Yeast Trm7 interacts with distinct proteins for critical modifications of the tRNA^{Phe} anticodon loop

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ABSTRACT

Post-transcriptional modification of the tRNA anticodon loop is critical for translation. Yeast Trm7 is required for 2'-O-methylation of C₃₂ and N₃₄ of tRNA^{Phe}, tRNA^{Trp}, and tRNA^{Leu(UAA)} to form Cm₃₂ and Nm₃₄, and *trm7-Δ* mutants have severe growth and translation defects, but the reasons for these defects are not known. We show here that overproduction of tRNA^{Phe} suppresses the growth defect of *trm7-Δ* mutants, suggesting that the crucial biological role of Trm7 is the modification of tRNA^{Phe}. We also provide in vivo and in vitro evidence that Trm7 interacts with ORF YMR259c (now named Trm732) for 2'-O-methylation of C₃₂, and with Rtt10 (named Trm734) for 2'-O-methylation of N₃₄ of substrate tRNAs and provide evidence for a complex circuitry of anticodon loop modification of tRNA^{Phe}, in which formation of Cm₃₂ and Gm₃₄ drives modification of m¹G₃₇ (1-methylguanosine) to yW (wyebutosine). Further genetic analysis shows that the slow growth of *trm7-Δ* mutants is due to the lack of both Cm₃₂ and Nm₃₄, and the accompanying loss of yW, because *trm732-Δ trm734-Δ* mutants phenocopy *trm7-Δ* mutants, whereas each single mutant is healthy; nonetheless, *TRM732* and *TRM734* each have distinct roles, since mutations in these genes have different genetic interactions with *trm1-Δ* mutants, which lack m^{2,2}G₂₆ in their tRNAs. We speculate that 2'-O-methylation of the anticodon loop may be important throughout eukaryotes because of the widespread conservation of Trm7, Trm732, and Trm734 proteins, and the corresponding modifications, and because the putative human *TRM7* ortholog *FTSJ1* is implicated in nonsyndromic X-linked mental retardation.

Keywords: *RTT10*; *TRM7*; tRNA^{Phe}; wyebutosine; *YMR259c*

INTRODUCTION

Post-transcriptional modifications of tRNA are numerous, widely conserved in nature, and critical for proper tRNA function (Agris et al. 2007; Phizicky and Hopper 2010). In the yeast *Saccharomyces cerevisiae*, 25 chemically distinct modifications are found among the 28 unique cytoplasmic tRNA species (of 42 total) that have been characterized for modifications, and at least 19 of these modifications are also known to occur in humans. Yeast cytoplasmic tRNAs have an average of 12.6 modifications, with 2.6 modifications

occurring within the anticodon loop (N₃₂–N₃₈) or its immediate vicinity (N₃₁, N₃₉, and N₄₀), and with the remaining 10 modifications in the main body of the tRNA, remote from the anticodon loop (Juhling et al. 2009).

Modifications in the main body of the tRNA have a number of different crucial roles in the cell. Lack of certain body modifications can provoke either of two quality-control pathways that degrade specific tRNAs at different stages of biosynthesis. Thus, a nuclear surveillance pathway targets pre-tRNA^{Met} lacking 1-methyladenosine (m¹A₅₈) for 3'-exonucleolytic degradation by the TRAMP complex, Rrp6, and the nuclear exosome (Kadaba et al. 2004, 2006; LaCava et al. 2005; Vanacova et al. 2005; Schneider et al. 2007), whereas a rapid tRNA decay (RTD) pathway targets several mature hypomodified tRNA species for 5' exonucleolytic degradation by Xrn1 and Rat1, due in large measure to destabilization of the acceptor and T-stem (Alexandrov et al. 2006; Chernyakov et al. 2008; Whipple et al. 2011). In addition to these quality control pathways, lack of m¹A₉ leads to misfolding of human mitochondrial

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tRNA^{Lys} (Helm et al. 1999), and lack of G₋₁ leads to the accumulation of uncharged tRNA^{His} (Gu et al. 2005).

Modifications in and around the anticodon loop of tRNA play several crucial roles in translation (Agris et al. 2007; Phizicky and Hopper 2010). Some anticodon modifications have a demonstrated role in ensuring charging fidelity; thus, for example, the presence of m¹G₃₇ on tRNA^{Asp} prevents mischarging by yeast arginyl-tRNA synthetase (ArgRS) (Putz et al. 1994), and the presence of lysidine on C₃₄ of *E. coli* tRNA^{Ile} promotes charging by IleRS and prevents mischarging by MetRS (Muramatsu et al. 1988).

Many anticodon loop modifications appear to affect decoding efficiency and/or accuracy, presumably by altering codon:anticodon interactions in the ribosome (Ogle et al. 2001; Murphy and Ramakrishnan 2004; Murphy et al. 2004; Selmer et al. 2006). Thus, in yeast, mcm⁵U₃₄ (5-methoxycarbonylmethyluridine) and ncm⁵U₃₄ (5-carbamoylmethyluridine) promote the reading of codons ending in G, whereas mcm⁵s²U₃₄ (5-methoxycarbonylmethyl-2-thiouridine) promotes reading of codons ending in either A or G (Johansson et al. 2008). Furthermore, these decoding properties likely explain the multiple phenotypes of yeast strains lacking the cm⁵ moiety of U₃₄, which are ascribed to reduced function of tRNA^{Lys}(UUU) and tRNA^{Gln}(UUG) (Esberg et al. 2006), and the lethality of yeast strains lacking the s² and mcm⁵ moieties of mcm⁵s²U₃₄, which is ascribed to reduced function of tRNA^{Lys}(UUU) (Bjork et al. 2007). In addition, yeast mutants lacking i⁶A₃₇ (N⁶-isopentenyladenosine) in their tRNAs have reduced nonsense suppression (Laten et al. 1978; Dihanich et al. 1987).

Several modifications in and near the anticodon loop also affect reading-frame maintenance. Thus, in bacteria, increased +1 frameshifting occurs with hypomodified tRNAs that have s²U₃₄ or mnm⁵U₃₄ (5-methylamino-methyluridine) instead of mnm⁵s²U₃₄, G₃₇ instead of m¹G₃₇, A₃₇ or i⁶A₃₇ instead of ms²io⁶A₃₇ (2-methylthio-N⁶-(cis-hydroxyisopentenyl) adenosine), and U₃₈ and U₃₉ instead of Ψ₃₈ and Ψ₃₉ (pseudouridine) (Urbonavicius et al. 2001). Similarly, in yeast, mutants with m¹G₃₇ instead of yW₃₇ (wyebutosine) in their tRNA^{Phe} have increased −1 frameshifting (Waas et al. 2007), mutants lacking t⁶A₃₇ (N⁶-threonylcarbamoyladenosine) in their tRNAs have increased

−1 frameshifting and +1 frameshifting, as well as increased recognition of GUG as an initiation codon (El Yacoubi et al. 2011), and mutants lacking Ψ₃₈ and Ψ₃₉ in their tRNAs have increased +1 and −1 frameshifting (Lecointe et al. 2002; Bekaert and Rousset 2005).

