# Conservation of an intricate circuit for crucial modifications of the tRNA$^{Phe}$ anticodon loop in eukaryotes

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**ABSTRACT**

**Post-transcriptional tRNA modifications are critical for efficient and accurate translation, and have multiple different roles. Lack of modifications often leads to different biological consequences in different organisms, and in humans is frequently associated with neurological disorders. We investigate here the conservation of a unique circuitry for anticodon loop modification required for healthy growth in the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* Trm7 interacts separately with Trm732 and Trm734 to 2′-*O*-methylate three substrate tRNAs at anticodon loop residues $C_{32}$ and $N_{34}$, and these modifications are required for efficient wybutosine formation at $m^1G_{37}$ of tRNA$^{Phe}$. Moreover, *trm7Δ* and *trm732Δ trm734Δ* mutants grow poorly due to lack of functional tRNA$^{Phe}$. It is unknown if this circuitry is conserved and important for tRNA$^{Phe}$ modification in other eukaryotes, but a likely human *TRM7* ortholog is implicated in nonsyndromic X-linked intellectual disability. We find that the distantly related yeast *Schizosaccharomyces pombe* has retained this circuitry for anticodon loop modification, that *S. pombe trm7Δ* and *trm734Δ* mutants have more severe phenotypes than the *S. cerevisiae* mutants, and that tRNA$^{Phe}$ is the major biological target. Furthermore, we provide evidence that Trm7 and Trm732 function is widely conserved throughout eukaryotes, since human *FTSJ1* and *THADA*, respectively, complement growth defects of *S. cerevisiae trm7Δ* and *trm732Δ* trm734Δ mutants by modifying $C_{32}$ of tRNA$^{Phe}$, each working with the corresponding *S. cerevisiae* partner protein. These results suggest widespread importance of 2′-*O*-methylation of the tRNA anticodon loop, implicate tRNA$^{Phe}$ as the crucial substrate, and suggest that this modification circuitry is important for human neuronal development.**

**Keywords: FTSJ1; TRM7; TRM732; TRM734; THADA; tRNA$^{Phe}$**

## INTRODUCTION

Post-transcriptional modification of tRNA is universally required for accurate and efficient translation. Modifications are found in all characterized tRNA species (Machnicka et al. 2013), and are highly conserved within each domain of life (Grosjean 2009). Modifications have a number of different roles, with well documented examples including modulating the efficiency and specificity of charging (Muramatsu et al. 1988; Pütz et al. 1994), altering the specificity of decoding (Johansson et al. 2008), maintaining the frame for decoding (Urbonavicius et al. 2001), and preventing decay of pre-tRNA (Kadaba et al. 2004) and mature tRNA (Alexandrov et al. 2006; Chernyakov et al. 2008).

Many tRNA modifications have a similar biological impact on different organisms. For example, the genes responsible for modification of residue $A_{34}$ to $I_{34}$ (inosine) in the wobble position of tRNAs are essential in the bacterium *Escherichia coli* (Wolf et al. 2002), in the yeast *Saccharomyces cerevisiae* (Gerber and Keller 1999), and in the distantly related yeast *Schizosaccharomyces pombe* (Kim et al. 2010), and RNAi against a putative homolog results in 29% embryonic lethality in the nematode *Caenorhabditis elegans* (Fernandez et al. 2005). Similarly, lack of the terminal methyl group of $mcm^5U_{34}$ (5-methoxycarbonylmethyluridine) in *S. cerevisiae* due to mutation of *TRM9* results in sensitivity to aminoglycosides (Kalhor and Clarke 2003) and DNA damaging agents (Begley et al. 2007) but no other growth defects, depletion of the human protein results in DNA damage sensitivity but no other growth defects (Fu et al. 2010), and mice lacking *TRM9* (*Alkbh8*$^{-/-}$) appear normal (Songe-Møller et al. 2010).

In contrast, many modifications often do not have precisely the same biological impact on different organisms. Thus, for example, the genes that specify the $m^1A_{58}$ (1-methyladensosine) modification found in many eukaryotic tRNAs are essential in *S. cerevisiae* (Anderson et al. 1998) due to turnover of pre-tRNA$_i^{Met}$ by the nuclear surveillance pathway (Kadaba et al. 2004), but, the corresponding gene knockouts are each

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viable in *S. pombe* (Kim et al. 2010), although one knockout strain that was examined grows slowly and is more sensitive to oxidative stress (Zuin et al. 2008). Similarly, the genes required for $t^6A_{37}$ ($N^6$-threonylcarbamoyladenosine) formation are essential in *E. coli* and *Haloferax volcanii*, but not in *S. cerevisiae*, although mutants grow poorly (El Yacoubi et al. 2011; Srinivasan et al. 2011; Naor et al. 2012).

Several modifications with more modest phenotypes in *S. cerevisiae* also appear to have a markedly different biological impact on different organisms. Thus, for example, lack of $i^6A_{37}$ ($N^6$-isopentenyladenosine) due to mutation of *MOD5* in *S. cerevisiae*, results in reduced nonsense suppression (Laten et al. 1978; Dihanich et al. 1987), but no obvious growth defect, whereas *S. pombe mod5*△ (*Sp tit1*△) mutants grow slowly on glycerol or rapamycin (Lamichhane et al. 2013), *C. elegans mod-5* mutants (*gro-1*) have slowed embryogenesis and development and an increased life span (Lemieux et al. 2001), and mutations in human *MOD5* (*TRIT1*) have been linked to encephalopathy and epilepsy due to mitochondrial defects (Yarham et al. 2014).

Although only a subset of tRNA substrates of a modification enzyme are often responsible for the known phenotypes of a mutation in the corresponding modification gene (Phizicky and Alfonzo 2010; Guy et al. 2012), it is unclear if the same subset of tRNA species are equally important in each organism. For example, in *S. cerevisiae* all phenotypes associated with mutations in the ELP complex (which forms the $cm^5U$ moiety found in $mcm^5U_{34}$, $ncm^5U_{34}$, [5-carbamoylmethyluridine], and $mcm^5s^2U_{34}$ [5-methoxycarbonylmethyl-2-thiouridine]) are due to lack of functional $tRNA^{Lys(UUU)}$, $tRNA^{Gln(UUG)}$, and $tRNA^{Glu(UUC)}$, although 11 tRNAs are targets of the ELP complex (Esberg et al. 2006; Johansson et al. 2008; Chen et al. 2011b); in contrast, it appears that only overexpression of $tRNA^{Lys(UUU)}$ is required in *S. pombe* to suppress most of the sensitivity to oxidative stress in an *Sp* elp3 mutant (which lacks both the $cm^5$ moiety and the $s^2$ moiety of $mcm^5s^2U$), although $tRNA^{Gln(UUG)}$ and $tRNA^{Glu(UUC)}$ have the same $mcm^5s^2U$ modification (Fernández-Vázquez et al. 2013).

The roles and biological effects of 2′-*O*-methylation of the anticodon loop are of particular interest because of prior work in *S. cerevisiae* showing a severe phenotype of mutants, specificity of that phenotype for modification of only one substrate, and an intricate circuitry for modification of tRNA substrates (Pintard et al. 2002; Guy et al. 2012). *S. cerevisiae* Trm7 is required for 2′-*O*-methylation of the anticodon loop of $tRNA^{Phe}$, $tRNA^{Trp}$, and $tRNA^{Leu(UAA)}$ at $C_{32}$ (forming 2′-*O*-methylcytidine, $Cm_{32}$) and at $N_{34}$ (forming $Gm_{34}$, $Cm_{34}$, and $ncm^5Um_{34}$, respectively); and *Sc trm7*△ mutants have a severe growth defect (Pintard et al. 2002). Interestingly, *Sc* Trm7 interacts with *Sc* Trm732 to form $Cm_{32}$, and separately interacts with *Sc* Trm734 to form $Nm_{34}$, and both modifications are required to efficiently drive formation of $yW_{37}$ (wybutosine) at $m^1G_{37}$ on $tRNA^{Phe}$ (Guy et al. 2012) by *Sc* Tyw1–Tyw4 (Fig. 1A; Noma et al. 2006). Furthermore, overexpression of only $tRNA^{Phe}$ fully suppresses the growth defect and the aminoglycoside sensitivity of *Sc trm7*△ mutants. In addition, the growth defect of an *Sc trm7*△ mutant requires loss of both the $Cm_{32}$ and $Gm_{34}$ modifications of $tRNA^{Phe}$ (and the accompanying loss of $yW_{37}$) since neither an *Sc trm732*△ nor an *Sc trm734*△ single mutant has any observable growth defect, whereas the double mutant is as sick as an *Sc trm7*△ mutant and is equally suppressed by overexpression of $tRNA^{Phe}$ (Guy et al. 2012).

The goal of the work described here is to determine if this intricate circuitry, the biologically significant tRNA target, and the importance of Trm7 modifications are conserved, focusing on *S. pombe*, whose lineage diverged from that of *S. cerevisiae* ~1.1 billion yr ago (Hedges 2002). Available evidence is equivocal for conservation of all these features among eukaryotes. Although Trm7 is highly conserved in each of 25 divergent eukaryotic genomes examined (Fig. 1B) (see Materials and Methods), Trm732 homologs can only be identified in 22 genomes, and Trm734 homologs only in 14 genomes, and both protein families have little overall sequence similarity, (~23% and 21% overall identity between the *Sc* proteins and their predicted human orthologs THADA [Trm732] [Fig. 1C] and WDR6 [Trm734], respectively [Shi et al. 2011]). Moreover, *S. cerevisiae* Trm734 has been implicated in regulation of Ty1 transposition (Nyswaner et al. 2008) and endoplasmic recycling (Shi et al. 2011), suggesting that Trm734 family proteins from other organisms might have other roles in addition to, or instead of, tRNA modification.

