Evolving specificity of tRNA 3-methyl-cytidine-32 (m^3C32) modification: a subset of tRNAs^Ser requires N^6-isopentenylation of A37

ANEESHKUMAR G. ARIMBASSERI,1,5 JAMES IBEN,1 FAN-YAN WEI,2 KESHAB RIJAL,1 KAZUHITO TOMIZAWA,2 MARKUS HAFNER,3 and RICHARD J. MARAIA4
1Intramural Research Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA
2Department of Molecular Physiology, Faculty of Life Sciences, Kumamoto University, 860-0862 Kumamoto, Japan
3National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892, USA
4Commissioned Corps, US Public Health Service, Washington, DC 20201, USA

ABSTRACT
Post-transcriptional modifications of anticodon loop (ACL) nucleotides impact tRNA structure, affinity for the ribosome, and decoding activity, and these activities can be fine-tuned by interactions between nucleobases on either side of the anticodon. A recently discovered ACL modification circuit involving positions 32, 34, and 37 is disrupted by a human disease-associated mutation to the gene encoding a tRNA modification enzyme. We used tRNA-HydroSeq (-HySeq) to examine 3-methyl-cytidine-32 (m^3C32), which is found in yeast only in the ACLs of tRNAs^Ser and tRNAs^Thr. In contrast to that reported for Saccharomyces cerevisiae in which all m^3C32 depends on a single gene, TRM140, the m^3C32 of tRNAs^Ser and tRNAs^Thr of the fission yeast S. pombe, are each dependent on one of two related genes, trm140^+ and trm141^+, homologs of which are found in higher eukaryotes. Interestingly, mammals and other vertebrates contain a third homolog and also contain m^3C at new sites, positions 32 on tRNAs^Arg and C47:3 in the variable arm of tRNAs^Ser. More significantly, by examining S. pombe mutants deficient for other modifications, we found that m^3C32 on the three tRNAs^Ser that contain anticodon base A36, requires N^6-isopentenyl modification of A37 (i6A37). This new C32–A37 ACL circuitry indicates that i6A37 is a pre- or corequisite for m^3C32 on these tRNAs. Examination of the tRNA database suggests that such circuitry may be more expansive than observed here. The results emphasize two contemporary themes, that tRNA modifications are interconnected, and that some specific modifications on tRNAs of the same anticodon identity are species-specific.

Keywords: anticodon loop; isopentenylation; tRNA-HySeq

INTRODUCTION
Nascent transcripts from tRNA genes undergo extensive post-transcriptional processing that includes nucleolytic cleavage, splicing, 3′ CCA addition, and chemical modifications of numerous nucleotides which play important roles in tRNA function (Phizicky and Hopper 2010). While modifications are observed throughout the tRNA, two nucleotides in the anticodon loop (ACL), at positions 34 (wobble) and 37 (the extended anticodon Yarus 1982), are the most variably and extensively modified as these contribute to decoding precision, efficiency, and reading frame maintenance during translation (Phizicky and Hopper 2010). Deamination of A34 to inosine expands codon recognition capability, especially in eukaryotes (Torres et al. 2014b). mcm^5U34 enhances decoding and is used to regulate translation during stress conditions (Begley et al. 2007). Modifications of A37 to N^6-isopentenyl-A37 (i6A37) and N^6-threonylcarbamoyl-A37 (t6A37) enhance tRNA activity (Lamichhane et al. 2011, 2013; Thiaville et al. 2015a). In some bacteria, yeasts, protists, and plants, t6A37 exists mostly as a cyclized ester with an oxazolone ring requiring the activity of the TCD enzymes (Miyauchi et al. 2013). Although several tRNA modification enzymes are nonessential in yeast, mutations in the human gene homologs are

© 2016 Arimbasseri et al. This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see http://rnajournal.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.
associated with diseases including cancer and neurodegeneration (Torres et al. 2014a).

Spatiotemporal order of tRNA processing and modification is dictated by subcellular location (Phizicky and Hopper 2010; Hopper 2013). The isopentenyltransferases (IPTase) responsible for i6A37 recognize single stranded, A36–A37–A38 in the context of a stem–loop motif (Motorin et al. 1997; Soderberg and Poulter 2000). However, some iA37-tRNAs are derived from precursor-tRNAs with introns inserted between A37 and A38 (Marck and Grosjean 2002); for this subset, modification must follow nuclear export because in budding and fission yeast, tRNA splicing occurs in the cytoplasm (Intine et al. 2002; Yoshihisa et al. 2003, 2007; Hopper et al. 2010; Cherkasova et al. 2011). Also, retrograde transport of spliced tRNAThr back into the nucleus is required to complete the G37 modification known as wybutosine (yW) (Shaheen and Hopper 2005; Hopper and Shaheen 2008; Ohira and Suzuki 2011).

Syntheses of some tRNA anticodon loop modifications are interdependent. Paabo and co-workers showed that Q formation in the anticodon of marsupial mitochondrial tRNAAsp occurs only after C to U editing elsewhere in the anticodon (Morl et al. 1995). In a cytosolic tRNAThr in Trypanosoma brucei, C to U editing at position 32 stimulates the efficiency of A to I editing at 34 (Rubio et al. 2006). In Thermus thermophilus, 7methyl-G46 (m7G46) positively affects Gm18 and m1G37 modifications (Tomikawa et al. 2010) whereas U55-pseudouridine negatively affects Gm18, m2sU54, and m1A58 modifications (Ishida et al. 2011). A three nucleotide circuitry was demonstrated in the ACL of S. cerevisiae tRNAAsp in which yW37 synthesis depends on 2'C-methylation of C32 and G34, which requires TRM7 (Guy et al. 2012). Genetic dissection of this ACL circuitry provided insight into the basis of a neurologic deficiency caused by heritable mutations in FTSJ1, encoding the human homolog of yeast Trm7 (Guy et al. 2015).