In a number of cases, however, it is not clear exactly which tRNAs are affected by lack of specific anticodon loop modifications, how the lack of modifications explains the observed phenotypes, and exactly how the modifications influence individual steps of translation. One such case is 2'-O-methylation of N₃₂ and N₃₄ to form Nm₃₂ and Nm₃₄ in the anticodon loop, which is critical for tRNA function, but largely unexplored. In yeast, 2'-O-methylation occurs on C₃₂ and N₃₄ of tRNA^{Phe}, tRNA^{Leu}(UAA), and tRNA^{Trp} (Fig. 1A) and requires Trm7 methyltransferase, mutants of which have a severe growth defect, presumably due to a 70% reduction in translation (Pintard et al. 2002). Trm7 and its modifications are of particular interest for two

FIGURE 1. tRNA^{Phe} is the Trm7 substrate that is important for healthy growth. (A) Schematic of anticodon loops of Trm7 substrate tRNAs. (B) Overexpression of tRNA^{Phe} suppresses slow growth of the trm7-Δ strain. Strains as indicated containing a [URA3 TRM7] plasmid or a [URA3] vector control, and a [LEU2] plasmid as indicated, were grown overnight in SD-Leu medium at 30°C, diluted to OD₆₀₀ of ~0.5 in H₂O, and serially diluted 10-fold in H₂O, and then 2 μL was spotted onto SD-Leu media containing 5-FOA to select for the LEU2 plasmid and against the URA3 plasmid. Cells were then grown for 2 d at 30°C. (C) tRNA^{Phe} levels in trm7-Δ mutants are not decreased. Wild-type and trm7-Δ mutant strains were grown at 30°C in YPD media, and RNA was isolated and analyzed by Northern blot as described in Materials and Methods. Numbers below lanes are levels of RNA normalized to 5S rRNA, and then normalized to wild type, itself normalized to 5S rRNA. (D) trm7-Δ mutants do not have an obvious tRNA^{Phe} charging defect. Strains were grown as in C, and RNA was isolated under acidic conditions and then analyzed by Northern blot as described in Materials and Methods. Control sample was treated with base prior to PAGE to deacylate the tRNA. Upper and lower arrows denote charged and uncharged tRNA species. Numbers indicate percentage of aminoacylated tRNA^{Phe}.

additional reasons. First, 2'-O-methylation of the anticodon loop is highly conserved in eukaryotes. Thus, all five sequenced eukaryotic tRNA^{Trp} species have Cm₃₂ and Cm₃₄, and all 17 sequenced eukaryotic tRNA^{Phe} genes have Cm₃₂, 16 of which also have Gm₃₄, including that of humans (Juhling et al. 2009). Moreover, Nm modifications in the anticodon loop appear to be widespread in other eukaryotic tRNA species since, for example, five of the 15 other sequenced human tRNA species contain Nm₃₂ or Nm₃₄ (Juhling et al. 2009). Second, *Trm7* and its modifications are associated with human disorders. Thus, mutations in the putative *TRM7* homolog *FTSJ1*, which result in truncated polypeptides, have been strongly associated with nonsyndromic X-linked mental retardation in multiple studies (Freude et al. 2004; Ramser et al. 2004; Froyen et al. 2007; Takano et al. 2008), and tRNA^{Phe} from neuroblastoma cells and Ehrlich ascites tumors has a significant portion of its tRNA^{Phe} lacking Cm₃₂ and Gm₃₄, and containing m¹G₃₇ instead of yW (Kuchino et al. 1982).

Because of the severe growth defect of *trm7*-Δ mutants, the widespread conservation of 2'-O-methylation of the anticodon loop, and the likely importance of *Trm7* and its modifications in humans, we sought to define the biology of *Trm7* and its modifications in yeast. We demonstrate here that tRNA^{Phe} is the *Trm7* target that affects growth and translation and provide evidence for a complex circuitry for anticodon loop modifications in tRNA^{Phe}, in which *Trm7* recruits one partner to methylate N₃₂ and another to methylate N₃₄ (both of which are important for different reasons), and in which lack of either of these modifications reduces formation of yW from m¹G₃₇.

RESULTS

The growth defect of *trm7*-Δ mutants is primarily due to hypomodified tRNA^{Phe}

The growth defect of *trm7*-Δ mutants could in principle be due to defective decoding of cognate codons by *Trm7* tRNA substrates, to defective decoding of noncognate codons by *Trm7* tRNA substrates, to a defect in some other step of translation, or to some unknown second function of *Trm7* unrelated to tRNA modification. We reasoned that if the *trm7*-Δ growth defect was due to poor decoding by one or more *Trm7* tRNA substrates (or to defects upstream of decoding), then overexpression of those tRNAs might suppress the slow-growth phenotype, as shown previously for phenotypes of other modification mutants (Alexandrov et al. 2006; Esberg et al. 2006). We tested this hypothesis by constructing a *trm7*-Δ strain harboring a *URA3* plasmid expressing *TRM7* (relevant genotype: *trm7*-Δ [*TRM7 URA3*]), and testing for growth after introduction of a high-copy (2μ) *LEU2* plasmid containing tRNA genes, and subsequent selection against the [*TRM7 URA3*] plasmid on media containing 5-fluoroorotic

acid (5-FOA). We find that overexpression of tRNA^{Phe} efficiently suppresses the slow growth of the *trm7*-Δ [*TRM7 URA3*] strain when plated to media containing 5-FOA, whereas overexpression of tRNA^{Leu(UAA)} or tRNA^{Trp} has no observable effect (Fig. 1B), suggesting that tRNA^{Phe} is the biologically important target of *Trm7*. Furthermore, we find that overexpression of tRNA^{Phe} completely suppresses the paromomycin sensitivity of *trm7*-Δ mutants (data not shown), which further suggests that the *trm7*-Δ growth defect is due to poor reading of Phe codons.

In this particular case, defective decoding of Phe codons in *trm7*-Δ mutants could be due to reduced decoding of Phe codons by hypomodified tRNA^{Phe} and/or to increased decoding of Phe codons by near-cognate tRNA^{Leu(UAA)}, which is also a *Trm7* substrate. Indeed, it has been speculated that the 2'-O-methyl group of U₃₄ might prevent tRNA^{Leu(UAA)} from reading UUX codons not ending in A (Johansson et al. 2008), suggesting that tRNA^{Leu(UAA)} in *trm7*-Δ mutants might decode the near-cognate UUU or UUC Phe codons (in addition to its cognate UUA Leu codon), which could then cause slow growth. However, since a *trm7*-Δ [2μ *LEU2* tRNA^{Leu(UAA)}] strain does not grow more poorly than a *trm7*-Δ [2μ *LEU2* vector] strain on SD-Leu media with 5-FOA (Fig. 1B), the slow growth of *trm7*-Δ mutants is not due to misreading of Phe codons by hypomodified tRNA^{Leu(UAA)}. Consistent with this interpretation, overexpression of tRNA^{Leu(UAA)} does not abrogate suppression of the growth defect of *trm7*-Δ mutants caused by overexpression of tRNA^{Phe} (Fig. 1B). Thus, we conclude that the growth defect of *trm7*-Δ mutants is due to poor decoding of Phe codons by hypomodified tRNA^{Phe}, or to some earlier step in the production of functional tRNA for translation. Since Northern analysis shows that *trm7*-Δ mutants have normal amounts of tRNA^{Phe} (Fig. 1C), and charged tRNA^{Phe} (Fig. 1D), we infer that the defect in *trm7*-Δ mutants occurs at some step between charging and decoding in the ribosome A-site.