It also appears that *TRM7* has an important but differing biological impact on different organisms, since a high throughput screen in *S. pombe* indicated that the putative *TRM7* gene was essential (Kim et al. 2010), and since mutations in a putative human *TRM7* homolog (*FTSJ1*) are associated with nonsyndromic X-linked intellectual disability (NSXLID) (Freude et al. 2004; Ramser et al. 2004; Froyen et al. 2007; Takano et al. 2008).

We report here that the circuitry and biologically important substrate for 2′-*O*-methylation of the tRNA anticodon loop are conserved in *S. pombe*, and that *S. pombe trm7Δ* and *trm734Δ* mutants are viable, but with more severe growth defects than in *S. cerevisiae*. Furthermore, we provide evidence that this circuitry is retained in other eukaryotes including humans, suggesting that defective 2′-*O*-methylation of tRNA is linked to NSXLID.

## RESULTS

### The putative *S. pombe trm7*$^+$ gene is required for Cm and Gm formation on $tRNA^{Phe}$ and mutants are barely viable

To determine if *S. cerevisiae* Trm7 function is conserved in eukaryotes, we generated and examined *S. pombe* strains lacking the likely *trm7*$^+$ gene (*SPAC4F10.03c*). Consistent with

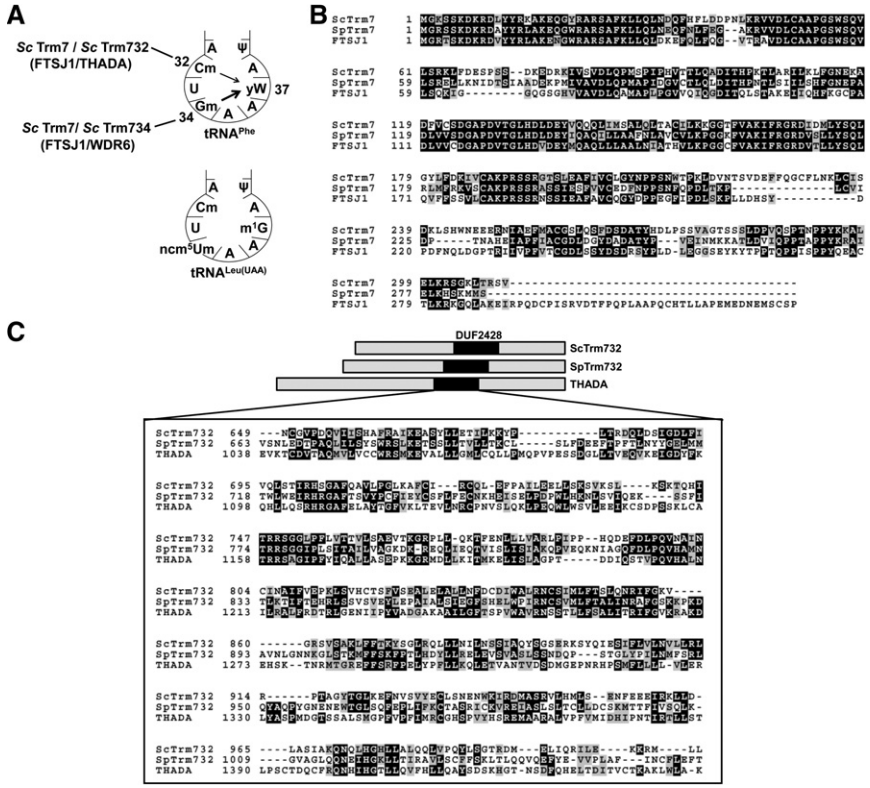


**FIGURE 1.** Trm7 modification machinery in eukaryotes. (*A*) Schematic of the anticodon loop of $tRNA^{Phe}$ and $tRNA^{Leu(UAA)}$ from *S. cerevisiae*. *S. cerevisiae* Trm7 (*Sc* Trm7) requires *Sc* Trm732 and *Sc* Trm734 to form $Cm_{32}$, and $Nm_{34}$, respectively, on $tRNA^{Phe}$ and $tRNA^{Leu(UAA)}$. $Cm_{32}$ and $Gm_{34}$ modification then drive yW formation from $m^1G$ on $tRNA^{Phe}$. Wider arrow for $Gm_{34}$ indicates that yW formation is more dependent on this modification than on $Cm_{32}$. Predicted human homologs of *Sc* Trm7, *Sc* Trm732, and *Sc* Trm734 are in brackets. (*B*) Amino acid sequence alignment of *Sc* Trm7 with putative homologs from *S. pombe* and *H. sapiens*. (*C*) Schematic representation of *Sc* Trm732 aligned with putative homologs from *S. pombe* and *H. sapiens*. *Inset* box is an amino acid alignment of the DUF2428 domain found in these proteins.

previous high throughput results (Kim et al. 2010), we were unable to generate *S. pombe trm7*Δ mutant haploids from a heterozygous diploid (relevant genotype: *spac4F10.03c*Δ*::kanMX*/*SPAC4F10.03c*) by sporulation and selection for the mutant haploid (data not shown). However, by first introducing a *ura4*$^+$ plasmid expressing *S. pombe trm7*$^+$ under control of the $P_{nmt1}$ promoter (no message in thiamine), we could recover an *Sp trm7*Δ [$P_{nmt1}$ *Sp trm7*$^+$ *ura4*$^+$] haploid after sporulation on Edinburgh minimal medium (EMM, permissive conditions) in the presence of selective drug; moreover, this *Sp trm7*Δ [$P_{nmt1}$ *Sp trm7*$^+$ *ura4*$^+$] haploid was viable (albeit barely) when plated to EMM containing 5-fluoroorotic acid (5-FOA) to select against the *ura4*$^+$ plasmid. Indeed, after streaking the *Sp trm7*Δ [$P_{nmt1}$ *Sp trm7*$^+$ *ura4*$^+$] strain to EMM containing 0.5 mg/L 5-FOA at 30°C, it took 11 d of growth to attain visible colonies, compared with 3 d for wild-type cells (data not shown). Control experiments demonstrated that the growth defect was due to the *trm7*Δ mutation of the *Sp trm7*Δ [$P_{nmt1}$ *Sp trm7*$^+$ *ura4*$^+$] strain, since introduction of a [$P_{nmt1^{**}}$ *Sp trm7*$^+$ *LEU2*] plasmid (expressing *trm7*$^+$ under control of the low strength no message in thiamine promoter), resulted in healthy growth after plating to EMM containing 5-FOA (Fig. 2A). The growth defect of *Sp trm7*Δ mutants thus appears to be more severe than that observed in the *S. cerevisiae trm7*Δ mutant, which is sick but viable (Pintard et al. 2002; Guy et al. 2012). Under repressive conditions in liquid YES medium, the *Sp trm7*Δ [$P_{nmt1^{**}}$ *Sp trm7*$^+$ *LEU2*] strain (although much healthier than the *Sp trm7*Δ haploid) had a generation time of 320 min, compared with 148 min for the wild-type strain (a difference of 2.16-fold) (Table 1), which was still more than the difference of 1.94-fold in generation times observed for an *S. cerevisiae trm7Δ* mutant compared with its wild-type control. Furthermore, this difference was even greater in selective EMM-Leu + thiamine, with a generation time of 603 min for the *Sp trm7*Δ [$P_{nmt1^{**}}$ *Sp trm7*$^+$ *LEU2*] strain, compared with 238 min for the wild-type control strain (a difference of 2.53-fold), whereas the *Sp trm7*Δ [$P_{nmt1^{**}}$ *Sp trm7*$^+$ *LEU2*] strain grew nearly as well as the wild-type strain in permissive conditions (EMM-Leu), with a difference in generation times of only 1.05-fold (Table 1).

Because it was extremely difficult to obtain and grow an *Sp trm7*Δ strain for analysis of modifications, we instead examined the effect of reduced levels of *Sp* Trm7 on modification of $tRNA^{Phe}$ by growing the *Sp trm7*Δ [$P_{nmt1^{**}}$ *Sp trm7*$^+$ *LEU2*] strain in EMM-Leu containing thiamine to repress *Sp* Trm7 expression, followed by purification of $tRNA^{Phe}$ and analysis of its nucleoside content by HPLC. Under these conditions, Cm levels of $tRNA^{Phe}$ were substantially reduced compared with those of a wild-type control strain grown in the same medium (0.13 versus 0.93 moles/mole) (Table 2; Fig. 2B), or to those when the *Sp trm7*Δ [$P_{nmt1^{**}}$ *Sp trm7*$^+$ *LEU2*] strain was grown under permissive (EMM-Leu) conditions (0.90 moles/mole). Similarly, Gm levels were reduced in $tRNA^{Phe}$ from the *Sp trm7*Δ [$P_{nmt1^{**}}$ *Sp trm7*$^+$ *LEU2*] strain grown in repressive conditions (EMM-Leu containing thiamine) relative to those from the wild-type strain grown under the same conditions (0.04 versus 0.99 moles/mole), or to those when the strain was grown under permissive (EMM-Leu) conditions (0.90 moles/mole). In contrast, the control modification Ψ (pseudouridine) showed little variation in these strains (Table 2).

