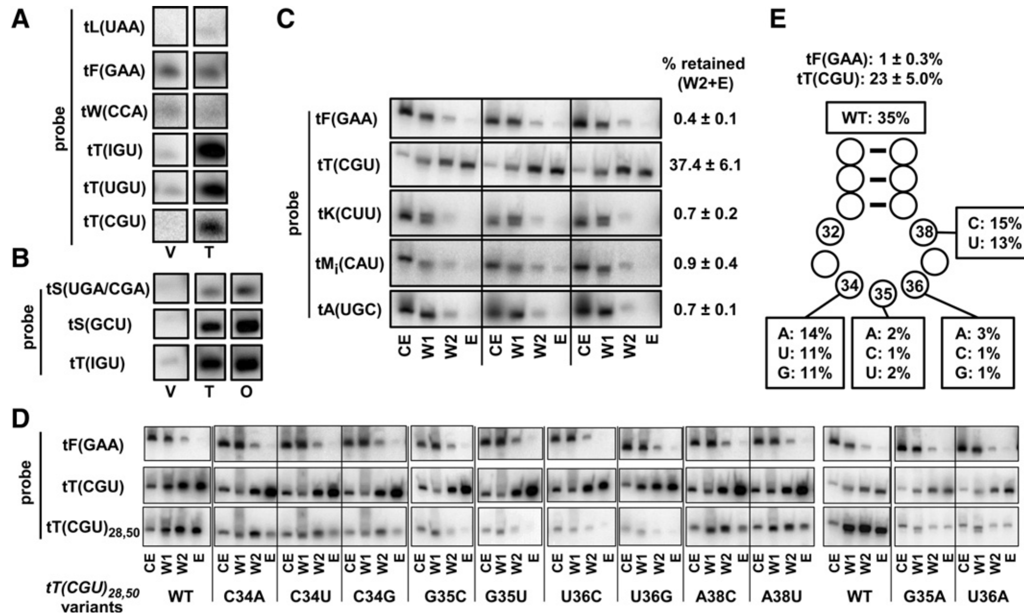
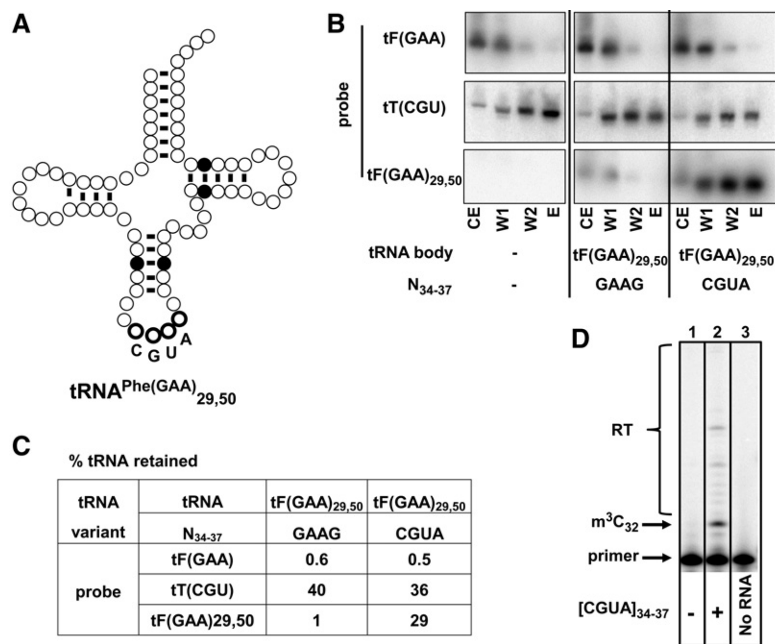


FIGURE 3. ORF240 binding of $tRNA^{Thr(CGU)}$ requires G_{35} and U_{36}. (*A*) Trm140 specifically binds $tRNA^{Thr}$ substrates. Trm140 was purified from a WT strain expressing *TRM140-PT* (or a vector control) by pull-down using IgG Sepharose, followed by a wash step and by elution of bound Trm140 by 3C protease treatment. Then, copurifying RNAs were resolved by PAGE, transferred to nitrocellulose, and analyzed by hybridization with probes as indicated. V, vector control; T, Trm140-PT. (*B*) ORF240 binds $tRNA^{Ser}$ substrates as efficiently as Trm140. Trm140 and ORF240 were purified from WT strains expressing *TRM140-PT* or *ORF240-PT* (or a vector control) by pull-down, and copurifying tRNAs were analyzed as in *A*. O, ORF240-PT. (*C*) ORF240 binds specifically and reproducibly to $tRNA^{Thr(CGU)}$ and not to four nonsubstrate tRNAs. *ORF240-PT* was expressed in WT cells in biological triplicate and purified by pull-down, two wash steps, and 3C protease elution of bound ORF240, and copurifying tRNAs were analyzed as in *A*. The percentage of tRNA retained in the combined second wash and the elution step, relative to that in the crude extract was calculated for each tRNA, as indicated. CE, crude extract (0.4% loaded); W1, wash 1 (10% loaded); W2, wash 2, (10% loaded); E, elution (10% loaded). (*D*) G_{35} and U_{36} of $tRNA^{Thr(CGU)}$ are important for binding of tRNA to ORF240. Strains containing integrated $tRNA^{Thr(CGU)}{}_{28,50}$ variants as indicated were grown and analyzed for ORF240 tRNA binding by the pull-down assay as described in *C*. (*E*) Schematic of pull-down results in *D*.

as the percentage of tRNA in the combined second wash and the elution step, relative to that in the crude extract, because it was apparent that $tRNA^{Thr(CGU)}$ was prominent in the second wash and the elution step, relative to the first wash, whereas the negative controls were much reduced in these fractions. These results establish that copurification of tRNA with Trm140 or ORF240 is a highly specific assay for substrate tRNA binding.

Consistent with our results from analysis of in vivo methyltransferase activity, we found that anticodon residues G_{35} and U_{36} were important for ORF240 binding as measured by this pull-down assay (Fig. 3D,E). Under these conditions, we observed reproducible copurification of wild-type $tRNA^{Thr(CGU)}$ (23 ± 5%) and reproducible lack of copurification of $tRNA^{Phe(GAA)}$ (1 ± 0.3%). For the $tRNA^{Thr(CGU)}{}_{28,50}$ variants, substitution of G_{35} or U_{36} with each of the other three nucleotides almost completely abolished the copurification of tRNA, whereas substitution of C_{34} with other nucleotides led to high levels of variant copurification. Based on these results, we conclude that Trm140 binds tRNAs with a G_{35}–U_{36} anticodon. Since these are the same residues that are important for m^3C modification, we infer that m^3C modification is driven in large part by Trm140 tRNA binding. Because we found that copurification of the C_{34} variants was distinctly more efficient than the other three variants (35% versus 11%–14%), we infer that C_{34} is a modestly preferred wobble base nucleotide for ORF240 recognition. Residue 38 is likewise modestly influential for Trm140 m^3C modification, since the $A_{38}U$ or $A_{38}C$ mutations reduced binding modestly, from 35% to 13%–15%, similar to the effects of C_{34} mutations.

