**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 (m3C) modification is ubiquitous in eukaryotic tRNA, widely found at C32 in the anticodon loop of tRNA\(^{\text{Thr}}\), tRNA\(^{\text{Ser}}\), and some tRNA\(^{\text{Arg}}\) species, as well as in the variable loop (V-loop) of certain tRNA\(^{\text{Ser}}\) species. In the yeast Saccharomyces cerevisiae, formation of m3C32 requires Trm140 for six tRNA substrates, including three tRNA\(^{\text{Thr}}\) species and three tRNA\(^{\text{Ser}}\) species, whereas in Schizosaccharomyces pombe, two Trm140 homologs are used, one for tRNA\(^{\text{Thr}}\) and one for tRNA\(^{\text{Ser}}\). 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 G35-U36-t6A37 of the anticodon loop of tRNA\(^{\text{Thr}}\) substrates, and this sequence is an identity element because it can be used to direct m3C modification of tRNA\(^{\text{Phe}}\). However, Trm140 recognition of tRNA\(^{\text{Ser}}\) substrates is different, since their anticodons do not share G35-U36 and do not have any nucleotides in common. Rather, specificity of Trm140 for tRNA\(^{\text{Ser}}\) is achieved by seryl-tRNA synthetase and the distinctive tRNA\(^{\text{Ser}}\) V-loop, as well as by t6A37 and i6A37. We provide evidence that all of these components are important in vivo and that seryl-tRNA synthetase greatly stimulates m3C modification of tRNA\(^{\text{Ser}}(\text{CGA})\) and tRNA\(^{\text{Ser}}(\text{UGA})\) 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 N32–N38 of the anticodon loop and the neighboring N31–N39 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 N34) or at N37 often contribute to accurate decoding and reading frame maintenance (Gerber and Keller 1999; Bjork et al. 2001, 2007; Urbanovicius 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 pus3Δ mutant, which lacks pseudouridine at U38 or U39, is temperature sensitive, primarily due to reduced tRNA\(^{\text{Gln(UUG)}}\) function (Han et al. 2015); and a yeast trm7Δ mutant, which lacks 2′-O-methylation at C32 (Cm) as well as Gm34, grows poorly due to reduced translation and reduced tRNA\(^{\text{Phe}}\) function (Pintard et al. 2002; Guy et al. 2012). N32 and N38 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 C32 in the anticodon loop of tRNAs and likely has an important role. m^3C is found at C32 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 C32, 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^3C32 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 C32. 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 trm140A 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^3C32 is underscored by the growth defect of S. cerevisiae trm140Δ trm1Δ 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^3G9 modification catalyzed by Trm10 for 13 of 24 species with G9 (Swinehart et al. 2013; Swinehart and Jackman 2015) and the Cm32 and Gm34 modification catalyzed by Trm7 on three tRNA species (Pintard et al. 2002), as well as the m^3C32 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 C32.

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^3C32 modification. For tRNA^Thr species, Trm140 reads the anticodon nucleotides and the t^A 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 t^A_37 or t^A_36.

RESULTS

An XGU anticodon and t^A_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: t^T(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)) with oligonucleotides spanning these residues (Fig. 1A). This variant was fully functional because an otherwise lethal t^T(CGU)Δ strain was completely rescued upon integration of t^T(CGU)) with

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**FIGURE 1.** The tRNA^Thr(CGU) scaffold used for analysis of tRNA^Thr variants is functional and efficiently modified to m^3C. (A) Schematic of tRNA^Thr(CGU). 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) is functional in vivo. t^T(CGU)Δ cells containing the integrated WT t^T(CGU) or t^T(CGU)) 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) is efficiently modified to m^3C32. Bulk RNA from cells containing the integrated WT t^T(CGU) or t^T(CGU)) 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) has m^3C32 based on the primer extension stop at U33, compared to the amount of read-through (RT).
no discernable growth defect in the range of 30°C to 37°C, compared to the corresponding strain bearing wild-type t\textsuperscript{T} (CGU) at the same locus (Fig. 1B). The tRNA\textsuperscript{Thr(CGU)}\textsubscript{28,50} variant was easily analyzed by primer extension independent of the wild-type tRNA\textsuperscript{Thr(CGU)} (Fig. 1C), and was efficiently modified to m\textsuperscript{3}C\textsubscript{32}, based on the prominent primer extension block at N\textsubscript{33}, compared to the relatively minor read-through signal of C\textsubscript{34} to other nucleotides resulted in retention of the major primer extension block at N\textsubscript{13}, similar to the amount of m\textsuperscript{3}C modification observed for wild-type tRNA\textsuperscript{Thr(CGU)} (Fig. 2).

Since the tRNA\textsuperscript{Thr} species bearing m\textsuperscript{3}C\textsubscript{32} all have similar anticodons (CGU, UGU, and IGU), we made and analyzed a set of yeast strains, each with an integrated tRNA\textsuperscript{Thr(CGU)}\textsubscript{28,50} variant bearing a single mutation in the anticodon loop (Fig. 2A,B). Primer extension analysis showed that alteration of C\textsubscript{32} to other nucleotides resulted in retention of the major primer extension block at N\textsubscript{13}, indicative of m\textsuperscript{3}C\textsubscript{32}. In contrast, substitution of residue G\textsubscript{35} or U\textsubscript{36} with each of the other nucleotides resulted in almost complete loss of m\textsuperscript{3}C\textsubscript{32}. These results suggest that the conserved G\textsubscript{35} and U\textsubscript{36} residues in the anticodons of tRNA\textsuperscript{Thr} species are required for m\textsuperscript{3}C formation. As expected, substitution of C\textsubscript{32} to U\textsubscript{32} resulted in no primer extension stop.

