Plasticity and diversity of tRNA anticodon determinants of substrate recognition by eukaryotic A37 isopentenyltransferases

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ABSTRACT

The N^6-(isopentenyl)adenosine (i^6A) modification of some tRNAs at position A37 is found in all kingdoms and facilitates codon-specific mRNA decoding, but occurs in different subsets of tRNAs in different species. Here we examine yeasts' tRNA isopentenyltransferases (i.e., dimethylallyltransferase, DMATase, members of the Δ^2-isopentenylpyrophosphate transferase, IPPT superfamily) encoded by *tit1*$^+$ in *Schizosaccharomyces pombe* and MOD5 in *Saccharomyces cerevisiae*, whose homologs are *Escherichia coli* miaA, the human tumor suppressor TRIT1, and the *Caenorhabditis elegans* life-span gene product GRO-1. A major determinant of miaA activity is known to be the single-stranded tRNA sequence, A36A37A38, in a stem–loop. $tRNA^{Trp}_{CCA}$ from either yeast is a Tit1p substrate, but neither is a Mod5p substrate despite the presence of A36A37A38. We show that Tit1p accommodates a broader range of substrates than Mod5p. $tRNA^{Trp}_{CCA}$ is distinct from Mod5p substrates, which we sort into two classes based on the presence of G at position 34 and other elements. A single substitution of C34 to G converts $tRNA^{Trp}_{CCA}$ to a Mod5p substrate in vitro and in vivo, consistent with amino acid contacts to G34 in existing Mod5p-$tRNA^{Cys}_{GCA}$ crystal structures. Mutation of Mod5p in its G34 recognition loop region debilitates it differentially for its G34 (class I) substrates. Multiple alignments reveal that the G34 recognition loop sequence of Mod5p differs significantly from Tit1p, which more resembles human TRIT1 and other DMATases. We show that TRIT1 can also modify $tRNA^{Trp}_{CCA}$ consistent with broad recognition similar to Tit1p. This study illustrates previously unappreciated molecular plasticity and biological diversity of the tRNA-isopentenyltransferase system of eukaryotes.

Keywords: codon; anticodon loop; tRNA modification; $tRNA^{Trp}$; wobble base; i^6A; (isopentenyl)adenosine

INTRODUCTION

The greatest diversity of tRNA modifications occurs on nucleotides 34 and 37 in the anticodon loop (ACL), which optimize codon:anticodon fit in the ribosome and promote translational fidelity (Gefter 1969; Vacher et al. 1984; Ericson and Bjork 1991; Jenner et al. 2010). Here we examine yeasts' tRNA isopentenyltransferases (i.e., dimethylallyltransferase, DMATase, members of the Δ^2-isopentenylpyrophosphate transferase, IPPT superfamily) encoded by *tit1*$^+$ in *Schizosaccharomyces pombe* and MOD5 in *Saccharomyces cerevisiae*, whose functional homologs are *Escherichia coli* miaA, human tumor suppressor TRIT1, and the *Caenorhabditis elegans* life-span gene product GRO-1, which convert A37 to N^6-(isopentenyl)adenosine (i^6A37) in their target tRNAs (Dihanich et al. 1987; Soderberg and Poulter 2000; Lemieux et al. 2001; Spinola et al. 2005). A major determinant of miaA activity is a single-stranded sequence A36A37A38 in a stem–loop (Motorin et al. 1997; Soderberg and Poulter 2000).

In *E. coli*, miaA creates i^6A37 that may be further modified, whereas eukaryotic tRNAs contain i^6A37 without further modification. The i^6A37 modification occurs on different subsets of tRNAs in different model organisms, largely consistent with the presence or absence of A36A37A38 in the tRNA. In bacteria, i^6A37 has been found on all tRNAs for codons with U in the first position—Cys, Leu, Phe, Ser, Trp, and Tyr (Bjork 1995; Persson et al. 1994), whereas in eukaryotes i^6A37 has been found only on a subset of those tRNAs, consistent with their lack of A36A37A38 in tRNAs for