To examine the sufficiency of G_{35}–U_{36}–t^6A_{37} for m^3C formation, we replaced the anticodon of the nonsubstrate $tRNA^{Phe(GAA)}$ with a CGU anticodon and a $G_{37}A$ mutation to allow for t^6A_{37} modification, and assayed the variant for m^3C modification and tRNA binding by ORF240. As we did for $tRNA^{Thr(CGU)}$ variants, we altered 2 bp in the stem to allow for unique detection of the $tRNA^{Phe(GAA)}$ variants; in this case, we flipped the 29:41 and 50:64 pairs to make $tRNA^{Phe(GAA)}{}_{29,50}$ (Fig. 4A). ORF240 pull-down experiments resulted in efficient copurification (29%) of the $tRNA^{Phe(GAA)}{}_{29,50}$ $[CGUA]_{34\text{-}37}$ variant, but only background copurification (1%) of $tRNA^{Phe(GAA)}{}_{29,50}$ $[GAAG]_{34\text{-}37}$ (Fig. 4B,C). Consistent with the binding results, primer extension of RNA from cells containing the $tRNA^{Phe(GAA)}{}_{29,50}$ $[CGUA]_{34\text{-}37}$ variant revealed a strong primer extension block at N_{33}, indicative of m^3C modification (Fig. 4D). These binding and primer extension results of $tRNA^{Phe(GAA)}{}_{29,50}$



tRNA variant	tRNA N_{34-37}	$tF(GAA)_{29,50}$ GAAG	$tF(GAA)_{29,50}$ CGUA
probe	tF(GAA)	0.6	0.5
	tT(CGU)	40	36
	tF(GAA)29,50	1	29

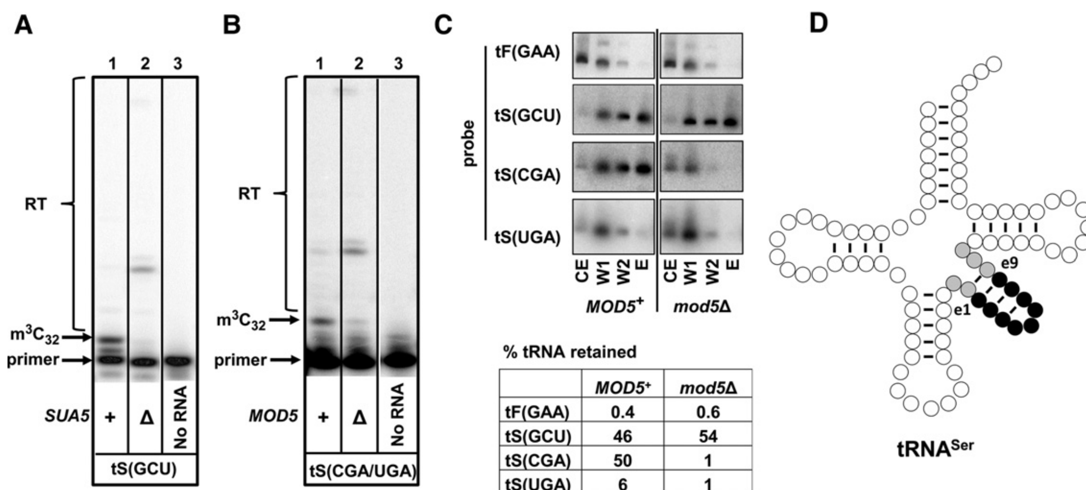
FIGURE 4. An XGU anticodon and t^6A_{37} are sufficient for ORF240 binding and for m^3C modification of $tRNA^{Phe}$. (*A*) Schematic of $tRNA^{Phe(GAA)}{}_{29,50}$ variant. The secondary structure of $tRNA^{Phe}$ is shown, with highlighting of base pairs 29:41 and 50:64 that were flipped, and bold outline of residues N_{34-37} that were changed from GAAG to CGUA. (*B*) A CGU anticodon and t^6A_{37} are sufficient for binding of $tRNA^{Phe}$ to ORF240. Strains with integrated *tF (GAA)*$_{29,50}$ variants as indicated were analyzed by the ORF240 tRNA pull-down assay as described in Figure 3C. (*C*) Summary of pull-down results in *B*. (*D*) A CGU anticodon and t^6A_{37} are sufficient for m^3C modification of $tRNA^{Phe}$. Bulk RNA from strains without (lane *1*) or with a $tRNA^{Phe(GAA)}{}_{29,50}$ $[CGUA]_{34-37}$ variant (lane *2*) was analyzed by primer extension for m^3C, using a primer complementary to residues 52–36 of the variant. Lane *3*, primer alone.

indicate that a CGU anticodon and A_{37} (presumably modified to t^6A) are sufficient for m^3C modification of $tRNA^{Phe(GAA)}$ and establish that this sequence is an identity element for the modification.

t^6A_{37} and i^6A_{37} are important for m^3C modification of $tRNA^{Ser}$ species

Unlike the three $tRNA^{Thr}$ Trm140 substrates, which all share the anticodon residues G_{35} and U_{36}, the three $tRNA^{Ser}$ substrates $tRNA^{Ser(CGA)}$, $tRNA^{Ser(UGA)}$, and $tRNA^{Ser(GCU)}$ do not share any anticodon residues with one another, and therefore the major Trm140 specificity determinant for $tRNA^{Ser}$ must be elsewhere. Furthermore, although t^6A_{37} is important for Trm140 modification of $tRNA^{Thr}$, only $tRNA^{Ser(GCU)}$ has t^6A, whereas $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ have i^6A_{37}.

Consistent with the importance of t^6A for m^3C modification of $tRNA^{Thr}$ species, we found that the m^3C modification level of $tRNA^{Ser(GCU)}$ was substantially lower in the *sua5*Δ strain compared to the WT strain (Fig. 5A). We also found that m^3C was substantially reduced in



	$MOD5^+$	mod5Δ
tF(GAA)	0.4	0.6
tS(GCU)	46	54
tS(CGA)	50	1
tS(UGA)	6	1

FIGURE 5. t^6A_{37} and i^6A_{37} are important for m^3C modification of $tRNA^{Ser}$ species. (*A*) t^6A_{37} is important for m^3C modification of $tRNA^{Ser(GCU)}$. Bulk RNA from $SUA5^+$ and *sua5*Δ cells was analyzed by primer extension with a primer annealing to residue e7–35 of $tRNA^{Ser(GCU)}$. (*B*) i^6A_{37} is important for m^3C modification of $tRNA^{Ser(UGA)}$ and $tRNA^{Ser(CGA)}$. Bulk RNA from $MOD5^+$ and *mod5*Δ cells was analyzed by primer extension with a primer annealing to residue e8–36 of $tRNA^{Ser(CGA)}$. (*C*) i^6A_{37} is important for $tRNA^{Ser(CGA)}$ binding to ORF240. *ORF240-PT* was expressed in $MOD5^+$ and *mod5*Δ strains, and ORF240 tRNA binding was analyzed by the IgG Sepharose pull-down assay as described in Figure 3C. (*D*) Schematic of $tRNA^{Ser}$ with highlighting of the V-loop containing residues N_{e1-e9} (black) and five other nucleotides (gray).

tRNA$^{Ser(CGA)/(UGA)}$ in a *mod5*Δ strain, which lacks the i^{6}A$_{37}$ modification (Fig. 5B; Dihanich et al. 1987), as also found for the m^{3}C modification of tRNA^{Ser} substrates in *S. pombe* (Arimbasseri et al. 2016). Thus, it appears that both t^{6}A and i^{6}A contribute to efficient m^{3}C modification.