In the ACL, base identities at one position covary with modifications at other positions as a mechanism to fine-tune or compensate for anticodon:codon base-pairing strengths so that all tRNAs achieve uniform binding affinity (Olejniczak et al. 2005; Ledoux and Uhlenbeck 2008). Identity and modification of the extended anticodon position 37 is tuned to the anticodon base identity at position 36 which pairs with the first nucleotide of the codon (Yarus 1982). With few exceptions, the first ACL nucleotide, position 32, is a pyrimidine, and 33 is almost always U (Fig. 1A; Olejniczak et al. 2005; Ledoux et al. 2009). Pyrimidines at 32 make a noncanonical hydrogen bond with the base at

---

**FIGURE 1.** The *S. pombe* tRNAs for serine and threonine have mC32 modification. (A) A cartoon of schematized tRNA anticodon loop with different nucleotide positions indicated as referred to in the text. (B) The tRNAAspUGA and tRNAThrUGU track profiles from IGV display software showing misincorporations as colored bars (Arimbasseri et al. 2015). IGV introduces color by default if ≥15% mismatch is detected relative to the reference gene sequence. Color key: green, A; red, T; orange, G; blue, C. Sequence read counts are indicated on the y-axis. (C) Plot showing C32 misincorporation levels for all *S. pombe* tRNAs that have C at position 32. Error bars indicate standard deviation of four replicates. The tRNAThr and tRNAAsp are further grouped according to base identity at position 36; note that three of the four tRNAThr contain A36 while the other tRNA contains U36 as do all of the tRNAAsp. (D) Heat map showing fractions of each nucleotide misincorporated at C32 for the seven tRNA anticodons to the right.
38 (Auffinger and Westhof 1999), the presence or absence of which in different tRNA contexts help fine-tune their affinity for the ribosome (Olejniczak et al. 2005). Known modifications at position 32 of eukaryotic tRNAs are pseudouridine, N3′-C methylation (m3C), or 2′-O-methylation (Cm) (Phizicky and Hopper 2010). mC32 has been observed only on tRNAsSer and tRNAsThr in yeast and these plus tRNAsArg in higher eukaryotes. Trm140 is responsible for mC32 in S. cerevisiae (D’Silva et al. 2011; Noma et al. 2011). While a trm140Δ mutant exhibits no significant phenotype, a trm140Δ trm1Δ double mutant (also lacking tRNA m3G26 modification) is sensitive to cycloheximide, suggesting impaired translocation on the ribosome (D’Silva et al. 2011).

We recently developed tRNA-HySeq, which detects certain base modifications as nucleotide misincorporations (Arimbasseri et al. 2015). By this approach, all tRNAsSer and tRNAsThr in S. pombe indicate mC32, which we confirmed here by deletion of the methyltransferase genes responsible. We report that unlike S. cerevisiae, in which a single gene, TRM140, is responsible for modifying both tRNAsThr and tRNAsSer, in S. pombe, two closely related genes, trm140+ and trm141+, are specific for tRNAsThr and tRNAsSer, respectively. A third homolog in mammals, METTL8, is correlated with emergence of mC32 in tRNAsArg. More significantly, analysis of other tRNAsSer with A at position 36 contains i6A37, while the tRNAsThr with U at position 36 contains i6A37, as do the tRNAsGln with U at 36 (Jühling et al. 2009), it was likely that these misincorporations reflected mC.

We developed tRNA-HySeq, which detects certain base modifications as nucleotide misincorporations (Arimbasseri et al. 2015). By this approach, all tRNAsSer and tRNAsThr in S. pombe indicate mC32, which we confirmed here by deletion of the methyltransferase genes responsible. We report that unlike S. cerevisiae, in which a single gene, TRM140, is responsible for modifying both tRNAsThr and tRNAsSer, in S. pombe, two closely related genes, trm140+ and trm141+, are specific for tRNAsThr and tRNAsSer, respectively. A third homolog in mammals, METTL8, is correlated with emergence of mC32 in tRNAsArg. More significantly, analysis of other S. pombe tRNA modification mutants uncovered a new ACl mC32 circuitry. All three tRNAsSer with A at position 36 and i6A37, show no mC32 in tRNA isopentenyltransferase-1 mutants (tit1Δ) which lack i6A37. Inspection of the tRNA modification database suggests that this circuitry may occur widely in nature and possibly extend to other A37 modifications.

RESULTS

tRNA-HySeq misincorporations indicate m3C32 in all tRNAsSer and tRNAsThr

Deep sequencing of full length tRNAs can be hindered by structures that impede adapter ligation as well as various base modifications that limit processivity of reverse transcriptase. Fragmentation of purified tRNAs by limited hydrolysis prior to adapter ligation can yield high yield tRNA sequencing by tRNA-HySeq (Arimbasseri et al. 2015). By this similar approaches, modifications that alter base-pairing are detected as mismatches (Arimbasseri et al. 2015 and references therein). Figure 1B shows tRNA-HySeq output data for tRNASerUGA and tRNAThrUGU using IGV display in which gray bars indicate match to genomic sequence and colored bars indicate mismatch at ≥15% (default setting of IGV program) (Arimbasseri et al. 2015). We note that these two tRNAs share mismatches at C32 reflecting their common mC32 modification but differ at other modification sites (Fig. 1B). When combined with deletion of a suspected modification enzyme gene, tRNA-HySeq can verify that a misincorporation is indeed due to the modification of interest (Arimbasseri et al. 2015).

Figure 1C shows the misincorporation levels for S. pombe tRNAs with C at position 32 which ranged from 11–65% for tRNAsSer and tRNAsThr but ≤1% for the 15 others (Fig. 1C, also see Supplemental Table 1). The known C32 modifications of eukaryotic tRNAs are 2′-O-ribose methylation (Cm) and N3′-methylation (m3C) (Phizicky and Hopper 2010). Since mC is predicted to interfere with Watson–Crick base-pairing and eukaryotic tRNAsSer and tRNAsThr were known to contain mC32 (Jühling et al. 2009), it was likely that these misincorporations reflected mC.

We recently developed tRNA-HySeq, which detects certain base modifications as nucleotide misincorporations (Arimbasseri et al. 2015). By this approach, all tRNAsSer and tRNAsThr in S. pombe indicate mC32, which we confirmed here by deletion of the methyltransferase genes responsible. We report that unlike S. cerevisiae, in which a single gene, TRM140, is responsible for modifying both tRNAsThr and tRNAsSer, in S. pombe, two closely related genes, trm140+ and trm141+, are specific for tRNAsThr and tRNAsSer, respectively. A third homolog in mammals, METTL8, is correlated with emergence of mC32 in tRNAsArg. More significantly, analysis of other S. pombe tRNA modification mutants uncovered a new ACl mC32 circuitry. All three tRNAsSer with A at position 36 and i6A37, show no mC32 in tRNA isopentenyltransferase-1 mutants (tit1Δ) which lack i6A37. Inspection of the tRNA modification database suggests that this circuitry may occur widely in nature and possibly extend to other A37 modifications.