Trm7 requires YMR259c to form Cm₃₂ and Rtt10 to form Nm₃₄ in vivo, and both are required for efficient synthesis of yW₃₇ from m¹G₃₇ of tRNA^{Phe}

Since high-throughput studies suggested that *Trm7* physically interacts with ORF YMR259c and Rtt10 (Krogan et al. 2006), we explicitly examined these interactions, and their consequences on 2'-O-methylation. Consistent with the high-throughput results, we find that *Trm7* interacts with ORF YMR259c and with Rtt10, since affinity purification of *Trm7* from a chromosomal *TRM7*-cMORF strain (carrying a C-terminal fusion of *TRM7* to a tandem affinity purification tag derived from the MORF collection) (Gelperin et al. 2005) results in substantial copurification of chromosomally tagged YMR259c-9myc (Fig. 2A, lane 5) or Rtt10-9myc (Fig. 2B, lane 5), whereas no copurification of either protein is

FIGURE 2. Trm7 forms a complex with ORF YMR259c and with Rtt10. (A) Chromosomally expressed YMR259c-9Myc and Trm7-cMORF form a complex. Soluble crude extract was prepared from the indicated strains, and proteins were purified using IgG-sepharose beads, followed by treatment with 3C protease as described in Materials and Methods. Extracts (input) were subjected to immunoblot analysis with anti-protein A and anti-(c-myc) antibody, and 3C-treated immunoprecipitates (IP) were analyzed by immunoblot with anti-(c-myc) antibody. C-terminal cMORF-tagged fusion proteins consist of ORF-His₆-HA-3C site-ZZ domain of protein A. (B) Chromosomally expressed Rtt10-9Myc and Trm7-cMORF form a complex. Indicated strains were analyzed as in A. The c-myc reactive band of molecular weight >200 kDa in the input (*) is likely Rtt10 aggregates.

observed with affinity-purified Trm8-cMORF (Fig. 2A, lane 6; Fig. 2B, lane 6).

To determine the effect of YMR259c and Rtt10 on 2'-O-methylation, we purified tRNAs from *trm7*-Δ, *rtt10*-Δ, and *ymr259c*-Δ mutants, and evaluated their nucleoside content by HPLC. As expected, we find that tRNA^{Leu(UAA)} from wild-type cells has Cm and ncm⁵Um (from residues 32 and 34, respectively), as well as Um (which presumably arises from incomplete modification of ncm⁵Um₃₄), whereas tRNA^{Leu(UAA)} from *trm7*-Δ mutants lacks any detectable

Cm, ncm⁵Um, or Um, and has instead ncm⁵U (Fig. 3A; Table 1). Strikingly, however, we find that tRNA^{Leu(UAA)} from *ymr259c*-Δ mutants has no detectable Cm (expected from residue 32), but has nearly the same amount of ncm⁵Um + Um (0.64 + 0.5 moles/mole) as found in wild-type cells at residue 34 (1.16 + 0.3 moles/mole). Conversely, tRNA^{Leu(UAA)} from *rtt10*-Δ mutants has no detectable ncm⁵Um (expected from residue 34), but instead has ncm⁵U (1.0 moles/mole) and has retained substantial amounts of Cm compared with that found in wild-type cells at residue 32 (0.36 vs. 0.61 moles/mole). Thus, it appears that YMR259c is required for formation of Cm₃₂ on tRNA^{Leu(UAA)}, and that Rtt10 is required for formation of ncm⁵Um₃₄ or Um₃₄. Consistent with this conclusion, we find that tRNA^{Trp} from *trm7*-Δ mutants lacks detectable Cm from Cm₃₂ and Cm₃₄, and that tRNA^{Trp} from *rtt10*-Δ mutants has ~50% of the normal amounts Cm (data not shown).

Surprisingly, examination of tRNA^{Phe} from *trm7*-Δ mutants reveals an additional 0.89 moles/mole of m¹G, in addition to the expected lack of detectable Cm and Gm (Fig. 3B; Table 2). m¹G is not normally present in tRNA^{Phe}, but might be present as the precursor to yW₃₇ (wyebuto-sine), which is normally generated from G₃₇ by Trm5-catalyzed methylation to form m¹G (Bjork et al. 2001), followed by the successive action of Tyw1, Tyw2, Tyw3, and Tyw4 (Noma et al. 2006). Consistent with this interpretation, we find that tRNA^{Phe} from *trm7*-Δ mutants lacks yW fluorescence at 425 nm, which is normally observed in wild-type tRNA^{Phe} (Fig. 3C); that tRNA^{Phe} from *tyw1*-Δ mutants contains both Gm (0.78 moles/mole) and m¹G (1.0 mole/mole) (Fig. 3B; Table 2) and lacks detectable yW fluorescence (Fig. 3C); and that tRNA^{Phe} from *trm7*-Δ *tyw1*-Δ mutants has the same amount of m¹G as *trm7*-Δ mutants (data not shown). Since mass spectrometry analysis shows explicitly that m¹G₃₇ is present in tRNA^{Phe} from *trm7*-Δ mutants, *trm7*-Δ *tyw1*-Δ mutants, and *tyw1*-Δ mutants, whereas yW₃₇ is present in tRNA^{Phe} from wild-type strains (data not shown), we conclude that the m¹G observed in HPLC analysis of tRNA^{Phe} from *trm7*-Δ mutants derives from the failure to complete yW synthesis after formation of the m¹G₃₇ intermediate.

