Identification of a Trm732 Motif Required for 2′-O-methylation of the tRNA Anticodon Loop by Trm7

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ABSTRACT: Posttranscriptional tRNA modifications are essential for proper gene expression, and defects in the enzymes that perform tRNA modifications are associated with numerous human disorders. Throughout eukaryotes, 2′-O-methylation of residues 32 and 34 of the anticodon loop of tRNA is important for proper translation, and in humans, a lack of these modifications results in non-syndromic X-linked intellectual disability. In yeast, the methyltransferase Trm7 forms a complex with Trm732 to 2′-O-methylate tRNA residue 32 and with Trm734 to 2′-O-methylate tRNA residue 34. Trm732 and Trm734 are required for the methylation activity of Trm7, but the role of these auxiliary proteins is not clear. Additionally, Trm732 and Trm734 homologs are implicated in biological processes not directly related to translation, suggesting that these proteins may have additional cellular functions. To identify critical amino acids in Trm732, we directly related to translation, suggesting that these proteins may have additional cellular functions. To identify critical amino acids in Trm732, we generated variants and tested their ability to function in yeast cells. We identified a conserved RRSAGLP motif in the conserved DUF2428 domain of Trm732 that is required for tRNA modification activity by both yeast Trm732 and its human homolog, THADA. The identification of Trm732 variants that lack tRNA modification activity will help to determine if other biological functions ascribed to Trm732 and THADA are directly due to tRNA modification or to secondary effects due to other functions of these proteins.

INTRODUCTION

tRNA from all organisms is extensively modified.1 These modifications are required for proper tRNA function and translation and therefore play an important role in gene expression. In the yeast Saccharomyces cerevisiae, a lack of cytoplasmic tRNA modifications causes varied phenotypes including slow growth, temperature sensitivity, and lethality.2,3 Likewise, defects in cytoplasmic tRNA modifications cause human neurological disorders, including familial dysautonomy4–6 and numerous types of intellectual disability (ID), often with accompanying disease phenotypes.7–23 Moreover, genes encoding tRNA modification enzymes or predicted modification enzymes have been linked to other diseases, including many mitochondrial disorders24,25 and cancer.26 Furthermore, modifications have also been shown to play a role in stem cell function,27–29 response to cellular stress,30–32 and host/pathogen interactions,33–36 among others.

One of the most common posttranscriptional tRNA modifications is 2′-O-methylation,37 which is found on residues 4, 18, 32, and 44 of certain yeast tRNAs.1 In yeast, 2′-O-methylation of residues 32 (Nm32) and 34 (Nm34) requires the methyltransferase Trm7.38 Lack of both Cm32 and Gm34 on tRNAile in trm7Δ mutants causes slow growth in both S. cerevisiae and Schizosaccharomyces pombe cells.38–40 The exact cause of this defect is not entirely clear. S. cerevisiae trm7Δ mutants grown in minimal media have a charging defect, but this defect is not observed in S. cerevisiae trm7Δ mutants grown in rich media nor in S. pombe trm7Δ mutants.41 In trm7Δ mutants from both yeast species, the general amino acid control (GAAC) pathway is constitutively active,41 suggesting that lack of Nm32 and Nm34 leads to translational stalling and ribosome collisions.42,43 Lack of these modifications also results in loss of wybutosine (yW) formation at the 1-methylguanosine residue found at position 37 (m1G37) on tRNAile.40

