Evolving specificity of tRNA 3-methyl-cytidine-32 (m³C32) modification: a subset of tRNAs^{Ser} requires N⁶-isopentenylolation of A37

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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 ³methyl-cytidine-32 (m³C32), 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 m³C32 depends on a single gene, *TRM140*, the m³C32 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 m³C 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 m³C32 on the three tRNAs^{Ser} that contain anticodon base A36, requires N⁶-isopentenyl modification of A37 (i⁶A37). This new C32–A37 ACL circuitry indicates that i⁶A37 is a pre- or corequisite for m³C32 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; isopentenylolation; 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). mcm⁵U34 enhances decoding and is used to regulate translation during stress conditions (Begley et al. 2007). Modifications of A37 to N⁶-isopentenyl-A37 (i⁶A37) and N⁶-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 (Miyachi et al. 2013). Although several tRNA modification enzymes are nonessential in yeast, mutations in the human gene homologs are

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Abbreviations: RNAP III, RNA polymerase III; m³C32, 3-methyl-cytosine-32; i⁶A37, isopentenyl-adenosine-37; yW, wybutosine; t⁶A37, threonyl-carbamoyl-adenosine-37

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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 i⁶A37-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 tRNA^{Phe} back into the nucleus is required to complete the G37 modification known as wybutosine (γW) (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 tRNA^{Asp} occurs only after C to U editing elsewhere in the anticodon (Morl et al. 1995). In a cytosolic tRNA^{Thr} 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, m⁵s2U54, and m¹A58 modifications (Ishida et al. 2011). A three nucleotide circuitry was demonstrated in the ACL of *S. cerevisiae* tRNA^{Phe} in which γW37 synthesis depends on 2'-O-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 m³C32 modification. (A) A cartoon of schematized tRNA anticodon loop with different nucleotide positions indicated as referred to in the text. (B) The tRNA^{Ser}UGA and tRNA^{Thr}UGU 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 tRNA^{Ser} and tRNA^{Thr} are further grouped according to base identity at position 36; note that three of the four tRNA^{Ser} contain A36 while the other tRNA^{Ser} contains U36 as do all of the tRNA^{Thr}. (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 (Oleńczak et al. 2005). Known modifications at position 32 of eukaryotic tRNAs are pseudouridine, N^3 -C methylation (m^3C), or 2'-O-methylation (Cm) (Phizicky and Hopper 2010). m^3C32 has been observed only on tRNAs^{Ser} and tRNAs^{Thr} in yeast and these plus tRNAs^{Arg} in higher eukaryotes. Trm140 is responsible for m^3C32 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 m^2G26 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 tRNAs^{Ser} and tRNAs^{Thr} in *S. pombe* indicate m^3C32 , 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 tRNAs^{Thr} and tRNAs^{Ser}, in *S. pombe*, two closely related genes, *trm140⁺* and *trm141⁺*, are specific for tRNAs^{Thr} and tRNAs^{Ser}, respectively. A third homolog in mammals, *METTL8*, is correlated with emergence of m^3C32 in tRNAs^{Arg}. More significantly, analysis of other *S. pombe* tRNA modification mutants uncovered a new ACL m^3C32 circuitry. All three tRNAs^{Ser} with A at position 36 and i⁶A37, show no m^3C32 in tRNA isopentenyl-transferase-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 m^3C32 in all tRNAs^{Ser} and tRNAs^{Thr}

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 generate high yield tRNA sequencing by tRNA-HySeq (Arimbasseri et al. 2015). By this and similar approaches, modifications that alter base-pairing are detected as misincorporations (Arimbasseri et al. 2015 and references therein). Figure 1B shows tRNA-HySeq output data for tRNA^{Ser}UGA and tRNA^{Thr}UGU using IGV display in which gray bars indicate match to genomic sequence and colored bars indicate mismatch at $\geq 15\%$ (default setting of IGV program) (Arimbasseri et al. 2015). We note that these two tRNAs share misincorporations at C32 reflecting their common m^3C32 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 tRNAs^{Ser} and tRNAs^{Thr} but $\leq 1\%$ 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 N^3 -methylation (m^3C) (Phizicky and Hopper 2010). Since m^3C is predicted to interfere with Watson–Crick base-pairing and eukaryotic tRNAs^{Ser} and tRNAs^{Thr} were known to contain m^3C32 (Jühling et al. 2009), it was likely that these misincorporations reflected m^3C . Consistent with this, tRNA^{Phe}GAA, which is known to contain Cm32 (McCutchan et al. 1978), did not show C32 misincorporation (Fig. 1C; Supplemental Table 1). The majority of C32 misincorporations were to T (Fig. 1D), as also detected by another tRNA-Seq method (Ryvkin et al. 2013).