We also found evidence that yW modification was reduced upon repression of *trm7*$^+$ expression in the *Sp trm7*Δ [$P_{nmt1^{**}}$ *Sp trm7*$^+$ *LEU2*] strain by growth in thiamine-containing

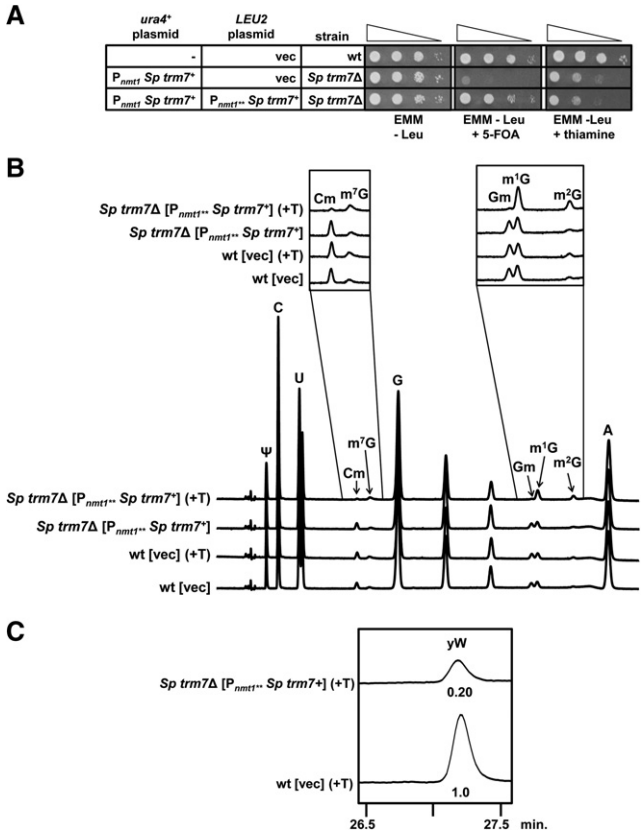


**FIGURE 2.** *S. pombe trm7Δ* mutants are barely viable, and *trm7Δ* [$P_{nmt1**}$ *Sp trm7*$^+$] strains grown in repressive conditions have reduced $Cm_{32}$, $Gm_{34}$, and $yW_{37}$ modification of $tRNA^{Phe}$. (*A*) *S. pombe trm7Δ* mutants are barely viable. *S. pombe* strains with the indicated plasmids were grown in EMM overnight, diluted to $OD_{600}$ of ~0.5 in $H_2O$, and serially diluted 10-fold in $H_2O$, and then 2 µL was spotted onto indicated media, followed by incubation for 4 d at 30°C. (*B*) HPLC traces of $tRNA^{Phe}$ from an *S. pombe trm7Δ* [$P_{nmt1**}$ *Sp trm7*$^+$] strain. $tRNA^{Phe}$ from indicated strains grown in EMM-Leu with thiamine (+T, repressive conditions) or without thiamine (permissive conditions) was digested to nucleosides and analyzed by HPLC as described in Materials and Methods. (*C*) Levels of yW on $tRNA^{Phe}$ substantially decrease in an *S. pombe trm7Δ* [$P_{nmt1**}$ *Sp trm7*$^+$] strain grown under repressive conditions. $tRNA^{Phe}$ from indicated strains grown in EMM-Leu with thiamine (+T) was analyzed as described in panel *B* under conditions optimized for detecting yW as described in Materials and Methods.

medium. Under these conditions, $m^1G$ levels of $tRNA^{Phe}$ increased substantially compared with those from the wild-type strain (1.80 versus 1.16 moles/mole), suggesting that yW formation from $m^1G$ was reduced (Table 2; Fig. 2B). Direct measurement by HPLC (Noma et al. 2006) confirmed that levels of yW were reduced in $tRNA^{Phe}$ from the *Sp trm7Δ* [$P_{nmt1**}$ *Sp trm7*$^+$ *LEU2*] strain grown under repressive conditions, to 20% of those from $tRNA^{Phe}$ of wild-type cells (Fig. 2C). Thus, just as in *S. cerevisiae* (Guy et al. 2012), *S. pombe* mutants lacking $Cm_{32}$ and $Gm_{34}$ due to loss of *trm7*$^+$ also appear to have reduced synthesis of yW from $m^1G$ in $tRNA^{Phe}$.

Curiously, levels of the control modifications $m^2G$ ($N^2$-methylguanosine) and $m^7G$ (7-methylguanosine) were unexpectedly reduced in $tRNA^{Phe}$ purified from different strains grown in EMM. $m^7G$ was reproducibly and significantly reduced whenever thiamine was missing from the medium, from 0.55 to 0.28 moles/mole for the wild-type strain, and from 0.46 to 0.25 moles/mole for the *Sp trm7Δ* [$P_{nmt1**}$ *Sp trm7*$^+$ *LEU2*] strain (Table 2; Fig. 2B). $m^2G$ levels in $tRNA^{Phe}$ were reproducibly different in different strains, with high levels in the *Sp trm7Δ* [$P_{nmt1**}$ *Sp trm7*$^+$ *LEU2*] strain in the presence of thiamine (0.75 moles/mole), and reduced levels when thiamine was absent (0.12 moles/mole), and with significantly lower levels in the wild-type strain (0.29 moles/mole), further reduced in the absence of thiamine (0.11 moles/mole).

## Overexpression of $tRNA^{Phe}$ suppresses the growth defect of *S. pombe trm7Δ* mutants

We also found that $tRNA^{Phe}$ was the important Trm7 substrate in *S. pombe*, since overexpression of this tRNA restored healthy growth to *Sp trm7Δ* mutants on 5-FOA medium, whereas overexpression of $tRNA^{Trp}$ or $tRNA^{Leu(UAA)}$, which were not 2′-*O*-methylated in *S. pombe* (data not shown), did not (Fig. 3A). Indeed, the *Sp trm7Δ* mutant overexpressing $tRNA^{Phe}$ grew nearly as well as the wild-type control strain on EMM at 25°C and 30°C (and a bit more poorly on YES medium), but was slightly temperature sensitive at 33°C and 37°C (Fig. 3B). As expected for a strain lacking Trm7, $tRNA^{Phe}$ from the *Sp trm7Δ* [$tRNA^{Phe}$] strain lacked detectable Cm and Gm (Table 2), had increased $m^1G$ levels compared with those from the wild-type strain overexpressing $tRNA^{Phe}$ (2.18 versus 1.62 moles/mole), and had undectable levels of yW (Fig. 3C). Thus, the important Trm7 substrate in *S. pombe* is $tRNA^{Phe}$, just as in *S. cerevisiae* (Guy et al. 2012).

**TABLE 1.** Comparison of generation times for *trm7Δ*, *trm732Δ*, and *trm734Δ* mutant strains from *S. pombe* and *S. cerevisiae* at 30°C

| Organism | Strain[a,b] | Growth medium | Generation time (min) |
|---|---|---|---|
| *S. pombe* | Wild type | YES | 148 ± 5 |
| *S. pombe* | *trm7Δ* [$P_{nmt1**}$ *trm7*$^+$] | YES | 320 ± 2 |
| *S. pombe* | *trm732Δ* | YES | 143 ± 11 |
| *S. pombe* | *trm734Δ* | YES | 258 ± 3 |
| *S. pombe* | Wild type [vec] | EMM-Leu | 315 ± 9 |
| *S. pombe* | Wild type [vec] | EMM-Leu + thiamine | 238 ± 3 |
| *S. pombe* | *trm7Δ* [$P_{nmt1**}$ *trm7*$^+$] | EMM-Leu | 330 ± 10 |
| *S. pombe* | *trm7Δ* [$P_{nmt1**}$ *trm7*$^+$] | EMM-Leu + thiamine | 603 ± 15 |
| *S. cerevisiae* | Wild type | YPD | 66 ± 2 |
| *S. cerevisiae* | *trm7Δ* | YPD | 128 ± 5 |
| *S. cerevisiae* | *trm732Δ* | YPD | 69 ± 6 |
| *S. cerevisiae* | *trm734Δ* | YPD | 70 ± 2 |

[a]Mean and standard deviation based on growth from three separate colonies.
[b]$P_{nmt1**}$: low strength no message in thiamine promoter.

**TABLE 2.** HPLC analysis of $tRNA^{Phe}$ nucleoside content from an *S. pombe trm7*△ strain

| Strain[a,b] | Medium | Cm | Gm | $m^1G$ | Ψ | $m^7G$ | $m^2G$ |
|---|---|---|---|---|---|---|---|
| Moles expected | | 1 | 1 | 1 | 4 | 1 | 1 |
| Wild type [vec] | EMM-Leu | 0.92 ± 0.02 | 0.90 ± 0.04 | 1.28 ± 0.05 | 3.85 ± 0.03 | 0.28 ± 0.02 | 0.11 ± 0.02 |
| Wild type [vec] | EMM-Leu + thiamine | 0.93 ± 0.03 | 0.99 ± 0.01 | 1.16 ± 0.01 | 3.90 ± 0.02 | 0.55 ± 0.03 | 0.29 ± 0.03 |
| *trm7*△ [$P_{nmt1**}$ *trm7*$^+$] | EMM-Leu | 0.90 ± 0.04 | 0.90 ± 0.03 | 1.27 ± 0.03 | 3.79 ± 0.14 | 0.25 ± 0.03 | 0.12 ± 0.04 |
| *trm7*△ [$P_{nmt1**}$ *trm7*$^+$] | EMM-Leu + thiamine | 0.13 ± 0.02 | 0.04 ± 0.01 | 1.80 ± 0.05 | 3.80 ± 0.06 | 0.46 ± 0.03 | 0.75 ± 0.04 |
| Wild type [$tRNA^{Phe}$] | EMM-Leu | 0.80 ± 0.02 | 0.70 ± 0.02 | 1.62 ± 0.11 | 3.83 ± 0.02 | 0.19 ± 0.04 | 0.10 ± 0.02 |
| *trm7*△ [$tRNA^{Phe}$] | EMM-Leu | <0.01 | <0.03 | 2.18 ± 0.04 | 3.90 ± 0.03 | 0.23 ± 0.01 | 0.33 ± 0.04 |

[a]Mean and standard deviation based on three individual growths and RNA preparations.
[b]$P_{nmt1**}$: low strength no message in thiamine promoter.

