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 \textit{Saccharomyces cerevisiae}. \textit{S. cerevisiae} \textit{Trm7} interacts separately with \textit{Trm732} and \textit{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\(^1\)G\(_{37}\) of tRNA\(^{Phe}\). Moreover, \textit{trm7}Δ and \textit{trm732}Δ \textit{trm734}Δ 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 \textit{TRM7} ortholog is implicated in nonsyndromic X-linked intellectual disability. We find that the distantly related yeast \textit{Schizosaccharomyces pombe} has retained this circuitry for anticodon loop modification, that \textit{S. pombe} \textit{trm7}Δ and \textit{trm732}Δ \textit{trm734}Δ mutants have more severe phenotypes than the \textit{S. cerevisiae} mutants, and that tRNA\(^{Phe}\) is the major biological target. Furthermore, we provide evidence that \textit{Trm7} and \textit{Trm732} function is widely conserved throughout eukaryotes, since human \textit{FTSJ1} and \textit{THADA}, respectively, complement growth defects of \textit{S. cerevisiae} \textit{trm7}Δ and \textit{trm732}Δ \textit{trm734}Δ mutants by modifying C\(_{32}\) of tRNA\(^{Phe}\), each working with the corresponding \textit{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: \textit{FTSJ1}; \textit{TRM7}; \textit{TRM732}; \textit{TRM734}; \textit{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 \textit{Escherichia coli} (Wolf et al. 2002), in the yeast \textit{Saccharomyces cerevisiae} (Gerber and Keller 1999), and in the distantly related yeast \textit{Schizosaccharomyces pombe} (Kim et al. 2010), and RNAi against a putative homolog results in 29% embryonic lethality in the nematode \textit{Caenorhabditis elegans} (Fernandez et al. 2005). Similarly, lack of the terminal methyl group of mcm\(^3\)U\(_{34}\) (5-methoxycarbonylmethyluridine) in \textit{S. cerevisiae} due to mutation of \textit{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 \textit{TRM9} (\textit{Alkbh8}−/−) appear normal (Sonne-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\(^1\)A\(_{38}\) (1-methyladenosine) modification found in many eukaryotic tRNAs are essential in \textit{S. cerevisiae} (Anderson et al. 1998) due to turnover of pre-tRNA\(^{Met}\) by the nuclear surveillance pathway (Kadaba et al. 2004), but, the corresponding gene knockouts are each...
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 Ψ^6^A_{32} (N^6^-threonylcarbamoylbenosine) 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 Ψ^6^A_{32} (N^6^-isopentenyladenosine) due to mutation of *MOD5* in *S. cerevisiae*, results in reduced nonsense suppression (Latanén et al. 1978; Dihanich et al. 1987), but no obvious growth defect, whereas *S. pombe* *mod5Δ* (Sp tit1Δ) 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* (*TRTI*) 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^5^U moiety found in cm^5^U_{34}, cm^5^U_{34}, [5-carbamoyl methyluridine], and cm^5^s^2^U_{34} [5-methoxy carbonylmethyl-2-thiouridine]) are due to lack of functional tRNA_{Lys(UU)}^{'}, tRNA_{Gln(UU)}^{'}, and tRNA_{Glu(UU)}^{'}, although 11 tRNAs are targets of the ELP complex (Essberg et al. 2006; Johansson et al. 2008; Chen et al. 2011b); in contrast, it appears that only overexpression of trNA^Lys(UU) 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 cm^5^s^2^U), although tRNA_{Gln(UU)} and tRNA_{Glu(UU)} have the same cm^5^s^2^U 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 cm^5^Um_{34}, respectively); and *Saccharomyces* *trm7Δ* mutants have a severe growth defect (Pintard et al. 2002). Interestingly, *S. cerevisiae* Trm7 interacts with *Sc* Trm732 to form Cm_{32}, and separately interacts with *Sc* Trm3734 to form N_{34}, and both modifications are required to efficiently drive formation of yW_{37} (wybutosine) at m^1^G_{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* trm7Δ mutants. In addition, the growth defect of an *Sc* trm7Δ 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* trm732Δ nor an *Sc* trm734Δ single mutant has any observable growth defect, whereas the double mutant is as sick as an *Sc* trm7Δ 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
The growth defect of Sp trm7Δ mutant thus appears to be more severe than that observed in the S. cerevisiae trm7Δ mutant, which is sick but viable (Pintard et al. 2002; Guy et al. 2012). Under repressive conditions in liquid YES medium, the Sp trm7Δ [P<sub>nmt1</sub>- Sp trm7<sup>+</sup> LEU2] strain (although much healthier than the Sp trm7Δ 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 trm7Δ [P<sub>nmt1</sub>- Sp trm7<sup>+</sup> LEU2] strain, compared with 238 min for the wild-type control strain (a difference of 2.53-fold), whereas the Sp trm7Δ [P<sub>nmt1</sub>- Sp trm7<sup>+</sup> 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 trm7Δ strain for analysis of modifications, we instead examined the effect of reduced levels of Sp Trm7 on modification of tRNA<sup>Phe</sup> by growing the Sp trm7Δ [P<sub>nmt1</sub>- Sp trm7<sup>+</sup> LEU2] strain in EMM-Leu containing thiamine to repress Sp Trm7 expression, followed by purification of tRNA<sup>Phe</sup> and analysis of its nucleoside content by HPLC. Under these conditions, Cm levels were reduced in tRNAPhe relative to those from the wild-type strain grown under the same conditions (0.99 versus 0.15 moles/mole), or to those when the mutant strain (although much healthier than 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). Similarly, Gm levels were reduced in tRNAPhe from the Sp trm7Δ [P<sub>nmt1</sub>- Sp trm7<sup>+</sup> 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<sup>+</sup> expression in the Sp trm7Δ [P<sub>nmt1</sub>- Sp trm7<sup>+</sup> LEU2] strain by growth in thiamine-containing growth defect was due to the mutant haploid (data not shown). Control experiments demonstrated that the growth defect was due to the trm7Δ mutation of the Sp trm7Δ [P<sub>nmt1</sub>- Sp trm7<sup>+</sup> LEU2] strain, since introduction of a [P<sub>nmt1</sub>- Sp trm7<sup>+</sup> LEU2] plasmid (expressing trm7<sup>+</sup> under control of the low strength no message in thiamine promoter), resulted in healthy growth after plating to EMM containing 5-FOA (Fig. 2A).

