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Article

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

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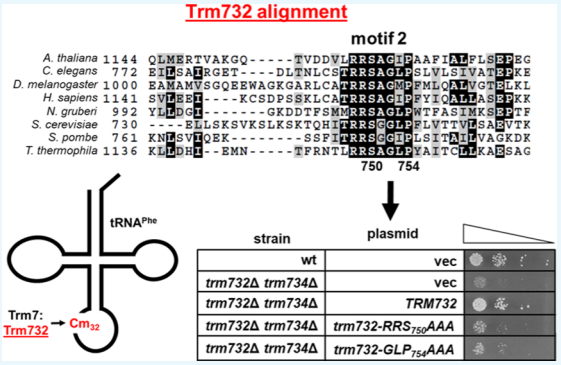
ABSTRACT: Posttranscriptional tRNA modifications are essential for proper gene expression, and defects in the enzymes that perform tRNA modifications are associated with numerous human disorders. Throughout eukaryotes, 2′-*O*-methylation of residues 32 and 34 of the anticodon loop of tRNA is important 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 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 dysautonomia[4−6] 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 disorders[24,25] 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,[1,37] which is found on residues 4, 18, 32, 34, and 44 of certain yeast tRNAs.[1] In yeast, 2′-*O*-methylation of residues 32 (Nm_{32}) and 34 (Nm_{34}) requires the methyltransferase Trm7.[38] Lack of both Cm_{32} and Gm_{34} on $tRNA^{Phe}$ in *trm7*Δ 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 trm7*Δ mutants grown in minimal media have a charging defect, but this defect is not observed in *S. cerevisiae trm7*Δ mutants grown in rich media nor in *S. pombe trm7*Δ mutants.[41] In *trm7*Δ mutants from both yeast species, the general amino acid control (GAAC) pathway is constitutively active,[41] suggesting that lack of Nm_{32} and Nm_{34} 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 (m^1G_{37}) on $tRNA^{Phe}$.[38−40]

In humans, defects in Nm_{32} and Nm_{34} 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

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the translation inhibitor paromomycin,[41] and are more sensitive to vaccinia virus infection.[34,45] Mice lacking *FTSJ1* show impaired learning, anxiety-like behavior, increased sensitivity to pain, metabolic differences, and other phenotypes.[46,47] The identity of the hypomodified tRNA(s) that causes these phenotypes in humans and mice lacking *FTSJ1* is likely $tRNA^{Phe}$ because loss of *FTSJ1* causes a reduction in steady-state levels of $tRNA^{Phe}$ in the brains of mice[47] and because decoding of Phe codons, and in particular UUU, is perturbed in both mice and in cultured human cells.[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 $tRNA^{Phe}$ lacking Gm_{34} was susceptible to fragmentation after heat shock.[48]

In the yeasts *S. cerevisiae* and *S. pombe*, Trm7 forms a complex with the protein Trm732 to form Cm_{32} and a complex with the protein Trm734 to form Nm_{34} on tRNA (Figure 1).[39,40] These partner proteins are required for Trm7 activity

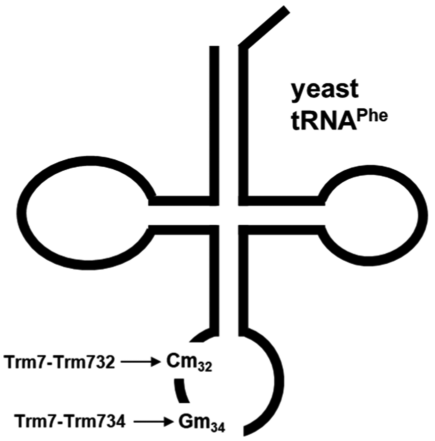

Figure 1. Schematic of 2′-*O*-methylation of the anticodon loop of $tRNA^{Phe}$ in yeast. In yeast, the Trm7−Trm732 complex forms Cm_{32} on $tRNA^{Phe}$, and the Trm7−Trm734 complex forms Gm_{34}.

because lack of Trm732 causes complete loss of Nm_{32}, and lack of Trm734 causes complete loss of Nm_{34}.[39,40] Trm7 forms distinct complexes with each protein, suggesting that the role of each is to direct Trm7 to a given nucleotide target.[40,49] Trm732 is an armadillo repeat protein which contains a DUF2428 domain (domain of unknown function),[40] whereas Trm734 is a WD40 protein.[49,50]