Since U\textsubscript{36} was important for m\textsuperscript{3}C modification activity, and U\textsubscript{36} is always followed by t\textsuperscript{6}A\textsubscript{37} (Machnicka et al. 2013) or, as frequently occurs in yeast and some other organisms, a cyclized derivative of t\textsuperscript{6}A called ct\textsuperscript{6}A (Miyauchi et al. 2013), it was possible that t\textsuperscript{6}A\textsubscript{37} or ct\textsuperscript{6}A\textsubscript{37} (collectively referred to as t\textsuperscript{6}A for simplicity) also had a role in m\textsuperscript{3}C formation. To examine the contribution of t\textsuperscript{6}A to m\textsuperscript{3}C modification, we analyzed m\textsuperscript{3}C levels in wild-type tRNA\textsuperscript{Thr} species from an sua5A strain, which lacks t\textsuperscript{6}A\textsubscript{37} (El Yacoubi et al. 2009). The m\textsuperscript{3}C levels of each of the three tRNA\textsuperscript{Thr} species from the sua5A strain were substantially reduced compared to those from the corresponding wild-type strain (Fig. 2C). This result suggested that t\textsuperscript{6}A\textsubscript{37} was important, but not absolutely required, for m\textsuperscript{3}C modification of the three tRNA\textsuperscript{Thr} species in vivo, along with the required G\textsubscript{35}–U\textsubscript{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\textsuperscript{Thr}(CGU), tRNA\textsuperscript{Thr}(UGU), and tRNA\textsuperscript{Thr}(CGU), relative to that in a vector control, whereas several nonsubstrate tRNAs examined did not copurify with Trm140, including tRNA\textsuperscript{Phe}(GAA), tRNA\textsuperscript{Phe}(GAA), and tRNA\textsuperscript{Phe}(GAA). 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\textsuperscript{Thr}(CGU), tRNA\textsuperscript{Thr}(CGU), and tRNA\textsuperscript{Thr}(CGU) (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\textsuperscript{Thr}(CGU) bound very efficiently and reproducibly, whereas four nonsubstrate tRNAs did not, including tRNA\textsuperscript{Phe}(GAA), tRNA\textsuperscript{Cys}(CGU), tRNA\textsuperscript{Met}(CAU), and tRNA\textsuperscript{Lys}(CGU) (Fig. 3C). For these and subsequent experiments we calculated binding
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 G35 and U36 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) variants, substitution of G35 or U36 with each of the other three nucleotides almost completely abolished the copurification of tRNA, whereas substitution of C34 with other nucleotides led to high levels of variant copurification. Based on these results, we conclude that Trm140 binds tRNAs with a G35→U36 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.

To examine the sufficiency of G35→U36→t^6A37 for m^3C formation, we replaced the anticodon of the nonsubstrate tRNA^Phe(GAA) with a CGU anticodon and a G37A mutation to allow for t^6A37 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[CGUA]34-37 (Fig. 4B,C). ORF240 pull-down experiments resulted in efficient copurification (29%) of the tRNA^Phe(GAA)_29,50[CGUA]34-37 variant, but only background copurification (1%) of tRNA^Thr(CGU)_29,50[GAAG]34-37 (Fig. 4B,C). Consistent with the binding results, primer extension of RNA from cells containing the tRNA^Phe(GAA)_29,50[CGUA]34-37 variant revealed a strong primer extension block at N33, indicative of m^3C modification (Fig. 4D). These binding and primer extension results of tRNA^Phe(GAA)_29,50[CGUA]34-37 variant revealed a strong primer extension block at N33, indicative of m^3C modification (Fig. 4D). These binding and primer extension results of tRNA^Phe(GAA)_29,50[CGUA]34-37 variant revealed a strong primer extension block at N33, indicative of m^3C modification (Fig. 4D).
indicate that a CGU anticodon and A_{37} (presumably modified to t^{6}A) are sufficient for m^{3}C modification of tRNA^{Phe} (GAA) and establish that this sequence is an identity element for the modification.

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

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^{6}A_{37} is important for Trm140 modification of tRNA^{Thr}, only tRNA^{Ser}(GCU) has t^{6}A, whereas tRNA^{Ser}(CGA) and tRNA^{Ser}(UGA) have i^{6}A_{37}.

Consistent with the importance of t^{6}A for m^{3}C modification of tRNA^{Thr} species, we found that the m^{3}C modification level of tRNA^{Ser}(GCU) was substantially lower in the sua5 strain compared to the WT strain (Fig. 5A). We also found that m^{3}C was substantially reduced in
tRNA_{\text{Ser}}^{\text{CGA}}/\text{tRNA}_{\text{Ser}}^{\text{UGA}} in a mod5Δ strain, which lacks the i^6A_{37} modification (Fig. 5B; Dihanich et al. 1987), as also found for the m^3C modification of tRNA_{\text{Ser}}^{\text{CGA}} substrates in S. pombe (Arimbasseri et al. 2016). Thus, it appears that both i^6A and i^6A contribute to efficient m^3C modification.