S. pombe has two distinct homologs of TRM140

The gene responsible for mC modification of tRNAsSer and tRNAsThr in S. cerevisiae is TRM140/ABP140 (D’Silva et al. 2011; Noma et al. 2011) which contains two open reading frames that produces a single polypeptide due to a +1 frame shift (Fig. 2A; Farabaugh et al. 2006). The N-terminal region is an actin binding domain (Asakura et al. 1998) that is dispensable for tRNA C32 methylase activity which is mediated by the AdoMet-MTase C-terminal domain (Fig. 2A; D’Silva et al. 2011; Noma et al. 2011). Deletion of TRM140 from S. cerevisiae abolished all detectable mC in total RNA (Noma et al. 2011).

BLASTp analysis of the S. cerevisiae Trm140 amino acid sequence against the S. pombe genome returned two highly significant homologies, SPBC21C.07c (hereafter, trm140+) and SPBC3H7.11 (hereafter, trm141+), neither with an actin binding domain (Fig. 2A), with e-values of 1 × 10−102 and 6 × 10−36, respectively, while the next gene homology value was 4.7. Sequence alignment with the AdoMet-MTase domain of S. cerevisiae Trm140 shows that D466 and D547, which are important for catalytic activity (Noma et al. 2011), were conserved in both of the S. pombe proteins (Fig. 2B). As will be shown in a later section, trm140+ and
trm141+ encode functionally distinct tRNA m^3C modification activities. We also performed more extensive analyses that led to the phylogenetic tree shown in Figure 2C.

**FIGURE 2.** Two sequence homologs for TRM140 in S. pombe. (A) Cartoon showing the general domain architecture of S. cerevisiae TRM140 and the two S. pombe homologs. Position of +1 frame shift in S. cerevisiae gene is indicated as +1 FS, which corresponds to amino acid 301; amino acid numbering is further indicated in panel B. (B) Alignment of the two S. pombe homologs of TRM140 with the S. cerevisiae protein. Only the C-terminal domain of the S. cerevisiae protein (starting from 301) is shown. Asterisks indicate amino acids D466, D547, and the horizontal line indicates the C-terminal 20 amino acids important for catalytic activity of TRM140 (Noma et al. 2011). (C) Phylogenetic tree of trm140+ and trm141+ homologs in different species generated from an alignment created using MUSCLE in Jalview (Edgar 2004; Waterhouse et al. 2009).

**Trm140+ duplication extends to other fission yeasts, worms, flies, and vertebrates**

We performed BLAST analysis of a range of species using S. pombe Trm141 and Trm140 amino acid sequences as queries (Supplemental Table 2). Trm141 and Trm140 yielded nearly identical results for several different budding yeasts (Supplemental Table 2) which indicate a single TRM140 gene (Noma et al. 2011). In contrast, all fission yeast and metazoan examined revealed distinct homologs for Trm141 and Trm140 (Supplemental Table 2).

A multiple sequence alignment was generated from extensive BLAST analyses (not shown) and used to generate a phylogenetic tree (Fig. 2C). This suggested three major branches comprised of Trm140 homologs, Trm141 plus metazoan METTL6 homologs, and the METTL2 and METTL8 homologs (Fig. 2C). As shown below, the fission yeast gene duplication yielded functionally distinct tRNA m^3C modification activities.

**Distinct m^3C modification systems for tRNAs^Ser\* and tRNAs^Thr\* in S. pombe**

We deleted trm140+ and trm141+ individually and in combination, and subjected total RNA from the deletion strains to mass spectrometric analysis for m^3C and m^5C. This revealed that trm140Δ and trm141Δ separately showed reduced m^3C/m^5C levels (40% and 37%, respectively; Fig. 3B,C) relative to the wild-type (WT) strain (Fig. 3A), whereas deletion of both trm140Δ and trm141Δ abolished all m^3C (Fig. 3D). We note that deletion of trm140+ and trm141+ each decreased m^3C/m^5C levels to <50% of WT levels. This and other observations noted below and in the Discussion suggest some cross-talk between these modification systems.

We performed rescue of the single trm140Δ and trm141Δ mutants as well as the trm140Δ trm141Δ double mutant using a pRep4X expression plasmid containing either trm140+ or trm141+ under the control of the nmt1+ promoter (Fig. 3E–H). Expressing trm140+ in the single trm140Δ mutant led to increased m^3C/m^5C levels relative to trm140Δ transformed with the empty pRep4X plasmid (compare m^3C/m^5C levels in Fig. 3B,E). Likewise, expressing trm141+ in trm141Δ led to increased m^3C/m^5C relative to trm141Δ with empty pRep4X (compare m^3C/m^5C levels in Fig. 3C,F). We note that while the expression plasmids more than doubled the m^3C/m^5C levels in the single deletion mutants, they did not restore levels to 100% relative to WT, perhaps because this is an ectopic expression system under control of a heterologous promoter. Expressing either trm140+ or trm141+ in the trm140Δ trm141Δ double mutant increased m^3C/m^5C levels from zero with empty pRep4X
to levels comparable to that found in each of the single mutants (compare Fig. 3G,H,D with B,C). The results fit a model in which Trm140 and Trm141 each have a limited capacity for m3C modification. Individual tRNA analysis in the next section revealed the tRNA-specific basis of this.

tRNA-HySeq reveals quantitative information on individual tRNAs including the modification efficiency of several methylated bases (Arimbasseri et al. 2015). For the present study, we used this to focus on m3C in the \( \text{trm140}\Delta \) and \( \text{trm141}\Delta \) strains. As expected, based on results from \( \text{S. cerevisiae} \), our analysis of \( \text{S. pombe} \) revealed that all of the m3C was found in tRNAsThr and tRNAsSer. Deletion of \( \text{trm140}\Delta \) abolished C32 misincorporations from the tRNAsThr but not the tRNAsSer, while deletion of \( \text{trm141}\Delta \) abolished misincorporations only from tRNAsSer (Fig. 3I). These data indicate that distinct but related modification enzymes are responsible for C32 methylation in \( \text{S. pombe} \). Moreover, this occurs in a tRNA-specific manner; \( \text{trm141}\Delta \) for tRNAsSer, and \( \text{trm140}\Delta \) for the tRNAsThr. Notably, while C32 misincorporations in the principal target tRNAs were eliminated in their respective mutants, misincorporations in the other tRNAs were nonuniformly decreased relative to WT (Fig. 3B), suggesting some cross-talk between the modification systems (see Discussion).