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Leu, Phe, and sometimes Cys. Specifically, yeasts' $tRNA^{Phe}$ and $tRNA^{Leu}$ contain G37 and are therefore not modified with i^6A37. $tRNA^{Cys}_{GCA}$ carries i^6A37 in *S. cerevisiae* (Holness and Atfield 1976) but not *S. pombe*, whose $tRNA^{Cys}_{GCA}$ has G37 (Table 1). However, in apparent discord is *S. cerevisiae* $tRNA^{Trp}_{CCA}$, which contains A36A37A38 but not i^6A37 (Keith et al. 1971; Juhling et al. 2009; this study). Thus, one cannot confidently predict which tRNAs will be substrates for i^6A37 modification based only on sequence, at least in yeast and maybe other eukaryotes. Better understanding of the molecular determinants of substrate specificity for DMATases may inform us about the evolution of this ancient activity. In addition, knowing which tRNAs are modified will be important toward deciphering codon-specific effects during translation in different model organisms. Yet, the basis for $tRNA^{Trp}_{CCA}$ discrimination in yeast is unknown, and the specificity of any eukaryotic DMATase has not been reported.

Synthetic RNA minihelices used to examine MiaA in vitro indicate that sequences required for activity reflect a consensus derived from natural tRNA substrates (Motorin et al. 1997; Soderberg and Poulter 2000). In addition to A36A37A38, miaA requires an anticodon stem of at least 5 bp (Motorin et al. 1997; Soderberg and Poulter 2000). MiaA is 316 amino acids in length, very similar to other bacterial DMATases, whereas eukaryotic DMATases contain an additional C-terminal domain of ~100 amino acids that includes a Zn-finger motif. Crystal structures of Mod5p bound to $tRNA^{Cys}_{GCA}$ reveal interaction of the Zn-finger motif with the top of the anticodon stem, somewhat distant from the catalytic site and adjacent region that contact other nucleotides in the ACL (Zhou and Huang 2008). In addition to A37, which is flipped out into a catalytic pocket similar to MiaA-$tRNA^{Phe}_{GAA}$ (Chimnaronk et al. 2009; Seif and Hallberg 2009), the structures also reveal Mod5p interactions with U33 and C35, as well as contacts to the G34 base (Zhou and Huang 2008).

Our interest in specificity arose as we identified $tRNA^{Trp}_{CCA}$ as a substrate of Tit1p in *S. pombe* and confirmed that $tRNA^{Trp}_{CCA}$ is apparently unmodified at N^6 of A37 in *S. cerevisiae* despite the presence of A36A37A38. Pursuing the basis of this, our data argued that differential tRNA trafficking in the two yeasts was not responsible. We then performed modification assays in vitro and in vivo for Tit1p and Mod5p and found that discrimination occurs at the level of substrate recognition. Moreover, discrimination by Mod5p can be reversed by changing a single base of $tRNA^{Trp}_{CCA}$, C34 to G. These observations are consistent with extensive differences in the amino acid sequences of Mod5p and Tit1p in the region of Mod5p that contacts the G34 base of tRNA seen in existing crystal structures. Mutation of Mod5p in this region led to selective debilitation of activity toward its G34-containing natural substrates. Multiple sequence alignments reveal significant diversity in this region of DMATases. Accordingly, the human TRIT1 protein also has broad substrate recognition, more similar to the *S. pombe* enzyme. Thus, insight into the molecular plasticity and biological diversity of the isopentenyltransferase-tRNA substrate system, which affects codon-specific translation, is revealed.

TABLE 1. Differences in i^6A37 among tRNAs in *S. cerevisiae* and *S. pombe*

Anticodon	*S. cerevisiae*	*S. pombe*
CysGCA	i^6A37	G37[a]
SerAGA	i^6A37	i^6A37
SerCGA	i^6A37	i^6A37
SerUGA	i^6A37	i^6A37
TyrGUA	i^6A37	i^6A37
TrpCCA	A37[b]	i^6A37

[a]G is the templated base at this position; G37 modifications may be present.
[b]As reported (Keith et al. 1971; Juhling et al. 2009); N^6 of A37 appears unmodified based on in vitro modification by Tit1p.