Two distinctions are notable at this point. Ser is coded for by six codons, four begin with U and two begin with A, whereas Thr is limited to four codons, and all begin with A. Three tRNAs decode the four U-beginning Ser codons and a different tRNA^{Ser} decodes the A-beginning codon, while three tRNAs^{Thr} decode all four of its A-beginning codons. The three tRNAs^{Thr} and one tRNA^{Ser} that decode A-beginning codons and thus share U as their position 36 base, showed higher levels of C32 misincorporations than the three tRNAs^{Ser} with A as their position 36 base (Fig. 1C). Second, the three tRNAs^{Ser} with A at position 36 contain i⁶A37, while the tRNA^{Ser} with U at position 36 contains t⁶A37, as do the tRNAs^{Thr} with U at 36 (Jühling et al. 2009).

S. pombe has two distinct homologs of *TRM140*

The gene responsible for m^3C modification of tRNAs^{Ser} and tRNAs^{Thr} 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 m^3C 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, *SPBC21C3.07c* (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

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*

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 *nmr1*⁺ promoter (Fig. 3E–H). Expressing *trm140*⁺ in the single

trm140Δ mutant led to increased $\text{m}^3\text{C}/\text{m}^5\text{C}$ levels relative to *trm140Δ* transformed with the empty pRep4X plasmid (compare $\text{m}^3\text{C}/\text{m}^5\text{C}$ levels in Fig. 3B,E). Likewise, expressing *trm141⁺* in *trm141Δ* led to increased $\text{m}^3\text{C}/\text{m}^5\text{C}$ relative to *trm141Δ* with empty pRep4X (compare $\text{m}^3\text{C}/\text{m}^5\text{C}$ levels in Fig. 3C,F). We note that while the expression plasmids more than doubled the $\text{m}^3\text{C}/\text{m}^5\text{C}$ 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 $\text{m}^3\text{C}/\text{m}^5\text{C}$ levels from zero with empty pRep4X

FIGURE 3. *S. pombe* *trm140Δ* and *trm141Δ* are each partially deficient in m³C and can be rescued by *trm140⁺* or *trm141⁺*. (A–H) Mass chromatograms of m³C and m⁵C from total RNA from WT (wild type), *trm140Δ*, *trm141Δ* and the double mutant, *trm140Δ trm141Δ*, cells transformed by empty plasmid pRep4X (A–D), or with Rep4X expressing *trm140⁺* or *trm141⁺* (E–H). The y-axes indicate abundance and the numbers associated with the peaks indicate quantities for m³C and m⁵C, which were used to calculate the m³C/m⁵C value given in each panel; the value of 0.2091 in the WT cells was set as 100%. (The high levels of both m³C and m⁵C in G reflect a generally higher concentration of free nucleosides in that digest as indicated by its adenosine content relative to others samples [not shown]). (I) Bar plot showing C32 misincorporations for individual tRNAs^{Ser} and tRNAs^{Thr} in *trm140Δ*, *trm141Δ*, and WT cells. Error bars indicate standard deviation of four replicates for WT and two replicates each for *trm140Δ* and *trm141Δ*.