We further showed that i^{6}A$_{37}$ was important for tRNA$^{Ser(CGA)}$ binding to ORF240 (Fig. 5C). While ORF240 bound 50% of the tRNA$^{Ser(CGA)}$ in a *MOD5*$^+$ strain, it only bound 1% of this tRNA in a *mod5*Δ strain. Similarly, although tRNA$^{Ser(UGA)}$ bound more weakly to ORF240 in a *MOD5*$^+$ strain (6%), this binding was undetectable in a *mod5*Δ strain. As expected, binding of tRNA$^{Ser(GCU)}$ was not affected in the *mod5*Δ strain, because this tRNA is not a Mod5 substrate. Because i^{6}A$_{37}$ is crucial for ORF240 binding and for m^{3}C modification of tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$, we infer that ORF240 binding is important for m^{3}C formation.

Although i^{6}A and t^{6}A are important for recognition of tRNA^{Ser} substrates by Trm140, they could not be the sole determinants for m^{3}C modification, since there are nine other *S. cerevisiae* tRNAs with both C$_{32}$ and t^{6}A$_{37}$ that lack the m^{3}C modification, and one other tRNA with C$_{32}$ and i^{6}A that lacks m^{3}C.

Ses1 copurifies with Trm140

One unique feature of tRNA^{Ser} species that could in principle be important for m^{3}C modification is the distinctive long V-loop. In yeast, the tRNA^{Ser} V-loops are all 14 nt long (Fig. 5D), whereas the only other tRNAs with a long V-loop are members of the tRNA^{Leu} family, with V-loops of 13 or 15 nt. Trm140 could recognize the distinctive tRNA^{Ser} V-loop, or Trm140 might cooperate with other proteins such as seryl-tRNA synthetase, Ses1, to recognize the V-loop. Ses1 is known to recognize a V-loop of the appropriate length for its charging activity (Himeno et al. 1997).

To define proteins that interact with Trm140, we purified ORF240 under identical conditions to those we used for copurification of tRNAs, and compared the copurifying polypeptides with those of the negative control glutaminyl-tRNA synthetase, encoded by *GLN4*. Coomassie staining revealed three polypeptide bands that copurified with ORF240, one of which, labeled A, appeared to be shared by Gln4 (Fig. 6A). Mass spectrometry analysis showed that the major proteins in band A from Ses1 and Gln4 were the same (heat shock protein Ssa1, Ssa2, and Ssc1), and that band C corresponded to ORF240 breakdown polypeptides. However, the major polypeptides in band B were Ses1 and YNL040W, with convincing Mascot scores of 1737 and 660, respectively, and these proteins were not found at all in the corresponding Gln4 purification. Examination of a *ynl040w*Δ strain showed no alteration of m^{3}C levels of tRNA$^{Thr(IGU)}$ or tRNA$^{Ser(UGA)/(CGA)}$. We therefore focused on Ses1.

We found that Trm140 interacted with Ses1 in wild-type cells, because affinity purification of Trm140 with a chromosomal C-terminal MORF tag (Trm140-cMORF) resulted in

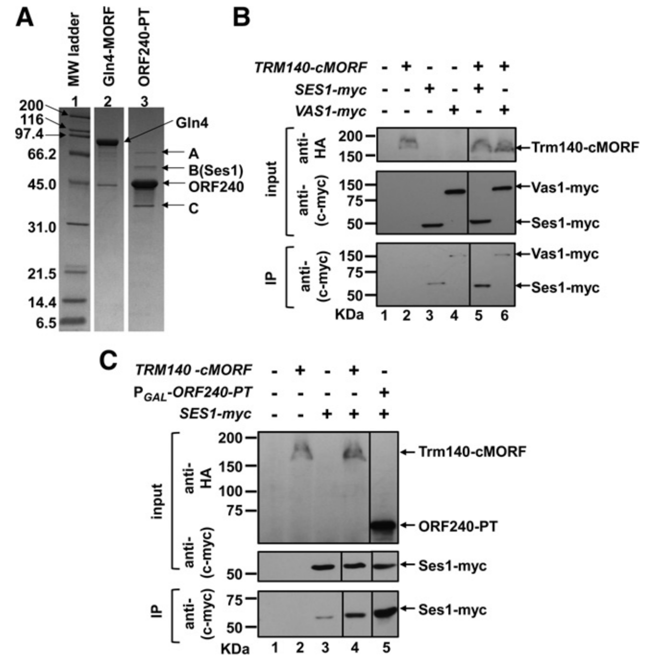


FIGURE 6. Trm140 interacts with Ses1. (*A*) SDS-PAGE analysis reveals three polypeptide bands that copurify with Trm140. Strains containing either P$_{GAL}$-*ORF240-PT* or P$_{GAL}$-*GLN4-MORF* were grown as described in Materials and Methods, ORF240 and Gln4 were affinity purified under conditions identical to those used for pull-down assays, and proteins were analyzed by SDS-PAGE and Coomassie staining. Three distinct copurifying bands that were found in the ORF240 preparation (lane *3*) are labeled A–C. (*B*) Chromosomally expressed Ses1-Myc and Trm140-cMORF interact. Trm140 was affinity purified from crude extracts of the indicated strains, using IgG Sepharose and 3C protease cleavage. Crude extract (input) was subjected to immunoblot analysis with anti-HA and anti-(c-myc) antibody, and IgG-purified protein (immunoprecipitate, IP) was analyzed by immunoblot with anti-(c-myc) antibody. (*C*) Overproduction of ORF240-PT results in increased copurification of Ses1. Trm140 was purified from a chromosomally tagged Trm140-cMORF strain, ORF240 was purified from a strain overexpressing ORF240-PT, and the amount of copurifying Ses1 was compared by immunoblot, as described in *B*.

copurification of chromosomally tagged Ses1-myc (Fig. 6B, cf. lanes 3 and 5), but no obvious copurification of the control Vas1-Myc (valyl-tRNA synthetase) (Fig. 6B, lanes 4,6). Moreover, overproduction of ORF240-PT under P$_{GAL}$ control resulted in copurification of substantially increased amounts of Ses1-myc (Fig. 6C, lanes 4,5). This interaction of Trm140 with Ses1 suggested the possibility that Ses1 might have a role in the m^{3}C modification reaction.