In humans and other multicellular eukaryotes, Trm732 and Trm734 orthologs are also involved in 2′-*O*-methylation of the anticodon loop by the Trm7 ortholog FTJS1. The predicted human ortholog of Trm732 is THADA (thyroid adenoma-associated protein), and overexpression of human THADA in yeast complements the lack of Trm732 by allowing the formation of Cm_{32} on $tRNA^{Phe}$.[39] However, the requirement of THADA for Nm_{32} formation in human cells has not been established. Likewise, *D. melanogaster* has a Trm732/THADA homolog,[48] but the role of this protein in Cm_{32} modification has not been determined. The Trm734 homolog in humans is WDR6, which forms a complex with FTSJ1 and is required with FTSJ1 to form Gm_{34} on $tRNA^{Phe}$ both in cells and in vitro.[44,47] Although the precise roles of Trm732 and Trm734 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.[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.[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[51] and has been shown to be involved in thermogenesis in *D. melanogaster*,[52] and in cold resistance in the model plant *Arabidopsis thaliana*.[53] A recent report also proposed a role for THADA as a regulator of programmed death-ligand 1 (PD-L1) maturation.[54] A genome wide association study (GWAS) also suggested that *THADA* plays a role in cold adaptation in humans.[55] Other GWAS analyses have implicated single nucleotide polymorphisms (SNPs) of *THADA* in polycystic ovary syndrome (PCOS),[56] prostate cancer,[57,58] and type 2 diabetes.[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,[34,45] 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.

RESULTS

A Conserved Motif in the DUF2428 Domain of Trm732 is Required for Cm_{32} Modification on $tRNA^{Phe}$. To study the role of Trm732 in formation of the Cm_{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 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.[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).

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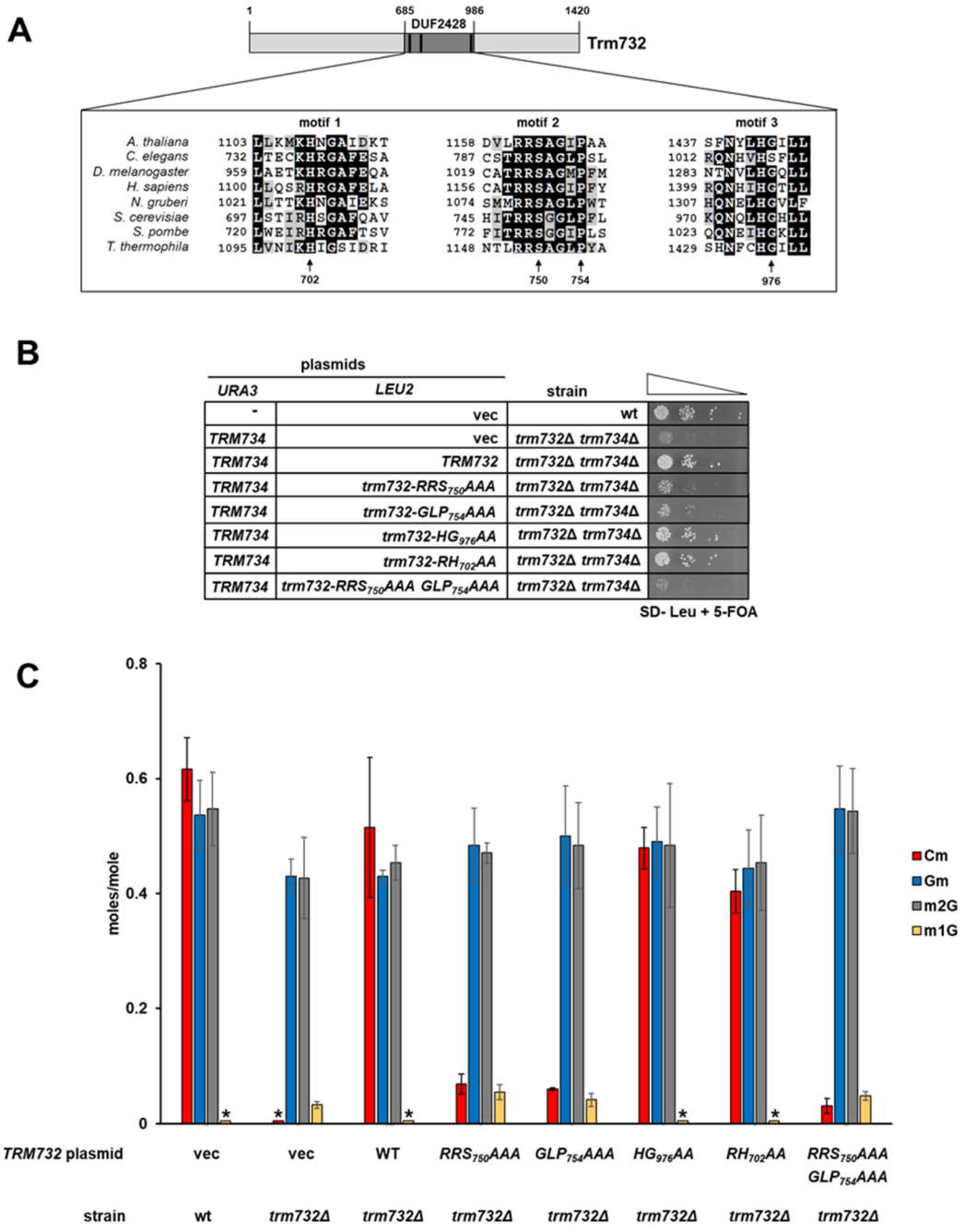