**Expansion of m3C in mammals to m3C32 in tRNAArgCCU and tRNAArgUCU, and m3C47:3 in the variable arms of tRNAsSer**

We analyzed mouse embryonic fibroblast (MEF) and human A549 cells using tRNA-HySeq. Unlike in yeast, vertebrate gene copies for a tRNA with any given anticodon tend to
contain one or more nucleotide differences, referred to as iso-decoders (Goodenbour and Pan 2006; Chan and Lowe 2015). In mouse, there are 26 distinct tRNAs of Ser and Thr identity all of which contain C at position 32. Moreover, there are 121 total distinct mouse tRNA sequences with C at position 32, thirty-four of which showed misincorporation at significant levels (Fig. 4A,B). In addition to all of the tRNAsSer and tRNAsThr, we found isodecoders for both tRNAsArg with U at position 36, tRNAArgCCU and tRNAArgUCU, with substantial C32 misincorporations (Fig. 4B), but no misincorporations at C32 for the many tRNAsArg, with G at position 36.

Similar to MEFs, human A549 cells also have m^3^C32 on tRNAArgCCU and tRNAArgUCU in addition to all of the tRNAsSer and tRNAsThr (Fig. 4C). While the total number of isodecoders is greater in human as compared to mouse for these tRNA species, the m^3^C32 was limited to the same tRNA identities as in mice, and no m^3^C32 on any of the many isodecoders of the three tRNAsArg with G at 36. The cumulative results are in good agreement with data from Bos taurus in which both tRNAArgCCU and tRNAArgUCU were found to have m^3^C32 (Keith 1984) while neither tRNAArgCCG from B. taurus (Miller et al. 1983) nor tRNAArgACG from mouse showed m^3^C32 (Jühling et al. 2009).

In S. pombe and other yeast, m^3^C32 is limited to tRNAsSer and tRNAsThr while we additionally found it on tRNAArgCCU and tRNAArgUCU in mouse and human cells, consistent with data from various higher eukaryotes (Ginsberg et al. 1971; Keith 1984; Capone et al. 1985; Cribbs et al. 1987). tRNA-HySeq of mouse and human tRNAs also found m^3^C in the variable arm, at position 47:3 of all four tRNAsSer (Fig. 4D) but not other tRNAs, consistent with prior data (Jühling et al. 2009). This revealed that all tRNAsSer with the UCU sequence at positions 47:2-47:3-47:4 show misincorporations at C47:3 in mouse and human cells.

**FIGURE 4.** m^3^C32 modification in mouse and human cells. (A) Heat map of 121 unique mouse embryonic fibroblast (MEF) tRNA sequences with C at position 32, 34 of which have significant, ≥0.1, misincorporation at position 32. (B) Bar plot of the 34 tRNAs from A that have ≥0.1 misincorporation at C32. (C) Bar plot for all unique tRNAs that show significant C32 misincorporation in human A549 cells. (D) Bar plots for all unique tRNAs from MEFs and A549 cells that show significant misincorporation at C47:3. Error bars indicate standard deviation of two replicates each for MEFs and A549.

**Loss of i^6^A37 from tRNAsSer causes their loss of m^3^C32**
i^6^A37 is synthesized by tRNA isopentenyltransferases in bacteria and eukaryotes. In S. pombe, tit1+ synthesizes i^6^A37 and tit1Δ deletion strains lacking it have been characterized (Lamichhane et al. 2011, 2013). The substrates of Tit1 include the three tRNAsSer with A at position 36 but not the tRNAsThr with U at position 36 nor the tRNAsThr (Lamichhane et al. 2011). N°-isopentenyl-A37 retains an N° hydrogent that can donate to base-pairing and does not cause misincorporation, as reported previously (Fig. 5A; Arimbasseri et al. 2015). Comparison of tit1+ and tit1Δ strains revealed loss of C32 misincorporation in tit1Δ for the usual Tit1 tRNAs substrate (compare WT and tit1Δ in Fig. 5A) but not for the non-Tit1 substrates, tRNAThr (compare WT and tit1Δ in Fig. 5B).

Compiled data for all seven tRNAs show that loss of m^3^C32 in tit1Δ was specific to the three tRNAsSer that are usually substrates for i^6^A37 modification (Fig. 5C, compare WT and tit1Δ). Because some tRNA modifying activities involve multisubunit complexes (e.g., see Guy et al. 2012, 2015) we asked if dependency of m^3^C32 on tit1+ requires i^6^A37 modification activity per se or just the presence of the Tit1 protein. For this, we expressed ectopic tit1+ or a previously characterized tit1-T12A point mutant that encodes a catalytically inactive protein (Lamichhane et al. 2011, 2013) in tit1Δ cells and performed tRNA-HySeq. Ectopic tit1+ restored m^3^C32 on the Tit1 targets while tit1-T12A did not (Fig. 5C). The data strongly suggest that i^6^A37 itself is required for m^3^C32 modification by the Trm141 enzyme on the Tit1 substrates. Notably, tRNA_GCU does not bear i^6^A37 (Lamichhane et al. 2011) and its m^3^C32 content was not significantly affected by tit1+ deletion (Fig. 5C).

Mass spec analysis of m^3^C in WT and tit1Δ RNA revealed that total m^3^C levels in tit1Δ were reduced to about one-third
of WT relative to m^3C (Fig. 5D), consistent with the results from tRNA-HySeq. Independent analysis by mass spec also confirmed the complete absence of i^6A in the \( \text{tit1}^{\Delta} \) cells as expected (not shown).

Finally, we report that deletion of \( \text{trm140}^+ \) and \( \text{trm141}^+ \) either separately or together had no overt phenotype nor caused loss of tRNA-mediated suppression (not shown). We could find no difference in growth relative to our wild-type \( S. \text{pombe} \) cells by serial dilution spot assays in rich or minimal media, nor with glycerol as the carbon source or in the presence of rapamycin, in each condition either at 30°C or 37°C (not shown).