In humans, defects in Nm32 and Nm34 caused by mutation of the human TRM7 ortholog FTSJ1 cause non-syndromic X-linked ID (NSXLID).14,16 Human cell lines lacking FTSJ1 exhibit a growth defect that is exacerbated in the presence of...
the translation inhibitor paromomycin,\textsuperscript{41} and are more sensitive to vaccinia virus infection.\textsuperscript{56,42} Mice lacking FTSJ1 show impaired learning, anxiety-like behavior, increased sensitivity to pain, metabolic differences, and other phenotypes.\textsuperscript{46,57} The identity of the hypomethylated tRNA(s) that causes these phenotypes in humans and mice lacking FTSJ1 is likely tRNA\textsuperscript{Phe} because loss of FTSJ1 causes a reduction in steady-state levels of tRNA\textsuperscript{Phe} in the brains of mice\textsuperscript{47} and because decoding of Phe codons, and in particular UUU, is perturbed in both mice and in cultured human cells.\textsuperscript{44,47} Interestingly, in Drosophila melanogaster, there are two Trm7/FTSJ1 paralogs, one of which modifies position 32 on substrate tRNAs and the other modifies position 34 on substrates. Flies lacking these tRNA modification genes showed a decreased size and lifespan and a decrease in defense against the Drosophila C virus, and their tRNA\textsuperscript{Phe} lacking Gm\textsubscript{34} was susceptible to fragmentation after heat shock.\textsuperscript{48}

In the yeasts S. cerevisiae and S. pombe, Trm7 forms a complex with the protein Trm732 to form Cm\textsubscript{32} and a complex with the protein Trm734 to formNm\textsubscript{34} on tRNA (Figure 1).\textsuperscript{39,40} These partner proteins are required for Trm7 activity because lack of Trm732 causes complete loss of Nm\textsubscript{34}, and lack of Trm734 causes complete loss of Nm\textsubscript{34}.\textsuperscript{39,40} Trm7 forms distinct complexes with each protein, suggesting that the role of each is to direct Trm7 to a given nucleotide target.\textsuperscript{39,49} Trm732 is an armadillo repeat protein which contains a DUF2428 domain (domain of unknown function),\textsuperscript{40} whereas Trm734 is a WD\textsubscript{40} protein.\textsuperscript{39,50}

In humans and other multicellular eukaryotes, Trm732 and Trm734 orthologs are also involved in 2’-O-methylation of the anticodon loop of tRNA\textsuperscript{Phe} in yeast. In yeast, the Trm7–Trm732 complex forms Cm\textsubscript{32} on tRNA\textsuperscript{Phe}, and the Trm7–Trm734 complex forms Gm\textsubscript{34}.

Figure 1. Schematic of 2’-O-methylation of the anticodon loop of tRNA\textsuperscript{Phe} in yeast. In yeast, the Trm7–Trm732 complex forms Cm\textsubscript{32} on tRNA\textsuperscript{Phe}, and the Trm7–Trm734 complex forms Gm\textsubscript{34}.

in tRNA methylation are not known, the analysis of the recently solved crystal structure of the yeast Trm7–Trm734 complex suggests that Trm734 is required to correctly position the substrate tRNA onto the Trm7–Trm734 enzyme.\textsuperscript{49} Moreover, human WDR6 by itself and the FTSJ1–WDR6 complex bind tRNA, whereas FTSJ1 alone does not, further indicating that WDR6 functions in tRNA binding.\textsuperscript{44}

THADA and WDR6 are also implicated in several biological processes not obviously related to tRNA modification. THADA was first identified as being associated with thyroid adenomas\textsuperscript{51} and has been shown to be involved in thermogenesis in D. melanogaster,\textsuperscript{52} and in cold resistance in the model plant Arabidopsis thaliana.\textsuperscript{53} A recent report also proposed a role for THADA as a regulator of programmed death-ligand 1 (PD-L1) maturation.\textsuperscript{54} A genome wide association study (GWAS) also suggested that THADA plays a role in cold adaptation in humans.\textsuperscript{55} Other GWAS analyses have implicated single nucleotide polymorphisms (SNPs) of THADA in polycystic ovary syndrome (PCOS),\textsuperscript{56} prostate cancer,\textsuperscript{57,58} and type 2 diabetes.\textsuperscript{59–62} Although WDR6 has not been implicated in human diseases, it was recently identified with FTSJ1 as a host range restriction factor for a mutant vaccinia virus,\textsuperscript{63,45} suggesting a possible role for WDR6 in host defense. Because none of these studies involving THADA or WDR6 in higher eukaryotes has included a tRNA modification analysis, it is not clear whether these additional biological roles are due to tRNA modification activity or other bona fide functions of the proteins.