Figure 2. Motif 2 of Trm732 is required for Cm_{32} formation on $tRNA^{Phe}$. (A) Schematic representation of the Trm732 sequence. Inset box is an amino acid alignment of regions of high sequence similarity between Trm732 proteins from eight eukaryotes. Arrows point to the amino acids changed in Trm732 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 Cm_{32} 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.

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 Cm_{32} on $tRNA^{Phe}$. 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.[40] Expression of functional Trm732 proteins in the double-mutant strain should lead to normal levels of Cm_{32} on $tRNA^{Phe}$, resulting in a $tRNA^{Phe}$ anticodon loop modification profile identical to the healthy growing *trm734Δ* strain.[40] 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 [*TRM734 URA3*] plasmid. *trm732Δ trm734Δ* mutant cells expressing wild-type Trm732 were healthy, as were the cells expressing the motif 1 variant $Trm732\text{-}RH_{702}AA$ and the motif 3 variant $Trm732\text{-}HG_{976}AA$

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ACS Omega 2022, 7, 13667−13675

(Figure 2B). In contrast, *trm732Δ trm734Δ* mutants expressing the motif 2 variants Trm732-RRS$_{750}$AAA or Trm732-GLP$_{754}$AAA grew only slightly better than mutants expressing a vector, indicating that motif 2 is important for Trm732 activity. Mutants expressing the Trm732-RRS$_{750}$AAA-GLP$_{754}$AAA 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[a]
wild type	vec	1.00 ± 0.14
trm732Δ trm734Δ	vec	0.096 ± 0.03
trm732Δ trm734Δ	*TRM732*	3.75 ± 0.57
trm732Δ trm734Δ	*TRM732-RRS$_{750}$AAA*	3.12 ± 0.16
trm732Δ trm734Δ	*TRM732-GLP$_{754}$AAA*	2.94 ± 0.28
trm732Δ trm734Δ	*TRM732-HG$_{976}$AA*	3.25 ± 0.82
trm732Δ trm734Δ	*TRM732-RH$_{702}$AA*	3.86 ± 0.43
trm732Δ trm734Δ	*TRM732-RRS$_{750}$AAA, GLP$_{754}$AAA*	3.70 ± 0.58
trm732Δ trm734Δ	*TRM732-R$_{748}$A*	4.44 ± 0.68
trm732Δ trm734Δ	*TRM732-S$_{750}$A*	2.88 ± 0.08
trm732Δ trm734Δ	*TRM732-G$_{752}$A*	2.47 ± 0.29
trm732Δ trm734Δ	*TRM732-L$_{753}$A*	2.91 ± 0.09
trm732Δ trm734Δ	*TRM732-P$_{754}$A*	4.18 ± 2.43