**DISCUSSION**

The major conclusion of this study is that m^3C32 modification of the three tRNAs^Ser with A at position 36, which decode Ser codons that begin with U, is specifically dependent on \( \text{A}^3 \text{A}^3 \text{A}^3 \) modification. This indicates a new ACL modification circuitry in addition to activities described including 2'-O-ribose methylation of C32 and G34, and modification of m^1G37 to yW of tRNA^Phe (Guy and Phizicky 2015; Guy et al. 2012, 2015). A second conclusion is that the genes responsible for tRNA m^3C32 modification evolved isoacceptor specificity in fission yeast such that \( \text{trm140}^+ \) and \( \text{trm141}^+ \) are principally responsible for tRNAs^Thr and tRNAs^Ser, respectively. Phylogenetics suggests that the one- versus two-enzyme system is a distinguishing feature of the lineages to the budding yeast and to the fission yeasts and animals, respectively.

The results emphasize a current theme, that tRNA modifications are interconnected, especially in the ACL. In addition, the results contribute to an appreciation of the species-specificity of tRNA modification patterns, even for tRNAs with the same anticodon identity.

**Evolution of C32 methyltransferases**

m^3C32 is not found on bacterial or archael tRNAs and thus appears to be eukaryote-specific (Jühling et al. 2009). A significant aspect of this work is the evolutionary divergence and expansion of C32 methyltransferases with distinct tRNA specificities in fission yeast (and presumably higher eukaryote) lineages as compared to one in budding yeast.
Another unexpected finding was dependence of the three tRNAs with m3C32 modification requires i6A37 in some tRNAs.

A new ACL modification circuit

Another unexpected finding was dependence of the three tRNA substrates of different eukaryotes. Since mammals have m3C32 on tRNAArgUCU and tRNAArgCCU in addition to tRNAser and tRNAThr, it is tempting to speculate that the third homolog may methylate the tRNAsArg. Primates have four sequence homologs, including two versions of METTL2, A and B. METTL2B appears to be a true homolog since its knockdown reduced m3C32 levels (Noma et al. 2011).

Trm140 and Trm141 may operate as multisubunit complexes with shared and distinct cofactors for tRNAser and tRNAThr. With regard to this, we note that deletion of either trm140+ or trm140Δ reduced m3C32 levels in the others’ substrates (Fig. 3B). In particular, tRNAThrAGT m3C32 was unusually and unexpectedly sensitive to trm141-deletion (Fig. 3B). Also, tRNAThrAGT m3C32 levels were unexpectedly lowered by both ectopic tit1 and tit1-T12A (Fig. 5C). These observations suggest dominant negative effects of ectopic Tit1 when overexpressed from the strong promoter used, consistent with involvement in a multisubunit complex. Finally, our unpublished data show that i6A37 levels measured by mass spectrometry were significantly increased in tRNAThrAGT m3C32 was unexpectedly lowered by both ectopic tit1 and tit1-T12A (Fig. 5C). These observations suggest dominant negative effects of ectopic Tit1 when overexpressed from the strong promoter used, consistent with involvement in a multisubunit complex. Finally, our unpublished data show that i6A37 alone is not a sufficient signal for m3C32 modification.

Further analysis revealed another finding of potential relevance. While tRNAThrAGT m3C32 also carry itA37, itA37 or their derivatives (Fig. 5E). However, it should be noted that several others that carry itA37, namely tRNAs for Met, Ile, Asn, Lys, and Arg (Thiaville et al. 2015b), do not contain m3C32 even though several have C at position 32 (see Fig. 1C), suggesting that itA37 alone is not a sufficient signal for m3C32 modification.

Finally, we note that the findings reported here may be relevant to a heritable disorder caused by mutation of human tRNA isopentenyl transferase, TRIT1 and associated i6A37 hypomodification of cytoplasmic and mitochondrial tRNA (Yarham et al. 2014; Lamichhane et al. 2016). The data suggest that human i6A37 hypomodification may be accompanied by m3C32 hypomodification. Notably, using yeast, Guy et al. (2015) could dissect the tRNAThr ACL yW37 modification pathway and discover an allele of FT SJ1, the human homolog of yeast TRM7, that provided unique insight into the circuitry wiring.

MATERIALS AND METHODS

S. pombe strains, growth conditions, and cloning

A list of S. pombe strains used is given in Table 1. Deletion of trm140+ and trm141+ was executed as described (Longtine et al. 1998; Thiaville et al. 2015b), and are known to contain itA37 (Jühling et al. 2009).

We therefore analyzed existing tRNA modification data (Jühling et al. 2009) and found that virtually all known tRNAs with m3C32 also carry i6A37, itA37 or their derivatives (Fig. 5E). However, it should be noted that several others that carry itA37, namely tRNAs for Met, Ile, Asn, Lys, and Arg (Thiaville et al. 2015b), do not contain m3C32 even though several have C at position 32 (see Fig. 1C), suggesting that itA37 alone is not a sufficient signal for m3C32 modification.
Table 1. Yeast strains used for experimental work in this study

| Species | Strain name | Genotype |
|---------|-------------|----------|
| S. pombe | yW1 (WT) (Bayfield and Mata 2009) | h−|leu1-32::BRNamSer7T-leu1+|ura4-D18|ade6-704 |
| S. pombe | yNB5 (t1Δ) (Lamichhane et al. 2011) | h−|leu1-32::BRNamSer7T-leu1+|ura4-D18|ade6-704|tit1Δ::KanMX6 |
| S. pombe | yAG503 (trm140Δ) This report | h−|leu1-32::BRNamSer7T-leu1+|ura4-D18|ade6-704|trm140Δ::KanMX6 |
| S. pombe | yAG504 (trm141Δ) This report | h−|leu1-32::BRNamSer7T-leu1+|ura4-D18|ade6-704|trm141Δ::KanMX6 |
| S. pombe | yAG505 (trm140Δ trm141Δ) This report | h−|leu1-32::BRNamSer7T-leu1+|ura4-D18|ade6-704|trm140Δ::KanMX6|trm141Δ::NatNT2 |

1998). The growth media used were EMM (minimal media) or YES (rich media).