We further showed that i^6A_{37} was important for tRNA_{\text{Ser}}^{\text{CGA}} binding to ORF240 (Fig. 5C). While ORF240 bound 50% of the tRNA_{\text{Ser}}^{\text{CGA}} in a MOD5+ strain, it only bound 1% of this tRNA in a mod5Δ strain. Similarly, although tRNA_{\text{Ser}}^{\text{UGA}} bound more weakly to ORF240 in a MOD5+ strain (6%), this binding was undetectable in a mod5Δ strain. As expected, binding of tRNA_{\text{Ser}}^{\text{GCU}} was not affected in the mod5Δ strain, because this tRNA is not a Mod5 substrate. Because i^6A_{37} is crucial for ORF240 binding and for m^3C modification of tRNA_{\text{Ser}}^{\text{CGA}} and tRNA_{\text{Ser}}^{\text{UGA}}, we infer that ORF240 binding is important for m^3C formation.

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

**Ses1 copurifies with Trm140**

One unique feature of tRNA_{\text{Ser}} species that could in principle be important for m^3C modification is the distinctive V-loop. In yeast, the tRNA_{\text{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^{\text{Leu}} family, with V-loops of 13 or 15 nt. Trm140 could recognize the distinctive tRNA_{\text{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 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. (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.

**Ses1 stimulates m^3C formation of tRNA_{\text{Ser}} species**

To determine if Ses1 had a role in m^3C modification, we examined the methyltransferase activity of Trm140 in vitro. We expressed and purified His6-ORF240 from *Escherichia coli* and assayed its activity with tRNA purified from a *trm140A* strain. After incubation of ORF240 with S-adenosylmethionine (SAM) and tRNA, we analyzed m^3C modification by primer extension in the presence of ddTTP, and calculated 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_{\text{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^3C modification reaction.
the efficiency of m^3C modification based on the intensities of the primer extension blocks at U33, due to m^3C32, and at A31, due to read-through and termination by ddTTP incorporation. Using this assay, we found that the three tRNAThr species were equally efficiently modified by ORF240 (Table 1; Fig. 7A,B), in each case requiring \(\sim 0.02 \, \mu M\) ORF240 for one-half-maximal modification (ORF240_{1/2}). In contrast, ORF240 was very inefficient at m^3C modification of tRNASer(CGA) and tRNASer(UGA), with ORF240_{1/2} values of \(\sim 15 \, \mu M\) and \(\sim 0.3 \, \mu M\), respectively, while the ORF240_{1/2} value was \(\sim 0.05 \, \mu M\) for tRNASer(GCU). As anticipated, no m^3C was detected upon ORF240 assay of tRNATyr(GUA), which normally bears an unmodified C32 (Table 1).

Consistent with an important biological role for the interaction of Trm140 with Ses1, we found that purified Ses1 stimulated the Trm140 m^3C modification activity on tRNAThr species in vitro. Using Ses1 purified from a \(\text{trm}140\Delta\) strain, to avoid Trm140 contamination, we found that 1.5 \(\mu M\) Ses1 reduced the ORF240_{1/2} of tRNAThr(CGA) from \(\sim 0.3 \, \mu M\) to \(\sim 0.02 \, \mu M\), of tRNAThr(UGA) from \(\sim 15 \, \mu M\) to \(\sim 0.3 \, \mu M\), and of tRNAThr(GCU) from 0.05 \(\mu M\) to 0.02 \(\mu M\), but had little or no effect on tRNAThr(IGU) or tRNAThr(UAA) (Table 1; Fig. 8A,B).

Consistent with these in vitro results, we observed Ses1 stimulation of m^3C modification of m^3C modification in vivo. To address this question, we assayed m^3C modification after overproduction of Ses1-MORF in a mod5Δ strain, in which m^3C_{32} modification of tRNASer(GCU)/(UGA) was reduced due to lack of i^6A (Fig. 5B). We found that overproduction of Ses1 resulted in a substantial increase in m^3C levels of tRNASer(GCU)/(UGA) relative to that in the vector control, from 2% to 24% (Fig. 9A). Overproduction of ORF240 also improved the m^3C modification, consistent with the fact that i^6A was important for ORF240 binding. As expected, the m^3C levels of tRNAThr(IGU), which has t^6A and not i^6A, were not affected in the mod5Δ strain or by overproduction of either Ses1 or ORF240.

To determine whether the important region for m^3C modification of tRNASer species was the V-loop, we tested the modification of a chimeric tRNAThr species with a V-loop based on tRNAThr. We constructed a variant with the body sequence of tRNAThr(UAA), the anticodon UGA, and A37, called variant M1 (Fig. 9B). This M1 variant was not a substrate for m^3C modification (Fig. 9C, lane 2). However, the variant M2, which differs from M1 due to replacement of the V-loop with that of tRNASer(GCU), was significantly modified to m^3C_{32} (Fig. 9C, lane 5). This result shows that the tRNASer 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^3C modification. Trm140 explicitly recognizes the XGU anticodon and t^6A_{37} of tRNAThr species as an identity element for m^3C modification in vivo. Thus, substitution of either G_{35} or U_{36} of tRNAThr(UAA) with any other residue resulted in the complete absence of m^3C modification, whereas introduction of a CGU anticodon and A_{37} to tRNAPhe resulted in efficient m^3C modification in vivo. Consistent with these
results, ORF240 expressed and purified from E. coli efficiently catalyzed modification of all three tRNA\textsuperscript{Thr} species, but not tRNA\textsuperscript{Tyr}.