[a]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 Cm$_{32}$ activity, we purified tRNA^{Phe} from a *trm732Δ* single mutant expressing Trm732 variants and analyzed the nucleoside content by ultra-pressure liquid chromatography (UPLC). As expected, tRNA^{Phe} 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 tRNA^{Phe} from *trm732Δ* strains expressing the motif 2 variants Trm732-RRS$_{750}$AAA and Trm732-GLP$_{754}$AAA had severely reduced levels of Cm, and strains expressing the motif 2 double-variant Trm732-RRS$_{750}$AAA-GLP$_{754}$AAA had even less Cm. In contrast, tRNA^{Phe} from *trm732Δ* strains expressing motif 1 or motif 3 variants had relatively high levels of Cm, nearly as high as those found on tRNA^{Phe} 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 Gm on tRNA^{Phe} from these strains were similar to those from tRNA^{Phe} from a wild-type strain regardless of the Trm732 plasmid expressed. Small, but detectable, levels of m^{1}G were observed on tRNA^{Phe} from *trm732Δ* strains expressing an empty vector or expressing Trm732 motif 2 variants (Figure 2C). The presence of m^{1}G on tRNA^{Phe} in certain mutants is most likely due to a defect in yW$_{37}$ formation because m^{1}G$_{37}$ is the precursor to yW$_{37}$, and *trm732Δ* mutants have been shown previously to have a defect in yW levels.[40] Thus, the defects in Cm$_{32}$ formation in mutants expressing Trm732 variants cause decreased yW$_{37}$ formation, resulting in detection of m^{1}G. Levels of 2-methylguanosine (m^{2}G) on tRNA^{Phe} 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 tRNA^{Phe} 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-R$_{748}$A variant showing a significant, reproducible suppression defect, especially at 25 °C (Figure 3). Indeed, *trm732Δ trm734Δ*

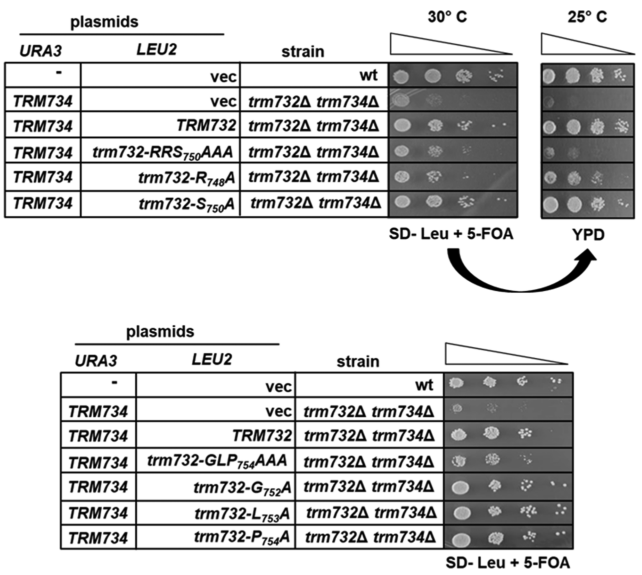

Figure 3. Requirement of individual motif 2 residues for Trm732 function. (A) Amino acid residue R748 is important for Trm732 function. Strains with plasmids as indicated were grown overnight in SD-Leu and analyzed as in Figure 2B, after incubation for 2 days at 30 °C. In the top panel, following growth on 5-FOA at 30 °C, cells were spotted on YPD at 25 °C and incubated for 2 days.

mutants expressing the Trm732-R$_{748}$A variant had a generation time of 289 min compared to 210 min for mutants expressing wild-type Trm32, when grown at 25 °C in minimal media (Table 2). The inability of the Trm732-R$_{748}$A variant to fully suppress the growth defect is almost certainly due to an intermediate level of Cm$_{32}$ on tRNA^{Phe}. Thus, it is likely that it is the combination of all three amino acid changes in each of the motif 2 variants that causes the bulk of loss of tRNA modification activity.