RESULTS

Mutants in *tit1*$^+$, formerly known as *sin1*$^+$, derived from a genetic screen after NaNO2-induced mutagenesis (Thuriaux et al. 1976), exhibit loss of codon-specific tRNA-mediated suppression (TMS) by *sup3-e* (and *sup9-e*), which encodes a serine-inserting $tRNA^{Ser}_{UCA}$ (Kohli et al. 1979; Rafalski et al. 1979; Egel et al. 1980; Janner et al. 1980). The multiple previously described *sin1* mutants were mapped genetically but were not sequenced, and the precise mutations remain unknown (Thuriaux et al. 1976; Kohli et al. 1989). Strain yYH1 contains a weak suppressor $tRNA^{Ser}_{UCA}$ allele (Huang et al. 2005, 2006) derived from *sup3-e* that suppresses a UGA nonsense codon in *ade6-704* and the accumulation of red pigment. Due to a partial suppression phenotype, yYH1 can report either gain or loss of TMS (Huang et al. 2005, 2006). Our approach was to target *tit1*$^+$ for deletion from yYH1. The resulting *tit-Δ* strain, designated yNB5, exhibited loss of TMS as reflected by more red pigment than yYH1 (Fig. 1B). Ectopic *tit1*$^+$ rescued this phenotype (Fig. 1A,B, yNB5 + *tit1*$^+$). Based on mutagenesis of threonine-19 of MiaA (Soderberg and Poulter 2001), which rendered it catalytically debilitated, and multiple sequence alignment, we mutated Tit1p threonine-12 to alanine. Indeed, *tit1-T12A* was inactive for TMS (Fig. 1A,B). Mid-Western blotting using anti-i^6A antibody detects the physical presence of this modification on tRNA (Benko et al. 2000). This confirmed absence of the i^6A modification in *tit1-Δ* and *tit1-T12A* tRNAs, while ectopic *tit1*$^+$ restored the modification (Fig. 1C,D). The presence of at least two i^6A-containing bands that migrated with the tRNAs suggests that substrates other than just the suppressor-$tRNA^{Ser}_{UCA}$, which is a minor $tRNA^{Ser}$ derived from the $tRNA^{Ser}_{UGA}$ gene (Rafalski et al. 1979; Janner et al. 1980), are modified by Tit1p. Western blotting showed that Tit1p-T12A was expressed at a level comparable to Tit1p

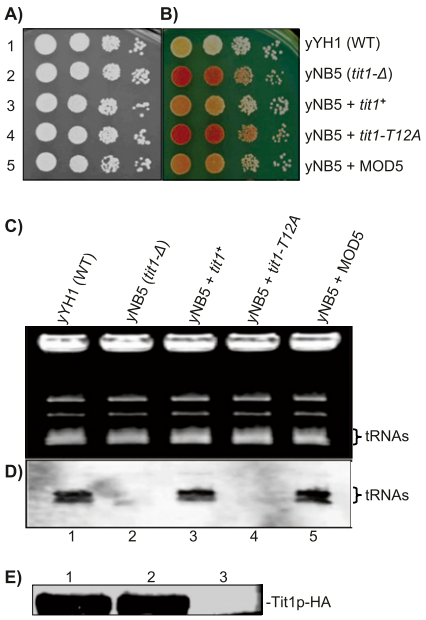

FIGURE 1. Deletion of *tit1*$^+$ causes loss of tRNA i^{6}A and tRNA-mediated suppression. Various strains growing in EMM with non-limiting (*A*) or limiting (*B*) adenine, the latter reflecting tRNA-mediated suppression (TMS) activity. yYH1 (WT, *tit1*$^+$), the parent strain of yNB5, was transformed with empty vector (row *1*); this strain exhibits partial TMS activity (Huang et al. 2005). yNB5 (*tit1-Δ*) was transformed with empty vector, *tit1*$^+$, *tit1-T12A*, or MOD5, as indicated for rows *2–5*. (*C*,*D*) Analysis of *S. pombe* strains in *A* and *B* for i^{6}A in tRNA by mid-Western blotting (Benko et al. 2000). (*C*) Ethidium bromide–stained total RNA in TBE-urea gel. (*D*) Blot of *C* after incubation with anti-i^{6}A antibody and secondary processing for chemiluminescence (only the tRNA region is shown). (*E*) The *tit1*$^+$ (lane *1*), *tit1-T12A* (lane *2*), and empty vector (lane *3*) transformed strains from above were analyzed by immunoblotting using anti-HA antibody.