### Figure 1

**Trm7 modification machinery in eukaryotes.**

(A) Schematic of the anticodon loop of tRNA<sup>Phe</sup> and tRNA<sup>Leu(UAA)</sup> from S. cerevisiae. S. cerevisiae Trm7 (Sc Trm7) requires Sc Trm732 and Sc Trm734 to form Cm32, and Nm34, respectively, on tRNA<sup>Phe</sup> and tRNA<sup>Leu(UAA)</sup>. Cm32 and Nm34 modification then drive yW formation from m1G on tRNAPhe. Wider arrow for Sp trm7<sup>+</sup>.

(B) Schematic representation of Sp Trm7 with putative homologs from S. pombe and H. sapiens. Box is an amino acid alignment of the DUF2428 domain found in these proteins.

(C) Schematic representation of Sc Trm732 aligned with putative homologs from S. pombe and H. sapiens. Insert box is an amino acid alignment of the DUF2428 domain found in these proteins.
serially diluted 10-fold in H2O, and then 2 µL was spotted onto indicated

medium. Under these conditions, m1G 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 m1G 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^vec]+ Sp trm7^+] 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 Cm32 and Gm34 due to loss of trm7^* also appear to have reduced synthesis of yW from m1G in tRNA^Phe.

Curiously, levels of the control modifications m2G (N2-methylguanosine) and m4G (7-methylguanosine) were unexpectedly reduced in tRNA^Phe purified from different strains grown in EMM. m3G 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^vec]+ Sp trm7^+ LEU2] strain (Table 2; Fig. 2B). m2G levels in tRNA^Phe were reproducibly different in different strains, with high levels in the Sp trm7Δ [P^vec]+ 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^{Tip} 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 m1G levels compared with those from the wild-type strain overexpressing tRNA^Phe (2.18 versus 1.62 moles/mole), and had undetectable 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).