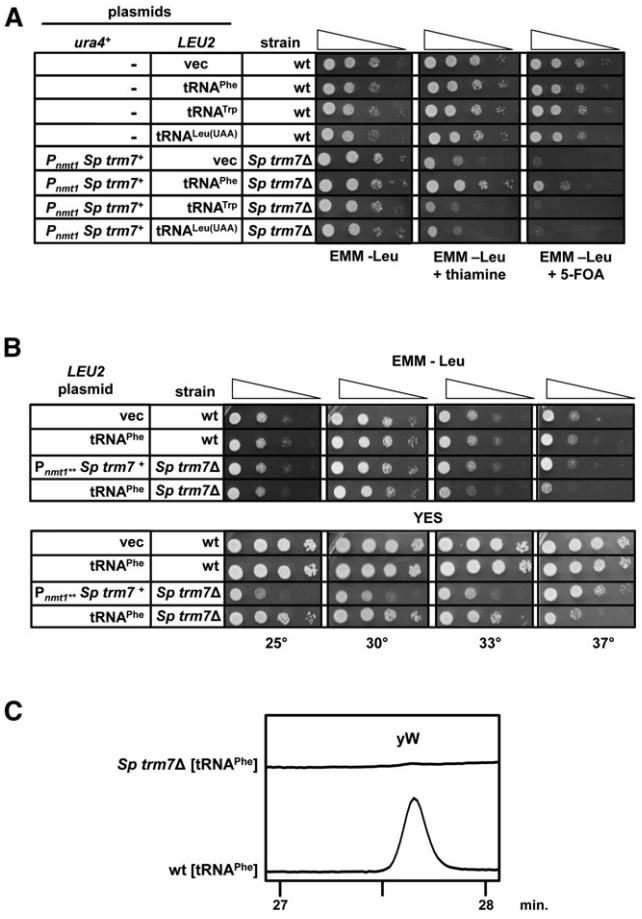


**FIGURE 3.** The biologically important *Sp* Trm7 tRNA substrate is $tRNA^{Phe}$. (*A*) Overexpression of $tRNA^{Phe}$ suppresses the near lethality of *S. pombe trm7*△ mutants. Strains with plasmids as indicated were grown overnight in EMM-Leu and analyzed as in Figure 2A, after incubation for 3 d at 30°C. (*B*) Overexpression of $tRNA^{Phe}$ in *S. pombe trm7*△ mutants does not completely restore wild-type growth at 37°C. Strains as indicated were purified on 5-FOA to ensure loss of the [$P_{nmt1**}$ *Sp trm7*$^+$ *ura4*$^+$] plasmid, grown overnight in EMM-Leu, and then growth was analyzed as indicated in Figure 2A. (*C*) $tRNA^{Phe}$ from the *S. pombe trm7*△ [$tRNA^{Phe}$] strain lacks yW. $tRNA^{Phe}$ purified from the indicated strains after growth in EMM-Leu was analyzed as described in Figure 2C.

## The putative *S. pombe trm732*$^+$ and *trm734*$^+$ genes are required for Cm and Gm formation, respectively, on $tRNA^{Phe}$

To further determine if the modification circuitry for the anticodon loop of $tRNA^{Phe}$ is conserved between *S. cerevisiae* and *S. pombe*, we analyzed the putative *S. pombe trm732*△ (*SPCC1494.07*) and *trm734*△ (*SPBC1306.02*) strains. An *Sp trm732*△ haploid mutant obtained by sporulation of the heterozygous diploid lacked detectable Cm in its $tRNA^{Phe}$ and had normal levels of Gm compared with that from wild type (0.88 versus 0.90 moles/mole) (Table 3; Fig. 4A) and did not have any obvious growth defect on plates or in liquid YES medium (Table 1; Fig. 4B), similar to the lack of an obvious growth defect observed for the *Sc trm732*△ mutant (Table 1; Guy et al. 2012).

However, an *Sp trm734*△ [*Sp trm734*$^+$ *ura4*$^+$] haploid obtained by sporulation of the heterozygous diploid containing the plasmid grew poorly when plated on EMM containing 5-FOA (Fig. 4C), and the resulting *Sp trm734*△ strain lacked $Gm_{34}$ in its $tRNA^{Phe}$, and had Cm levels comparable to those of wild type (0.86 versus 0.91 moles/mole) (Table 3; Fig. 4A). The *Sp trm734*△ mutant had a significant growth defect on YES medium on plates or in liquid (generation time of 258 min, compared with 148 min for wild type) (Table 1; Fig. 4D), whereas the *Sc trm734*△ mutant lacked any obvious growth defect on plates or in liquid medium (generation time of 70 min, compared with 66 min for wild type) (Table 1; Guy et al. 2012), demonstrating that Trm734 has a more prominent role in *S. pombe* than in *S. cerevisiae*. Furthermore, since overexpression of $tRNA^{Phe}$ nearly completely suppressed the slow growth phenotype of the *Sp trm734*△ strain over a range of temperatures (Fig. 4E), we conclude that lack of $Gm_{34}$ of $tRNA^{Phe}$ is the cause of the *Sp trm734*△ defect.

In addition, our analysis showed that, as in *S. cerevisiae* (Guy et al. 2012), yW formation was impaired in the *Sp trm734*△ mutant (44% of wild-type levels) and to a lesser extent in the *Sp trm732*△ mutant (73%) (Fig. 4F), consistent with the increased $m^1G$ levels (Table 3; Fig. 4A). We also note that $m^2G$ levels on $tRNA^{Phe}$ are substantially higher in the *Sp trm734*△ mutant compared with those from a wild-type strain when grown in EMM (0.61 versus 0.19 moles/mole).

**TABLE 3.** HPLC analysis of $tRNA^{Phe}$ nucleoside content from *S. pombe trm732*△ and *S. pombe trm734*△ strains

| Strain[a] | Medium | Cm | Gm | $m^1G$ | Ψ | $m^7G$ | $m^2G$ |
|---|---|---|---|---|---|---|---|
| Moles expected | | 1 | 1 | 1 | 4 | 1 | 1 |
| Wild type | YES | 0.88 ± 0.05 | 0.90 ± 0.03 | 0.98 ± 0.10 | 3.99 ± 0.08 | 0.32 ± 0.01 | 0.62 ± 0.05 |
| *trm732*△ | YES | <0.01 | 0.88 ± 0.04 | 1.19 ± 0.07 | 3.81 ± 0.17 | 0.31 ± 0.03 | 0.64 ± 0.07 |
| Wild type [vec] | EMM-Ura | 0.90 ± 0.04 | 0.92 ± 0.02 | 1.20 ± 0.21 | 3.94 ± 0.10 | 0.26 ± 0.12 | 0.14 ± 0.02 |
| *trm734*△ [*trm734*$^+$] | EMM-Ura | 0.75 ± 0.18 | 0.85 ± 0.06 | 1.24 ± 0.13 | 3.84 ± 0.07 | 0.23 ± 0.05 | 0.16 ± 0.05 |
| Wild type | EMM | 0.91 ± 0.05 | 0.89 ± 0.06 | 1.14 ± 0.10 | 3.98 ± 0.04 | 0.21 ± 0.03 | 0.19 ± 0.12 |
| *trm734*△ | EMM | 0.86 ± 0.04 | <0.03 | 1.57 ± 0.02 | 4.01 ± 0.06 | 0.31 ± 0.05 | 0.61 ± 0.11 |

[a]Mean and standard deviation based on three individual growths and RNA preparations.