(Fig. 1E), providing evidence that *tit1-T12A* produces an inactive enzyme.

Substrate specificity of Tit1p

To set up an in vitro modification assay similar to that used for MiaA (Soderberg and Poulter 2000, 2001), we used purified recombinant Tit1p and 17-nt or 19-nt synthetic oligo-RNAs representing ASLs as minihelix analogs of tRNAs (Soderberg and Poulter 2000, 2001), ^{14}C-DMAPP (Dihanich et al. 1987) (dimethylallyl pyrophosphate), and 250 nM Tit1p, which was determined to robustly modify one of our best substrate minihelices, SerAGA-19. We examined stem lengths for TyrGUA and SerAGA ASLs as depicted in Figure 2A. Of these, only the ASLs with a predicted stem of 6 bp were efficiently modified by Tit1p (Fig. 2B). Although natural tRNA substrates have only a 5-bp AC stem, these data suggest that the eukaryotic enzyme requires more contact with the upper part of the AC stem than does MiaA (Motorin et al. 1997; Soderberg and Poulter 2000).

To examine specificity for A36A37A38, we assayed ASLs with substitutions as in Ser-G36, Ser-G38, Ser-G37, and Ser-G36,G38 (Fig. 2C). Position 36 and 37 substitutions greatly diminished activity (Fig. 2C, lanes 2,3). However, Ser-G38 was modified by Tit1p (Fig. 2C, lane 4), unexpected because MiaA exhibits much reduced activity on similar substrates (Soderberg and Poulter 2000). Ser-C38 and Ser-U38 were not modified (Fig. 2C, lanes 6,7).

Upon finding that Tit1p can modify substrates with G38 ASL, we surveyed the *S. pombe* tRNA genome database (Lowe 2011). Significantly, no tRNAs that contain A36A37G38 were found among the 186 *S. pombe* tRNA sequences. Thus, although Tit1p may be able to modify substrates containing A36A37G38, no such natural tRNA exists. We were unable to determine if Tit1p could modify a tRNA$^{Ser}_{UCA}$-G38 in vivo because the mutant tRNA$^{Ser}_{UCA}$-G38 failed to accumulate in *S. pombe* presumably due to rapid decay or counterselection (data not shown).

The C-terminal Zn finger of Tit1p is important for activity in vitro and in vivo

To try to understand the apparent difference between MiaA and Tit1p in ASL stem length, we focused on the eukaryote-specific C-terminal Zn motif. We deleted 55 amino acids from the C terminus of Tit1p to make Tit1p(1-379) and also mutated both cysteines of the C2H2 Zn finger to alanine (Tit1p-ZnAA). We examined these purified Tit1p proteins by in vitro modification of ASL minihelices, in vitro modification of hypomodified tRNAs isolated from *tit1-Δ* cells, and in vivo by TMS. The mutated proteins were