The genes trm140+ and trm141+ were amplified from the S. pombe chromosomal DNA of the wild-type strain yA99 and cloned into the XhoI/BamHI sites of pRep4X, which puts their expression under the control of the trm141+ promoter. The oligo-DNA primers used for amplification cloning were: TRM140-FOR: 5′-AGTGGAGATCtctcg-agATGATACAAACCCACACAGAATTTTC-3′; TRM140-REV: 5′-AGATTTGAGATCtctgc-TCAGACCTTTTTTCCAACTCCTT-3′. The constructs were confirmed by restriction digestion analysis and sequencing.

“Mouse embryonic fibroblasts” and A549 cells were grown in DMEM media (Gibco) with 10% serum at 37°C in 5% CO2. A549 cells were obtained from the Division of Cancer Treatment and Diagnosis, NCI Tumor Repository (https://dtp.cancer.gov/organization/btb/docs/DCTDTumorRepositoryCatalog.pdf). Cells were grown in 150 mm2 flasks to ∼70% confluency before harvesting for RNA isolation.

tRNA isolation

Total RNA from S. pombe was isolated as described previously (Arimbasseri et al. 2015) using hot-phenol. Total RNA from mammalian cells was prepared using Tripure RNA isolation reagent (Roche, Basel).

Mass spectrometry

Total RNAs isolated from WT (wild type), tit1Δ, trm140Δ, trm141Δ, and trm140Δ trm141Δ cells were digested with 2.5 U of Nuclease P1 (Sigma-Aldrich) and 0.2 U alkaline phosphatase (Takara) in 5 mM ammonium acetate pH 5.3 and 20 mM HEPES-KOH pH 7.0 for 3 h at 37°C. Nucleotides were separated by C18 reverse-phase column (GL Science) and directly injected to Agilent 6460 Triple Quadrupole Mass spectrometry. MRM parameters for m3C and m5C were as follows: m3C/m5C: precursor ion, m/z 258, product ion, m/z 126.

tRNA-HySeq

A high-throughput tRNA sequencing library was prepared from gel-purified tRNA as previously described (Arimbasseri et al. 2015) and modified from Karaca et al. (2014). tRNA was excised after electrophoretic resolution on 6% urea–polyacrylamide gels, and subjected to the crush and soak extraction method in 0.3 M NaCl at 4°C. The eluate was filtered and RNA was precipitated using ethanol. In short, purified tRNA was subjected to partial alkaline hydrolysis in 10 mM bicarbonate pH 9.7 at 90°C for 5 min. After quenching on ice for 2 min, the RNA fragments were subjected to dephosphorylation to remove 3′ phosphates with calf intestinal phosphatase (CIP; NEB). The 5′ ends were then phosphorylated using T4 polynucleotide kinase (T4 PNK; NEB). To the 5′ phosphorylated fragments, preadenylated, 3′ blocked 3′ adapters were ligated using the truncated T4 RNA ligase known as T4 RNL2K27Q (NEB). The ligated fragments were gel purified from 15% polyacrylamide–urea gel followed by ligation of 5′ adapters using T4 RNA ligase 1 (Thermo Scientific). Ligated fragments were gel purified and subjected to reverse transcription using SSIII RT (Life Technologies) followed by PCR amplification of the library and sequencing using an Illumina HiSeq machine.

Analysis of sequence data

 Reads were mapped to a reference library comprised of all predicted tRNA genes in S. pombe, mouse or human. Misincorporations were calculated from the alignment files for the positions studied (Arimbasseri et al. 2015).

Phylogenetics

To identify C32 methyltransferases in fission yeast, BLASTp analysis of S. cerevisiae Trm140 protein sequence was queried against the S. pombe genome using the default settings at NCBI. For trm140+ and trm141+, we analyzed genomes of budding (ncbi taxid:4892) and fission yeast (Schizosaccharomyces; taxid: 4895), Caenorhabditis elegans (taxid: 6239), Drosophila melanogaster (taxid:7227), Danio rerio (taxid:7955), Xenopus laevis (taxid:8355), Gallus gallus (taxid:9031), Mus musculus, chimp (taxid:9598), and human (taxid:9606) using NCBI BLAST with Trm140 or Trm141 protein sequence as query with default settings. Sequences were aligned using MUSCLE (Edgar 2004) and the output was used to create a phylogenetic tree using Jalview software (average distance calculation from the alignment files for the positions studied). Sequences were aligned using MUSCLE (Edgar 2004) and the output was used to create a phylogenetic tree using Jalview software (average distance calculation from the alignment files for the positions studied). Sequences were aligned using MUSCLE (Edgar 2004) and the output was used to create a phylogenetic tree using Jalview software (average distance calculation from the alignment files for the positions studied). Sequences were aligned using MUSCLE (Edgar 2004) and the output was used to create a phylogenetic tree using Jalview software (average distance calculation from the alignment files for the positions studied). Sequences were aligned using MUSCLE (Edgar 2004) and the output was used to create a phylogenetic tree using Jalview software (average distance calculation from the alignment files for the positions studied). Sequences were aligned using MUSCLE (Edgar 2004) and the output was used to create a phylogenetic tree using Jalview software (average distance calculation from the alignment files for the positions studied).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

A.G.A. thanks S. Mattijssen for cell lines and advice with tissue culture, and M. Blum for media preparation. This work was supported...
by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, and National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health.

Received February 6, 2016; accepted May 24, 2016.

REFERENCES

Arimbasseri AG, Blewett NH, Iben JR, Lamicichane TN, Cherkasova V, Hafner M, Maria RJ. 2015. Global RNA polymerase III activity impacts a functional tRNA modification. PLoS Genet 11: e1005671.

Asakura T, Sasaki T, Nagano F, Satoh A, Obashi H, Nishioka H, Imamura H, Hotta K, Tanaka K, Nakanishi H, et al. 1998. Isolation and characterization of a novel actin filament-binding protein from Saccharomyces cerevisiae. Oncogene 16:121–130.

Auffinger P, Westhof E. 1999. Singly and bifurcated hydrogen-bonded base-pairs in tRNA anticodon hairpins and ribozymes. J Mol Biol 292:467–483.

Bayfield MA, Maria RJ. 2009. Precursor-product discrimination by La protein during tRNA metabolism. Nat Struct Mol Biol 16:430–437.