(Fig. 3D) 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 *trm140Δ* and *trm141Δ* strains. As expected, based on results from *S. cerevisiae*, our analysis of *S. pombe* revealed that all of the m³C was found in tRNAs^{Thr} and tRNAs^{Ser}. Deletion of *trm140⁺* abolished C32 misincorporations from the tRNAs^{Thr} but not the tRNAs^{Ser}, while deletion of *trm141⁺* abolished misincorporations only from tRNAs^{Ser} (Fig. 3I). These data indicate that distinct but related modification enzymes are responsible

for C32 methylation in *S. pombe*. Moreover, this occurs in a tRNA-specific manner; *trm141⁺* for tRNAs^{Ser}, and *trm140⁺* for the tRNAs^{Thr}. 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 tRNA^{Arg}CCU and tRNA^{Arg}UCU, and m³C47:3 in the variable arms of tRNAs^{Ser}

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 isodecoders (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 tRNAs^{Ser} and tRNAs^{Thr}, we found isodecoders for both tRNAs^{Arg} with U at position 36, tRNA^{Arg}CCU and tRNA^{Arg}UCU, with substantial C32 misincorporations (Fig. 4B), but no misincorporations at C32 for the many tRNAs^{Arg}, with G at position 36.

Similar to MEFs, human A549 cells also have m³C32 on tRNA^{Arg}CCU and tRNA^{Arg}UCU in addition to all of the tRNAs^{Ser} and tRNAs^{Thr} (Fig. 4C). While the total number of isodecoders is greater in human as compared to mouse for these tRNA species, the m³C32 was limited to the same tRNA identities as in mice, and no m³C32 on any of

the many isodecoders of the three tRNAs^{Arg} with G at 36. The cumulative results are in good agreement with data from *Bos taurus* in which both tRNA^{Arg}CCU and tRNA^{Arg}UCU were found to have m³C32 (Keith 1984) while neither tRNA^{Arg}CCG from *B. taurus* (Miller et al. 1983) nor tRNA^{Arg}ACG from mouse showed m³C32 (Jühling et al. 2009).

In *S. pombe* and other yeast, m³C32 is limited to tRNAs^{Ser} and tRNAs^{Thr} while we additionally found it on tRNA^{Arg}CCU and tRNA^{Arg}UCU 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 m³C in the variable arm, at position 47:3 of all four tRNAs^{Ser} (Fig. 4D) but not other tRNAs, consistent with prior data (Jühling et al. 2009). This revealed that all tRNAs^{Ser} with the UCU sequence at positions 47:2-47:3-47:4 show misincorporations at C47:3 in mouse and human cells.

FIGURE 4. m³C32 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 i⁶A37 from tRNAs^{Ser} causes their loss of m³C32

i⁶A37 is synthesized by tRNA isopentenyltransferases in bacteria and eukaryotes. In *S. pombe*, *tit1*⁺ synthesizes i⁶A37 and *tit1Δ* deletion strains lacking it have been characterized (Lamichhane et al. 2011, 2013). The substrates of Tit1 include the three tRNAs^{Ser} with A at position 36 but not the tRNA^{Ser} with U at position 36 nor the tRNAs^{Thr} (Lamichhane et al. 2011). N⁶-isopentenyl-A37 retains an N⁶ hydrogen 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 tRNA^{Ser} substrates (compare WT and *tit1Δ* in Fig. 5A) but not for the non-Tit1 substrates, tRNA^{Thr} (compare WT and *tit1Δ* in Fig. 5B).

Compiled data for all seven tRNAs show that loss of m³C32 in *tit1Δ* was specific to the three tRNAs^{Ser} that are usually substrates for i⁶A37 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 m³C32 on *tit1*⁺ requires i⁶A37 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 m³C32 on the Tit1 targets while *tit1-T12A* did not (Fig. 5C). The data strongly suggest that i⁶A37 itself is required for m³C32 modification by the Trm141 enzyme on the Tit1 substrates. Notably, tRNA^{Ser}GCU does not bear i⁶A37 (Lamichhane et al. 2011) and its m³C32 content was not significantly affected by *tit1*⁺ deletion (Fig. 5C).