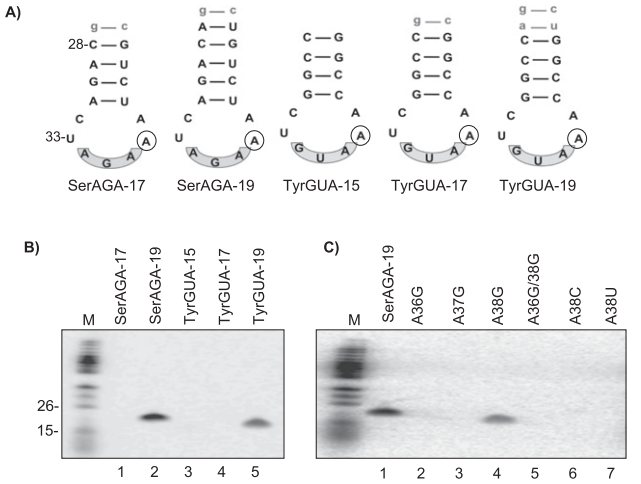

FIGURE 2. Stem length of anticodon stem–loop (ASL) substrates of Tit1p. (*A*) Predicted structures of ASL oligos representing tRNA^{Ser} and tRNA^{Tyr} of various lengths; A37 is circled. Nucleotide positions 28 and 33 are indicated for SerAGA-17; numbering is the same for all others. For some, an extra closing G-C base pair was added (lowercase). For TyrGUA-19, an A-U base pair was added for comparison to SerAGA-19. (*B*) In vitro modification of the ASLs in *A* using recombinant Tit1p and ^{14}C-DMAPP. (*C*) SerAGA-19 (lane *1*) and its derivative ASLs with the substitutions indicated *above* lanes *2–7* were assayed as in *B*.

inactive on all of the ASL minihelices tested (data not shown).

The purified proteins were examined for activity on tRNAs isolated from *tit-Δ* yNB5 cells (Fig. 3A,B). Wild-type Tit1p produced two bands (Fig. 3A, lane 1) that reflect tRNAs^{Ser} and tRNA^{Tyr} based on their sizes of 82 and 74 nt, respectively, and other characteristics described below. Tit1p(1-379) and Tit1-ZnAA were inactive (Fig. 3A, lanes 2,4). Tit1p suffered no loss of activity when mixed with Tit1p(1-379) or Tit1-ZnAA, indicating that the mutated proteins did not contain an inhibitor (Fig. 3A, lanes 3,5).

For in vivo analysis, the proteins were expressed with HA tags. The mutated proteins were inactive for TMS (Fig. 3C) even though they accumulated in *S. pombe* as did Tit1p-HA (Fig. 3D). The cumulative results with these mutants revealed very good agreement of data obtained with ASLs and cellular tRNAs in vitro, and TMS in vivo.

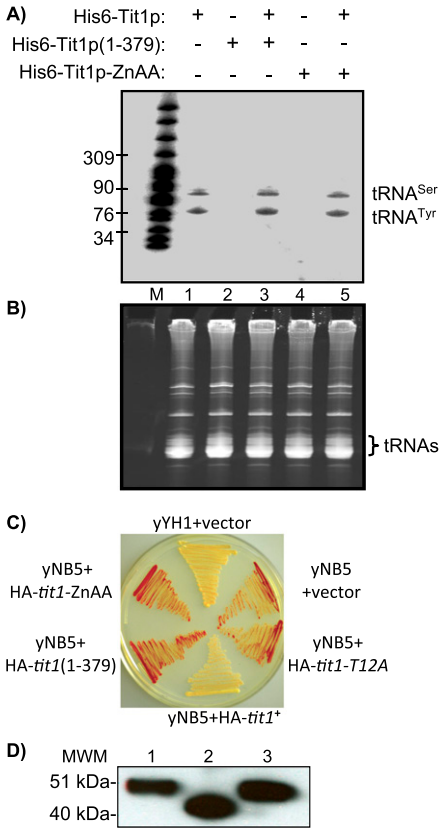

FIGURE 3. The Tit1p extended C terminus including Zn finger is critical for activity in vitro and in vivo. (*A*) Equal amounts of purified recombinant proteins were tested for in vitro activity using ^{14}C-DMAPP and total RNA from yNB5 (*tit1-Δ*) as substrate, with protein(s) as indicated *above* the lanes. (*B*) The gel in *A* stained with ethidium bromide. (*C*) tRNA-mediated suppression assay of the strains transformed with the expression plasmids indicated. (*D*) Proteins from the yNB5 strains in *C* transformed with HA-*tit1*$^+$ (lane *1*), HA-*tit1*(1-379) (lane *2*), and HA-*tit1*-ZnAA (lane *3*) were examined by immunoblotting using anti-HA antibody.