With this interpretation of the m¹G levels in tRNA^{Phe} of *trm7*-Δ mutants, our results show that YMR259c and Rtt10 have similar effects on 2'-O-methylation of tRNA^{Phe} to those observed with tRNA^{Leu(UAA)}. Thus, tRNA^{Phe} from *ymr259c*-Δ mutants lacks any detectable Cm (expected from residue 32) (Fig. 3B; Table 2), but has comparable levels of Gm to that in wild-type cells at residue 34 (0.77 compared with 0.83 moles/mole), and in addition, has moderate levels of m¹G (0.41 moles/mole), consistent with its comparable reduced fluorescence from yW (Fig. 3C). Similarly, tRNA^{Phe} from *rtt10*-Δ mutants has nearly the same amount of Cm (0.77 moles/mole) as found in wild-type cells at residue 32 (0.83 moles/mole), but lacks any

FIGURE 3. ORF YMR259c is required for 2'-O-methylation of C₃₂, and Rtt10 is required for 2'-O-methylation of N₃₄ in vivo. (A) HPLC traces of tRNA^{Leu(UAA)} from *ymr259c-Δ* cells and *rtt10-Δ* cells. tRNA^{Leu(UAA)} isolated from the indicated strains was digested to nucleosides and analyzed by HPLC as described in Materials and Methods. 2'-O-methylated nucleosides are underlined. (B) HPLC traces of tRNA^{Phe} from *ymr259c-Δ* cells and *rtt10-Δ* cells. tRNA^{Phe} was analyzed as in A. (C) tRNA^{Phe} from *trm7-Δ* mutants lacks yW. Emission spectra of tRNA^{Phe} purified from wild-type cells, *tyw1-Δ* mutants, *trm7-Δ* mutants, *rtt10-Δ* mutants, or *ymr259c-Δ* mutants were determined after excitation at 320 nm.

detectable Gm, and has 0.62 moles of m¹G and substantially reduced levels of yW (Fig. 3B,C; Table 2). Our data therefore show that YMR259c is required with Trm7 to 2'-O-methylate C₃₂ of substrate tRNAs, and Rtt10 is required with Trm7 to modify N₃₄, and suggest that yW formation requires Gm₃₄ more than it requires Cm₃₂. Moreover, the demonstration that Trm7 can independently 2'-O-methylate C₃₂ with YMR259c, and N₃₄ with Rtt10, suggests that these protein pairs might act independently in vivo.

Consistent with independent function for each Trm7 partner protein, our data suggest that Trm7 forms independent complexes with YMR259c and Rtt10. Thus, we find that Rtt10-9myc does not copurify with chromosomally tagged YMR259c-cMORF (Fig. 4, lane 5), whereas Trm7-9myc does copurify, as expected (Fig. 4, lane 6). Furthermore, no complex is detected between YMR259c-9myc

and Rtt10-MORF when Rtt10-MORF is overexpressed 600-fold on a 2μ plasmid under control of the galactose promoter (data not shown). These results indicate that it is unlikely that a ternary Trm732/Trm7/Trm734 complex exists, unless it is inherently unstable under our purification conditions.

Trm7 requires YMR259c to catalyze Cm₃₂ formation and Rtt10 to catalyze Nm₃₄ formation in vitro

To more rigorously evaluate the effect of YMR259c and Rtt10 on Trm7 modification activity, we examined extracts from strains deleted for each of these components. As expected, extracts from wild-type cells catalyze formation of both Cm and Gm on tRNA^{Phe} (Fig. 5A,B, panel 2), and extracts from *trm7-Δ* cells do not catalyze formation of either Cm or Gm (Fig. 5A,B, panel 3), although *trm7-Δ* extracts are fully functional for formation of m⁵C and m^{2,2}G. Consistent with in vivo results, *ymr259c-Δ* extracts do not catalyze any detectable Cm formation on tRNA^{Phe} (Fig. 5A, panel 5), whereas *rtt10-Δ* extracts have nearly identical Cm formation activity to that of wild type extracts (Fig. 5A, cf. panels 2 and 4). As expected for specific modification activity, no Cm formation is observed with tRNA^{Thr(IGU)} (Fig. 5A, panel 6), which does not normally have Cm. Similarly, *rtt10-Δ* extracts do not catalyze detectable formation of Gm (Fig. 5B, panel 4), whereas *ymr259c-Δ* extracts have very similar Gm formation activity to that of wild-type extracts (Fig. 5B, cf. panels 2 and 5).

Thus, we conclude that Trm7 requires YMR259c (now named Trm732) for Cm₃₂ formation, and requires Rtt10 (now named Trm734) for Nm₃₄ formation both in vivo and in vitro, and our data suggest that the two activities are independent of each other.

Slow growth of *trm7-Δ* mutants is due to lack of both Cm₃₂ and Nm₃₄

The finding that Cm₃₂ formation requires Trm732 (YMR259c) and that Nm₃₄ formation requires Trm734 (Rtt10) allowed us to determine the contribution of each 2'-O-methylated residue to the slow growth phenotype of *trm7-Δ* mutants by examining the phenotypes of each reconstructed single mutant and the corresponding double

TABLE 1. Quantification of HPLC analysis of tRNA^{Leu(UAA)}

Modification	Moles expected	Wild type ^a	<i>trm7-Δ</i> ^a	<i>ymr259c-Δ</i> ^a	<i>rtt10-Δ</i> ^b
Cm	1	0.61 ± 0.04	<0.01	<0.01	0.36 ± 0.02
ncm ⁵ Um	1	1.16 ± 0.48	0.02 ± 0.02	0.64 ± 0.04	0.03 ± 0.03
Um	0	0.3 ± 0.14	0.01 ± 0.01	0.5 ± 0.05	0.01 ± 0.01
ncm ⁵ Um + Um	1	1.46 ± 0.34	0.02 ± 0.03	1.14 ± 0.09	0.04 ± 0.04
ncm ⁵ U	0	0.02 ± 0.03	1.0 ± 0.04	0.06 ± 0.03	1.0 ± 0.09
Ψ	2	2.6 ± 0.36	2.32 ± 0.17	2.29 ± 0.02	2.37 ± 0.16
m ⁵ C	1	0.76 ± 0.03	0.8 ± 0.04	0.87 ± 0.01	0.8 ± 0.02
m ¹ G + Gm	2	1.93 ± 0.04	1.93 ± 0.04	1.87 ± 0.01	1.88 ± 0.01
ac ⁴ C	1	0.91 ± 0.03	0.91 ± 0.01	0.84 ± 0.01	0.89 ± 0.01
m ² G	1	1.06 ± 0.06	1.03 ± 0.02	1.0 ± 0.01	1.0 ± 0.01

^aMean and standard deviation based on two individual growths and RNA preparations.^bMean and standard deviation based on three individual growths and RNA preparations.

mutant. We find that neither *trm732-Δ* mutants nor *trm734-Δ* mutants have any obvious growth defect on media containing 5-FOA (Fig. 6A), whereas the *trm732-Δ trm734-Δ* [TRM734 URA3] strain grows as poorly as the *trm7-Δ* [TRM7 URA3] strain when plated to media containing 5-FOA (Fig. 6A). Control experiments demonstrate that *tyw1-Δ* mutants (Noma et al. 2006), *trm732-Δ tyw1-Δ* mutants, and *trm734-Δ tyw1-Δ* mutants are each as healthy as wild-type cells (Fig. 6A). These results show that, in effect, the slow growth of *trm7-Δ* mutants is actually a negative synthetic effect due to the lack of Cm₃₂ and Gm₃₄, accompanied by lack of γW₃₇, on tRNA^{Phe}, all due to lack of Trm7.

Consistent with our conclusion that the *trm732-Δ trm734-Δ* strain phenocopies the *trm7-Δ* mutant strain we find that overexpression of tRNA^{Phe} efficiently suppresses the slow growth of a *trm732-Δ trm734-Δ* mutant (Fig. 6B). In contrast, overproduction of Trm7 does not suppress the slow growth of a *trm732-Δ trm734-Δ* mutant. Since Trm7 levels are about 100-fold greater when expressed from the [2μ P_{GAL} TRM7] plasmid than from the chromosomal copy (Fig. 6C, cf. lane 4 with lanes 5–8),

we infer that Trm732 and Trm734 provide specific functions to promote modification that cannot be bypassed by Trm7 alone.