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

| Organism       | Strain                       | Growth medium | Generation time (min) |
|----------------|------------------------------|---------------|-----------------------|
| S. pombe       | Wild type                    | YES           | 148 ± 5               |
| S. pombe       | trm7Δ [P^vec]+ trm7^+]       | YES           | 320 ± 2               |
| S. pombe       | trm732Δ                      | YES           | 143 ± 11              |
| S. pombe       | trm734Δ                      | YES           | 258 ± 3               |
| S. pombe       | Wild type [vec]              | EMM-Leu       | 313 ± 9               |
| S. pombe       | Wild type [vec]              | EMM-Leu + thiamine | 238 ± 3         |
| S. pombe       | trm7Δ [P^vec]+ trm7^+]       | EMM-Leu       | 330 ± 10              |
| S. pombe       | trm7Δ [P^vec]+ 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                |

*Mean and standard deviation based on growth from three separate colonies.

^p^vec+/: low strength no message in thiamine promoter.
The putative *S. pombe trm732* and *trm734* genes are required for Cm and Gm formation, respectively, on tRNA\textsubscript{Phe}.

To further determine if the modification circuitry for the anticodon loop of tRNA\textsubscript{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\textsubscript{trm732}△ haploid mutant obtained by sporulation of the heterozygous diploid lacked detectable Cm in its tRNAPhe 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\textsubscript{trm734}△ [Sp\textsubscript{trm734}+ ura4+] haploid obtained by sporulation of the heterozygous diploid lacking detectable Cm in its tRNA\textsubscript{Phe} and had normal levels of Gm compared with that from wild type (0.86 versus 0.91 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\textsubscript{trm734}△ mutant (Table 1; Guy et al. 2012).

Furthermore, since overexpression of tRNA\textsubscript{Phe} nearly completely suppressed the slow growth phenotype of the Sp\textsubscript{trm734}△ strain over a range of temperatures (Fig. 4E), we conclude that lack of Gm\textsubscript{34} of tRNAPhe is the cause of the Sp\textsubscript{trm734}△ defect.

In addition, our analysis showed that, as in *S. cerevisiae* (Guy et al. 2012), yW formation was impaired in the *Sp trm734*△ [tRNAPhe] strain lacking Gm\textsubscript{34} in its tRNAPhe (44% of wild-type levels) and to a lesser extent in the *Sp trm732*△ mutant (73%) (Fig. 4F), consistent with the increased m\textsuperscript{2}G levels (Table 3; Fig. 4A). We also note that m\textsuperscript{3}G levels on tRNA\textsubscript{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).

| Strain\textsuperscript{a,b} | Medium | Cm  | Gm  | m\textsuperscript{1}G | Ψ   | m\textsuperscript{2}G | m\textsuperscript{3}G |
|-----------------------------|--------|-----|-----|----------------|-----|----------------|----------------|
| Moles expected              | 1      | 1   | 1   | 4              | 1   | 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 |
| *trm732* [P\textsuperscript{vec} trm737\textsuperscript{+}] | 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 |
| *trm737* [P\textsuperscript{vec} trm737\textsuperscript{+}] | 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 [tRNAPhe]         | 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 |
| *trm737* [tRNAPhe]          | EMM-Leu | <0.01 | <0.03 | 2.18 ± 0.04 | 3.90 ± 0.03 | 0.23 ± 0.01 | 0.33 ± 0.04 |

\textsuperscript{a}Mean and standard deviation based on three individual growths and RNA preparations.

\textsuperscript{b}P\textsubscript{mnt1}: low strength no message in thiamine promoter.
TABLE 3. HPLC analysis of tRNA<sup>Phe</sup> nucleoside content from <i>S. pombe</i> trm732<sup>Δ</sup> and <i>S. pombe</i> trm734<sup>Δ</sup> strains

| Strain | Medium  | Cm | Gm | m<sup>1</sup>G | Ψ | m<sup>2</sup>G | m<sup>3</sup>G |
|--------|---------|----|----|----------------|---|-------------|-------------|
| 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 |
| trm732Δ | 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 |
| trm734Δ [trm734<sup>Δ</sup>] | 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 |
| trm734Δ | EMM | 0.86 ± 0.04 | <0.03 | 1.57 ± 0.02 | 4.01 ± 0.06 | 0.31 ± 0.05 | 0.61 ± 0.11 |

*Mean and standard deviation based on three individual growths and RNA preparations.