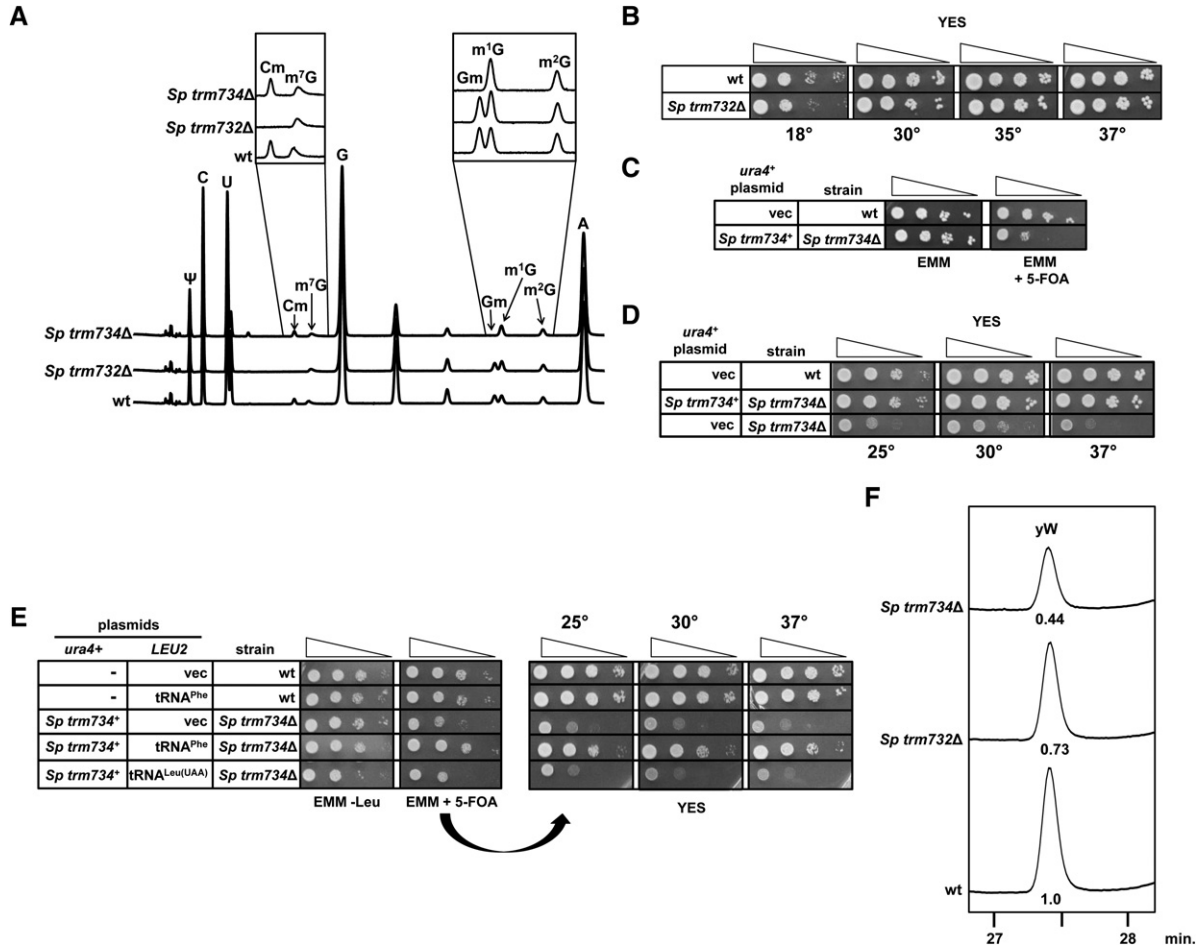


**FIGURE 4.** *S. pombe trm734*△ mutants are sick due to hypomodified $tRNA^{Phe}$. (*A*) HPLC traces of $tRNA^{Phe}$ from *S. pombe trm732*△ and *S. pombe trm734*△ mutants. $tRNA^{Phe}$ was purified from the indicated strains grown in YES medium and then digested to nucleosides and analyzed by HPLC. (*B*) *S. pombe trm732*△ mutants are healthy. Indicated strains were grown in YES medium overnight, and analyzed on YES plates as described in Figure 2A. (*C*) *S. pombe trm734*△ mutants have a growth defect on EMM. Indicated strains were grown overnight in EMM, diluted, and plated on EMM as indicated. (*D*) *S. pombe trm734*△ mutants have a substantial growth defect on rich medium at various temperatures. Indicated strains were transformed with indicated *ura4*$^+$ plasmids, grown in EMM-Ura, diluted, and plated on YES medium at the indicated temperatures after 3 d. (*E*) Overexpression of $tRNA^{Phe}$ suppresses the slow growth of *S. pombe trm734*△ mutants. Strains with plasmids as indicated were grown in EMM-Leu overnight, and analyzed as in Figure 2A, after incubation for 3 d at 30°C. Strains were then purified on medium containing 5-FOA, grown overnight in EMM-Leu, diluted, spotted, and analyzed at the indicated temperatures on YES medium. (*F*) Levels of yW in $tRNA^{Phe}$ are decreased in *S. pombe trm732*△ and *S. pombe trm734*△ mutants. $tRNA^{Phe}$ was purified from the indicated strains after growth in YES medium and analyzed as described in Figure 2C.

### FTSJ1 is the human Trm7 ortholog and requires *S. cerevisiae* Trm732 to catalyze $Cm_{32}$ modification on $tRNA^{Phe}$ in *S. cerevisiae*

To investigate the conservation of function of metazoan genes involved in 2′-*O*-methylation of the tRNA anticodon loop, we examined complementation of the corresponding *S. cerevisiae* mutants by introduction of appropriate constructs expressing metazoan *TRM7* and/or its metazoan *TRM732* and *TRM734* partners. To identify *TRM7* homologs, we examined *Sp trm7*$^+$ and predicted *TRM7* orthologs from *D. melanogaster* and humans by introduction of a high copy [2μ *LEU2* $P_{GAL}$ *TRM7*] plasmid expressing the corresponding ortholog under galactose control to an *Sc* trm7Δ [*Sc* TRM7 URA3] strain, followed by analysis of growth after plating on medium containing 5-FOA and galactose. We found that the slow growth phenotype of *Sc trm7*Δ mutants was efficiently suppressed by expression of *Sp* Trm7 (~61% identical to *Sc* Trm7), *D. melanogaster* ORF *CG5220* (*Dm TRM7*, ~43% identical to *Sc* Trm7), and human *FTSJ1* (~50% identical to *Sc* Trm7) but not human *FTSJ2* (~34% identical to *Sc* Trm7) (Fig. 5A), after selection on medium containing 5-FOA. Furthermore, the resulting *Sc* trm7Δ [2μ *LEU2* $P_{GAL}$ *FTSJ1*] strain grew nearly as well as the wild-type *S. cerevisiae* strain (Fig. 5A, right panel), strongly suggesting that FTSJ1 is the human ortholog of Trm7.

The ability of human *FTSJ1* and the other tested *TRM7* orthologs to complement the growth defect of an *S. cerevisiae trm7*Δ mutant strain was surprising because *Sc* Trm7 and *Sp* Trm7 have a strict requirement for their corresponding Trm732 and Trm734 partner proteins to generate $Cm_{32}$ and $Nm_{34}$, respectively (Guy et al. 2012). Because $tRNA^{Phe}$ is the *Sc* Trm7 substrate that must be modified for healthy growth in *S. cerevisiae* (Guy et al. 2012), we examined 2′-*O*-methylation of $tRNA^{Phe}$ from the *Sc trm7*Δ [2μ *LEU2* $P_{GAL}$ *FTSJ1*] strain. We found that $tRNA^{Phe}$ purified from this strain had high levels of Cm (0.89 versus 0.90 moles/mole in wild type), but no detectable Gm (Table 4), suggesting that human FTSJ1 is working in concert with *Sc* Trm732 to modify $tRNA^{Phe}$. The healthy growth of this *Sc trm7*Δ [2μ *LEU2* $P_{GAL}$ *FTSJ1*] strain is consistent with our previous observation that the $Cm_{32}$ modification of $tRNA^{Phe}$ is sufficient for healthy growth in *S. cerevisiae* (Guy et al. 2012).

The occurrence of Cm in $tRNA^{Phe}$ from an *Sc trm7*Δ [2μ *LEU2* $P_{GAL}$ *FTSJ1*] strain suggested either that FTSJ1 does not require an interacting partner for $Cm_{32}$ catalysis, or that it works with *S. cerevisiae* Trm732. Since the slow growth phenotype of the *Sc trm7*Δ *trm732*Δ strain was not suppressed by introduction of the [2μ *LEU2* $P_{GAL}$ *FTSJ1*] plasmid and no Cm formation was observed on $tRNA^{Phe}$ purified from this strain (Table 4; Fig. 5B), we infer that FTSJ1 works with *Sc* Trm732 for formation of $Cm_{32}$ on $tRNA^{Phe}$. Since $tRNA^{Leu(UAA)}$ from an *Sc trm7*Δ [2μ *LEU2* $P_{GAL}$ *FTSJ1*] strain lacked both Cm and $ncm^5Um$ (Table 5; Fig. 1A), the activity of FTSJ1 with Trm732 in *S. cerevisiae* does not extend to all *Sc* Trm7 substrates.

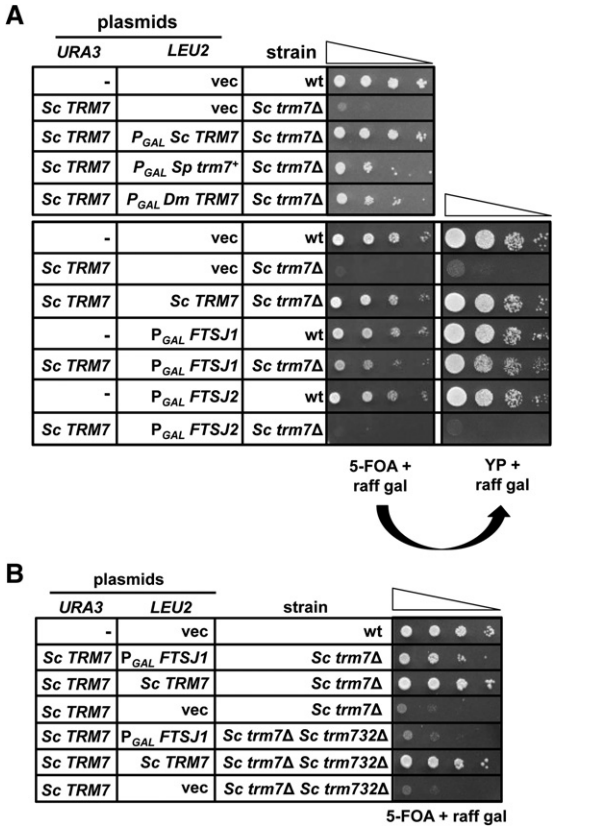


**FIGURE 5.** FTSJ1 is the human ortholog of *S. cerevisiae* Trm7, and its function in *S. cerevisiae* requires Trm732 for $Cm_{32}$ modification. (*A*) Expression of *S. pombe trm7*$^+$, *D. melanogaster TRM7*, or human *FTSJ1* suppresses the slow growth *S. cerevisiae trm7*△ mutants. Strains with plasmids as indicated were grown overnight in S-Leu medium containing raffinose and galactose, diluted to $OD_{600}$ of ~0.5 in $H_2O$, and serially diluted 10-fold in $H_2O$, and then 2 μL was spotted onto S medium containing 5-FOA, raffinose, and galactose, followed by incubation for 3 d at 30°C. (*Right* panel) Selected strains were then purified on medium containing 5-FOA, grown overnight in S-Leu medium containing raffinose and galactose, and analyzed by spotting to YP medium containing raffinose and galactose. (*B*) *FTSJ1* requires *Sc TRM732* to suppress the slow growth of *Sc trm7*△ mutants. Strains were analyzed as in *A*.

We note that because FTSJ1 is expressed from a high copy $P_{GAL}$ plasmid in these experiments, it is formally possible that FTSJ1 is not the true Trm7 homolog. However, this seems unlikely due to the high sequence similarity between FTSJ1 and Trm7, and the requirement of *Sc* Trm732 function for FTSJ1 activity in *S. cerevisiae*.