The synthetic phenotype of a *trm7-Δ trm1-Δ* strain is due to lack of Nm₃₄, but not Cm₃₂

Since previous high-throughput experiments suggested that *trm7-Δ trm1-Δ* mutants, which also lack m^{2,2}G₂₆ on their tRNAs (Ellis et al. 1986), have a synthetic growth defect (Wilmes et al. 2008), we examined this defect to explore its cause. Consistent with the earlier report, we find that *trm7-Δ*

trm1-Δ [TRM7 URA3] mutants do not detectably grow when plated on media containing 5-FOA, whereas *trm1-Δ* mutants are healthy (Fig. 7). Strikingly, we find strong evidence that the contribution of the *trm7-Δ* mutation to the *trm7-Δ trm1-Δ* lethal phenotype is almost entirely due to lack of Nm₃₄. Thus, *trm734-Δ trm1-Δ* [TRM1 URA3] mutants are extremely sick when plated on media containing 5-FOA, whereas *trm732-Δ trm1-Δ* [TRM1 URA3] mutants and *tyw1-Δ trm1-Δ* [TRM1 URA3] mutants have no obvious growth defect. If, as seems likely, the lethal phenotype of *trm7-Δ trm1-Δ* mutants is primarily due to lack of m^{2,2}G₂₆ and Nm₃₄ (and possibly the accompanying substantial reduction in γW₃₇), this finding also demonstrates that Cm₃₂ and Nm₃₄ have separate functions, even though they appear to make equivalent contributions to the *trm7-Δ* mutant phenotype (Fig. 6A).

DISCUSSION

We have shown here that *trm7-Δ* mutants grow poorly primarily because of decreased tRNA^{Phe} function (Fig. 1B).

TABLE 2. Quantification of HPLC analysis of tRNA^{Phe}

Modification	Moles expected	Wild type ^a	<i>trm7-Δ</i> ^b	<i>ymr259c-Δ</i> ^c	<i>rtt10-Δ</i> ^d	<i>tyw1-Δ</i> ^e
Cm	1	0.83 ± 0.01	0.01 ± 0.01	<0.01	0.77 ± 0.03	0.78
Gm	1	0.83 ± 0.05	n/d ^f	0.77 ± 0.02	n/d ^f	0.78
m ¹ G	0	n/d ^f	0.89 ± 0.03	0.41 ± 0.04	0.62 ± 0.02	1.0
Ψ	2	2.27 ± 0.19	2.31 ± 0.14	2.15 ± 0.05	2.14 ± 0.08	2.23
m ⁵ C	2	1.74 ± 0.17	1.67 ± 0.07	1.66 ± 0.08	1.66 ± 0.09	1.60
m ² G	1	0.83 ± 0.02	0.82 ± 0.04	0.85 ± 0.01	0.83 ± 0.03	0.82

^aFour individual growths and RNA preparations.^bSix individual growths and RNA preparations.^cTwo individual growths and RNA preparations.^dThree individual growths and RNA preparations.^eOne RNA preparation.^fNot detected.

FIGURE 4. Chromosomally expressed YMR259c-cMORF interacts with Trm7-9myc, but not with Rtt10-9myc. Indicated strains were analyzed as in Figure 2A. The c-myc reactive band of molecular weight >200 kDa in the input (*) is likely Rtt10 aggregates.

Our data suggest a complex mechanism of anticodon loop modification of tRNA^{Phe}, in which the Trm7 methyltransferase interacts with Trm732 for 2'-O-methylation of C₃₂ and with Trm734 for 2'-O-methylation of G₃₄, and these modifications in turn drive modification of m¹G₃₇ to yW (Fig. 8). Furthermore, we have provided strong evidence that the slow growth phenotype of *trm7-Δ* mutants (Pintard et al. 2002) requires lack of both Cm₃₂ and Gm₃₄ of tRNA^{Phe} (Fig. 6A), although the specific functions of Cm₃₂ and Gm₃₄ are different, based on differential genetic interactions with *trm1-Δ* mutants (Fig. 7).

Our observation that Gm₃₄ of tRNA^{Phe} (and to a lesser extent Cm₃₂) drives formation of yW₃₇ from m¹G explains previous systems biology data showing that *trm7-Δ* mutants have reduced levels of yW (Chan et al. 2010). These results suggest strongly that Tyw1 requires Gm₃₄ and Cm₃₂ for the next catalytic step in yW formation from m¹G₃₇ (Noma et al. 2006). This dependence of yW₃₇ formation on the presence of Gm₃₄ and Cm₃₂ is consistent with the circuitous tRNA^{Phe} maturation pathway, which involves splicing in the cytoplasm, followed by Trm7 modification in the cytoplasm, retrograde tRNA nuclear import (Murthi et al. 2010), m¹G₃₇ modification by Trm5, and subsequent re-export for yW₃₇ formation (Ohira and Suzuki 2011). Furthermore, the requirement of Gm₃₄ and Cm₃₂ modification for yW modification of tRNA^{Phe} represents the first documented example in eukaryotes of a tRNA modification on one residue directing modification on a different residue, similar to an earlier report describing a tRNA modification network in *Thermus thermophilus* that is dependent on m⁷G (Tomikawa et al. 2010).

The Trm7 requirement for Trm732 to form Cm₃₂ and for Trm734 to form Nm₃₄ (Figs. 3, 5; Tables 1, 2) adds to an emerging theme of complexes that are required for apparently simple tRNA methylation reactions; in yeast, these include Trm6/Trm61 for formation of m¹A₅₈ (Anderson et al. 1998), Trm8/Trm82 for formation of m⁷G₄₆ (Alexandrov et al. 2002), Trm11/Trm112 for formation of m²G₁₀ (Purushothaman et al. 2005), and Trm9/Trm112 for the terminal methylation of mcm⁵U₃₄ (Studte et al. 2008). In the case of Trm7 methylation, it is clear both in vivo and in vitro that Trm732 and Trm734 impart specificity to Trm7 for methylation of the corresponding residues (Figs. 3, 5; Tables 1, 2), much as Pho85 kinase specificity is modulated by partner proteins (Dephoure et al. 2005; Huang et al. 2007). Indeed, the requirement for different Trm7 partners for each modification may be explained in part by the fact that the 2'-O-methyl groups of C₃₂ and G₃₄ are 12.6 Å apart in the tRNA^{Phe} crystal structure (PDB 1EHZ), with that of C₃₂ buried, and that of G₃₄ solvent exposed (Shi and Moore 2000). However, we

FIGURE 5. ORF YMR259c is required for formation of Cm₃₂ and Rtt10 is required for Gm₃₄, on substrate tRNA^{Phe} in vitro. (A) ORF YMR259c is required for formation of Cm on tRNA^{Phe} in yeast extracts. A total of 50 μg of crude extracts from the indicated strains were 10-fold serially diluted and assayed for Cm methyltransferase activity with in vitro-transcribed [α -³²P]CTP-labeled tRNA^{Phe}, and the products were digested and analyzed by thin layer chromatography as described in Materials and Methods. (B) Rtt10 is required for formation of Gm on tRNA^{Phe} in yeast extracts. Crude extracts were assayed for Gm methyltransferase activity using [α -³²P]GTP-labeled tRNA^{Phe} as described in A.