FIGURE 4. <i>S. pombe</i> trm734<sup>Δ</sup> mutants are sick due to hypomodified tRNA<sup>Phe</sup>. (A) HPLC traces of tRNA<sup>Phe</sup> from <i>S. pombe</i> trm732<sup>Δ</sup> and <i>S. pombe</i> trm734<sup>Δ</sup> mutants. tRNA<sup>Phe</sup> was purified from the indicated strains grown in YES medium and then digested to nucleosides and analyzed by HPLC. (B) <i>S. pombe</i> trm732<sup>Δ</sup> mutants are healthy. Indicated strains were grown in YES medium overnight, and analyzed on YES plates as described in Figure 2A. (C) <i>S. pombe</i> trm734<sup>Δ</sup> mutants have a growth defect on EMM. Indicated strains were grown overnight in EMM, diluted, and plated on EMM as indicated. (D) <i>S. pombe</i> trm734<sup>Δ</sup> mutants have a substantial growth defect on rich medium at various temperatures. Indicated strains were transformed with indicated <i>ura4</i><sup>+</sup> plasmids, grown in EMM-Ura, diluted, and plated on YES medium at the indicated temperatures after 3 d. (E) Overexpression of tRNA<sup>Phe</sup> suppresses the slow growth of <i>S. pombe</i> trm734<sup>Δ</sup> 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<sup>Phe</sup> are decreased in <i>S. pombe</i> trm732<sup>Δ</sup> and <i>S. pombe</i> trm734<sup>Δ</sup> mutants. tRNA<sup>Phe</sup> 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 Cm32 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 an appropriate construct expressing metazoan TRM7 and/or its metazoan TRM732 and TRM734 partners. To identify TRM7 homologs, we examined \( \text{Sp} \) \( \text{trm7}^+ \) and predicted TRM7 orthologs from \( \text{D. melanogaster} \) and humans by introduction of a high copy \( [\text{2µ LEU2 P}_{\text{GAL}} \text{TRM7}] \) plasmid expressing the corresponding ortholog under galactose control to an \( \text{Sc} \) \( \text{trm7} \Delta \) \( [\text{Sc TRM7 URA3}] \) strain, followed by analysis of growth after plating on medium containing 5-FOA and galactose. We found that the slow growth phenotype of \( \text{Sc} \) \( \text{trm7} \Delta \) mutants was efficiently suppressed by expression of \( \text{Sp} \) \( \text{Trm7} \) \( (\sim 61\%) \) identical to \( \text{Sc} \) \( \text{Trm7} \), \( \text{D. melanogaster} \) ORF CG5220 \( (\text{Dm} \text{TRM7}, \sim 43\%) \) identical to \( \text{Sc} \) \( \text{Trm7} \), and human FTSJ1 \( (\sim 50\%) \) identical to \( \text{Sc} \) \( \text{Trm7} \) but not human FTSJ2 \( (\sim 34\%) \) identical to \( \text{Sc} \) \( \text{Trm7} \) (Fig. 5A), after selection on medium containing 5-FOA. Furthermore, the resulting \( \text{Sc} \) \( \text{trm7} \Delta \) \( [\text{2µ LEU2 P}_{\text{GAL}} \text{FTSJ1}] \) strain grew nearly as well as the wild-type \( \text{S. cerevisiae} \) 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 \( \text{S. cerevisiae} \) \( \text{trm7} \Delta \) mutant strain was surprising because \( \text{Sc} \) \( \text{Trm7} \) and \( \text{Sp} \) \( \text{Trm7} \) have a strict requirement for their corresponding \( \text{Trm732} \) and \( \text{Nm34} \) partner proteins to generate Cm32 modification (Trm732 and Trm7 have a strict requirement for their corresponding orthologs to complement the growth defect of an \( \text{Sc} \) \( \text{trm7} \Delta \) strain (Guy et al. 2012). Because tRNA^Phe is the \( \text{Sc} \) Trm7 substrate that must be modified for healthy growth in \( \text{S. cerevisiae} \) (Guy et al. 2012), we examined 2'-O-methylation of tRNA^Phe from the \( \text{Sc} \) \( \text{trm7} \Delta \) \( [\text{2µ LEU2 P}_{\text{GAL}} \text{FTSJ1}] \) 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 Cm (Table 4), suggesting that human FTSJ1 is working in concert with \( \text{Sc} \) Trm732 to modify tRNA^Phe. The healthy growth of this \( \text{Sc} \) \( \text{trm7} \Delta \) \( [\text{2µ LEU2 P}_{\text{GAL}} \text{FTSJ1}] \) strain is consistent with our previous observation that the Cm32 modification of tRNA^Phe is sufficient for healthy growth in \( \text{S. cerevisiae} \) (Guy et al. 2012).