In contrast, the CGA, UGA, and GCU anticodons of the three tRNA\textsuperscript{Ser} species that are modified with m\textsuperscript{3}C do not have a common feature, and Trm140 recognition of these species is instead propelled by interaction with Ses1 and the tRNA\textsuperscript{Ser} V-loop, as well as by i\textsuperscript{6}A\textsubscript{37} of tRNA\textsuperscript{Ser}(CGA) and tRNA\textsuperscript{Ser}(UGA), and t\textsuperscript{6}A\textsubscript{37} of tRNA\textsuperscript{Ser}(GCU). Thus, purified Ses1 stimulated the efficiency of ORF240 m\textsuperscript{3}C modification of tRNA\textsuperscript{Ser}(CGA) compared to tRNA\textsuperscript{Ser}(UGA) by ∼50-fold. Furthermore, this Ses1 stimulation of m\textsuperscript{3}C modification was also observed in vivo, since Ses1 overproduction significantly increased m\textsuperscript{3}C modification of tRNA\textsuperscript{Ser}(CGA) and tRNA\textsuperscript{Ser}(UGA) relative to that of tRNA\textsuperscript{Ser}(GCU).

Our results suggest that m\textsuperscript{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\textsuperscript{Thr} anticodon loop variants tracked perfectly with m\textsuperscript{3}C modification, since all six possible variants with mutations in G\textsubscript{35} or U\textsubscript{36} lacked modification and also did not bind, whereas each of the three variants with mutations in C\textsubscript{34} were modified and bound efficiently, although not as efficiently as with C\textsubscript{34}. Furthermore, ORF240 bound tRNA\textsuperscript{Phe} efficiently only when its anticodon was altered to CGU-A\textsubscript{37}, correlated with m\textsuperscript{3}C modification, and i\textsuperscript{6}A\textsubscript{37} was important for binding of tRNA\textsuperscript{Ser}(CGA) to ORF240 as well as for m\textsuperscript{3}C modification. These results suggest a modular binding mechanism to explain substrate specificity, in which contributions are made by G\textsubscript{35}, U\textsubscript{36}, i\textsuperscript{6}A\textsubscript{37}, or t\textsuperscript{6}A\textsubscript{37}, and the V-loop, as well as C\textsubscript{34}. C\textsubscript{34} is favored over other N\textsubscript{34} residues based on preferential binding of tRNA\textsuperscript{Thr} C\textsubscript{34} variants and preferential binding of tRNA\textsuperscript{Ser}(CGA) compared to tRNA\textsuperscript{Ser}(UGA) since these two tRNAs are identical other than U\textsubscript{34} and the N\textsubscript{28}–N\textsubscript{42} pair. This preferential binding of tRNA\textsuperscript{Ser}(CGA) is also consistent with the preferential ORF240 modification activity of tRNA\textsuperscript{Ser}(CGA) compared to tRNA\textsuperscript{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\textsuperscript{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\textsuperscript{Thr} compared to tRNA\textsuperscript{Ser}(CGA) and tRNA\textsuperscript{Ser}(UGA) also appear to involve common elements including C\textsubscript{34} and i\textsuperscript{6}A\textsubscript{37} or t\textsuperscript{6}A\textsubscript{37}, as well as the specific involvement of Ses1 for tRNA\textsuperscript{Ser} and G\textsubscript{35}–U\textsubscript{36} for tRNA\textsuperscript{Thr}.

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

**FIGURE 8.** Ses1 stimulates ORF240 m\textsuperscript{3}C modification of tRNA\textsuperscript{Ser}(UGA) and tRNA\textsuperscript{Ser}(CGA). (A) Effect of Ses1 on the titration of ORF240 m\textsuperscript{3}C methyltransferase activity on tRNA\textsuperscript{Ser}(CGA) and tRNA\textsuperscript{Ser}(UGA). Of note, 0.35 pmol of tRNA\textsuperscript{Ser}(UGA) and tRNA\textsuperscript{Ser}(CGA) purified from a trm140Δ strain was assayed for m\textsuperscript{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\textsuperscript{3}C modification as a function of the concentration of ORF240 in A.
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′C_{32} (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^{3}A/\text{m}^{3}C 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′C 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 coxcrystal structures of both *Thermus thermophilus* SerRS-tRNA_{Ser} and human SerRS-tRNA_{Ser} 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′C modification, but not casting light on its mechanism of m′C 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 bayanus, Kluyveromyces lactis, Ashbya gossypii, Saccharomyces kluyveri, Kluyveromyces waltii, Aspergillus nidulans, Aspergillus oryzae, Neurospora crassa, Coccidioides posadasii, Coccidioides immitis, Coprinopsis cinerea, Phanerochaete chrysosporium, Uncinocarpus reesi, 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′C_{32} or with m′C in the V-loop (Arimbasseri et al. 2016). In view of our findings, we speculate that tRNA_{Ser} m′C 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′C 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
Nm52 formation, Trm7/Trm734 for Nm53 formation (Guy
et al. 2012; Guy and Phizicky 2015), and Kre33/Tan1 for
ac€C12 formation (Johansson and Bystrom 2004; Sharma
et al. 2015). The finding of Ses1 as an interacting partner for
Trm140 m3C modification is also another example of the re-
markable 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
determine precisely how Ses1 recognizes Trm140 to stim-
ulate m3C modification activity of tRNA\textsuperscript{Ser}, and the connec-
tion between tRNA\textsuperscript{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 purifi-
cation were derivatives of YLH126.