To further understand the role of Trm732 in the Trm7 methyltransferase reaction, we sought to identify regions of this protein important for 2’-O-methylation of tRNA in yeast. We report the identification of an important motif in Trm732 that is required for tRNA modification activity. This motif is also required for the activity of human THADA. Our identification of residues required for Trm732/THADA activity should allow for experiments to determine whether the roles of this protein in diverse biological processes are dependent on tRNA modification activity or on other functions of the protein.

\section*{RESULTS}

A Conserved Motif in the DUF2428 Domain of Trm732 is Required for Cm\textsubscript{32} Modification on tRNA\textsuperscript{Phe}.

To study the role of Trm732 in formation of the Cm\textsubscript{32} modification, we sought to identify amino acid residues important for Trm732 function. Trm732 proteins are large and consist of armadillo repeats (Figure 2A), with the S. cerevisiae protein containing 1420 amino acids, including 312 conserved amino acids. The largest stretch of amino acid conservation among Trm732 proteins that may be required for tRNA modification activity or other bona fide functions of the proteins.

Figure 2A. A Conserved Motif in the DUF2428 Domain of Trm732 is Required for Cm\textsubscript{32} Modification on tRNA\textsuperscript{Phe}. To study the role of Trm732 in formation of the Cm\textsubscript{32} modification, we sought to identify amino acid residues important for Trm732 function. Trm732 proteins are large and consist of armadillo repeats (Figure 2A), with the S. cerevisiae protein containing 1420 amino acids, including 312 conserved amino acids comprising the DUF2428 domain. There is little detectable sequence homology among Trm732 proteins, except for the DUF2428 domain and small regions near the C-terminus. Even the DUF2428 domain, which has the highest amount of conservation, is only around 30% identical between the human and S. cerevisiae proteins.\textsuperscript{39} To identify regions of conservation among Trm732 proteins that may be required for tRNA modification activity, we performed an amino acid alignment with S. cerevisiae and S. pombe Trm732, human THADA, and five other putative Trm732 proteins from divergent eukaryotic species. We identified three motifs of conserved amino acids. The largest stretch of amino acid similarity was found in motif 2, comprising residues 748–754 in the DUF2428 domain of S. cerevisiae Trm732 with a strong consensus sequence of RRSAGLP (Figure 2A).
To test the requirement of these motifs for Trm732 function, we generated variants that replaced conserved amino acids with alanine residues, expressed them in yeast from a low-copy CEN plasmid, and tested their ability to form Cm₃₂ on tRNAPhe. First, we tested whether Trm732 variants with amino acid changes in each motif could rescue the slow growth of trm₇₃₂Δ trm₇₃₄Δ double-mutant cells. We used the trm₇₃₂Δ trm₇₃₄Δ double mutant because trm₇₃₂Δ single mutants do not have an obvious growth phenotype. Expression of functional Trm732 proteins in the double-mutant strain should lead to normal levels of Cm₃₂ on tRNAPhe, resulting in a tRNAPhe anticodon loop modification profile identical to the healthy growing trm₇₃₄Δ strain. We therefore transformed plasmids expressing Trm732 variants into a trm₇₃₂Δ trm₇₃₄Δ [TRM734 URA3] strain and then tested growth after plating on media containing 5-fluoroorotic acid (5-FOA) to select against the URA3 plasmid. Cells were grown for 2 days at 30 °C. (C) Conserved amino acids in Trm732 are required for Cm₃₂ formation on tRNA^{Phe} in yeast. Quantification of nucleosides by UPLC from tRNA^{Phe} purified from indicated yeast strains, (*) levels below the threshold of detection.