The occurrence of Cm in tRNA^Phe from an \( \text{Sc} \) \( \text{trm7} \Delta \) \( [\text{2µ LEU2 P}_{\text{GAL}} \text{FTSJ1}] \) strain suggested either that FTSJ1 does not require an interacting partner for Cm32 catalysis, or that it works with \( \text{S. cerevisiae} \) Trm732. Since the slow growth phenotype of the \( \text{Sc} \) \( \text{trm7} \Delta \) \( \text{trm732} \Delta \) strain was not suppressed by introduction of the \( [\text{2µ LEU2 P}_{\text{GAL}} \text{FTSJ1}] \) plasmid and no Cm formation was observed on tRNA^Phe purified from this strain (Table 4; Fig. 5B), we infer that FTSJ1 works with \( \text{Sc} \) Trm732 for formation of Cm32 on tRNA^Phe. Since tRNA^Leu(UAA) from an \( \text{Sc} \) \( \text{trm7} \Delta \) \( [\text{2µ LEU2 P}_{\text{GAL}} \text{FTSJ1}] \) strain lacked both Cm and ncm5Um (Table 5; Fig. 1A), the activity of FTSJ1 with Trm732 in \( \text{S. cerevisiae} \) does not extend to all \( \text{Sc} \) Trm7 substrates.

We note that because FTSJ1 is expressed from a high copy \( P_{\text{GAL}} \) 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 \( \text{Sc} \) Trm732 function for FTSJ1 activity in \( \text{S. cerevisiae} \).

THADA is the human homolog of Trm732 and works with Trm7 for Cm32 modification

Although Trm732 homologs have only \( \sim 20\% \) sequence identity, we find that the putative human Trm732 homolog THADA functions efficiently to replace \( \text{Sc} \) Trm732. Thus, the slow growth of an \( \text{Sc} \) \( \text{trm7} \Delta \) \( \text{trm732} \Delta \) strain was
suppressed by expression of FTSJ1 and THADA, but not by FTSJ1 alone (Fig. 6A). Since tRNA\textsuperscript{Phe} from this Sc trm7\textsuperscript{Δ} trm732\textsuperscript{Δ} [2μ P\textsubscript{GAL} FTSJ1 P\textsubscript{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 tRNALeu(UAA) Cm levels to that from the wild-type strain (0.90 moles/mole) 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\textsubscript{32} formation, since expression of THADA suppressed the slow growth of an Sc trm732\textsuperscript{Δ} trm734\textsuperscript{Δ} strain (Fig. 6A), resulting in tRNA\textsuperscript{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\textsubscript{32} on tRNA\textsuperscript{Phe} in S. cerevisiae. S. cerevisiae Trm7 also appears to work with S. pombe Trm32, because expression of Sp trm32\textsuperscript{+} suppressed the slow growth of the Sc trm732\textsuperscript{Δ} trm34\textsuperscript{Δ} strain (Fig. 6B).

**DISCUSSION**

Our results demonstrate that the entire circuitry for tRNA\textsuperscript{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\textsubscript{23}, and with Trm734 to 2′-O-methylate G\textsubscript{34}, leading to efficient conversion of m\textsubscript{1}G\textsubscript{37} to y\textsubscript{W}. 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\textsubscript{32} of tRNA\textsuperscript{Phe} (Table 4). THADA and S. pombe trm32\textsuperscript{+} each complemented the Trm732 defect of Sc trm732\textsuperscript{Δ} trm734\textsuperscript{Δ} 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 4. HPLC analysis of tRNA\textsuperscript{Phe} nucleoside content from S. cerevisiae trm7\textsuperscript{Δ} [FTSJ1] and S. cerevisiae trm7\textsuperscript{Δ} trm732\textsuperscript{Δ} [FTSJ1 THADA] strains