FIGURE 6. A *trm732-Δ trm734-Δ* mutant phenocopies the growth phenotypes of a *trm7-Δ* mutant. (A) Lack of both Cm₃₂ and Nm₃₄ are required for slow growth of *trm7-Δ* mutants. Strains were grown overnight at 30°C in YPD medium, adjusted to an OD₆₀₀ of ~0.5, and serially diluted 10-fold; then, 2 μL was spotted onto media containing 5-FOA and incubated for 2 d at 30°C. (B) Overexpression of tRNA^{Phe} suppresses the slow growth of *trm732-Δ trm734-Δ* mutants. Strains with plasmids as indicated were grown overnight in S-Leu medium containing raffinose and galactose and analyzed by spotting to S media containing 5-FOA, raffinose, and galactose, and incubated for 2 d at 30°C. (C) Trm7 is overproduced more than 100-fold when expressed from a [2μ P_{GAL}] plasmid. Soluble crude extract was prepared from indicated strains and then subjected to immunoblot analysis with anti-protein A antibody.

note that each subunit of the Trm7 complexes may also contribute in other ways, as shown for Trm6 and Trm61 variants in m¹A modification (Ozanick et al. 2007).

It is unclear why 2'-O-methylation of C₃₂ and G₃₄ of tRNA^{Phe} is critical for healthy growth of yeast. Since overexpression of tRNA^{Phe} suppresses the slow-growth phenotype of *trm7-Δ* mutants, but levels of charged tRNA^{Phe} appear normal (Fig. 1D), a critical defect of *trm7-Δ* mutants is likely at a step after charging, and at or before ribosome A-site decoding, since if the defect was in translocation, P-site interactions, or E-site interactions, additional tRNA^{Phe}

copies would not be expected to overcome the defect. In principle, Cm₃₂ and/or Gm₃₄ of tRNA^{Phe} might influence any step of tRNA^{Phe} function during the transfer of charged tRNA to EF-1A, during delivery to the ribosome, or at any translation step before peptide bond formation (Gromadski et al. 2007; Zaher and Green 2009). We note that since the *trm7-Δ* slow-growth phenotype is really a synthetic genetic interaction that requires loss of both Cm₃₂ and Nm₃₄ (and yW), and since each 2'-O-methylation has a distinct role, only one defect of *trm7-Δ* mutants needs to be corrected by overexpression of tRNA^{Phe} to overcome the slow-growth phenotype.

It is not immediately obvious how Cm₃₂ and Gm₃₄ contribute to tRNA^{Phe} function. Although 2'-O-methylation is known to stabilize the C3'-endo form of uridine (Kawai et al. 1992) and to increase stacking (Drake et al. 1974), it is unclear how these properties contribute to tRNA^{Phe} anticodon loop function. One possible role of Cm₃₂ in tRNA^{Phe} function could be to influence the interaction of Cm₃₂ with A₃₈. In a number of tRNA structures, including that of yeast tRNA^{Phe}, a bifurcated hydrogen bond is formed between O2 of C₃₂ (or U₃₂, or the spatially equivalent O4 of Ψ) and the exocyclic amine of N₃₈ (Auffinger and Westhof 1999). Furthermore, the identity of the N₃₂:N₃₈ pair influences codon:anticodon recognition in prokaryotes (Olejnickak et al. 2005) and affects translational fidelity (Ledoux et al. 2009; Murakami et al. 2009). Thus, it is possible that 2'-O-methylation of C₃₂ influences the C₃₂:A₃₈ pair to affect one or more of the steps of translation before peptide bond formation (Zaher and Green 2009).

One possible role of Gm₃₄ of tRNA^{Phe} might be to promote cognate codon:anticodon interactions in the yeast ribosome A-site, perhaps due to its improved stacking and pairing properties, although no specific role is ascribed to the 2'-oxygen of G₃₄ in the crystal structure of bacterial ribosomes complexed with the yeast anticodon stem-loop

FIGURE 7. A *trm7-Δ trm1-Δ* mutation is lethal primarily due to the lack of Nm₃₄ and m^{2,2}G₂₆. Strains with plasmids as indicated were grown overnight at 30°C in YPD medium, and analyzed as in Figure 6A.

FIGURE 8. Summary of the proteins required for 2'-O-methylation of the anticodon loop of yeast tRNA^{Phe}. A schematic of the anticodon loop of tRNA^{Phe} is shown, together with the proteins required for formation of Cm₃₂ and Gm₃₄. Arrows indicate that yW₃₇ formation requires Gm₃₄, and to a lesser extent Cm₃₂.

(Ogle et al. 2001). Gm₃₄ might also conceivably affect interactions of tRNA^{Phe} with its synthetase PheRS, since G₃₄ is a known determinant for charging (Sampson et al. 1989), and since completely unmodified tRNA^{Phe} is less efficiently charged than fully modified tRNA^{Phe} in vitro (Sampson and Uhlenbeck 1988), although our northern analysis indicates that levels of charged tRNA^{Phe} are nearly normal in *trm7-Δ* mutants (Fig. 1D).

It is unclear from our results how 2'-O-methylation of the anticodon loops of tRNA^{Leu(UAA)} and tRNA^{Trp} by Trm7 contributes to their function. Since overexpression of tRNA^{Phe} completely suppresses the slow growth phenotype of *trm7-Δ* mutants (Fig. 1B), 2'-O-methylation of tRNA^{Leu(UAA)} and tRNA^{Trp} is not measurably important under these growth conditions. Although it is possible that the Cm₃₂ and Nm₃₄ modifications of tRNA^{Leu(UAA)} and tRNA^{Trp} have an important role under other conditions, it is also possible that these tRNAs are modified by Trm7 due to overlapping substrate specificity with tRNA^{Phe} rather than because of an inherent biological necessity, as has been speculated for tRNA modification phenotypes associated with the rapid tRNA decay pathway and the nuclear surveillance pathway (Phizicky and Alfonzo 2010), and appears to explain the multiple phenotypes associated with mutations in the ELP complex (Esberg et al. 2006; Chen et al. 2011).

It remains to be determined whether the other documented phenotypes of *trm734-Δ* mutants are related to tRNA biology. Since both *trm734-Δ* (*rtt10-Δ*) mutants and *trm7-Δ* mutants were identified in a screen for mutated genes that promote Ty1 transposition (Nyswaner et al. 2008), it is plausible that hypomodified tRNA causes a decrease in expression of specific Phe-rich genes important for Ty1 transposition. Trm734 (Rtt10, Ere2) was also recently identified as having a role in retromer-dependent endoplasmic recycling, because *trm734-Δ* (*rtt10-Δ*, *ere2-Δ*) mutants have increased canavanine resistance due to a defect in Can1 recycling (Shi et al. 2011). It is unclear at present whether the Trm734 2'-O-methylation function is responsible for this phenotype, or if this phenotype represents a second role of Trm734.