Ses1 stimulates m^{3}C formation of tRNA^{Ser} species

To determine if Ses1 had a role in m^{3}C modification, we examined the methyltransferase activity of Trm140 in vitro. We expressed and purified His$_6$-ORF240 from *Escherichia coli* and assayed its activity with tRNA purified from a *trm140*Δ strain. After incubation of ORF240 with *S*-adenosylmethionine (SAM) and tRNA, we analyzed m^{3}C modification by primer extension in the presence of ddTTP, and calculated

TABLE 1. Summary of the efficiency of ORF240 m^{3}C methyltransferase activity on tRNA^{Thr} and tRNA^{Ser} substrates

tRNA[a]	ORF240$_{1/2}$ (μM)	ORF240$_{1/2}$ (μM) with 1.5 μM Ses1
tRNA$^{Thr(IGU)}$	0.02	0.02
tRNA$^{Thr(UGU)}$	0.02	–
tRNA$^{Thr(CGU)}$	0.02	–
tRNA$^{Ser(GCU)}$	0.05	0.02
tRNA$^{Ser(CGA)}$	0.3	0.02
tRNA$^{Ser(UGA)}$	~15[b]	0.3
tRNA$^{Tyr(GUA)}$	>30[b]	>30[b]

[a]0.35 pmol assayed in 20 μL.
[b]Estimated from activity at 2.5 μM ORF240.

the efficiency of m^{3}C modification based on the intensities of the primer extension blocks at U_{33}, due to m$^3C_{32}$, and at A_{31}, due to read-through and termination by ddTTP incorporation. Using this assay, we found that the three tRNA^{Thr} species were equally efficiently modified by ORF240 (Table 1; Fig. 7A,B), in each case requiring ~0.02 μM ORF240 for one-half-maximal modification (ORF240$_{1/2}$). In contrast, ORF240 was very inefficient at m^{3}C modification of tRNA$^{Ser(UGA)}$ and tRNA$^{Ser(CGA)}$, with ORF240$_{1/2}$ values of ~15 μM and ~0.3 μM, respectively, while the ORF240$_{1/2}$ value was ~0.05 μM for tRNA$^{Ser(GCU)}$. As anticipated, no m^{3}C was detected upon ORF240 assay of tRNA$^{Tyr(GUA)}$, which normally bears an unmodified C_{32} (Table 1).

Consistent with an important biological role for the interaction of Trm140 with Ses1, we found that purified Ses1 stimulated the Trm140 m^{3}C modification activity on tRNA^{Ser} species in vitro. Using Ses1 purified from a *trm140*Δ strain, to avoid Trm140 contamination, we found that 1.5 μM Ses1 reduced the ORF240$_{1/2}$ of tRNA$^{Ser(CGA)}$ from ~0.3 μM to ~0.02 μM, of tRNA$^{Ser(UGA)}$ from ~15 μM to ~0.3 μM, and of tRNA$^{Ser(GCU)}$ from 0.05 μM to 0.02 μM, but had little or no effect on tRNA$^{Thr(IGU)}$ or tRNA$^{Tyr(GUA)}$ (Table 1; Fig. 8A,B).

Consistent with these in vitro results, we observed Ses1 stimulation of m^{3}C modification in vivo. To address this question, we assayed m^{3}C modification after overproduction of Ses1-MORF in a *mod5*Δ strain, in which m$^3C_{32}$ modification of tRNA$^{Ser(CGA)/(UGA)}$ was reduced due to lack of i^{6}A (Fig. 5B). We found that overproduction of Ses1 resulted in a substantial increase in m^{3}C levels of tRNA$^{Ser(CGA)/(UGA)}$ relative to that in the vector control, from 2% to 24% (Fig. 9A). Overproduction of ORF240 also improved the m^{3}C modification, consistent with the fact that i^{6}A was important for ORF240 binding. As expected, the m^{3}C levels of tRNA$^{Thr(IGU)}$, which has t^{6}A and not i^{6}A, were not affected in the *mod5*Δ strain or by overproduction of either Ses1 or ORF240.

To determine whether the important region for m^{3}C modification of tRNA^{Ser} species was the V-loop, we tested the modification of a chimeric tRNA^{Leu} species with a V-loop based on tRNA^{Ser}. We constructed a variant with the body sequence of tRNA$^{Leu(UAA)}$, the anticodon UGA, and A_{37}, called variant M1 (Fig. 9B). This M1 variant was not a substrate for m^{3}C modification (Fig. 9C, lane 2). However, the variant M2, which differs from M1 due to replacement of the V-loop with that of tRNA$^{Ser(GCU)}$, was significantly modified to m$^3C_{32}$ (Fig. 9C, lane 5). This result shows that the tRNA^{Ser} V-loop is sufficient to confer m$^3C_{32}$ modification of the M1 variant in the context of this tRNA species.

DISCUSSION

We have provided evidence here that *S. cerevisiae* Trm140 has two strikingly different recognition modes for tRNA m^{3}C modification. Trm140 explicitly recognizes the XGU anticodon and t$^6A_{37}$ of tRNA^{Thr} species as an identity element for m^{3}C modification in vivo. Thus, substitution of either G_{35} or U_{36} of tRNA$^{Thr(CGU)}$ with any other residue resulted in the complete absence of m^{3}C modification, and removal of t^{6}A substantially reduced m^{3}C modification, whereas introduction of a CGU anticodon and A_{37} to tRNA^{Phe} resulted in efficient m^{3}C modification in vivo. Consistent with these

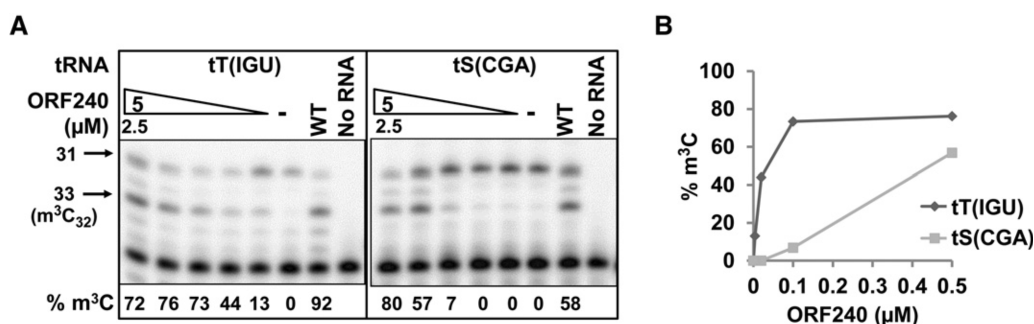


FIGURE 7. ORF240 catalyzes efficient m^{3}C modification of tRNA$^{Thr(IGU)}$ but inefficient modification of tRNA$^{Ser(CGA)}$. (*A*) Titration of ORF240 m^{3}C methyltransferase activity on tRNA$^{Thr(IGU)}$ and tRNA$^{Ser(CGA)}$. Of note, 0.35 pmol of tRNA$^{Thr(IGU)}$ or tRNA$^{Ser(CGA)}$ purified from a *trm140*Δ strain was incubated with serial fivefold dilutions of ORF240 (2.5, 0.5, 0.1, 0.02, 0.004 μM) purified from *E. coli* or with buffer (−) in the presence of SAM at 30°C for 1 h, and tRNA was analyzed for m^{3}C by primer extension in the presence of ddTTP, as described in Materials and Methods. (*B*) Plot of m^{3}C modification as a function of the concentration of ORF240 in *A*.