| Modification | Wild type [vec] | trm7\textsuperscript{Δ} [vec] | trm7\textsuperscript{Δ} [TRM7] | trm7\textsuperscript{Δ} [FTSJ1] | trm7\textsuperscript{Δ} trm732\textsuperscript{Δ} [vec] | trm7\textsuperscript{Δ} trm32\textsuperscript{Δ} [FTSJ1 THADA] |
|--------------|----------------|-----------------|-----------------|----------------|----------------|----------------|
| Cm           | 0.90 ± 0.16    | <0.01           | 0.89 ± 0.22     | <0.01          | 0.76 ± 0.13   | 0.68 ± 0.16   |
| Gm           | 0.81 ± 0.03    | <0.03           | 0.47 ± 0.13     | <0.03          | 0.66 ± 0.19   | 0.76 ± 0.09   |
| m\textsuperscript{1}G | <0.03         | 0.99 ± 0.09     | 2.13 ± 0.14     | 2.08 ± 0.05    |               |               |
| m\textsuperscript{2}G | 0.90 ± 0.09   | 2.11 ± 0.16     | 1.85 ± 0.09     | 1.76 ± 0.09    |               |               |
| m\textsuperscript{5}C | 1.75 ± 0.09   | 1.83 ± 0.06     | 1.86 ± 0.06     | 0.89 ± 0.16    |               |               |
| m\textsuperscript{5}Um | 0.98 ± 0.05   | 0.96 ± 0.08     | 0.95 ± 0.05     |               |               |               |

*Mean and standard deviation based on three individual growths and RNA preparations.*

### TABLE 5. HPLC analysis of tRNA\textsuperscript{Leu(UAA)} nucleoside content from an S. cerevisiae trm7\textsuperscript{Δ} [FTSJ1] strain

| Modification | wt [vec] | trm7\textsuperscript{Δ} [vec] | trm7\textsuperscript{Δ} [TRM7] | trm7\textsuperscript{Δ} [FTSJ1] | trm7\textsuperscript{Δ} trm732\textsuperscript{Δ} [vec] | trm7\textsuperscript{Δ} trm32\textsuperscript{Δ} [FTSJ1 THADA] |
|--------------|----------|-----------------|-----------------|----------------|----------------|----------------|
| Cm           | 0.58     | 0.02            | 0.57            | 0.03           | 0.02           | 0.02           |
| Gm           | 0.02     | 1.43            | 0.03            | 0.01           | 1.13           | 1.0            |
| m\textsuperscript{1}G | 1.8        | 1.97            | 1.8             | 1.96           | 1.52           | 1.48           |
| m\textsuperscript{2}G | 2.16       | 2.13            | 2.14            | 2.3            | 2.14           | 2.0            |
| m\textsuperscript{5}C | 2.16       | 2.13            | 2.14            | 2.3            | 2.14           | 2.0            |
| m\textsuperscript{5}U | 2.16       | 2.13            | 2.14            | 2.3            | 2.14           | 2.0            |

*Mean and standard deviation based on three individual growths and RNA preparations.*
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. 2007), or by a Box C/D guide RNA (Joardar et al. 2007).

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 trm7Δ trm73Δ 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 Gm34 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 O2yW37 (peroxywybutosine) on tRNAPhe also lack Cm32 and Gm34 modifications on tRNAPhe (Kuchino et al. 1982), consistent with the requirement for 2′-O-methylation of the anticodon loop of tRNA^{Phe} as a prerequisite for yW37 formation, and fueling speculation that these tRNA defects arise from defective FTSJ1 function.

Our data also provide further evidence indicating that levels of tRNA modifications are regulated by cellular growth conditions. Thus, we found that m2G and m2G 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 m2G levels of tRNA^{Phe} are near normal in the Sp trm7Δ and Sp trm734Δ mutants (compared with those from wild-type cells grown in EMM) implies the existence of a compensatory modification mechanism, similar to that observed in trn9Δ mutants, which have acquired ncm3U (and ncm5s2U) in the absence of mcm3U34 (and mcm5s3U) (Chen et al. 2011a), but in this case the m2G 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 C32 of tRNA^{Phe} in S. cerevisiae, and that tRNA^{Phe} from 16 of 17 eukaryotes examined contains Cm32 and Gm34 (Machnicka et al. 2013). However, we note that overexpression of tRNA^{Phe} did not completely suppress the growth defect of Sp trm7Δ 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 trm734Δ mutants at all temperatures, the defect is almost certainly caused by loss of Gm34 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 trm7Δ [Pnmt1Δ; Sp trm7Δ LEU2] strain (although itself much healthier than the Sp trm7Δ 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 FTS1 as the human Sc TRM7 ortholog suggests that defects in the human gene result in a relatively mild, albeit medically serious, condition, since FTS1 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 Nm32 modification remains to be determined.