Figure 2. Motif 2 of Trm732 is required for Cm₃₂ formation on tRNAPhe. (A) Schematic representation of the Trm732 sequence. Inset box is an amino acid alignment of regions of high sequence similarity between Trm732 proteins from eight eukaryotes. Arrows point to the amino acids changed in Trm732 variants tested in this study. (B) Several conserved amino acids in Trm732 are required for suppression of the slow growth of trm₇₃₂Δ trm₇₃₄Δ mutants. Indicated strains containing URA3 and LEU2 plasmids were grown overnight in SD − Leu medium, diluted to an OD₆₀₀ of ~0.5, serially diluted 10-fold, and then spotted on medium containing 5-FOA to select against the URA3 plasmid. Cells were grown for 2 days at 30 °C. (C) Conserved amino acids in Trm732 are required for Cm₃₂ formation on tRNAPhe in yeast. Quantification of nucleosides by UPLC from tRNA^{Phe} purified from indicated yeast strains, (*) levels below the threshold of detection.
(Figure 2B). In contrast, trm732Δ trm734Δ mutants expressing the motif 2 variants Trm732-RRS750AAA-GLP754AAA double variant grew as poorly as cells expressing only a vector (Figure 2B), further demonstrating the importance of motif 2 for modification activity. To verify that the TRM732 genes were transcribed, we performed quantitative real-time PCR (qRT-PCR) and found that mRNA was expressed for each gene construct (Table 1). Thus, loss of complementation is likely due to loss of Trm732 function, although we note the possibility that it could be due to loss of protein stability.

Table 1. Relative mRNA Levels of Mutant TRM732 Genes

| strain       | plasmid        | relative level |
|--------------|----------------|----------------|
| wild type    | vec            | 1.00 ± 0.14    |
| trm732Δ      | vec            | 0.996 ± 0.03   |
| trm732Δ      | TRM732         | 3.75 ± 0.57    |
| trm732Δ      | TRM732-RRS750AAA| 3.12 ± 0.16    |
| trm732Δ      | TRM732-GLP754AAA| 2.94 ± 0.28    |
| trm732Δ      | TRM732-HG754AAA| 3.25 ± 0.82    |
| trm732Δ      | TRM732-RH754AAA| 3.86 ± 0.43    |
| trm732Δ      | TRM732-RS750AAA, GLP754AAA| 3.70 ± 0.58    |
| trm732Δ      | TRM732-R748A   | 4.44 ± 0.68    |
| trm732Δ      | TRM732-S750A   | 2.88 ± 0.08    |
| trm732Δ      | TRM732-G752A   | 2.47 ± 0.29    |
| trm732Δ      | TRM732-L753A   | 2.91 ± 0.09    |
| trm732Δ      | TRM732-P754A   | 4.18 ± 2.43    |

Relative to TRM732 in wild-type cells after normalization to ACT1. Values are from three independent growths.

To determine if the inability of Trm732 motif 2 variants to rescue the slow growth of the trm732Δ trm734Δ strain was due to loss of Cm32 activity, we purified tRNA32 from a trm732Δ single mutant expressing Trm732 variants and analyzed the nucleoside content by ultra-pressure liquid chromatography (UPLC). As expected, tRNA32 from trm732Δ strains expressing wild-type Trm732 had levels of Cm similar to those from a wild-type strain, whereas trm732Δ strains without a Trm732 expression plasmid had no detectable Cm (Figure 2C). We found that tRNA32 from trm732Δ strains expressing motif 2 variants Trm732-RRS750AAA and Trm732-GLP754AAA had severely reduced levels of Cm, and strains expressing the motif 2 double-variant Trm732-RRS750AAA-GLP754AAA had even less Cm. In contrast, tRNA32 from trm732Δ strains expressing motif 1 or motif 3 variants had relatively high levels of Cm, nearly as high as those found on tRNA32 from trm732Δ strains expressing wild-type Trm732 (Figure 2C).