TRM140 was deleted by PCR amplification of the \textit{trm140\textsuperscript{Δ}}::ble\textsuperscript{R}
cassette from ySD179 using primers containing sequences 5’ and
3’ of \textit{TRM140} (\textit{TRM140} − 409 and \textit{TRM140} + 307), followed by trans-
formation, selection on YPD media containing 8 mg/L
Bleocin and verification by PCR using appropriate primers.

MOD5 was deleted in a similar fashion.

The \textit{tT(CGU)}\textit{Δ}[\textit{CEN} URA\textit{3 tT(CGU)}] strain was constructed by trans-
formation of BY4741 with the [\textit{CEN} URA\textit{3 tT(CGU)}] plasmid
containing the \textit{tT(CGU)} gene with its own flanking sequence,
followed by PCR amplification of the ble\textsuperscript{R} marker and linear transfor-
mation to delete the \textit{tT(CGU)} gene.

Strains with the chromosomal cMORF tag (His\textsubscript{6}-HA-3C site-ZZ
domain of protein A) were generated by PCR amplification of
a gene-specific product from a cMORF:URA\textit{3} 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\textsuperscript{R} cassette (Janke et al. 2004).

Plasmids

Plasmids used in this study are listed in Table 3. Plasmids express-
ing tRNAs were constructed by ligation-independent cloning (LIC)
of a tRNA with its own flanking sequence into the [2\mu LEU\textit{2}] LIC
vector pAVA577. LIC was also used to build the [CEN URA\textit{3 MOD5}]
plasmid. Integration vectors for tRNA variants were made by insertion of a BglIII, XhoI fragment encoding the tRNA
variant into plasmid pAB230-1, as previously described (Guy
et al. 2014).

ORF240 was cloned by LIC into a [2\mu URA\textit{3} P\textsubscript{GAL1,10} expression vector, in which ORF240 is expressed under
\textit{P\textsubscript{GAL1}} control with a C-terminal PT tag (as ORF240-3C site-HA
epitope-His\textsubscript{6}-ZZ domain of protein A) essentially as previously de-
scribed (Quartley et al. 2009).

Expression and affinity purification of Ses1-MORF from yeast

To purify Ses1 from yeast without interacting Trm140, strain
YLH974-1 (\textit{trm140\textsuperscript{Δ}}) was transformed with a [2\mu URA\textit{3} P\textsubscript{GAL1,10}-SSES1-MORF] plasmid encoding a SSES1-MORF fusion protein
(Ses1-His\textsubscript{6}-HA-3C site-ZZ domain of protein A), and the resulting
strain was grown in selective media containing raffinose and in-
duced for 6 h by addition of one-half volume of 3\% YP media con-
taining 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 contain-
ing 50\% glycerol, 20 mM Tris–Cl pH 7.5, 150 mM NaCl, 1 mM
MgCl\textsubscript{2}, and 1 mM DTT, as previously described (Quartley et al.
2009).

| TABLE 2. Strains used in this study |
|-----------------------------------|
| Strain | Genotype | Source |
|-----------------|-----------------|-----------------|
| BY4741 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Open Biosystems |
| BY4742 | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Open Biosystems |
| BCY123 | MATa pep4Δ::His5 prbΔ::EU2 bar1Δ::HISG lys2Δ::GAL1/10-GAL4 can1 ade2 ura3 leu2-3, 112 | Macbeth et al. (2005) |
| YLH562-1 | BY4741 \textit{tT(CGU)}Δ::ble\textsuperscript{R} \{\textit{CEN} URA\textit{3 tT(CGU)}\} | 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::EU2 | Na et al. (1992) |
| YLH126 | leu2Δ trp1Δ ura3Δ prb1-1122 pep4-1 his3Δ::P\textsubscript{GAL1,10}-GAL4 | Erin O’Shea |
| ySD844 | BY4741 mod5Δ::kan\textsuperscript{R} | This study |
| ySD356 | BCY123 TRM140-cMORF-URA3 | This study |
| YLH1302-1 | BCY123 SES1-1Mycc7His::kan\textsuperscript{R} | This study |
| YLH1305-1 | BCY123 VAS1-1Mycc7His::kan\textsuperscript{R} | This study |
| YLH1307-1 | BCY123 TRM140-cMORF::URA3 SES1-1Mycc7His::kan\textsuperscript{R} | This study |
| YLH1310-1 | BCY123 TRM140-cMORF::URA3 VAS1-1Mycc7His::kan\textsuperscript{R} | This study |
| yMG814-1 | BY4741 trm732Δ::ble\textsuperscript{R} | Guy et al. (2012) |
| ySD179 | BY4742 trm140::ble\textsuperscript{R} | D’Silva et al. (2011) |
| YLH974-1 | YLH126 trm140::ble\textsuperscript{R} | This study |
TABLE 3. Plasmids used in this study