It also remains to be determined the extent to which the Trm7 anticodon loop modification circuitry described here is conserved in other eukaryotes and the extent to which Trm7 modifications are important for tRNA^{Phe} or other tRNAs in other eukaryotes. Available evidence suggests that Trm7 and Trm734 are widely conserved in eukaryotes and that Trm734 contains WD40 repeats (Feder et al. 2003; Shi et al. 2011), and our BLAST (<http://blast.ncbi.nlm.nih.gov/>) and InterProScan (<http://ebi.ac.uk/Tools/pfa/iprscan/>) analyses indicate that Trm732 is widely conserved, contains a DUF2428 domain (domain of unknown function), and contains Armadillo repeats (data not shown). Since both WD40 repeats (Stirnemann et al. 2010) and Armadillo repeats (Tewari et al. 2010) are typically found in scaffolding proteins, it seems plausible that these domains are important for interactions with Trm7 in eukaryotes. In addition, 16 of 17 sequenced eukaryotic tRNA^{Phe} species are known to have Cm₃₂ and Gm₃₄, all five sequenced eukaryotic tRNA^{Trp} species have Cm₃₂ and Cm₃₄ (Juhling et al. 2009), and a substantial portion of the tRNA^{Phe} from neuroblastoma cells and Ehrlich ascites tumors lacks Cm₃₂ and Gm₃₄, and has m¹G₃₇ instead of yW₃₇ (Kuchino et al. 1982). Furthermore, high-throughput analysis suggests that the putative *Schizosaccharomyces pombe* *TRM7* gene is essential (Kim et al. 2010), and numerous studies implicate the putative human homolog *FTSJ1* in nonsyndromic X-linked mental retardation (Freude et al. 2004; Ramser et al. 2004; Froyen et al. 2007; Takano et al. 2008). Based on these observations, it is tempting to speculate that the circuitry for Trm7 modification of the anticodon loop is conserved and that these modifications are widely important for tRNA^{Phe} function.

MATERIALS AND METHODS

Yeast strains

Yeast strains are listed in Table 3. The BY4741 *trm7-Δ::ble^R* [2μ URA3 P_{GAL}TRM7-MORF] strain (yMG78-2) and the *trm7-Δ::ble^R* [TRM7 URA3 CEN] strain (yMG348-1) were constructed by standard methods (Chernyakov et al. 2008). All double-mutant *trm7-Δ* strains were constructed by PCR amplification of DNA from the appropriate YKO collection *kanMX* strain (Open Biosystems), followed by transformation of the DNA into a *trm7-Δ::ble^R* [URA3 TRM7] strain. Other double-mutant strains were constructed similarly. C-terminally tagged ORF-9myc strains were generated by transformation of parent strain BCY123 with a gene-specific PCR product of the appropriate fragment of the pYM18 9myc::kanMX cassette (Janke et al. 2004). C-terminally tagged ORF-cMORF strains (tagged with a cassette comprised of a His₆-HA-3C site-ZZ domain of protein A from the MORF collection) (Gelperin et al. 2005) were generated in a similar fashion from the cMORF::URA3 cassette of pAVA0258. All strain constructions were verified by PCR confirmation using appropriate oligonucleotides.

TABLE 3. Strains used in this study

Strain	Genotype	Source
BY4741	<i>MATa his3-Δ 1 leu2-Δ0 met15-Δ0 ura3-Δ0</i> (wild type)	Open Biosystems
yMG78-2	BY4741, <i>trm7-Δ::ble^R</i> [<i>2μ URA3 P_{GAL} TRM7-MORF</i>]	This study
yMG348-1	BY4741, <i>trm7-Δ::ble^R</i> [<i>CEN URA3 TRM7</i>]	This study
yMG105	BY4741, <i>trm7-Δ::ble^R</i>	This study
BCY123	<i>MATa pep4-Δ::HIS3 prb-Δ::LEU2 bar1-Δ::HISG</i> <i>lys2-Δ::GAL1/10-GAL-Δ4 can1 ade2 ura3 leu2-3, 112</i>	(Macbeth et al. 2005)
yMG744-1	BCY123, <i>RTT10-9myc::kanMX</i>	This study
yMG797-1	BCY123, <i>TRM7-HA-3C site-protein A::URA3</i>	This study
yMG798-1	BCY123, <i>TRM8-HA-3C site-protein A::URA3</i>	This study
yMG799-1	yMG744-1, <i>TRM7-HA-3C site-protein A::URA3</i>	This study
yMG800-1	yMG744-1, <i>TRM8-HA-3C site-protein A::URA3</i>	This study
yMG724-5	BY4741, <i>rtt10-Δ::ble^R</i>	This study
yMAB109	BY4741, <i>tyw1-Δ::kanMX</i>	This study
yMG814-1	BY4741, <i>YMR259c-Δ::ble^R</i>	This study
yMG857-1	BCY123, <i>YMR259c-9myc::kanMX</i>	This study
yMG843-1	yMG797-1, <i>YMR259c-9myc::kanMX</i>	This study
yMG858-1	yMG798-1, <i>YMR259c-9myc::kanMX</i>	This study
yMG839-1	BCY123, <i>YMR259c-HA-3C site-protein A::URA3</i>	This study
yMG859-1	yMG839-1, <i>RTT10-9myc::kanMX</i>	This study
yMG842-1	BCY123, <i>TRM7-9myc::kanMX</i>	This study
yMG885-1	yMG842-1, <i>YMR259c-HA-3C site-protein A::URA3</i>	This study
yMG861-4	yMG842-1, <i>RTT10-HA-3C site-protein A::URA3</i>	This study
yBP67A	yMG724-5, [<i>CEN URA3 RTT10</i>]	This study
yMG818-1	yBP67A, <i>YMR259c-Δ::kanMX</i>	This study
yMG349-1	BY4741, <i>trm7-Δ::ble^R, trm1-Δ::kanMX</i> [<i>TRM7 URA3 CEN</i>]	This study
yLN005	BY4741, <i>trm1-Δ::ble^R</i>	This study
yLN059	yLN005, [<i>CEN URA3 TRM1</i>]	This study
yMG819-2	yLN059, <i>YMR259c-Δ::kanMX</i>	This study
yBP48D	yLN059, <i>rtt10-Δ::kanMX</i>	This study
yBP155B	yMG814-1, [<i>CEN URA3 YMR259c</i>]	This study
yMG799-1	yBP155B, <i>tyw1-Δ::kanMX</i>	This study
yMG800-3	yLN059, <i>tyw1-Δ::kanMX</i>	This study
yMG956-1	yBP67A, <i>tyw1-Δ::kanMX</i>	This study