As expected, because we did these experiments in trm732Δ single mutants, which express Trm734, levels of Cm on tRNA32 from these strains were similar to those from tRNA32 from a wild-type strain regardless of the Trm732 plasmid expressed. Small, but detectable, levels of mG were observed on tRNA32 from trm732Δ strains expressing an empty vector or expressing Trm732 motif 2 variants (Figure 2C). The presence of mG on tRNA32 in certain mutants is most likely due to a defect in ywW7 formation because mG is the precursor to ywW7, and trm732Δ mutants have been shown previously to have a defect in ywW levels. Thus, the defects in Cm32 formation in mutants expressing Trm732 variants cause decreased yW37 formation, resulting in detection of mG. Levels of 2-methylguanosine (m2G) on tRNA32 from each strain were similar, as expected for a control modification that is not formed or influenced by Trm7 (Figure 2C). Overall, these results demonstrate that for the Trm732 variants tested, a lack of Cm levels on tRNA32 corresponded with an inability to rescue the slow growth of a trm732Δ trm734Δ strain and that motif 2 is required for Trm732 tRNA modification activity.

To further determine which individual residues of motif 2 are most important for Trm732 tRNA modification activity, we generated 5 of 6 possible single amino acid variants and tested their ability to rescue the slow growth of the trm732Δ trm734Δ strain. We found that expression of four of these single mutant variants tested suppressed the growth defect of the trm732Δ trm734Δ strain, with the Trm732-R748A variant showing a significant, reproducible suppression defect, especially at 25 °C (Figure 3). Indeed, trm732Δ trm734Δ
In contrast to what THADA rescued the slow growth of the double mutant media with 5-FOA. As expected, we found that wild-type the slow growth of the THADA severely impaired the ability of the protein to rescue function, although we note as above that amino acid changes in RH1105AA) only partially rescued the slow growth of the yeast Trm732, expression of human trm734 and then incubated for 3 days at 30 °C. Therefore, we generated the S medium containing ra

Figure 4. Human THADA requires motif 2 for complementation of the yeast trm732Δ mutant. Indicated strains were grown overnight in the S medium containing raffinose, galactose, and 5-FOA; spotted to medium containing raffinose, galactose, and 5-FOA; and then incubated for 3 days at 30 °C.

THADA severely impaired the ability of the protein to rescue the slow growth of the trm732Δ trm734Δ mutants (THADA-RRS1161AAA and THADA-GIP1165AAA). In contrast to what we observed for the yeast Trm732, expression of human THADA with amino acid changes in motif 1 (THADA-RH1105AAA) only partially rescued the slow growth of the trm732Δ trm734Δ mutant (Figure 4). To verify that the THADA genes were expressed, we performed qRT-PCR and found that mRNA was expressed for each gene construct (Table 3). Because THADA expression has previously been shown to restore Cm32 to tRNA\textsubscript{Phe} in yeast lacking TRM732,\textsuperscript{39} these results strongly suggest that failure of THADA variants to restore growth is due to the lack of Cm32 formation on tRNA\textsubscript{Phe}. Partial rescue by a motif 1 variant may be due to the fact that the analysis of THADA variants in yeast is a more sensitive assay than the analysis of yeast Trm732 variants. We conclude that the importance of motif 2 is conserved in both Trm732/THADA, likely similarly affecting THADA protein function, although we note as above that amino acid changes in

| strain | TRM732 plasmid | generation time (min) |
|--------|----------------|------------------------|
| wild type | vec | 206 ± 12 |
| trm732Δ trm734Δ | vec | 484 ± 19 |
| trm732Δ trm734Δ | wild type | 210 ± 6 |
| trm732Δ trm734Δ | RRS1161AAA | 444 ± 31 |
| trm732Δ trm734Δ | S750A | 289 ± 29 |
| trm732Δ trm734Δ | S750A | 217 ± 7 |

"Mean and standard deviations based on growth from three separate colonies.