Begley U, Dyavaiah M, Patil A, Rooney JP, Direnzo D, Young CM, Mirzajani M, Lee DS, Zitomer RS, Begley TJ. 2007. Trm9-catalyzed tRNA modifications link translation to the DNA damage response. Mol Cell 28:860–870.

Capone JP, Sharp PA, RajBhandary UL. 1985. Amber, ochre and opal suppressor tRNA genes derived from a human serine tRNA gene. EMBO J 4:213–221.

Chan PF, Lowe TM. 2015. GrtRNAdb 2.0: an expanded database of transfer RNA genes identified in complete and draft genomes. Nucleic Acids Res 44:D184–D189.

Cherkasova V, Bähler J, Backova D, Priddham K, Maria RJ. 2011. Altered nuclear tRNA metabolism in La-deleted S. pombe is accompanied by a nutritional stress response involving Atf1p and Pcr1p. Mol Biol Cell 12:2543–2547.

Cribbs DL, Gillam IC, Tenger GM. 1987. Nucleotide sequences of three tRNAs in the order Saccharomycetales. J Mol Evol 36:4–13.

D’Silva S, Haider SJ, Phizicky EM. 2011. A domain of the actin binding protein Abp1p40 is the yeast methyltransferase responsible for 3-methylcytidine modification in the tRNA anti-codon loop. RNA 17:1100–1110.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797.

Farabaugh PJ, Kramer E, Vallabhaneni H, Raman A. 2006. Evolution of +1 programmed frameshifting signals and frameshift-regulating tRNAs in the order Saccharomycetaceae. J Mol Evol 63:545–561.

Ginsberg T, Rugg H, Staehelin M. 1971. Nucleotide sequences of rat liver serine-tRNA. 3. The partial enzymic of serine-tRNA and derivation of its total primary structure. Eur J Biochem 21:249–257.

Goodenour JM, Pan T. 2006. Diversity of tRNA genes in eukaryotes. Nucleic Acids Res 34:6137–6146.

Guy MP, Phizicky EM. 2015. Conservation of an intricate circuit for crucial modifications of the tRNA\textsuperscript{tm} anticodon loop in eukaryotes. RNA 21:61–74.

Guy MP, Podyma BM, Preston MA, Shaheen HH, Krivos KL, Limbach PA, Hopper AK, Phizicky EM. 2012. Yeast Trm7 interacts with distinct proteins for critical modifications of the tRNA\textsuperscript{tm} anticodon loop. RNA 18:1921–1933.

Guy MP, Shaw M, Weiner CL, Hobsom L, Stark Z, Rose K, Kalscheuer VM, Gezcz J, Phizicky EM. 2015. Defects in tRNA anticodon loop 2’-O-methylation are implicated in nonsyndromic X-linked intellectual disability due to mutations in FTSJ1. Hum Mutat 36:1176–1187.

Hopper AK. 2013. Transfer RNA post-transcriptional processing, turnover, and subcellular dynamics in the yeast Saccharomyces cerevisiae. Genetics 194:43–67.

Hopper AK, Huang HY. 2015. Quality control pathways for nucleus-encoded eukaryotic tRNA biosynthesis and subcellular trafficking. Mol Cell Biol 35:2052–2058.

Hopper AK, Shaheen HH. 2008. A decade of surprises for tRNA nucleotycystolic dynamics. Trends Cell Biol 18:98–104.

Hopper AK, Pai DA, Engelke DR. 2010. Cellular dynamics of tRNAs and their genes. FEBS Lett 584:310–317.

Intine RV, Dunda M, Misteli T, Maria RJ. 2002. Aberrant nuclear trafficking of La protein leads to disordered processing of associated precursor tRNA. Mol Cell 9:1113–1123.

Ishida K, Kimubayashi T, Tomikawa C, Ochi A, Kanai T, Hirata A, Iwashita C, Hori H. 2011. Pseudouridine at position 55 in tRNA controls the contents of other modified nucleotides for low-temperature adaptation in the extreme-thermophilic eubacterium Thermus thermophilus. Nucleic Acids Res 39:2304–2318.

Jühling F, Mörl M, Hartmann RK, Sprinzl M, Stadler PF, Pütz J. 2009. tRNAdb 2009: compilation of tRNA sequences and tRNA genes. Nucleic Acids Res 37:D159–D162.

Karaca E, Wetzler S, Pehlivan D, Shiraishi H, Gogakos T, Hanada T, Jhanguin SN, Witsniewski W, Withers M, Campbell IM, et al. 2014. Human CLP1 mutations alter tRNA biogenesis, affecting both peripheral and central nervous system function. Cell 157:636–650.

Keith G. 1984. The primary structures of two arginine tRNAs (anticodos C-C-U and mcm5a2U-C-) and of glutamine tRNA (anticodon C-C-C) from bovine liver. Nucleic Acids Res 12:483.

Kimura S, Suzuki T. 2013. A cyclic form of tRNA Ser from Saccharomyces cerevisiae. Oncogene 32:6137–6146.

Kalscheuer VM, Gecz J, Phizicky EM. 2015. Defects in tRNA anticodon loop 2’-O-methylation in tRNA\textsuperscript{tm} from Saccharomyces cerevisiae. Eur J Biochem 249:249–257.

Kishii K, Kitamura S, Suzuki T. 2013. A cyclic form of tRNA Ser from bovine liver. Nucleic Acids Res 41:3046–3049.

Ledoux S, Blewett NH, Maria RJ. 2011. Plasticity and diversity of tRNA anticodon determinants of substrate recognition by eukaryotic A37 isopentenyltransferases. RNA 17:1846–1857.

Lamicichane TN, Blewett NH, Crawford AK, Cherkasova VA, Iben JR, Begley TJ, Farabaugh PJ, Maria RJ. 2013. Lack of tRNA modification isoCm5a2U37 alters mcm5a2U37 modification and causes metabolic deficiencies in fission yeast. Mol Cell Biol 33:2918–2929.

Lamicichane TN, Arimbasseri AG, Rajk J, Iben JR, Wei FY, Tomizawa K, Maria RJ. 2016. Lack of tRNA\textsuperscript{a6a} modification causes mitochondrial-like metabolic deficiency in S. pombe by limiting activity of cytosolic tRNA\textsuperscript{a6a}. Front Cell Neurosci 10:783–596.