Human THADA Requires Motif 2 for Complementation of the Yeast *trm732Δ* Mutant. To determine if motif 2 is also required for the activity of Trm732 from another organism, we determined whether the corresponding motif 2 residues are required for Cm formation activity by human THADA. Additionally, to determine the effect of changing the corresponding amino acid residues in human THADA that

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ACS Omega 2022, 7, 13667−13675

Table 2. Comparison of Generation Times for *trm732Δ trm734Δ* Mutants Expressing Trm732 Variants in Minimal Media at 25 °C

strain[a]	*TRM732* plasmid	generation time (min)
wild type	vec	206 ± 12
trm732Δ trm734Δ	vec	484 ± 19
trm732Δ trm734Δ	wild type	210 ± 6
trm732Δ trm734Δ	$RRS_{750}AAA$	444 ± 31
trm732Δ trm734Δ	$R_{748}A$	289 ± 29
trm732Δ trm734Δ	$S_{750}A$	217 ± 7

[a]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 $tRNA^{Phe}$.[39] Therefore, we generated variants of full-length isoform A of human THADA on high copy (2μ) expression plasmids under control of the P_{GAL} 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

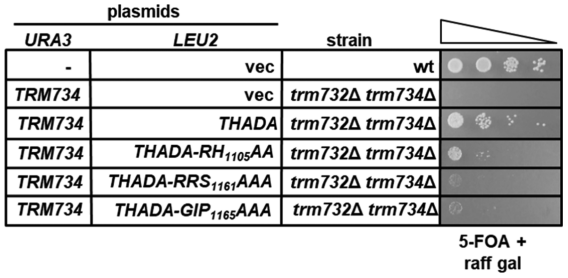

Figure 4. Human THADA requires motif 2 for complementation of the yeast *trm732Δ* mutant. Indicated strains were grown overnight in the S medium containing raffinose and galactose; diluted as in Figure 2B; spotted to medium containing raffinose, galactose, and 5-FOA; and then incubated for 3 days at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

THADA severely impaired the ability of the protein to rescue the slow growth of the *trm732Δ trm734Δ* mutants (THADA-RRS_{1161}AAA and THADA-GIP_{1165}AAA). In contrast to what we observed for the yeast Trm732, expression of human THADA with amino acid changes in motif 1 (THADA-RH_{1105}AA) 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 Cm_{32} to $tRNA^{Phe}$ in yeast lacking TRM732,[39] these results strongly suggest that failure of THADA variants to restore growth is due to the lack of Cm_{32} formation on $tRNA^{Phe}$. 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

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

strain	plasmid	relative level[a]
trm732Δ trm734Δ	*THADA*	34.4 ± 8.8
trm732Δ trm734Δ	vec	<0.01
trm732Δ trm734Δ	$THADA\text{-}RH_{1105}AA$	52.0 ± 11.0
trm732Δ trm734Δ	$THADA\text{-}RRS_{1161}AAA$	41.7 ± 4.8
trm732Δ trm734Δ	$THADA\text{-}GIP_{1165}AAA$	40.3 ± 1.9

[a]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 Cm_{32} on $tRNA^{Phe}$ and have shown that this same motif is required for the activity of the human Trm732 ortholog THADA. Substitution of the $RRSAGLP_{754}$ residues of motif 2 of yeast Trm732 with alanine residues resulted in a nearly complete lack of Cm modification on $tRNA^{Phe}$ (Figure 2) and a growth defect similar to a vector control in *trm732Δ trm734Δ* strains expressing the variant (Figure 2). Replacing either RRS_{750} or GLP_{754} 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 formation of Cm_{32} is not clear. One possible role for motif 2 residues could be tRNA binding and/or proper positioning of the tRNA in the active site to ensure that residue 32 is modified, with the arginine residues possibly interacting directly with the tRNA, and other residues involved in important hairpin turns common to armadillo proteins, which are known to be involved in RNA binding.[63] Another plausible explanation for loss of activity in motif 2 variants is that these amino acids are critical for protein−protein interactions between Trm732 and Trm7. Additional biochemical experiments could help determine the role(s) these residues play in tRNA modification.

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 Cm_{32} on $tRNA^{Phe}$ led to detectable rescue of slow growth. For instance, $tRNA^{Phe}$ from *trm732Δ* mutants expressing the Trm732 RRS_{750} and GLP_{754} variants had levels of Cm_{32} 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

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ACS Omega 2022, 7, 13667−13675