were not required for yeast Trm732 activity, we also tested a THADA motif 1 variant. We previously showed that expression of human THADA from a high copy plasmid rescues the slow growth of the yeast trm732Δ trm734Δ mutant by forming Cm on tRNA\textsubscript{Phe}.\textsuperscript{39} Therefore, we generated variants of full-length isoform A of human THADA on high copy (2μ) expression plasmids under control of the PGAL promoter and tested their ability to rescue the trm732Δ trm734Δ [TRM734 URA3] strain when plated on galactose media with 5-FOA. As expected, we found that wild-type THADA rescued the slow growth of the double mutant (Figure 4). Change of conserved amino acids in motif 2 of

Table 3. Relative mRNA Levels of Mutant THADA Genes Expressed in Yeast

| strain | plasmid | relative level |
|--------|---------|---------------|
| trm732Δ trm734Δ | THADA | 34.4 ± 8.8 |
| trm732Δ trm734Δ | vec | <0.01 |
| trm732Δ trm734Δ | TRM734Δ | 52.0 ± 11.0 |
| trm732Δ trm734Δ | TRM734Δ | 41.7 ± 4.8 |
| trm732Δ trm734Δ | TRM734Δ | 40.3 ± 1.9 |

"Relative to S. cerevisiae TUB1 after normalization of both genes to ACT1. Values are from three independent growths.

THADA could cause loss of protein stability, thereby causing loss of complementation by the variants in yeast.

**DISCUSSION**

In this study, we have identified an amino acid motif in yeast Trm732 that is required for Trm7-dependent formation of Cm32 on tRNA\textsubscript{Phe} and have shown that this same motif is required for the activity of the human Trm732 ortholog THADA. Substitution of the RRSAGLP754 residues of motif 2 of yeast Trm732 with alanine residues resulted in a nearly complete lack of Cm modification on tRNA\textsubscript{Phe} (Figure 2) and a growth defect similar to a vector control in trm732Δ trm734Δ strains expressing the variant (Figure 2). Replacing either RRS750 or GLP754 residues in this motif with alanine residues also resulted in a significant loss of modification activity (Figure 2), indicating that both of these stretches of amino acids are important for modification activity. Our finding that Trm732 variants with individual amino acid substitutions complemented, or mostly complemented, the growth defect of the trm732Δ trm734Δ mutant (Figure 3) suggests that none of the residues we tested are directly involved in catalysis but rather involved in other functions such as protein/tRNA interaction or are required for protein/protein interactions.

Motif 2 is highly conserved in Trm732 proteins throughout eukaryotes (Figure 2), and changes in residues of this motif in THADA also resulted in loss of activity (Figure 4). Because the structure of the Trm7–Trm732 complex has not been solved, the role of Trm732 motif 2 amino acid residues for the catalytic activity. Our results also shed some light on the levels of 2′-O-methylation in the anticodon loop required for proper growth in yeast. Somewhat surprisingly, we found that low levels of Cm32 on tRNA\textsubscript{Phe} led to detectable rescue of slow growth. For instance, tRNA\textsubscript{Phe} from trm732Δ mutants expressing the Trm732 RRS750 and GLP754 variants had levels of Cm32 approximately 10% of that from trm732Δ mutants expressing wild-type Trm732 (Figure 2), but trm732Δ cells expressing these variants still showed a detectable improvement in growth over cells expressing an empty vector (Figure 2). The role of
Nm13 on tRNA is not known, but our results suggest that it has an important role in S. cerevisiae because low levels of this modification fix some of the growth defects of the trn732Δ trn734Δ mutants. We note that Cm13 also likely has a role in S. pombe based on the severe growth defect of S. pombe trn7Δ mutants and the more mild growth defect of S. pombe trn734Δ mutants.