NSXLID associated with defective FTS1 (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 A34 deaminase (Alazami et al. 2013); a frameshift mutation in hTRMT1 (Najmabadi et al. 2011), which has tRNA m2,2G26 (N2,N2-dimethylguanosine) methyltransferase activity (Liu and Strâby 2000); mutations in NSUN2 (Abbasi-Moheb et al. 2012; Khan et al. 2012; Martinez et al. 2011c), which modifies C34, C48, C49, and C50 on target tRNAs to m5C; and mutations in 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 Nm32 modification remains to be determined.

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 Amoebozoa (Dictostelium discoideum), Archeplastida (Arabidopsis thaliana, Cyanidioschyzon merolae, Ostreococcus tauri, Oryza sativa, and Zea mays), Excavata (Giardia intestinalis, Naegleria gruberi), Ophistokonta (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 paramecum, Guillardia theta, Phytophthora infestans, Tetrahymana thermophila, Thalassiosira pseudonana, Toxoplasma gondii, and Trypanosoma brucei).

Yeast strains

Yeast strains are listed in Table 6. The S. pombe haploid Sp trm7Δ::kanMX [Pnmt1 Sp trm7+ ura4+] (yMG1052A) strain was generated by transformation of the Sp trm7Δ::kanMX heterozygous diploid with pMG360A [Pnmt1 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 heterozygous 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 [Psp trm734 Sp trm734+ ura4+] mutant strain (yMG1289-1) was generated by transformation of the Sp trm732Δ::kanMX heterozygous diploid with a LEU2 tRNAphe 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 (Psp trm734 Sp trm734+ ura4+), followed by selection of leu-/ura+ colonies, and PCR confirmation. The S. cerevisiae trm7Δ::bleR [URA3 Sc Trm7] (yMG348-1), Sc trm732Δ::bleR (yMG814-1), Sc trm734Δ::bleR (yMG724-5), and Sc 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 trm7Δ under control of the Pnmt1 (no message in thiamine) (pMG360A) and Pnmt1+ (low strength no message in thiamine) promoters (pMG327B) were constructed by PCR of the Sp trm7+ cDNA ORF from an S. pombe cDNA library (Fikes et al. 1990) and insertion into the Xhol and Smal sites of pREP4X or pREP81X vectors, respectively. The S. pombe vector expressing Sp trm732Δ under control of the native Sp trm734Δ promoter (pMG426G) was constructed by PCR of Sp trm734+ from S. pombe genomic DNA and insertion at the PstI and Xhol sites of pREP4X to

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 Amoebozoa (Dictostelium discoideum), Archeplastida (Arabidopsis thaliana, Cyanidioschyzon merolae, Ostreococcus tauri, Oryza sativa, and Zea mays), Excavata (Giardia intestinalis, Naegleria gruberi), Ophistokonta (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 paramecum, Guillardia theta, Phytophthora infestans, Tetrahymana thermophila, Thalassiosira pseudonana, Toxoplasma gondii, and Trypanosoma brucei).
remove the P*<sub>nmt1</sub>* promoter sequence. tRNA expression vectors were generated by PCR of the appropriate tRNA fragment from genomic DNA and insertion at the Pst1 and Xhol 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

### TABLE 6. Strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| SP286 (wild-type S. pombe diploid) | ade-6/M210/ade-6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 | Bioneer |
| yMG957B (wild-type haploid) | SP286 | This study |
| spc4F10.03Δ (Sp trm7Δ heterozygous diploid) | SP286, spc4F10.03Δ::kanMX/SPAC4F10.03Δc | Bioneer |
| yMG1052A | SP286, spc4F10.03Δ::kanMX [P<sub>nmt1</sub> SPAC4F10.03Δc ura4] | This study |
| spec1306.02Δ (Sp trm734Δ heterozygous diploid) | SP286, spbc1306.02Δ::kanMX/SPBC1306.02Δc | This study |
| yMG1289-1 | SP286, spbc1306.02Δ::kanMX [P<sub>nmt1</sub> SPBC1306.02Δc ura4] | Bioneer |
| yMG1291 | SP286, spbc1306.02Δ::kanMX | This study |
| spec1494.07Δ (Sp trm732Δ heterozygous diploid) | SP286, spec1494.07Δ::kanMX/SPCC1494.07Δc | This study |
| yMG958B | SP286, spec1494.07Δ::kanMX | This study |
| BY4741 (wild S. cerevisiae haploid) | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Open Biosystems |
| yMG348-1 | BY4741, Sc trm7Δ::ble<sup>+</sup> [Sc TRM7 URA3 CEN] | Guy et al. (2012) |
| yMG105 | BY4741, Sc trm7Δ::ble<sup>R</sup> | Guy et al. (2012) |
| yMG724-5 | BY4741, Sc trm734Δ::ble<sup>R</sup> | Guy et al. (2012) |
| yMG814-1 | BY4741, Sc trm734Δ::ble<sup>R</sup> | 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<sup>+</sup>, Sc trm732Δ::kanMX [Sc TRM734 URA3 CEN] | Guy et al. (2012) |