| Plasmid    | Parent               | Description                                               | Source            |
|------------|----------------------|---------------|--------------------------------------------------|
| BG2663     | BG2663               | 2µ URA3 P<sub>CAI</sub> TRM140-PT                        | Quartley et al. (2009) |
| pSD142     | BG2663               | 2µ URA3 P<sub>CAI</sub>-TRM140-PT                        | D’Silva et al. (2011) |
| pSD252     | BG2663               | 2µ URA3 P<sub>CAI</sub>-TRM240-PT                        | This study        |
| pAB230-1   | pW132                | ade2::5’His<sub>GUG</sub> G2::Flac                       | Guy et al. (2014)  |
| pEH248-1   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU)                    | This study        |
| pEH249-1   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U | This study        |
| pEH307-1   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U C32U | This study        |
| pEH308-1   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U C34A | This study        |
| pEH309-4   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U C34U | This study        |
| pEH310-4   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U C34G | This study        |
| pEH311-1   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U G35A | This study        |
| pEH312-2   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U G35U | This study        |
| pEH313-4   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U G35C | This study        |
| pEH314-2   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U U36A | This study        |
| pEH315-2   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U U36C | This study        |
| pEH316-5   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U U36G | This study        |
| pEH317-1   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U A38U | This study        |
| pEH318-1   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tT(CGU) C2BG–G42C U50A–64U A38C | This study        |
| pEH328-2   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tG(CAA) A29U–U41A U50A–64U | This study        |
| pEH329-2   | pAB230-1             | ade2::5’His<sub>GUG</sub> G2::tG(CAA) A29U–U41A U50A–64U CGUA[34–37] | This study        |
| pJE832A    | pAVA421              | His<sub>G</sub>-GLN4-MORF                                 | Grant et al. (2012) |
| pYM46      | pAVA421              | 1Myc-7His::kan<sup>R</sup>                                | Janke et al. (2004) |
| pAVA577    | 2µ LEU2 LIC vector   |                                                           | Alexandrov et al. (2006) |
| pJW038     | pAVA577              | 2µ LEU2 IS(CGA)                                          | Whipple et al. (2011) |
| pKD9094    | pAVA577              | 2µ LEU2 tT(CGU)                                           | Han et al. (2015)  |
| pJW043     | pAVA577              | 2µ LEU2 IS(UCA)                                          | Han et al. (2015)  |
| pEH106-1   | pAVA577              | 2µ LEU2 tT(CGU)                                           | Han et al. (2015)  |
| pEH419-1   | pAVA577              | 2µ LEU2 IS(UGC)<sup>F</sup>                               | This study        |
| pAVA579    | pAVA579              | CEN URA3 LIC vector                                      | Quartley et al. (2009) |
| pELN010    | pAVA579              | CEN URA3 MOD5                                            | This study        |
| pAVA0258   | pAVA421              | His<sub>G</sub>-HA-3C-protein A::URA3 cassette             | Gelperin et al. (2005) |
| pJE1256A   | pAVA421              | P<sub>CAI</sub>-SES1-MORF                                 | Gelperin et al. (2005) |
| pAVA421    | pAVA421              | pT7-His<sub>G</sub>-3C site-ORF LIC vector                | Quartley et al. (2009) |
| pSD248     | pAVA421              | His<sub>G</sub>-3C-ATG-ORF240                             | D’Silva et al. (2011) |

Growth and affinity purification of His<sub>6</sub>-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 His<sub>6</sub>-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<sub>600</sub> 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<sub>2</sub> at 50°C–55°C for 1 h. For reactions containing ddTTP, ddTTP 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<sub>CAI</sub>-ORF240-PT or P<sub>CAI</sub>-TRM140-PT plasmid were grown in selective media containing raffinose to OD<sub>600</sub> ~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 OD600 1–2.5, and crude extracts were made from 500–600 OD 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 anti-Cellulose membrane (Bio-Rad), and probed with appropriate anti-
The reaction mixtures (20 µL) contained 60–67.5 mM NaCl, 50 mM Hepes, pH 7.5, 3 mM MgCl2, 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 strain, ORF240 purified from E. coli, and where indicated, Sex1 purified from a trm140 strain or compensating buffer. Reactions were incubated at 30°C for 1 h, stopped by phenol extraction, and then RNA was precipitated with ethanol, resuspended in water, and subjected to primer extension analysis to analyze m3C32.

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**REFERENCES**

Achsel T, Gross HJ. 1993. Identity determinants of human tRNAser sequence elements necessary for serylation and maturation of a tRNA with a long extra arm. EMBO J 12: 3333–3338.

Agris PF, Vendex FA, Graham WD. 2007. tRNA’s wobble decoding of the genome: 40 years of modification. J Mol Biol 366: 1–13.

Alexandrov A, Chernyakov I, Gu W, Hiley SL, Hughes TR, Grayhack EL, Phizicky EM. 2006. Rapid tRNA decay can result from lack of non-essential modifications. Mol Cell 21: 87–96.

Arimbasseri AG, Iben J, Wei FY, Rijal K, Tomizawa K, Hafner M, Maira RJ. 2016. Evolving specificity of tRNA 3-methyl-cytidine-32 (m3C32) modification: a subset of tRNAs requires N6-isopentenyltation of A37. RNA 22: 1400–1410.

Auffinger P, Westhof E. 1999. Singly and bifurcated hydrogen-bonded base-pairs in tRNA anticodon hairpins and ribozymes. J Mol Biol 292: 467–483.

Biou V, Yaremchuk A, Tukalo M, Cusack S. 1994. The 2.9 Å crystal structure of T. thermophiles seryl-tRNA synthetase complexed with tRNAmet. Science 263: 1404–1410.

Björk GR, Jacobsson K, Nilsson K, Johansson MJ, Bystrom AS, Persson OP. 2001. A primordial tRNA modification required for the evolution of life? EMBO J 20: 231–239.

Björk GR, Huang B, Persson OP, Byström AS. 2007. A conserved modified wobble nucleoside (mcm5’sU) in Iysyl-tRNA is required for viability in yeast. RNA 13: 1245–1255.

Chen C, Huang B, Eliasson M, Ryden P, Bystrom AS. 2011. Elongator complex influences telomeric gene silencing and DNA damage response by its role in wobble uridine tRNA modification. PLoS Genet 7:e1002258.