Mass spec analysis of m³C in WT and *tit1Δ* RNA revealed that total m³C levels in *tit1Δ* were reduced to about one-third

FIGURE 5. Loss of i⁶A37 from tRNAs^{Ser} causes their loss of m³C32. (A,B) IGV display tracks for tRNA^{Ser}UGA (A) and tRNA^{Thr}UGU (B); colors as in Figure 1; note the absence of C32 misincorporations from the *tit1Δ* cells of A but not B. (C) Bar plot of C32 misincorporations in the mutants indicated. Brackets with asterisks indicate the three Tit1 substrates, all of which have A at position 36 and differ from the four other m³C32-containing tRNAs which contain U at position 36. Error bars indicate standard deviations of four replicates each for WT and *tit1Δ*, and two replicates each for *tit1Δ+tit1-T12A* and *tit1Δ+tit1+*. (D) Mass chromatograms of m³C and m⁵C in WT and *tit1Δ* cells; numbers indicate abundances. (E) Pie chart showing the A37 modification status of tRNAs that carry m³C32; compiled from data in the tRNA modification database (UK, unknown) (Jühling et al. 2009).

of WT relative to m⁵C (Fig. 5D), consistent with the results from tRNA-HySeq. Independent analysis by mass spec also confirmed the complete absence of i⁶A in the *tit1Δ* cells as expected (not shown).

Finally, we report that deletion of *trm140*⁺ and *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. 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 m³C32 modification of the three tRNAs^{Ser} with A at position 36, which decode Ser codons that begin with U, is specifically dependent on i⁶A37 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 m¹G37 to yW of tRNA^{Phe} (Guy and Phizicky 2015;

Guy et al. 2012, 2015). A second conclusion is that the genes responsible for tRNA m³C32 modification evolved isoacceptor specificity in fission yeast such that *trm140*⁺ and *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

m³C32 is not found on bacterial or archaeal 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

(Fig. 2C; D'Silva et al. 2011; Noma et al. 2011). The ability of tRNA-HySeq to monitor m³C32 of individual tRNAs combined with gene deletion and mass spec clearly indicates related but distinct m³C32-methyltransferases in *S. pombe*: *trm140*⁺ for tRNAs^{Thr} and *trm141*⁺ for tRNAs^{Ser}. BLAST analyses found both homologs in the three other fission yeast and multiple invertebrates examined, and three homologs in vertebrates. Since mammals have m³C32 on tRNA^{Arg}UCU and tRNA^{Arg}CCU in addition to tRNAs^{Ser} and tRNAs^{Thr}, it is tempting to speculate that the third homolog may methylate the tRNAs^{Arg}. 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 tRNAs^{Ser} and tRNAs^{Thr}. With regard to this, we note that deletion of either *trm141*⁺ or *trm140*⁺ reduced m³C32 levels in the others' substrates (Fig. 3B). In particular, tRNA^{Thr}AGT m³C32 was unusually and unexpectedly sensitive to *trm141*-deletion (Fig. 3B). Also, tRNA^{Thr}AGT 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 i⁶A levels measured by mass spectrometry were significantly increased in *trm141Δ* and *trm140Δ* deletion strains relative to wild type, but returned to wild type levels in the *trm141Δ trm140Δ* double deletion mutant.

A new ACL modification circuit

Another unexpected finding was dependence of the three tRNA^{Ser} substrates of *trm141*⁺, each with A at position 36, on i⁶A37 in the same tRNAs. A strength of the data is the specificity provided by tRNA^{Ser}GCU, which is a substrate of *trm141*⁺ but naturally does not carry i⁶A37, and does not lose m³C32 upon *tit1*⁺ deletion (Fig. 5C). We can exclude a differential role for introns in this specificity because tRNA^{Ser}GCU, tRNA^{Ser}CGA, and tRNA^{Ser}UGA contain introns while tRNA^{Ser}AGA does not; from this can also be inferred that m³C32 modification must occur in the cytoplasm or require retrograde transport of spliced tRNA to the nucleus (Hopper and Huang 2015). Specificity provided by tRNA^{Ser}GCU allows another conclusion. Because tRNA^{Ser}GCU is a Trm141 substrate but retains m³C32 in *tit1Δ* (Fig. 5C), we can conclude that i⁶A37 is not required for Trm141 activity per se.