### THADA is the human homolog of Trm732 and works with ScTrm7 for $Cm_{32}$ modification

Although Trm732 homologs have only ~20% sequence identity, we find that the putative human Trm732 homolog THADA functions efficiently to replace *Sc* Trm732. Thus, the slow growth of an *Sc trm7*Δ *trm732*Δ strain was

**TABLE 4.** HPLC analysis of $tRNA^{Phe}$ nucleoside content from *S. cerevisiae trm7Δ* [*FTSJ1*] and *S. cerevisiae trm7Δ trm732Δ* [*FTSJ1 THADA*] strains

| Strain[a] | Cm | Gm | $m^1G$ | Ψ | $m^5C$ | $m^2G$ |
|---|---|---|---|---|---|---|
| Moles expected | 1 | 1 | 0 | 2 | 2 | 1 |
| Wild type [vec] | 0.90 ± 0.16 | 0.81 ± 0.03 | <0.03 | 2.07 ± 0.12 | 1.75 ± 0.09 | 0.86 ± 0.05 |
| *trm7Δ* [vec] | <0.01 | <0.03 | 0.99 ± 0.09 | 2.11 ± 0.16 | 1.83 ± 0.06 | 0.98 ± 0.08 |
| *trm7Δ* [*TRM7*] | 1.0 ± 0.18 | 0.86 ± 0.08 | <0.03 | 2.13 ± 0.05 | 1.77 ± 0.10 | 0.96 ± 0.08 |
| *trm7Δ* [*FTSJ1*] | 0.89 ± 0.22 | <0.03 | 0.47 ± 0.13 | 2.13 ± 0.14 | 1.85 ± 0.09 | 0.95 ± 0.05 |
| *trm7Δ trm732Δ* [vec] | <0.01 | <0.03 | 0.91 ± 0.15 | 2.11 ± 0.07 | 1.82 ± 0.05 | 0.89 ± 0.21 |
| *trm7Δ trm732Δ* [*FTSJ1*] | <0.01 | <0.03 | 0.93 ± 0.06 | 2.10 ± 0.04 | 1.82 ± 0.02 | 1.01 ± 0.03 |
| *trm7Δ trm732Δ* [*FTSJ1* + *THADA*] | 0.90 ± 0.20 | <0.03 | 0.63 ± 0.06 | 2.11 ± 0.05 | 1.86 ± 0.04 | 0.99 ± 0.07 |
| *trm732Δ trm734Δ* [vec] | <0.01 | <0.03 | 1.03 ± 0.03 | 2.13 ± 0.08 | 1.86 ± 0.06 | 0.98 ± 0.04 |
| *trm732Δ trm734Δ* [*THADA*] | 0.76 ± 0.13 | <0.03 | 0.66 ± 0.19 | 2.08 ± 0.05 | 1.76 ± 0.09 | 0.89 ± 0.16 |

[a]Mean and standard deviation based on three individual growths and RNA preparations.

suppressed by expression of *FTSJ1* and *THADA*, but not by *FTSJ1* alone (Fig. 6A). Since $tRNA^{Phe}$ from this *Sc trm7Δ trm732Δ* [2μ $P_{GAL}$ *FTSJ1* $P_{GAL}$ *THADA*] strain had identical Cm levels to that from the wild-type strain (0.90 moles/mole) (Table 4) and no detectable Gm, and since $tRNA^{Leu(UAA)}$ from this strain had substantial Cm modification (0.36 moles/mole) and no $ncm^5Um$ (Table 5), we conclude that THADA is the human Trm732 homolog and functionally interacts with FTSJ1. The ability of FTSJ1 to modify $C_{32}$ of $tRNA^{Leu(UAA)}$ in combination with THADA but not with *Sc* Trm732 may be due to higher levels of THADA relative to endogenous levels of yeast Trm732, and/or more efficient partnering of FTSJ1 and THADA.

We also find evidence that *S. cerevisiae* Trm7 can interact with human THADA for $Cm_{32}$ formation, since expression of *THADA* suppressed the slow growth of an *Sc trm732Δ trm734Δ* strain (Fig. 6A), resulting in $tRNA^{Phe}$ with 0.76 moles/mole Cm (Table 4). Thus, our data demonstrate that human (FTSJ1) or *S. cerevisiae* Trm7 can each utilize either human (THADA) or *S. cerevisiae* Trm732 to catalyze formation of $Cm_{32}$ on $tRNA^{Phe}$ in *S. cerevisiae*. *S. cerevisiae* Trm7 also appears to work with *S. pombe* Trm732, because expression of *Sp trm732*$^+$ suppressed the slow growth of the *Sc trm732Δ trm734Δ* strain (Fig. 6B).

## DISCUSSION

Our results demonstrate that the entire circuitry for $tRNA^{Phe}$ anticodon loop modification established for *S. cerevisiae* has been retained in the yeast *S. pombe*, requiring Trm7 to act with Trm732 to 2′-*O*-methylate $C_{32}$, and with Trm734 to 2′-*O*-methylate $G_{34}$, leading to efficient conversion of $m^1G_{37}$ to yW. Furthermore, we have provided strong evidence that FTSJ1 and THADA are the human Trm7 and Trm732 orthologs, and that the human and *S. cerevisiae* proteins can act interchangeably together to catalyze 2′-*O*-methylation of $C_{32}$ of $tRNA^{Phe}$ (Table 4). *THADA and S. pombe trm732*$^+$ each complemented the Trm732 defect of *Sc trm732Δ trm734Δ* mutants, but have little overall conservation (~20% identity, mostly clustered in the small DUF2428 region) (Fig. 1C), suggesting that there may be substantial structural homology in less conserved regions, such as in the predicted armadillo repeats (Tewari et al. 2010).

**TABLE 5.** HPLC analysis of $tRNA^{Leu(UAA)}$ nucleoside content from an *S. cerevisiae trm7Δ* [*FTSJ1*] strain

| Modification | Moles expected | wt [vec] | *trm7Δ* [vec] | *trm7Δ* [*TRM7*] | *trm7Δ* [*FTSJ1*] | *trm7Δ trm732Δ* [vec] | *trm7Δ trm732Δ* [*FTSJ1*] | *trm7Δ trm732Δ* [*FTSJ1 THADA*] |
|---|---|---|---|---|---|---|---|---|
| Cm | 1 | 0.58 | 0.02 | 0.57 | 0.03 | 0.02 | 0.02 | 0.36 |
| $ncm^5Um$ | 1 | 1.52 | 0.03 | 1.43 | 0.03 | 0.01 | <0.01 | 0.02 |
| $ncm^5U$ | 0 | <0.01 | 0.94 | <0.01 | 1.16 | 1.13 | 1.0 | 0.97 |
| $m^1G + Gm$ | 2 | 1.8 | 1.86 | 1.97 | 1.8 | 1.96 | 1.52 | 1.48 |
| Ψ | 2 | 2.16 | 2.17 | 2.13 | 2.14 | 2.3 | 2.14 | 2.0 |
| $m^5C$ | 1 | 0.82 | 0.83 | 0.92 | 0.74 | 1.02 | 0.77 | 0.97 |
| $ac^4C$ | 1 | 0.71 | 0.81 | 0.83 | 0.74 | 0.71 | 0.35 | 0.35 |
| $m^2G$ | 1 | 0.84 | 0.95 | 1.02 | 0.93 | 1.04 | 0.78 | 0.92 |
| $m^{2,2}G$ | 1 | 0.88 | 0.88 | 0.89 | 0.89 | 0.75 | 0.96 | 0.83 |

Since the *S. cerevisiae* and *S. pombe* lineages diverged from one another ~1.1 billion yr ago, and since humans and fungal lineages diverged ~1.6 billion yr ago (Hedges 2002), it seems likely that this anticodon loop modification circuitry is widely conserved among eukaryotes. The absence of identifiable Trm732 and Trm734 orthologs in some eukaryotes may indeed be due to lack of universal conservation of the corresponding genes in some organisms, perhaps because of increased amounts of $tRNA^{Phe}$ in those organisms. Alternatively the lack of identifiable Trm732 and Trm734 orthologs may reflect the poor sequence conservation of these proteins, or may be due to alternative 2′-*O*-methylation pathways, such as by a phylogenetically distinct anticodon loop 2′-*O*-methyltransferase family (Tkaczuk et al. 2007), or by a Box C/D guide RNA (Joardar et al. 2011).

We note that experiments to test the function of WDR6, the predicted human Trm734 homolog, by complementation of the appropriate *S. cerevisiae* mutants have been inconclusive. Coexpression of human *WDR6* with *FTSJ1* does not suppress the slow growth of a *Sc trm7*Δ *trm732*Δ strain (data not shown), but WDR6 was not expressed well in *S. cerevisiae* (data not shown). Nonetheless, because Trm734 function is conserved in *S. pombe* and *S. cerevisiae*, it seems plausible that WDR6 and the other Trm734 family members will be required for $Gm_{34}$ formation on $tRNA^{Phe}$ in their corresponding organisms.

Further evidence that this $tRNA^{Phe}$ anticodon loop modification circuitry is conserved in humans derives from the observation that Ehrlich ascites tumors and neuroblastoma cells lacking $O_2yW_{37}$ (peroxywybutosine) on $tRNA^{Phe}$ also lack $Cm_{32}$ and $Gm_{34}$ modifications on $tRNA^{Phe}$ (Kuchino et al. 1982), consistent with the requirement for 2′-*O*-methylation of the anticodon loop of $tRNA^{Phe}$ as a prerequisite for $yW_{37}$ formation, and fueling speculation that these tRNA defects arise from defective FTSJ1 function.