Dibonacci ME, Najarian D, Clark R, Gillman EC, Martin NC, Hopper AK. 1987. Isolation and characterization of MOD5, a gene required for isopentenyltation of cytoplasmic and mitochondrial tRNAs of Saccharomyces cerevisiae. Mol Cell Biol 7: 177–184.

D’Silva S, Haider SJ, Phizicky EM. 2011. A domain of the actin binding protein Abp140 is the yeast methyltransferase responsible for 3-methylcytidine modification in the tRNA anti-codon loop. RNA 17: 1100–1110.

El Yacoubi B, Lyons B, Cruz Y, Reddy R, Nordin B, Agnellii F, Williamson JR, Schimmel P, Swaraj MA, de Creyc-Lagarv. 2009. The universal YrdC/Sua5 family is required for the formation of trheonylcarbamoylaladinosine in tRNA. Nucleic Acids Res 37: 2894–2909.

El Yacoubi B, Hatin I, Deutsch C, Kahveci T, Rousset JP, Iwata-iyuly D, Murzin AG, de Crécy-Lagarv. 2011. A role for the universal Kae1/Qri7/YgjD (COG0533) family in tRNA modification. EMBO J 30: 882–893.

Esberg A, Huang B, Johansson MJ, Bystrom AS. 2006. Elevated levels of two tRNA species bypass the requirement for elongator complex in transcription and exocytosis. Mol Cell 24: 139–148.

Fleming IM, Paris Z, Gaston KW, Balakrishnan R, Frettick K, Rubio MA, Alfonzo JD. 2016. A tRNA methyltransferase paralog is important for ribosome stability and cell division in Trypanosoma brucei. Sci Rep 6: 21438.

Gelperin DM, White MA, Wilkinson ML, Kon Y, Kung LA, Wise KJ, Lopez-Hoyo N, Jiang L, Piccirillo S, Yu H, et al. 2005. Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. Genes Dev 19: 2816–2826.

Gerber AP, Keller W. 1999. An adenosine deaminase that generates inosine at the wobble position of tRNAs. Science 286: 1146–1149.

Grant TD, Snell EH, Luft JR, Quartley E, CorretoRE S, Wobley JR, Snell ME, Hadd A, Perona JI, Phizicky EM, et al. 2012. Structural conservation of an ancient tRNA sensor in eukaryotic glutaminyl-tRNA synthetase. Nucleic Acids Res 40: 3723–3731.

Guo M, Yang XL, Schimmel P. 2010. New functions of aminoacyl-tRNA synthetases beyond translation. Nat Rev Mol Cell Biol 11: 668–674.

Guy MP, Phizicky EM. 2014. Two-subunit enzymes involved in eukaryotic post-transcriptional tRNA modification. RNA Biol 11: 1608–1618.
Guy MP, Phizicky EM. 2015. Conservation of an intricate circuit for crucial modifications of the tRNA^Phe anticodon loop in eukaryotes. RNA 21: 61–74.

Guy MP, Podzynsk B, Preston MA, Shaheen HH, Krivos KL, Limbach PA, Hopper AK, Phizicky EM. 2012. Yeast Trm7 interacts with distinct proteins for critical modifications of the tRNA^Phe anticodon loop. RNA 18: 1921–1933.

Guy MP, Young DL, Payea MJ, Zhang X, Kon Y, Dean KM, Grayhack EJ, Mathews DH, Fields S, Phizicky EM. 2014. Identification of the determinants of tRNA function and susceptibility to rapid tRNA decay by high-throughput in vivo analysis. Genes Dev 28: 1721–1732.

Guy MP, Shaw M, Weiner CL, Hobson L, Stark Z, Rose K, Kalscheuer VM, Han L, Kon Y, Phizicky EM, Anderson J, Hobson L, Kon Y, Phizicky EM. 2015. Functional importance of the yeast gene encoding the tRNA m1G methyltransferase. Hum Mutat 36: 1167–1187.

Hall TM. 2005. Multiple modes of RNA recognition by zinc finger proteins. Curr Opin Struct Biol 15: 367–373.

Han L, Searles MA, Klug A. 2003. Crystal structure of a zinc-finger-RNA complex. RNA 9: 712–719.

Herbert CJ, Labouesse M, Dujardin G, Slonimski PP. 1988. The NAM2 codon loop determines ability of anticodon UCC to discriminate among glycine codons. Proc Natl Acad Sci USA 85: 3343–3347.

Macbeth MR, Schubert HL, Vandemark AP, Lingam AT, Hill CP, Bass BL. 2005. Inositol hexakisphosphate is bound in the ADAR2 core and required for RNA editing. Science 309: 1534–1539.

Machnicka MA, Milanowska K, Osman Oglou O, Purta E, Kurkowska M, Ochowick A, Januszewski W, Kalinowski S, Dunin-Horkawicz S, Rother KM, et al. 2013. MORDOMICS: a database of RNA modification pathways–2013 update. Nucleic Acids Res 41: D262–D267.

Miyazaki K, Kimura S, Suzuki T. 2013. A cyclic form of N^6-threonylcarbamoyladenosoine as a widely distributed tRNA hypermodification. Nat Chem Biol 9: 105–111.

Moitso de Vargas L, Pargellig CA, Hasan NM, Bushman EW, Landy A. 1988. Autonomous DNA binding domains of λ integrase recognize two different sequence families. Cell 54: 923–929.