One possibility is that m³C32 became uniquely dependent on i⁶A37 in the tRNAs^{Ser} that naturally bear i⁶A37, perhaps associated with the evolutionary divergence of the two *TRM140* tRNA substrate specializations reported here. There is support for this but to a limited extent. In vitro modification using T7 RNA polymerase-transcribed tRNA^{Thr} as

substrate showed that recombinant *S. cerevisiae* Trm140 could modify C32 indicating no absolute prerequisite for i⁶A37 (D'Silva et al. 2011; Noma et al. 2011). Other attempts using T7-transcribed tRNA^{Ser} were unsuccessful whereas T7-transcribed tRNA^{Thr} was less efficiently methylated when compared to tRNA substrate purified from Trm140-deficient *S. cerevisiae*; this indeed led the authors to suggest that another modification may positively affect C32 methylation (Noma et al. 2011). However, we emphasize that these studies were done with *S. cerevisiae* Trm140.

Another possibility is that *S. cerevisiae* Trm140 is dependent on either of two bulky A37 modifications, i⁶A37 or t⁶A37 or their derivatives, for efficient methyltransferase activity, and that as part of their substrate specificity, *S. pombe* Trm140 and Trm141 became dependent on either A37 modification. The tRNAs^{Thr} and tRNA^{Ser}GCU share U at position 36, part of the U36–A37–A38 consensus for t⁶A37 (Morin et al. 1998; Thiaville et al. 2015b), and are known to contain t⁶A37 (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, t⁶A37 or their derivatives (Fig. 5E). However, it should be noted that several others that carry t⁶A37, 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 t⁶A37 alone is not a sufficient signal for m³C32 modification.

Further analysis revealed another finding of potential relevance. While tRNA^{Ser}GCU shares U36 and t⁶A37 with tRNAs^{Thr}, it is a Trm141 substrate, while the tRNAs^{Thr} are Trm140 substrates. A more unique distinction for tRNA^{Ser}GCU is that of all seven tRNAs with m³C32, it is the only one with C rather than G in anticodon position 35 (see Fig. 1D). In this regard, it matches the higher eukaryote m³C32 expansion substrates, tRNA^{Arg}CCU and tRNA^{Arg}UCU at positions C35, U36, and t⁶A37 (Thiaville et al. 2015b).

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 tRNA^{Phe} ACL γW37 modification pathway and discover an allele of *FTSJ1*, 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.

TABLE 1. Yeast strains used for experimental work in this study

Species	Strain name	Genotype
<i>S. pombe</i>	yYH1 (WT) (Bayfield and Maraia 2009)	<i>h-leu1-32::[tRNA^{Ser}7T-leu1+] ura4-D18 ade6-704</i>
<i>S. pombe</i>	yNB5 (<i>tit1Δ</i>) (Lamichhane et al. 2011)	<i>h-leu1-32::[tRNA^{Ser}7T-leu1+] ura4-D18 ade6-704 tit1Δ::KanMX6</i>
<i>S. pombe</i>	yAG503 (<i>trm140Δ</i>) This report	<i>h-leu1-32::[tRNA^{Ser}7T-leu1+] ura4-D18 ade6-704 trm140Δ::KanMX6</i>
<i>S. pombe</i>	yAG504 (<i>trm141Δ</i>) This report	<i>h-leu1-32::[tRNA^{Ser}7T-leu1+] ura4-D18 ade6-704 trm141Δ::KanMX6</i>
<i>S. pombe</i>	yAG505 (<i>trm140Δ trm141Δ</i>) This report	<i>h-leu1-32::[tRNA^{Ser}7T-leu1+] ura4-D18 ade6-704 trm140Δ::KanMX6 trm141Δ::NatNT2</i>

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 yAS99 and cloned into the XhoI/BamHI sites of pRep4X, which puts their expression under the control of the *nmt1*⁺ promoter. The oligo-DNA primers used for amplification cloning were: *TRM140*-FOR: 5'-AGTCGAGATCctcgagATGGATACAACACCAGATAATTC-3'; *TRM140*-REV: 5'-AGATTGACAggagc-cTCATTTTGAACCTGGCTTGTA-3'; *TRM141*-FOR: 5'-AGTCGAGATCctcgag-ATGAGTGGTATATCAAAATTAA G-3'; *TRM141*-REV: 5'-AGATTGACAggagc-TCACAGCTTTT CCAAACTCCTTG-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 RNL2K227Q (NEB). The ligated fragments were gel purified from 15% polyacrylamide-urea gel followed by ligation of 5' adapters using T4 RNA ligase I (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 using BLOSUM62) (Waterhouse et al. 2009).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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