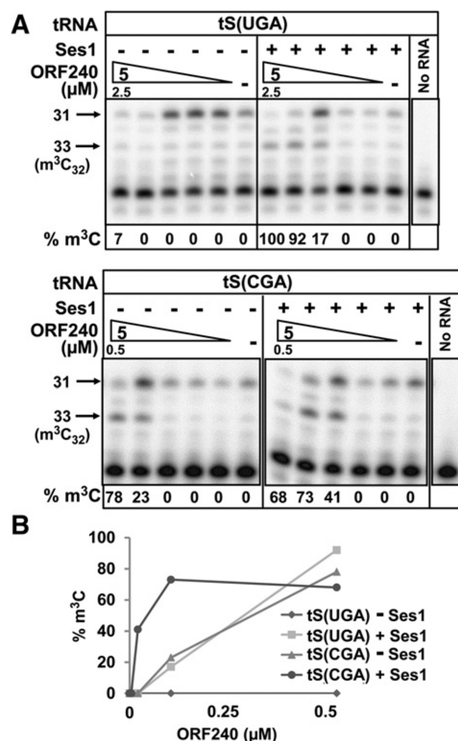


FIGURE 8. Ses1 stimulates ORF240 m^{3}C modification of tRNA$^{Ser(UGA)}$ and tRNA$^{Ser(CGA)}$. (*A*) Effect of Ses1 on the titration of ORF240 m^{3}C methyltransferase activity on tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$. Of note, 0.35 pmol of tRNA$^{Ser(UGA)}$ and tRNA$^{Ser(CGA)}$ purified from a *trm140Δ* strain was assayed for m^{3}C formation by ORF240 in the presence or absence of 1.5 µM Ses1 purified from a *trm140Δ* strain, as described in Figure 7A. (*B*) Plot of m^{3}C modification as a function of the concentration of ORF240 in *A*.

results, ORF240 expressed and purified from *E. coli* efficiently catalyzed modification of all three tRNA^{Thr} species, but not tRNA^{Tyr}.

In contrast, the CGA, UGA, and GCU anticodons of the three tRNA^{Ser} species that are modified with m^{3}C$_{32}$ do not have a common feature, and Trm140 recognition of these species is instead propelled by interaction with Ses1 and the tRNA^{Ser} V-loop, as well as by i^{6}A$_{37}$ of tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$, and t^{6}A$_{37}$ of tRNA$^{Ser(GCU)}$. Thus, purified Ses1 stimulated the efficiency of ORF240 m^{3}C modification of tRNA$^{Ser(CGA)}$, decreasing ORF240$_{1/2}$ by 20-fold, to a value comparable to that of tRNA^{Thr} substrates. Similarly, Ses1 stimulated the efficiency of ORF240 m^{3}C modification of tRNA$^{Ser(UGA)}$ by ∼50-fold. Furthermore, this Ses1 stimulation of m^{3}C modification was also observed in vivo, since Ses1 overproduction significantly increased m^{3}C modification of tRNA$^{Ser(CGA)/(UGA)}$ in a strain lacking i^{6}A.

Our results suggest that m^{3}C modification activity is driven in part by binding. We showed that all six known Trm140 substrate tRNAs copurified with Trm140 or ORF240, whereas each of six tested nonsubstrate tRNAs did not copurify. We also showed that ORF240 binding of tRNA^{Thr} anticodon loop variants tracked perfectly with m^{3}C modification, since all six possible variants with mutations in G$_{35}$ or U$_{36}$ lacked modification and also did not bind, whereas each of the three variants with mutations in C$_{34}$ were modified and bound efficiently, although not as efficiently as with C$_{34}$. Furthermore, ORF240 bound tRNA^{Phe} efficiently only when its anticodon was altered to CGU-A$_{37}$, correlated with m^{3}C modification, and i^{6}A$_{37}$ was important for binding of tRNA$^{Ser(CGA)}$ to ORF240 as well as for m^{3}C modification. These results suggest a modular binding mechanism to explain substrate specificity, in which contributions are made by G$_{35}$, U$_{36}$, i^{6}A$_{37}$, or t^{6}A$_{37}$, and the V-loop, as well as C$_{34}$. C$_{34}$ is favored over other N$_{34}$ residues based on preferential binding of tRNA^{Thr} C$_{34}$ variants and preferential binding of tRNA$^{Ser(CGA)}$ compared to tRNA$^{Ser(UGA)}$ since these two tRNAs are identical other than U$_{34}$ and the N$_{28}$–N$_{42}$ pair. This preferential binding of tRNA$^{Ser(CGA)}$ relative to that of tRNA$^{Ser(UGA)}$ is also consistent with the preferential ORF240 modification activity of tRNA$^{Ser(CGA)}$ compared to tRNA$^{Ser(UGA)}$.

Proteins like Trm140 that bind nucleic acids by two very different modes are rare. Notable examples include TFIIIA, which recognizes 5S promoter DNA and 5S RNA using distinctive zinc fingers (Nolte et al. 1998; Lu et al. 2003; Hall 2005), and phage λ integrase protein, which recognizes two different DNA sequences with different domains (Moitoso de Vargas et al. 1988). It is intriguing that bovine mitochondrial Ses1 recognizes tRNA^{Ser} substrates with and without a D-stem–loop by different mechanisms, but involving the same region of the T-loop (Shimada et al. 2001); the different modes of Trm140 recognition of tRNA^{Thr} compared to tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$ also appear to involve common elements including C$_{34}$ and i^{6}A$_{37}$ or t^{6}A$_{37}$, as well as the specific involvement of Ses1 for tRNA^{Ser} and G$_{35}$–U$_{36}$ for tRNA^{Thr}.

The observation that i^{6}A and t^{6}A are important for m^{3}C modification is a clear demonstration of ordered modification circuitry in the anticodon loops of tRNA^{Thr} and tRNA^{Ser}, in which either A$_{37}$ modification drives m^{3}C formation. Maraia and coworkers recently observed that i^{6}A modification was important for m^{3}C$_{32}$ modification in tRNA^{Ser} species of *S. pombe* and speculated that t^{6}A modification would be important for m^{3}C$_{32}$ modification of tRNA^{Thr} and tRNA^{Ser} substrates with U$_{36}$ (Arimbasseri et al. 2016). Because we observed that i^{6}A is important for both tRNA binding and m^{3}C modification, this suggests that tRNA binding efficiency is at least partially responsible for the observed ordering of these two modifications. It remains to be determined how both t^{6}A and i^{6}A drive the same m^{3}C modification reaction, and in particular if t^{6}A$_{37}$ stimulates m^{3}C modification due to increased Trm140 tRNA binding. Our preliminary attempts to express ORF240 in an *sua5* mutant strain have been unsuccessful.