Murphy FV, Ramkrishnan V. 2004. Structure of a purine–purine wobble base pair in the decoding center of the ribosome. Nat Struct Mol Biol 11: 1251–1252.

Na JG, Pinto I, Hampsey M. 1992. Isolation and characterization of SUA5, a novel gene required for normal growth in Saccharomyces cerevisiae. Genetics 131: 791–801.

Nakamura A, Nemoto T, Heinemann IU, Yamashita K, Sonoda T, Komoda K, Tanaka I, Soll D, Yao M. 2013. Structural basis of reverse nucleotide polymerization. Proc Natl Acad Sci USA 110: 20970–20975.

Nolte RT, Conlin RM, Harrison SC, Brown RS. 1998. Differing roles for zub proteins in DNA recognition: structure of a six-finger transcription factor IIA complex. Proc Natl Acad Sci USA 95: 2938–2943.

Noma A, Yi S, Kataj T, Takai Y, Suzuki T, Suzuki T. 2011. Actin-binding protein ABP140 is a methyltransferase for 3'-methylcytidine at position 32 of tRNAs in Saccharomyces cerevisiae. RNA 17: 1111–1119.

Olejniczak M, Uhlenbeck OC. 2006. tRNA residues that have coevolved with their anticodon to ensure uniform and accurate codon recognition. Biochemistry 45: 943–950.

Olejniczak M, Dale T, Fahman RP, Uhlenbeck OC. 2005. Idiosyncratic tuning of tRNAs to achieve uniform ribosome binding. Nat Struct Mol Biol 12: 788–793.

Pintard L, Lecointe F, Bujnicki JM, Bonnerot G, Grosjean H, Lapeyre B. 2002. Trm7p catalyses the formation of two 2'-O-methylribosides in yeast tRNA anticodon loop. EMBO J 21: 1007–1014.

Jackman JE, Montange RK, Malik HS, Phizicky EM. 2003. Identiﬁcation of the yeast gene encoding the tRNA mG methyltransferase responsible for modification at position 9. RNA 9: 574–585.

Janke C, Magiera MM, Rathfelder N, Taxis C, Reber S, Maekawa H, Moreno-Borchard A, Doenges G, Schwoeb E, Schiebel E, et al. 2004. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21: 947–962.

Johansson MJ, Bystrom AS. 2004. The Saccharomyces cerevisiae TANI gene is required for N^6-acetylcytidine formation in tRNA. RNA 10: 712–719.

Johansson MJ, Esberg A, Huang B, Bjork GR, Bystrom AS. 2008. Eukaryotic wobble uridine modifications promote a functionally redundant decoding system. Mol Cell Biol 28: 3301–3312.

Juhling F, Morl H, Hartmann RK, Sprinzl M, Stadler PF, Putz J. 2009. tRNAdb 2009: compilation of tRNA sequences and tRNA genes. Nucleic Acids Res 37: D1159–D162.

Kadaba S, Krueger A, Trice T, Kreic AM, Hinnebusch AG, Anderson J. 2004. Nuclear surveillance and degradation of hypomodified initiator tRNA^Met in S. cerevisiae. Genes Dev 18: 1227–1240.

Ledoux S, Olejniczak M, Uhlenbeck OC. 2009. A sequence element that tunes Escherichia coli tRNA^Glu^Glu to ensure accurate decoding. Nat Struct Mol Biol 16: 359–364.

Lu D, Searles MA, Klug A. 2003. Crystal structure of a zinc-finger-RNA complex reveals two modes of molecular recognition. Nature 426: 96–100.

Lustig F, Boren T, Claesson C, Simonsson C, Barciszewksa M, Lagerkvist U. 1993. The nucleotide in position 32 of the tRNA anticodon loop determines ability of anticodon UCC to discriminate among glycine codons. Proc Natl Acad Sci 90: 3343–3347.
Urbonavicius J, Qian Q, Durand JM, Hagervall TG, Bjork GR. 2001. Improvement of reading frame maintenance is a common function for several tRNA modifications. EMBO J 20: 4863–4873.
Waas WF, Druzina Z, Hanan M, Schimmel P. 2007. Role of a tRNA base modification and its precursors in frameshifting in eukaryotes. J Biol Chem 282: 26026–26034.
Wakasugi K, Schimmel P. 1999. Two distinct cytokines released from a human aminocyl-tRNA synthetase. Science 284: 147–151.
Wang C, Guo Y, Tian Q, Jia Q, Gao Y, Zhang Q, Zhou C, Xie W. 2015. SerRS-tRNA\textsuperscript{Sec} complex structures reveal mechanism of the first step in selenocysteine biosynthesis. Nucleic Acids Res 43: 10534–10545.
Weissenbach J, Kiraly I, Dirheimer G. 1977. Primary structure of tRNA Thr 1a and b from brewer’s yeast. Biochimie 59: 381–391.
Weixlbaumer A, Murphy FVt, Dziergowska A, Malkiewicz A, Vendeix FA, Agris PF, Ramakrishnan V. 2007. Mechanism for expanding the decoding capacity of transfer RNAs by modification of uridines. Nat Struct Mol Biol 14: 498–502.
Whipple JM, Lane EA, Chernyakov I, D’Silva S, Phizicky EM. 2011. The yeast rapid tRNA decay pathway primarily monitors the structural integrity of the acceptor and T-stems of mature tRNA. Genes Dev 25: 1173–1184.
Yao P, Fox PL. 2013. Aminocyl-tRNA synthetases in medicine and disease. EMBO Mol Med 5: 332–343.