Leducx S, Uhlenbeck OC. 2008. Different aa-tRNAs are selected uniformly on the ribosome. Mol Cell 31:114–123.

Leducx S, Olenjczak M, Uhlenbeck OC. 2009. A sequence element that tunes Escherichia coli tRNA\textsuperscript{a6a} to ensure accurate decoding. Nat Struct Mol Biol 16:359–364.

Longtine MS, McKenzie A III, Demarini DJ, Shah NG, Wach A, Brachat A, Philippson P, Pringle JR. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14:953–961.

Moric C, Grosjean H. 2002. tRNomics: analysis of tRNA genes from 50 genomes of Eukarya, Archaea, and Bacteria reveals anticodon-sparing strategies and domain-specific features. RNA 8:1189–1232.

Mocchutan T, Silverman S, Kohli J, Soll D. 1978. Nucleotide sequence of phenylalanine transfer RNA from Schizosaccharomyces pombe: implications for transfer RNA recognition by yeast phenylalanyl-tRNA synthetase. Biochemistry 17:1622–1628.

Miller EK, Pirlle IL, Dudock BS, Pirlle RM. 1983. The nucleotide sequence of arginine tRNA\textsuperscript{CGG} from bovine liver. Nucleic Acids Res 11:1933–1936.

Miyauchi K, Kimura S, Suzuki T. 2013. A cyclic form of N\textsuperscript{6}-threonylcarbamoyladenosine as a widely distributed tRNA hypermodification. Nat Chem 5:105–111.

Moric A, Grosjean H. 1998. Structural requirements for enzymatic formation of threonylcarbamoyladenosine (c\textsuperscript{T}) in tRNA: an in vivo study with Xenopus laevis oocytes. RNA 4:24–37.

Mörn M, Dorner M, Pabo S. 1995. C to U editing and modifications during the maturation of the mitochondrial tRNA\textsuperscript{mth} in marsupials. Nucleic Acids Res 23:3380–3384.

Motorin Y, Bec G, Tewari R, Grosjean H. 1997. Transfer RNA recognition by the Escherichia coli Δ-isopentenyl-1-pyrophosphate:tRNA
Δ^2-isopentenyl transferase: dependence on the anticodon arm structure. RNA 3: 721–733.

Noma A, Yi S, Katai T, Takai Y, Suzuki T, Suzuki T. 2011. Actin-binding protein ABP140 is a methyltransferase for 3-methylcytidine at position 32 of tRNAs in Saccharomyces cerevisiae. RNA 17: 1111–1119.

Ohira T, Suzuki T. 2011. Retrograde nuclear import of tRNA precursors is required for modified base biogenesis in yeast. Proc Natl Acad Sci 108: 10502–10507.

Olejniczak M, Dale T, Fahlman RP, Uhlenbeck OC. 2005. Idiosyncratic tuning of tRNAs to achieve uniform ribosome binding. Nat Struct Mol Biol 12: 788–793.

Phizicky EM, Hopper AK. 2010. tRNA biology charges to the front. Genes Dev 24: 1832–1860.

Rubio MA, Ragone FL, Gaston KW, Ibba M, Alfonzo JD. 2006. C to U editing stimulates A to I editing in the anticodon loop of a cytoplasmic threonyl tRNA in Trypanosoma brucei. J Biol Chem 281: 115–120.

Ryvin P, Leung YY, Silverman IM, Childress M, Valladares O, Dragomir I, Gregory BD, Wang LS. 2013. HAMR: high-throughput annotation of modified ribonucleotides. RNA 19: 1684–1692.

Shaheen HH, Hopper AK. 2005. Retrograde movement of tRNAs from the cytoplasm to the nucleus in Saccharomyces cerevisiae. Proc Natl Acad Sci 102: 11290–11295.

Soderberg T, Poulter CD. 2000. Escherichia coli dimethylallyl diphosphate:tRNA dimethylallyltransferase: essential elements for recognition of tRNA substrates within the anticodon stem-loop. Biochemistry 39: 6546–6553.

Thiaville PC, Iwata-Reuyl D, De Crécy-Lagard V. 2015a. Diversity of the biosynthesis pathway for threonylcarbamoyladenosine (t^A) , a universal modification of tRNA. RNA Biol 11: 1529–1539.

Thiaville PC, Legendre R, Rojas-Benítez D, Baudin-Bailleur A, Hatin I, Chalancon G, Glavic A, Namy O, de Crécy-Lagard V. 2015b. Global translational impacts of the loss of the tRNA modification t^A in yeast. Microb Cell 3: 29–45.

Tomikawa C, Yokogawa T, Kanai T, Hori H. 2010. N^2-Methylguanine at position 46 (m^G46) in tRNA from Thermus thermophilus is required for cell viability at high temperatures through a tRNA modification network. Nucleic Acids Res 38: 942–957.

Torres AG, Batlle E, Ribas de Pouplana L. 2014a. Role of tRNA modifications in human diseases. Trends Mol Med 20: 306–314.

Torres AG, Piñeyro D, Filonava L, Stracker TH, Batlle E, Ribas de Pouplana L. 2014b. A-to-I editing on tRNAs: biochemical, biological and evolutionary implications. FEBS Lett 588: 4279–4286.

Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 25: 1189–1191.

Yarham JW, Lamichhane TN, Pyle A, Mattijssen S, Baruffini E, Bruni F, Donnini C, Vassilev A, He L, Blakely EL, et al. 2014. Defective i^6A37 modification of mitochondrial and cytosolic tRNAs results from pathogenic mutations in TRIT1 and its substrate tRNA. PLoS Genet 10: e1004424.

Yarus M. 1982. Translational efficiency of transfer RNA’s: uses of an extended anticodon. Science 218: 646–652.

Yoshiiha T, Yunoki-Esaki K, Ohshima C, Tanaka N, Endo T. 2003. Possibility of cytoplasmic pre-tRNA splicing: the yeast tRNA splicing endonuclease mainly localizes on the mitochondria. Mol Biol Cell 14: 3266–3279.

Yoshiiha T, Ohshima C, Yunoki-Esaki K, Endo T. 2007. Cytoplasmic splicing of tRNA in Saccharomyces cerevisiae. Genes Cells 12: 285–297.