Nm_{32} 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 *trm732Δ trm734Δ* mutants. We note that Cm_{32} also likely has a role in *S. pombe* based on the severe growth defect of *S. pombe trm7Δ* mutants and the more mild growth defect of *S. pombe trm734Δ* 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 Nm_{32} formation in multicellular eukaryotes has not been established, although the ability of human THADA to complement yeast *trm732Δ* mutants by interacting with yeast Trm7 strongly suggests that its tRNA modification function will also be conserved.[39] Our finding that yeast Trm732 and human THADA variants with a mutated motif 2 lack tRNA activity makes it possible to determine if the thermogenesis phenotype in *D. melanogaster THADA* mutants[52] and the PD-L1 phenotype in human cells[54] are due to the lack of tRNA modification activity or uncharacterized protein activity. Likewise, the recent finding that human WDR6 is required for Nm_{34} activity in human cells,[41] that it is required for in vitro activity,[47] and that it forms a complex with FTSJ1[41,44] further shows the conserved and critical role of Trm734/WDR6 proteins in tRNA modification. Further experiments using THADA and WDR6 variants with impaired tRNA modification activity could help clarify the role of these proteins in other biological processes.

METHODS

Yeast Strains and Plasmids. Yeast strains are listed in Table 4. All yeast strains were constructed using standard techniques, as described previously.[40] Plasmids are listed in Table 5. The *CEN LEU2 TRM732* expression plasmid was constructed by ligation-independent cloning (LIC) into pAVA581.[64] Plasmids expressing Trm732 and full-length human isoform A THADA variants were generated by QuickChange PCR (Stratagene) or Q5 site-directed mutagenesis (New England Biolabs). All plasmids were confirmed by sequencing prior to use.

Table 4. Strains Used in This Study

strain	genotype	source
BY4741	*MATa his3-Δ1 leu2Δ0 met15-Δ0 ura3-Δ0*	
yMG814-1	BY4741, *trm732Δ::ble*^{R}	ref 40
yMG818-1	BY4741, *trm734Δ::ble*^{R} *trm732Δ::kanMX* [*CEN URA3 TRM734*]	ref 40

Isolation of RNA from Yeast Cells. *S. cerevisiae trm732Δ* 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]

Quantitative Real-Time PCR. RNA was treated with RQ1 RNase-free DNase (Promega), followed by reverse transcription using a Verso cDNA Kit (Thermo Scientific) with a 3:1 (v/v) mix of random hexamers and anchored oligo-dT primers. After reverse transcription, DNA was PCR-amplified using DyNAmo HS SYBR Green qPCR Kit (Thermo Scientific) master mix using primers specific to indicated genes. RNA levels were normalized to *ACT1*.

Purification of tRNA and Analysis of Modified Nucleosides by UPLC. Specific tRNA was purified using complementary biotinylated oligos, followed by digestion of tRNA to nucleosides using P1 nuclease and phosphatase as previously described.[65] After purification, tRNA from yeast was analyzed by UPLC using a 50 mm HSS T3 C_{18} 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..

Table 5. Plasmids Used in This Study

plasmid	parent	description	source
pBP2A		*CEN URA3 TRM734*	ref 40
pAVA581		*CEN LEU2 LIC*	ref 64
pMG586	pMG586	*CEN LEU2 TRM732*	this study
pMG581	pMG586	*CEN LEU2 TRM732-RRS*$_{750}$*AAA*	this study
pMG582	pMG586	*CEN LEU2 TRM732-GLP*$_{754}$*AAA*	this study
pMG584	pMG586	*CEN LEU2 TRM732-HG*$_{976}$*AA*	this study
pMG585	pMG586	*CEN LEU2 TRM732-RH*$_{702}$*AA*	this study
pMG619A	pMG582	*CEN LEU2 TRM732-RRS*$_{750}$*AAA, GLP*$_{754}$*AAA*	this study
pMG739C	pMG586	*CEN LEU2 TRM732-R*$_{748}$*A*	this study
pMG741B	pMG586	*CEN LEU2 TRM732-S*$_{750}$*A*	this study
pMG742E	pMG586	*CEN LEU2 TRM732-G*$_{752}$*A*	this study
pMG743A	pMG586	*CEN LEU2 TRM732-L*$_{753}$*A*	this study
pMG744E	pMG586	*CEN LEU2 TRM732-P*$_{754}$*A*	this study
pMG245A		*2μ LEU2* P_{GAL} *THADA*	ref 39
pMG643A	pMG245A	*2μ LEU2* P_{GAL} *THADA-RH*$_{1105}$*AA*	this study
pMG644	pMG245A	*2μ LEU2* P_{GAL} *THADA-RRS*$_{1161}$*AAA*	this study
pMG645A	pMG245A	*2μ LEU2* P_{GAL} *THADA-GIP*$_{1165}$*AAA*	this study

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