Plasmids

Plasmids used in this study are listed in Table 4. *CEN* plasmids were constructed by ligation-independent cloning (LIC) of appropriate DNA fragments into pAVA581 (*LEU2*) and pAVA579 (*URA3*) (Quartley et al. 2009). [*2μ LEU2* tRNA] expression plasmids were constructed by insertion of the appropriate tRNA sequence into the *XhoI* *BglII* site of a tRNA expression plasmid (pMAB813A), which harbors a *BamHI* fragment containing the tRNA^{His(GUG)} gene (tH(GUG)G2) and flanking sequences, with a *XhoI* site 22 bp 5' of the +1 site of the mature tRNA sequence, and a *BglII* site 21 bp 3' of residue 73. Dual tRNA expression plasmids were constructed by PCR amplification of the appropriate tRNA fragment and ligation into the multicloning site of the [*2μ LEU2* tRNA^{Phe}] plasmid (pMG16A) derived from pMAB813A. Plasmids for ORF expression in yeast are [*2μ P_{GALI,10}*] dual ORF expression vectors, designed to express ORFs under *P_{GALI}* control with a C-terminal PT tag (ORF-3C site-HA epitope-His₆-ZZ domain of protein A), cloned by LIC after digestion with *BbrP1* and *Pacl*, and to express ORFs under *P_{GALI10}* control with no tag, cloned by LIC after digestion with *SwaI*, essentially as described previously (Quartley et al. 2009). All plasmids were confirmed by sequencing before use.

Northern blot analysis

Yeast strains were grown to an OD₆₀₀ of ~1 and RNA was prepared using the hot phenol method, or under acidic conditions to preserve aminoacylation, and RNA was then analyzed by Northern blot as previously described (Alexandrov et al. 2006) using appropriate probes.

Growth and lysis of strains for biochemical analysis

Yeast strains were grown in YPD to an OD₆₀₀ of ~2, or if they harbored a [*2μ P_{GALI,10}*] expression plasmid, were grown in S dropout media containing raffinose and galactose to an OD₆₀₀ of ~1. Yeast crude extracts were prepared by bead beating in the presence of protease inhibitors as described previously (Quartley et al. 2009).

Affinity purification of MORF-tagged proteins

cMORF-tagged proteins were purified by affinity purification using IgG sepharose, elution with GST-3C protease, and then removal of the protease with glutathione sepharose resin as described previously (Quartley et al. 2009).

TABLE 4. Plasmids used in this study

Plasmid	Parent	Description	Source
pMG26A		2 μ <i>URA3</i> P _{GAL} <i>TRM7-MORF</i>	(Gelperin et al. 2005)
pug66		<i>bIe^R</i> cassette	(Gueldener et al. 2002)
pAVA581		<i>CEN LEU2</i> LIC	(Quartley et al. 2009)
pMG12	pAVA581	<i>CEN LEU2 TRM7</i>	This study
pMAB813A		2 μ <i>LEU2</i> tRNA ^{His} (GUG)	This study
pMG16A	pMAB813A	2 μ <i>LEU2</i> tRNA ^{Phe} (GAA)	This study
pMG18B	pMAB813A	2 μ <i>LEU2</i> tRNA ^{Trp} (CCA)	This study
pMG24A	pMAB813A	2 μ <i>LEU2</i> tRNA ^{Leu} (UAA)	This study
pAVA579		<i>CEN URA3</i> LIC	(Quartley et al. 2009)
pMG13	pAVA579	<i>CEN URA3 TRM7</i>	This study
pMG100A	pMG16A	2 μ <i>LEU2</i> tRNA ^{Phe} (GAA) tRNA ^{Trp} (CCA)	This study
pMG101A	pMG16A	2 μ <i>LEU2</i> tRNA ^{Phe} (GAA) tRNA ^{Leu} (UAA)	This study
pMG102A	pMG16A	2 μ <i>LEU2</i> tRNA ^{Phe} (GAA) tRNA ^{His} (GUG)	This study
pAVA0258		HA-3C site-protein A:: <i>URA3</i> cassette	(Gelperin et al. 2005)
pYM18		9myc <i>kanMX</i> cassette	(Janke et al. 2004)
pBG2619		2 μ <i>LEU2</i> P _{GAL1,10} LIC	(Quartley et al. 2009)
pBP2A	pAVA579	<i>CEN URA3 RTT10</i>	This study
pELN001	pAVA579	<i>CEN URA3 TRM1</i>	This study
pMG240A	pBG2619	2 μ <i>LEU2</i> P _{GAL10} <i>TRM7</i>	This study

tetraacetic acid (EDTA), and emission from 350 to 550 nm was measured in 1-nm increments after excitation at 320 nm, and plotted after subtracting the spectrum from tRNA^{Trp}.

In vitro transcription and methyltransferase assays

Plasmids containing tRNA^{Phe} (pEMP1577) and tRNA^{Thr}(IGU) (pEMP1568) were digested with BstN1 and transcribed with T7 polymerase in the presence of 50 μ Ci [α -³²P]CTP or [α -³²P]GTP (3000 Ci/mmol, Perkin Elmer), and tRNA was purified by PAGE and assayed for methyltransferase activity with crude extract, followed by P1 nuclease digestion (Jackman et al. 2003), and resolution of nucleotides by cellulose thin-layer chromatography developed in isobutyric acid:ammonia:H₂O (66:1:33).

Immunoblot analysis

Yeast crude extracts and affinity-purified samples were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membrane (Bio-Rad), and probed with the appropriate antibodies. The 9myc tag was detected with mouse monoclonal anti-[c-myc] (Roche), followed by incubation with goat anti-mouse IgG-HRP (Bio-Rad), and visualization with Amersham ECL Plus (GE Healthcare). MORF-tagged constructs were detected with rabbit polyclonal anti-protein A (Sigma), followed by goat anti-rabbit IgG-HRP (Bio-Rad), and visualization.

Isolation and purification of tRNA

Yeast strains were grown at 30°C in YPD to mid-log phase. Bulk low-molecular weight RNA was extracted from 300 OD pellets, and the appropriate 5' biotinylated oligonucleotides were used to purify tRNA as previously described (Jackman et al. 2003).

HPLC analysis of tRNA

Purified tRNA from yeast was digested with P1 nuclease and phosphatase as previously described (Jackman et al. 2003), and nucleosides were subjected to HPLC analysis essentially as previously described (Jackman et al. 2003) for tRNA^{Leu}(UAA) and tRNA^{Trp}. tRNA^{Phe} was analyzed similarly, except that the HPLC buffers were at pH 7.0 and the gradients were adjusted to a maximize separation of Gm and m¹G. At a flow rate of 0.75 mL/min, the gradient was as follows: 100% buffer A (10 mM (NH₄)H₂PO₄, 2.5% methanol) for 14.4 min; a gradient to achieve 10% buffer B (10mM (NH₄)H₂PO₄, 20% methanol) at 24 min; and a gradient to achieve 25% buffer B at 45 min.

Detection of wye base by fluorescence

tRNA^{Phe} purified from appropriate strains was diluted to 10 μ g/mL in 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediamine-

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