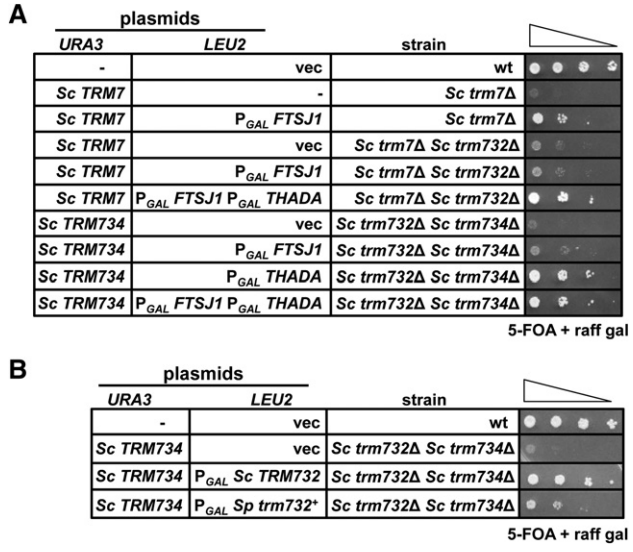


**FIGURE 6.** *S. cerevisiae* Trm7 works with human THADA or *S. pombe* Trm732 for function. (*A*) *THADA* is the human ortholog of *S. cerevisiae TRM7*. Strains with plasmids as indicated were analyzed as in Figure 5A. (*B*) *S. pombe trm732*$^+$ suppresses the slow growth of *S. cerevisiae trm732*△ *trm734*△ mutants. Strains were analyzed as in Figure 5A.

Our data also provide further evidence indicating that levels of tRNA modifications are regulated by cellular growth conditions. Thus, we found that $m^7G$ and $m^2G$ levels on $tRNA^{Phe}$ are decreased in *S. pombe* cells grown in EMM in the absence of thiamine (Table 2), similar to the changes in tRNA modification levels observed in *S. cerevisiae* cells grown under cellular stress conditions or in cells that have undergone growth arrest (Chan et al. 2010; Chan et al. 2012; Preston et al. 2013). Furthermore, the finding that $m^2G$ levels of $tRNA^{Phe}$ are near normal in the *Sptrm7*Δ and *Sptrm734*Δ mutants (compared with those from wild-type cells grown in EMM) implies the existence of a compensatory modification mechanism, similar to that observed in *trm9Δ* mutants, which have acquired $ncm^5U$ (and $ncm^5s^2U$) in the absence of $mcm^5U_{34}$ (and $mcm^5s^2U$) (Chen et al. 2011a), but in this case the $m^2G$ is likely on a different residue.

Our finding that $tRNA^{Phe}$ is the biologically important Trm7 substrate in both *S. cerevisiae* and *S. pombe* (Fig. 3; Guy et al. 2012) suggests that $tRNA^{Phe}$ may be the important substrate throughout eukaryotes. This is consistent with our finding that human FTSJ1 and THADA readily modified $C_{32}$ of $tRNA^{Phe}$ in *S. cerevisiae*, and that $tRNA^{Phe}$ from 16 of 17 eukaryotes examined contains $Cm_{32}$ and $Gm_{34}$ (Machnicka et al. 2013). However, we note that overexpression of $tRNA^{Phe}$ did not completely suppress the growth defect of *Sp trm7*△ mutants, particularly at high temperature (Fig. 3). This could occur if there are other tRNA species that require modification for full function at this temperature, if the $tRNA^{Phe}$ is not sufficiently overexpressed at this temperature to overcome the defect in decoding, or if part of the defect in $tRNA^{Phe}$ occurs at a step after binding of the tRNA to the A-site of the ribosome, since additional copies of hypomodified tRNAs should not affect translation after this step. Because overexpression of $tRNA^{Phe}$ nearly completely suppressed the slow growth phenotype of *Sp trm734*△ mutants at all temperatures, the defect is almost certainly caused by loss of $Gm_{34}$ on $tRNA^{Phe}$ (Fig. 4E).

Although there is conservation of the Trm7 circuitry for $tRNA^{Phe}$ anticodon loop modification and for the importance of these modifications in *S. pombe* and *S. cerevisiae*, there are two crucial differences in the biological consequences of mutations in the corresponding genes in the two organisms. First, a *trm7Δ* mutation was more deleterious to growth in *S. pombe* than in *S. cerevisiae*; thus the *Sp trm7*Δ [$P_{nmt1**}$ *Sp trm7*$^+$ *LEU2*] strain (although itself much healthier than the *Sp trm7*Δ haploid) still had a generation time 2.16-fold higher than the wild-type strain (Table 1), which was slightly more than the difference of 1.94-fold observed for an *S. cerevisiae trm7Δ* mutant compared with its wild-type control. Second, an *S. pombe trm734Δ* mutant had a severe growth defect, with a 1.7-fold increased generation time relative to

the wild type strain, whereas an *S. cerevisiae trm734Δ* mutant had 1.06-fold difference in generation time. Furthermore, our identification of *FTSJ1* as the human *Sc TRM7* ortholog suggests that defects in the human gene result in a relatively mild, albeit medically serious, condition, since *FTSJ1* splice site, nonsense, and deletion mutations are consistently associated with NSXLID (Freude et al. 2004; Ramser et al. 2004; Froyen et al. 2007; Takano et al. 2008). NSXLID may occur in these patients because specific human tRNAs have a greater requirement for Trm7 modification in development of the central nervous system (CNS) than in other tissues; indeed, recent results suggest that mutation of a tRNA isodecoder expressed specifically in the CNS can lead to ribosome stalling and contribute to neurodegeneration in certain mutant mouse strains (Ishimura et al. 2014).

Our results demonstrating that THADA is the human Trm732 ortholog further suggest that 2′-*O*-methylation of the tRNA anticodon loop may be associated with human health. *THADA* is associated with epithelial thyroid adenomas (Rippe et al. 2003); and genome wide association studies have implicated *THADA* alleles in type 2 diabetes (Zeggini et al. 2008) and polycystic ovary syndrome (Chen et al. 2011c). However, the biological significance of these associations is not known, and the linkage to $Nm_{32}$ modification remains to be determined.

NSXLID associated with defective *FTSJ1* (Freude et al. 2004; Ramser et al. 2004; Froyen et al. 2007; Takano et al. 2008) adds to a growing list of neurological disorders associated with defective tRNA modification. This list includes intellectual disability associated with a point mutation in *hADAT3* , the predicted homolog of a subunit of the yeast tRNA $A_{34}$ deaminase (Alazami et al. 2013); a frameshift mutation in *hTRMT1* (Najmabadi et al. 2011), which has tRNA $m^{2,2}G_{26}$ ($N^2$,$N^2$-dimethylguanosine) methyltransferase activity (Liu and Strâby 2000); mutations in *NSUN2* (Abbasi-Moheb et al. 2012; Khan et al. 2012; Martinez et al. 2012), which modifies $C_{34}$, $C_{48}$, $C_{49}$, and $C_{50}$ on target tRNAs to $m^5C$; and mutations in *hELP2* (Najmabadi et al. 2011), a member of the ELP complex responsible for formation of the $cm^5U$ moiety found on $mcm^5U_{34}$, $ncm^5U_{34}$, $mcm^5s^2U_{34}$ and related modifications. In addition, familial disautonomia is associated with mutations in *hELP1* (*IKBAKP*) (Anderson et al. 2001). The ELP complex has been reported to have functions in addition to tRNA modification in humans (Creppe et al. 2009; Okada et al. 2010), suggesting the possibility that these disease associations could be due to non-tRNA related defects. Regardless, it is notable that many of the associations between defective tRNA modification and neurological disorders are linked to wobble residue 34, which is required for efficient and accurate decoding of mRNA (Agris et al. 2007; Johansson et al. 2008), suggesting that neurological development is extremely sensitive to defects in translation. It remains to be seen whether *FTSJ1*-associated NSXLID is specifically due to $Nm_{34}$ defects, to $Nm_{32}$ defects, or to both.

## MATERIALS AND METHODS

### Identification of Trm7, Trm732, and Trm734 sequence homologs from diverse eukaryotic genomes

BLAST searches (http://blast.ncbi.nlm.nih.gov/) for Trm7, Trm732, and Trm734 homologs were performed against sequenced genomes from a diverse set of eukaryotes including representatives from all five eukaryotic supergroups (Adl et al. 2012), including Amoebazoa (*Dictyostelium discoideum*), Archaeplastida (*Arabidopsis thaliana, Cyanidioschyzon merolae, Ostreococcus tauri*, *Oryza sativa*, and *Zea mays*), Excavata (*Giardia intestinalis*, *Naegleria gruberi*), Opisthokonta (*Bombyx mori, Caenorhabditis elegans, Danio rerio, Drosophila melanogaster, Homo sapiens, Monosiga brevicollis, Mus musculus, Saccharomyces cerevisiae, Schizosaccharomyces pombe,* and *Xenopus tropicalis*), and SAR (Stramenopiles + Alveolates + Rhizaria) (*Cryptomonas paramaecium, Guillardia theta*, *Phytophthora infestans, Tetrahymena thermophile, Thalassiosira pseudonana, Toxoplasma gondii*, and *Trypanosoma brucei*).