The ordered modification circuitry described above for m^{3}C modification of tRNA^{Ser} and tRNA^{Thr} is reminiscent of the conserved ordered modification circuitry found in

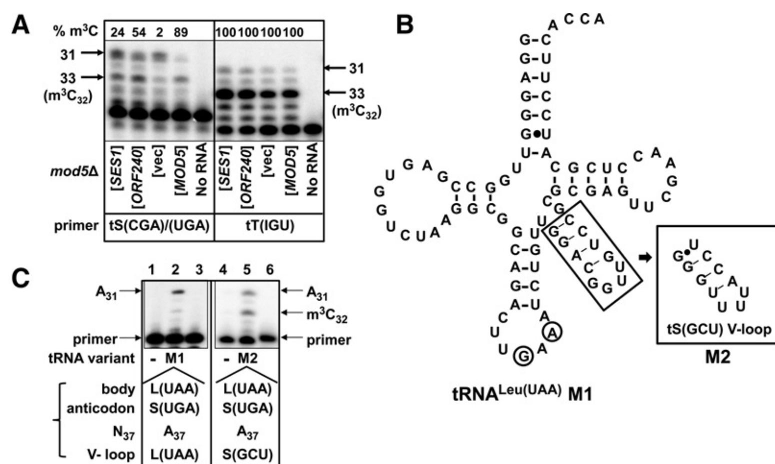


FIGURE 9. Ses1 stimulates m^3C modification of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ in vivo and the unique $tRNA^{Ser}$ V-loop that Ses1 recognizes is important for m^3C modification. (*A*) Ses1 stimulates m^3C formation in vivo. Bulk RNA from *mod5*Δ cells expressing *SES1*, *ORF240*, vector, or *MOD5* as indicated was analyzed for m^3C of $tRNA^{Ser(CGA)/(UGA)}$ or $tRNA^{Thr(IGU)}$ by primer extension in the presence of ddTTP, as described in Materials and Methods. (*B*) Schematic of $tRNA^{Leu(UAA)}$ M1 and M2 variants. The secondary structure of $tRNA^{Leu(UAA)}$ M1 is shown, with anticodon loop mutations as indicated (circled). Nucleotides in the V-loop that were changed to the V-loop of $tRNA^{Ser(GCU)}$ to make the $tRNA^{Leu(UAA)}$ M2 variant are boxed. (*C*) The variable loop of $tRNA^{Ser(GCU)}$ is sufficient to confer m^3C modification in vivo of $tRNA^{Leu(UAA)}$ with G_{35} and A_{37} mutations. Variants described in *B* were integrated into a *trm732*Δ strain, to eliminate the possibility of 2′-*O*-methylation at C_{32} (Guy et al. 2012), and bulk RNA from strains containing either variants M1 or M2 was analyzed for m^3C by primer extension, using ddTTP and primers extending from residue e8 to 36 of M1 and from residue 47 to 36 of M2.

the anticodon loop of $tRNA^{Phe}$. We previously showed that complete modification of the anticodon loop of *S. cerevisiae* $tRNA^{Phe}$ required 2′-*O*-methylation of C_{32} and G_{34} to drive efficient formation of wybutosine at m^1G_{37} (Guy et al. 2012), and that this circuitry for $tRNA^{Phe}$ anticodon loop modification was conserved in *S. pombe* and human lymphoblastoid cell lines (Guy and Phizicky 2015; Guy et al. 2015). The similar conservation of modification order for i^6A/t^6A and m^3C in the anticodon loops of $tRNA^{Thr}$ and $tRNA^{Ser}$ species in *S. cerevisiae* and *S. pombe* suggests that this ordered modification circuitry will be preserved in other eukaryotes, perhaps by conserved mechanisms, and suggests the existence of other circuits for anticodon loop modifications.

The stimulation by Ses1 of Trm140 m^3C modification activity that we observe in vitro and in vivo could occur in two ways. One possibility is that Trm140, Ses1, and tRNA form three binary interactions, which might stabilize weak enzyme–substrate binding. We have shown that Ses1-myc copurified with Trm140-cMORF, although it remains to be determined whether the interaction is tRNA mediated. Alternatively, it is possible that binding of $tRNA^{Ser}$ to Ses1 rearranges the local conformation of the anticodon loop to facilitate Trm140 recognition of C_{32}, although the cocrystal structures of both *Thermus thermophilus* SerRS-$tRNA^{Ser}$ and human SerRS-$tRNA^{Sec}$ lack any direct interaction between the synthetases and the anticodon loop (Biou et al. 1994; Wang et al. 2015). It is known that *S. cerevisiae* Ses1 recognizes the V-loop of $tRNA^{Ser}$ for its synthetase activity (Himeno et al. 1997), providing a probable explanation for the V-loop dependence of m^3C modification, but not casting light on its mechanism of m^3C stimulation.

The two modes of tRNA substrate recognition for *S. cerevisiae* Trm140 seems likely to be found widely in the Saccharomycotina and Pezizomycotina subdivisions of the phylum Ascomycota, and to a more limited extent in Basidiomycota, based on the occurrence of a single highly similar *TRM140* homolog in a large fraction of these organisms that we examined (*Candida albicans*, *Candida tropicalis*, *Yarrowia lipolytica*, *Saccharomyces castellii*, *Debaryomyces hansenii*, *Candida glabrata*, *Saccharomyces mikatae*, *Saccharomyces paradoxus*, *Saccharomyces bayanus*, *Kluyveromyces lactis*, *Ashbya gossypii*, *Saccharomyces kluyveri*, *Kluyveromyces waltii*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Neurospora crassa*, *Coccidioides posadasii*, *Coccidioides immitis*, *Coprinopsis cinerea*, *Phanerochaete chrysosporium*, *Uncinocarpus reesii*. *Chaetomium globosum*, *Fusarium verticillioides*, *Botrytis cinerea*). However, split substrate recognition by Trm140 and related proteins is also likely widely found since *S. pombe* was recently shown to have separate *trm140+* and *trm141+* (*METTL6*) homologs dedicated to $tRNA^{Thr}$ and to $tRNA^{Ser}$ substrates, respectively, and multiple homologs appear to be the rule rather than the exception in metazoans, plants, and other groups of fungi (Arimbasseri et al. 2016). Thus, a large number of eukaryotes, including vertebrates, have two or three *TRM140* family members, drawn from the phylogenetic clades of *TRM140*, *METTL6*, *METTL2*, and *METTL8* homologs, which might be used for modification of specific tRNA species with m^3C_{32} or with m^3C in the V-loop (Arimbasseri et al. 2016). In view of our findings, we speculate that $tRNA^{Ser}$ m^3C modification activity in other organisms might also be stimulated by SerRS. In support of this, we note that the human SerRS has the same V-loop recognition element as yeast Ses1 (Achsel and Gross 1993). Alternatively, it is possible that the different homologs have evolved separate tRNA recognition elements in *S. pombe* and other organisms. The recent finding that the METTL6 homolog TbMTase37 of *T. brucei* is important for ribosome stability and cytokinesis emphasizes the importance of this family of proteins, although the proximate cause is not yet known (Fleming et al. 2016).