### TABLE 7. Plasmids used in this study

| Plasmid | Parent | Description | Source |
|---------|--------|-------------|--------|
| pREP4X | pREP4X | P<sub>nmt1</sub> ura4<sup>+</sup> | Forsburg (1993) |
| pMG360A | pREP4X | P<sub>nmt1</sub> SPAC4F10.03Δc cDNA ura4<sup>+</sup> | This study |
| pREP81X | pREP4X | P<sub>nmt1</sub> LEU2 <sup>(low strength P<sub>nmt1</sub>)</sup> | Forsburg (1993) |
| pMG527B | pREP81X | P<sub>nmt1</sub> SPAC4F10.03Δc cDNA LEU2 | This study |
| pREP3X | pREP3X | P<sub>nmt1</sub> LEU2 | This study |
| pMG308C | pREP3X | LEU2 rRNA<sub>Phe</sub> | This study |
| pMG309A | pREP3X | LEU2 rRNA<sub>TP</sub> | This study |
| pMG310A | pREP3X | LEU2 rRNA<sub>Lig/AAA</sub> | This study |
| pMG426G | pREP4X | P<sub>S</sub>SPC1306.02Δc SPBC1306.02Δc ura4<sup>+</sup> | Quartley et al. (2009) |
| pAVAS79 | CEN URA3 LIC | This study |
| pMG13 | pAVAS79 | CEN URA3 Sc TRM7 | Guy et al. (2012) |
| pBG2619 | pBG2619 | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 Lic | Quartley et al. (2009) |
| pMG240A | pBG2619 | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 Sc TRM7 | Guy et al. (2012) |
| pMG298B | pBG2619 | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 SPAC4F10.03Δc PT cDNA | This study |
| pOT2-CCG220 | pBG2619 | pOT2-CCG220 cDNA | Drosophila Genomics Resource Center |
| pMG291A | pBG2619 | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 CCG220 | This study |
| pOT77 FT5I | pOT2-FT5I | pOT7 FT5I cDNA | Open Biosystems |
| pCR-BluntII-TOPO FT5I | pCR-BluntII-TOPO FT5I cDNA | Open Biosystems |
| pMG133A | pBG2619 | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 FT5I | This study |
| pMG135A | pBG2619 | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 FT5I | This study |
| pBG2619 | pAVA579 | CEN URA3 Sc TRM734 | Guy et al. (2012) |
| pMG140A | pBG2619 | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 FT5I-PT | This study |
| pENTR223.1 THADA | pENTR223.1 (THADA cDNA | Open Biosystems |
| pMG244A | pMG140A | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 FT5I-PT P<sub>GAL</sub>1,10 THADA | This study |
| pMG245A | pBG2619 | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 THADA | This study |
| pMG299A | pBG2619 | 2<sub>μ</sub> LEU2 P<sub>GAL</sub>1,10 SPCC1494.07Δc 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 trn7Δ [P_mnt−; Sp trn7″ LEU2] and wild-type strains under repressive conditions, thiamine was added to EMM-Leu at 5 mg/L. For detection of yW, HPLC was performed with buffers and gradients essentially as previously described (Jackman et al. 2003). For detection of yW, HPLC was done at pH 7.0 to maximize separation of tRNAPhe, HPLC was done at pH 7.0 to maximize separation of tRNAPhe, and nucleosides were subjected to HPLC analysis essentially as previously described (Jackman et al. 2003). For detection of yW, HPLC was performed with buffers and gradients essentially as previously described (Jackman et al. 2003).

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