### Yeast strains

Yeast strains are listed in Table 6. The *S. pombe* haploid *Sp trm7Δ::kanMX* [$P_{nmt1}$ *Sp trm7*$^+$ *ura4*$^+$] (yMG1052A) strain was generated by transformation of the *Sp trm7Δ::kanMX* heterozygous diploid with pMG360A [$P_{nmt1}$ *Sp trm7*$^+$*ura4*$^+$], followed by sporulation on EMM lacking uracil supplemented with 500 mg/L G418, selection of haploids, and PCR confirmation of the knockout. The haploid *S. pombe trm732Δ::kanMX* mutant strain (yMG958B) was generated by sporulation of the heterzygous diploid on yeast extract with 3% dextrose (YE medium) with 200 mg/L G418, selection of haploids, and PCR confirmation. The haploid *S. pombe trm734Δ* [$P_{Sp\ trm734}$ *Sp trm734*$^+$ *ura4*$^+$] mutant strain (yMG1289-1) was generated by transformation of the *Sp trm734Δ::kanMX* heterozygous diploid with a *LEU2* $tRNA^{Phe}$ plasmid (pMG308C), followed by sporulation on YE medium with 200 mg/L G418, selection of haploids and PCR verification. This strain was then transformed with pMG426G ($P_{Sp\ trm734}$ *Sp trm734*$^+$ *ura4*$^+$), followed by selection of *leu*$^-$/*ura*$^+$ colonies, and PCR confirmation. The *S. cerevisiae trm7Δ::ble*$^R$ [*URA3 Sc TRM7*] (yMG348-1), *Sc trm732Δ::ble*$^R$ (yMG814-1), *Sc trm734Δ::ble*$^R$ (yMG724-5), and *Sc trm734Δ::ble*$^R$ *trm732Δ::kanMX* [*Sc TRM734 URA3 CEN*] (yMG818-1) strains were described previously (Guy et al. 2012). Double mutant *S. cerevisiae trm7Δ* strains were constructed by PCR amplification of DNA from the appropriate YKO collection *kanMX* strain (Open Biosystems), followed by transformation of the DNA into yMG348-1.

### Plasmids

Plasmids used in this study are listed in Table 7. The *S. pombe* vector expressing *S. pombe trm7*$^+$ under control of the $P_{nmt1}$ (no message in thiamine) (pMG360A) and $P_{nmt1**}$ (low strength no message in thiamine) promoters (pMG527B) were constructed by PCR of the *Sp trm7*$^+$ cDNA ORF from an *S. pombe* cDNA library (Fikes et al. 1990) and insertion into the XhoI and SmaI sites of pREP4X or pREP81X vectors, respectively. The *S. pombe* vector expressing *Sp trm734*$^+$ under control of the native *Sp trm734*$^+$promoter (pMG426G) was constructed by PCR of *Sp trm734*$^+$ from *S. pombe* genomic DNA and insertion at the PstI and XhoI sites of pREP4X to

**TABLE 6.** Strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| SP286 (wild-type *S. pombe* diploid) | *ade6*-M210/*ade6*-M216 *ura4*-D18/*ura4*-D18 *leu1-32*/*leu1-32* | Bioneer |
| yMG957B (wild-type haploid) | SP286 | This study |
| *spac4F10.03c*Δ (*Sp trm7*△ heterozygous diploid) | SP286, *spac4F10.03cΔ::kanMX/SPAC4F10.03c* | Bioneer |
| yMG1052A | SP286, *spac4F10.03cΔ::kanMX* [$P_{nmt1}$ *SPAC4F10.03c ura4*$^+$] | This study |
| *spcc1306.02*Δ (*Sp trm734*Δ heterozygous diploid) | SP286, *spbc1306.02Δ::kanMX/SPBC1306.02* | Bioneer |
| yMG1289-1 | SP286, *spbc1306.02Δ::kanMX* [$P_{SPBC1306.02}$ *SPBC1306.02*$^+$ *ura4*$^+$] | This study |
| yMG1291 | SP286, *spbc1306.02Δ::kanMX* | This study |
| *spcc1494.07*Δ (*Sp trm732*Δ heterozygous diploid) | SP286, *spcc1494.07Δ::kanMX/SPCC1494.07* | Bioneer |
| yMG958B | SP286, *spcc1494.07Δ::kanMX* | This study |
| BY4741 (wild *S. cerevisiae* haploid) | *MAT*a *his3-Δ1 leu2Δ0 met15-Δ0 ura3-Δ0* | Open Biosystems |
| yMG348-1 | BY4741, *Sc trm7Δ::ble*$^R$ [*Sc TRM7 URA3 CEN*] | Guy et al. (2012) |
| yMG105 | BY4741, *Sc trm7Δ::ble*$^R$ | Guy et al. (2012) |
| yMG724-5 | BY4741, *Sc trm734Δ::ble*$^R$ | Guy et al. (2012) |
| yMG814-1 | BY4741, *Sc trm732Δ::ble*$^R$ | Guy et al. (2012) |
| yMD1-4 | yMG348-1, *Sc trm734Δ::kanMX* | This study |
| yMD2-4 | yMG348-1, *Sc trm732Δ::kanMX* | This study |
| yMG818-1 | BY4741, *Sc trm734Δ::ble*$^R$, *Sc trm732Δ::kanMX* [*Sc TRM734 URA3 CEN*] | Guy et al. (2012) |

remove the $P_{nmt1}$ promoter sequence. tRNA expression vectors were generated by PCR of the appropriate tRNA fragment from genomic DNA and insertion at the Pst1 and XhoI sites of pREP3X. For expression in *S. cerevisiae*, human *FTSJ1* and *THADA* ORFs were cloned from cDNA plasmids (Open Biosystems), as was *D. melanogaster* ORF *CG5220* (*Dm TRM7*) (Drosophila Genomics Resource Center). *S. pombe trm732*$^+$ ORF was cloned from an *S. pombe* cDNA library (Fikes et al. 1990). ORFs were then inserted by ligation independent cloning (LIC) into [2μ $P_{GAL1,10}$] *S. cerevisiae* dual ORF expression vectors which express ORFs under $P_{GAL1}$ control with a C-terminal PT tag (ORF-3C site-HA epitope-His6- ZZ domain of protein A), and ORFs under $P_{GAL10}$ control with no tag, essentially as described previously (Quartley et al. 2009; Guy et al. 2012). All plasmids were confirmed by sequencing before use.

**TABLE 7.** Plasmids used in this study

| Plasmid | Parent | Description | Source |
|---|---|---|---|
| pREP4X | | $P_{nmt1}$ *ura4*$^+$ | Forsburg (1993) |
| pMG360A | pREP4X | $P_{nmt1}$ *SPAC4F10.03*$^+$ cDNA *ura4*$^+$ | This study |
| pREP81X | | $P_{nmt1**}$ *LEU2* (low strength $P_{nmt1}$) | Forsburg (1993) |
| pMG527B | pREP81X | $P_{nmt1**}$ *SPAC4F10.03*$^+$ cDNA *LEU2* | This study |
| pREP3X | | $P_{nmt1}$ *LEU2* | Forsburg (1993) |
| pMG308C | pREP3X | *LEU2* $tRNA^{Phe}$ | This study |
| pMG309A | pREP3X | *LEU2* $tRNA^{Trp}$ | This study |
| pMG310A | pREP3X | *LEU2* $tRNA^{Leu(UAA)}$ | This study |
| pMG426G | pREP4X | $P_{SPBC1306.02}$ *SPBC1306.02*$^+$ *ura4*$^+$ | This study |
| pAVA579 | | *CEN URA3* LIC | Quartley et al. (2009) |
| pMG13 | pAVA579 | *CEN URA3 Sc TRM7* | Guy et al. (2012) |
| pBG2619 | | 2μ *LEU2* $P_{GAL1,10}$ LIC | Quartley et al. (2009) |
| pMG240A | pBG2619 | 2μ *LEU2* $P_{GAL10}$ *Sc TRM7* | Guy et al. (2012) |
| pMG298B | pBG2619 | 2μ *LEU2* $P_{GAL10}$ *SPAC4F10.03*$^+$*-PT* cDNA | This study |
| pOT2-*CG5220* | | pOT2-*CG5220* cDNA | Drosophila Genomics Resource Center |
| pMG291A | pBG2619 | 2μ *LEU2* $P_{GAL10}$ *CG5220* | This study |
| pOTB7 *FTSJ1* | | pOTB7 *FTSJ1* cDNA | Open Biosystems |
| pCR-BluntII-TOPO *FTSJ2* | | pCR-BluntII-TOPO *FTSJ2* cDNA | Open Biosystems |
| pMG133A | pBG2619 | 2μ *LEU2* $P_{GAL10}$ *FTSJ1* | This study |
| pMG135A | pBG2619 | 2μ *LEU2* $P_{GAL10}$ *FTSJ2* | This study |
| pBP2A | pAVA579 | *CEN URA3 Sc TRM734* | Guy et al. (2012) |
| pMG140A | pBG2619 | 2μ *LEU2* $P_{GAL1}$ *FTSJ1-PT* | This study |
| pENTR223.1 *THADA* | | pENTR223.1 *THADA* cDNA | Open Biosystems |
| pMG244A | pMG140A | 2μ *LEU2* $P_{GAL1}$ *FTSJ1-PT* $P_{GAL10}$*-THADA* | This study |
| pMG245A | pBG2619 | 2μ *LEU2* $P_{GAL10}$ *THADA* | This study |
| pMG299A | pBG2619 | 2μ *LEU2* $P_{GAL10}$ *SPCC1494.07*$^+$*-PT* cDNA | This study |

## Isolation and purification of tRNA

*S. pombe* strains were grown at 30°C to mid-log phase in YE medium supplemented with 225 mg/L adenine, lysine, histidine, leucine, and uracil (YES), or in EMM with appropriate supplements at 225 mg/L. For analysis of tRNA from the *Sp trm7*Δ [$P_{nmt1^{**}}$ *Sp trm7*$^{+}$ *LEU2*] and wild-type strains under repressive conditions, thiamine was added to EMM-Leu at 5 mg/L. *S. cerevisiae* strains were grown at 30°C to mid-log phase in S dropout medium containing 2% raffinose and 2% galactose. Bulk low molecular weight RNA was extracted from 300 OD-mL pellets, and appropriate 5′ biotinylated oligonucleotides were used to purify tRNA as previously described (Jackman et al. 2003).

## HPLC and analysis of tRNA

Purified tRNA was digested with P1 nuclease and phosphatase as previously described, and nucleosides were subjected to HPLC analysis essentially as previously described (Jackman et al. 2003). For $tRNA^{Phe}$, HPLC was done at pH 7.0 to maximize separation of Gm and $m^1G$ as previously described (Guy et al. 2012). For detection of yW, HPLC was performed with buffers and gradients essentially as previously described (Noma et al. 2006).

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