The Ses1 requirement for efficient Trm140 m^3C modification of $tRNA^{Ser}$ species adds to a small subset of the two-subunit modification enzymes (Guy and Phizicky 2014) in which one interacting partner seemingly directs the enzyme to

different residues or substrates, including Trm7/Trm732 for Nm_{32} formation, Trm7/Trm734 for Nm_{34} formation (Guy et al. 2012; Guy and Phizicky 2015), and Kre33/Tan1 for ac^4C_{12} formation (Johansson and Bystrom 2004; Sharma et al. 2015). The finding of Ses1 as an interacting partner for Trm140 m^3C modification is also another example of the remarkable range of different noncanonical functions of tRNA synthetases (Wakasugi and Schimmel 1999; Guo et al. 2010; Smirnova et al. 2012; Yao and Fox 2013), at least some of which exert these roles through RNA interactions (Herbert et al. 1988; Sampath et al. 2004; Sarkar et al. 2012). It remains to be determined precisely how Ses1 recognizes Trm140 to stimulate m^3C modification activity of $tRNA^{Ser}$, and the connection between $tRNA^{Ser}$ charging and modification.

MATERIALS AND METHODS

Yeast strains

Strains (listed in Table 2) used for genetic tests and/or analysis of tRNA were derivatives of strain BY4741 or BY4742, strains used for immunoblotting analysis were derivatives of BCY123, and strains used for pull-down experiments and protein purification were derivatives of YLH126.

TRM140 was deleted by PCR amplification of the *trm140Δ::ble*^{R} cassette from ySD179 using primers containing sequences 5′ and 3′ of *TRM140* (TRM140 − 409 and TRM140 + 307), followed by transformation, selection on YPD media containing 8 mg/L Bleocin and verification by PCR using appropriate primers. *MOD5* was deleted in a similar fashion.

The *tT(CGU)*Δ[*CEN URA3 tT(CGU)*] strain was constructed by transformation of BY4741 with the [*CEN URA3 tT(CGU)*] plasmid containing the *tT(CGU)* gene with its own flanking sequence, followed by PCR amplification of the *ble*^{R} marker and linear transformation to delete the *tT(CGU)* gene.

Strains with the chromosomal cMORF tag (His_6-HA-3C site-ZZ domain of protein A) were generated by PCR amplification of a gene-specific product from a cMORF::*URA3* cassette of pAVA0258, followed by linear transformation and selection (Gelperin et al. 2005; Guy et al. 2012). C-terminal myc-tagged strains were generated in a similar fashion from the pYM46 1myc-7His::*Kan*^{R} cassette (Janke et al. 2004).

Plasmids

Plasmids used in this study are listed in Table 3. Plasmids expressing tRNAs were constructed by ligation-independent cloning (LIC) of a tRNA with its own flanking sequence into the [2μ *LEU2*] LIC vector pAVA577. LIC was also used to build the [*CEN URA3 MOD5*] plasmid. Integration vectors for tRNA variants were made by insertion of a BglII, XhoI fragment encoding the tRNA variant into plasmid pAB230-1, as previously described (Guy et al. 2014). ORF240 was cloned by LIC into a [2μ *URA3* $P_{GAL1,10}$] expression vector, in which ORF240 is expressed under P_{GAL1} control with a C-terminal PT tag (as ORF240-3C site-HA epitope-His6-ZZ domain of protein A) essentially as previously described (Quartley et al. 2009).

Expression and affinity purification of Ses1-MORF from yeast

To purify Ses1 from yeast without interacting Trm140, strain YLH974-1 (*trm140Δ*) was transformed with a [2μ *URA3* P_{GAL}-*SES1-MORF*] plasmid encoding a *SES1-MORF* fusion protein (Ses1-His_6-HA-3C site-ZZ domain of protein A), and the resulting strain was grown in selective media containing raffinose and induced for 6 h by addition of one-half volume of 3× YP media containing 6% galactose and 6% raffinose. Then Ses1-MORF was affinity purified using IgG Sepharose chromatography, followed by Ses1 elution with GST-3C protease, removal of the protease with glutathione Sepharose resin, and dialysis into buffer containing 50% glycerol, 20 mM Tris–Cl pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT, as previously described (Quartley et al. 2009).

TABLE 2. Strains used in this study

Strain	Genotype	Source
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY4742	*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems
BCY123	*MAT*a *pep4Δ::HIS3 prbΔ::LEU2 bar1Δ::HISG lys2Δ::GAL1/10-GALΔ4 can1 ade2 ura3 leu2-3, 112*	Macbeth et al. (2005)
YLH562-1	BY4741 *tT(CGU)Δ::ble*^{R} [*CEN URA tT(CGU)K*]	This study
T14	*cyc1-1019 cyc7-67 ura3-52 leu2-3, 112 cyh2*	Na et al. (1992)
YJN64	*cyc1-1019 cyc7-67 ura3-52 leu2-3, 112 cyh2, sua5::LEU2*	Na et al. (1992)
YLH126	*leu2Δ trp1Δ ura3Δ prb1-1122 pep4-1 his3Δ::*P_{GAL10}-*GAL4*	Erin O'Shea
ySD844	BY4741 *mod5Δ::kan*^{R}	This study
ySD356	BCY123 *TRM140-cMORF-URA3*	This study
YLH1302-1	BCY123 *SES1-1Myc-7His::kan*^{R}	This study
YLH1305-1	BCY123 *VAS1-1Myc-7His::kan*^{R}	This study
YLH1307-1	BCY123 *TRM140-cMORF::URA3 SES1-1Myc-7His::kan*^{R}	This study
YLH1310-1	BCY123 *TRM140-cMORF::URA3 VAS1-1Myc-7His::kan*^{R}	This study
yMG814-1	BY4741 *trm732Δ::ble*^{R}	Guy et al. (2012)
ySD179	BY4742 *trm140::ble*^{R}	D'Silva et al. (2011)
YLH974-1	YLH126 *trm140::ble*^{R}	This study