Our results and other recent findings further support the idea that the primary function of Trm732 and Trm734 and their orthologs in other eukaryotes is likely tRNA modification. The role of THADA in Nm32 formation in multicellular eukaryotes has not been established, although the ability of human THADA to complement yeast trn732Δ trn734Δ mutants and the more mild growth defect of human THADA to complement yeast trn732Δ trn734Δ mutants and the more mild growth defect of human THADA to complement yeast trn732Δ trn734Δ mutants and the more mild growth defect of human THADA to complement yeast trn732Δ trn734Δ mutants.

Table 5. Plasmids Used in This Study

| plasmid       | parent          | description                  | source |
|---------------|-----------------|------------------------------|--------|
| pBP2A         | CEN URA3 TRM732 | ref 40                       |        |
| pAVAS81       | CEN LEU2 LIC    | ref 64                       |        |
| pMG586        | CEN LEU2 TRM732 | this study                   |        |
| pMG581        | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG582        | CEN LEU2 TRM732-GLP555AAA | this study |        |
| pMG584        | CEN LEU2 TRM732-HG555AAA | this study |        |
| pMG585        | CEN LEU2 TRM732-RH555AAA | this study |        |
| pMG619A       | CEN LEU2       | this study                   |        |
| pMG739C       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG741B       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG742E       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG743A       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG744E       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG245A       | 2μ LEU2 Pgal THADA | ref 39                      |        |
| pMG643A       | 2μ LEU2 Pgal THADA | ref 39                      |        |
| pMG644        | 2μ LEU2 Pgal THADA | ref 39                      |        |
| pMG645A       | 2μ LEU2 Pgal THADA | ref 39                      |        |

Techniques, as described previously. Plasmids are listed in Table 5. The CEN LEU2 TRM732 expression plasmid was constructed by ligation-independent cloning (LIC) into pAVAS81.64 Plasmids expressing Trm732 and full-length human isoform A THADA variants were generated by QuickChange PCR (Stratagene) or QS site-directed mutagenesis (New England Biolabs). All plasmids were confirmed by sequencing prior to use.

Isolation of RNA from Yeast Cells. S. cerevisiae trn732Δ trn734Δ strains harboring CEN plasmids expressing Trm732 variants were grown in liquid dropout media to an OD of ~2. RNA was extracted using the hot phenol method.65 After purification, tRNA from yeast was analyzed by UPLC using a 50 mm HSS T3 column with a 1.8 μm particle size. The buffer system consisted of buffer A (5 mM NaOAc pH 7.1 + 0.1% Acetonitrile) and buffer B (60% ACN). At a flow rate of 0.46 mL/min, the gradient was as follows: 98% buffer A for 8.92 min, a gradient to achieve 10% buffer B at 15.45 min, and a gradient to achieve 25% buffer B at 29.73 min, followed by 100% buffer B for 2 min.

Methods

Yeast Strains and Plasmids. Yeast strains are listed in Table 4. All yeast strains were constructed using standard techniques, as described previously.60 Plasmids are listed in Table 5. The CEN LEU2 TRM732 expression plasmid was constructed by ligation-independent cloning (LIC) into pAVAS81.64 Plasmids expressing Trm732 and full-length human isoform A THADA variants were generated by QuickChange PCR (Stratagene) or QS site-directed mutagenesis (New England Biolabs). All plasmids were confirmed by sequencing prior to use.

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| pMG582        | CEN LEU2 TRM732-GLP555AAA | this study |        |
| pMG584        | CEN LEU2 TRM732-HG555AAA | this study |        |
| pMG585        | CEN LEU2 TRM732-RH555AAA | this study |        |
| pMG619A       | CEN LEU2       | this study                   |        |
| pMG739C       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG741B       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG742E       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG743A       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG744E       | CEN LEU2 TRM732-RRS750AAA | this study |        |
| pMG245A       | 2μ LEU2 Pgal THADA | ref 39                      |        |
| pMG643A       | 2μ LEU2 Pgal THADA | ref 39                      |        |
| pMG644        | 2μ LEU2 Pgal THADA | ref 39                      |        |
| pMG645A       | 2μ LEU2 Pgal THADA | ref 39                      |        |

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