TABLE 3. Plasmids used in this study

Plasmid	Parent	Description	Source
BG2663		2μ *URA3* P_{GAL}	Quartley et al. (2009)
pSD142	BG2663	2μ *URA3* P_{GAL}-*TRM140-PT*	D'Silva et al. (2011)
pSD252	BG2663	2μ *URA3* P_{GAL}-*ORF240-PT*	This study
pAB230-1	pJW132	*ade2::5′tH(GUG)G2::Fluc*	Guy et al. (2014)
pELH248-1	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU)*	This study
pELH249-1	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U*	This study
pELH307-1	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U C32U*	This study
pELH308-1	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U C34A*	This study
pELH309-4	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U C34U*	This study
pELH310-4	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U C34G*	This study
pELH311-1	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U G35A*	This study
pELH312-2	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U G35U*	This study
pELH313-4	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U G35C*	This study
pELH314-2	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U U36A*	This study
pELH315-2	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U U36C*	This study
pELH316-5	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U U36G*	This study
pELH317-1	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U A38U*	This study
pELH318-1	pAB230-1	*ade2::5′tH(GUG)G2::tT(CGU) C28G–G42C U50A–A64U A38C*	This study
pELH328-2	pAB230-1	*ade2::5′tH(GUG)G2::tF(GAA) A29U–U41A U50A–A64U*	This study
pELH329-2	pAB230-1	*ade2::5′tH(GUG)G2::tF(GAA) A29U–U41A U50A–A64U CGUA[34–37]*	This study
pJE832A		His_6-*GLN4-MORF*	Grant et al. (2012)
pYM46		*1Myc-7His::kan*^{R}	Janke et al. (2004)
pAVA577		2μ *LEU2* LIC vector	Alexandrov et al. (2006)
pJW038	pAVA577	2μ *LEU2 tS(CGA)*	Whipple et al. (2011)
pEKD9094	pAVA577	2μ *LEU2 tT(CGU)*	Han et al. (2015)
pJW043	pAVA577	2μ *LEU2 tS(UGA)*	Han et al. (2015)
pELH106-1	pAVA577	2μ *LEU2 tT(UGU)*	Han et al. (2015)
pELH419-1	pAVA577	2μ *LEU2 tS(GCU)F*	This study
pAVA579		*CEN URA3* LIC vector	Quartley et al. (2009)
pELN010	pAVA579	*CEN URA3 MOD5*	This study
pAVA0258		His_6-HA-3C-protein A::*URA3* cassette	Gelperin et al. (2005)
pJE1256A		P_{GAL}-*SES1-MORF*	Gelperin et al. (2005)
pAVA421		pT7-His_6-3C site-ORF LIC vector	Quartley et al. (2009)
pSD248	pAVA421	His_6-3C-ATG-*ORF240*	D'Silva et al. (2011)

Growth and affinity purification of His6-ORF240 from *E. coli*

The *E. coli* expression plasmid pSD248, which expresses the entire C-terminal domain of *TRM140* (residues 277–628, called ORF240) as a His6-3C-ORF240 fusion, was transformed into pLys(S)BL21 (DE3) cells. Transformants were grown, induced, and harvested, and then tagged protein was purified using immobilized metal ion affinity chromatography (IMAC), followed by imidazole elution, cleavage of the affinity tag with protease 3C, removal of contaminants by passage through the same IMAC resin, and dialysis into buffer containing 50% glycerol, 20 mM Tris–Cl, pH 7.5, 200 mM NaCl, and 1 mM DTT, essentially as previously described (D'Silva et al. 2011).

Extraction of bulk RNA from yeast and purification of tRNA

Strains were grown to an OD_{600} 1–2, and bulk RNA was extracted from ∼300 OD pellets (for tRNA purification) or from ∼3 OD pellets (for primer extension analysis) using hot phenol. tRNA was purified from ∼1.25 mg bulk RNA using 5′-biotinylated oligonucleotides (Integrated DNA Technologies), as previously described (Jackman et al. 2003).

Primer extension assays

Primers were 5′ end labeled and purified as previously described (D'Silva et al. 2011). In a 5 µL annealing reaction, 0.25–1 pmol of labeled primers were annealed to 0.4–3 µg of bulk RNA or ∼3 ng of purified tRNA by incubation for 3 min at 95°C followed by slow cooling and incubation for 30 min at 50°C–55°C. The annealing product was then extended using 64 U Superscript III (Invitrogen) in a 10 µL reaction containing 1× First Strand buffer, 1 mM of each dNTP, and 10 mM $MgCl_2$ at 50°C–55°C for 1 h. For reactions containing ddTTP, dTTP was replaced by 2 mM ddTTP, and other dNTPs were reduced to 0.5 mM. Reactions were stopped by addition of 2× RNA loading dye containing 98% formamide, 10 mM EDTA, 1 mg/mL bromophenol blue, and 1 mg/mL xylene cyanol, resolved on a 7M urea–15% polyacrylamide gel, and the dried gel was imaged on a Typhoon phosphorimager and quantified as previously described (Jackman et al. 2003).

ORF240 and Trm140 pull-down assays of tRNA binding

Strains expressing P_{GAL}-*ORF240-PT* or P_{GAL}-*TRM140-PT* plasmid were grown in selective media containing raffinose to OD_{600} ∼0.75 and induced for 6 h with one-half volume 3× YP media

containing 6% galactose and 6% raffinose. Then tagged proteins were affinity purified from 320–360 OD pellets using IgG Sepharose, followed by one or two washes with 1 mL buffer and 3 min of mixing, and then overnight incubation with GST-3C protease to release bound protein and copurifying tRNAs. Then RNA was purified from each fraction, resolved by PAGE, and tRNAs were analyzed by hybridization, as previously described (Alexandrov et al. 2006).

Mass spectrometry

For mass spectrometry analysis of ORF240 binding proteins, ORF240-PT purification was done as for a pull-down assay, proteins were analyzed by SDS-PAGE, and copurifying polypeptides were analyzed by the Mass Spectrometry Resource Center of the University of Rochester Medical Center.

Immunoblotting analysis

Yeast strains with C-terminal chromosomal tags (cMORF or myc) were grown in YPD to OD_{600} 1–2.5 and crude extracts were made from 500–600 OD-mL pellets, followed by IgG Sepharose affinity purification and elution of bound protein with GST-3C protease. Then samples were subject to SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad), and probed with appropriate antibodies: mouse monoclonal anti-[c-myc] (Roche), followed by goat anti-mouse IgG-HRP (Bio-Rad); or rat anti-HA (Roche), followed by goat anti-rat IgG-HRP (Bio-Rad). Strains containing a P_{GAL}-*ORF240-PT* plasmid were grown as described in pull-down experiment.

Assay of m^3C methyltransferase activity in vitro

Reaction mixtures (20 µL) contained 60–67.5 mM NaCl, 50 mM Hepes, pH 7.5, 3 mM $MgCl_2$, 55–60 µg/mL BSA, 50 µM EDTA, 2.5–3.5 mM Tris–Cl pH 7.5, 2.5–5% glycerol, 0.5 mM SAM, 50 µg/mL Poly(A), 10 ng of purified and refolded tRNA from a *trm140*Δ strain, ORF240 purified from *E. coli*, and where indicated, Ses1 purified from a *trm140*Δ strain or compensating buffer. Reactions were incubated at 30°C for 1 h, stopped by phenol extraction, and then tRNA was precipitated with ethanol, resuspended in water, and subjected to primer extension analysis to analyze m^3C_{32}.

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