tRNA$^{Trp}_{CCA}$ is a substrate of Tit1p in vitro

Although tRNA$^{Trp}_{CCA}$'s from *S. pombe* and *S. cerevisiae* contain A36A37A38, *S. cerevisiae* tRNA$^{Trp}_{CCA}$ was found to contain unmodified A37 (Keith et al. 1971; Juhling et al. 2009). Two 19-nt ASLs representing tRNA$^{Trp}_{CCA}$ from *S. cerevisiae* and *S. pombe* (Fig. 4A), which migrated with different mobilities, were modified by Tit1p (Fig. 4B, lanes 2,3). These ASLs were resynthesized and again migrated with the relative mobilities seen in Figure 4B even before modification (data not shown).

We next performed the in vitro ^{14}C modification assay on tRNAs isolated from *tit1-Δ* cells before and after antisense oligo-DNA annealing followed by RNase H treatment to identify, by elimination, individual bands on the gel as specific tRNA isoacceptor substrates of Tit1p (Fig. 4C). tRNA from yYH1 (*tit1*$^+$) was not a substrate (Fig. 4C, lane 1), as expected if the tRNAs already contain i^{6}A37 or another modification to the N^6 of A37, whereas tRNA from yNB5 (*tit1-Δ*) was modified (Fig. 4C, lane 2). The electrophoresis used for Figure 4C resolved the yNB5 tRNA into three bands of Tit1p products (Fig. 4C, lane 2). In Figure 4C, lanes 3–10, antisense oligo-DNAs spanning the anticodon loop (ACL) of the specific tRNAs targeted for degradation were incubated with the cellular tRNA and subsequently treated with RNase H, then purified prior to addition of ^{14}C-DMAPP and Tit1p. The *S. pombe* genome harbors seven genes for tRNA$^{Ser}_{AGA}$, three genes for tRNA$^{Ser}_{UGA}$, and one gene for tRNA$^{Ser}_{CGA}$, all of which contained A36A37A38 (Lowe 2011). Targeting tRNA$^{Ser}_{AGA}$ with antisense oligo and RNase H reduced the amount of upper band but not the two lower bands (Fig. 4C, lane 3). Targeting tRNA$^{Ser}_{UGA}$ or tRNA$^{Ser}_{CGA}$ alone had negligible effect on the amount of upper band (Fig. 4C, lanes 4,5). When tRNA$^{Ser}_{AGA}$ and tRNA$^{Ser}_{UGA}$ or tRNA$^{Ser}_{CGA}$ were targeted simultaneously, the upper band was most diminished (Fig. 4C, lanes 6,7). Thus, as expected based on size, the three potential tRNAs^{Ser} substrates of Tit1p migrate as the single upper band. The four genes encoding *S. pombe* tRNA^{Tyr} are of identical sequence (Lowe 2011). Targeting tRNA$^{Tyr}_{GUA}$ caused disappearance of only the lowest band (Fig. 4C, lane 8). The three genes encoding *S. pombe* tRNA$^{Trp}_{CCA}$ are of identical sequence (Lowe 2011). Targeting tRNA$^{Trp}_{CCA}$ led to disappearance of the middle band only (Fig. 4C, lane 9). Targeting both tRNA$^{Tyr}_{GUA}$ and tRNA$^{Trp}_{CCA}$ left only the upper band (Fig. 4C, lane 10). These data suggest that tRNAs^{Ser}, tRNA$^{Tyr}_{GUA}$, and tRNA$^{Trp}_{CCA}$ are substrates for Tit1p in vitro and in *S. pombe*, and that they exhibit the relative mobilities indicated to the right of Figure 4C.

tRNA$^{Trp}_{CCA}$ is a substrate of Tit1p in vivo

Indirect evidence, based on altered chromatographic mobility, had suggested that *S. pombe* tRNA$^{Trp}_{CCA}$ was a substrate of *tit1* (then *sin1*), although detection of i^{6}A37, which was confirmed for tRNAs^{Ser} and tRNA^{Tyr}, was not reported for

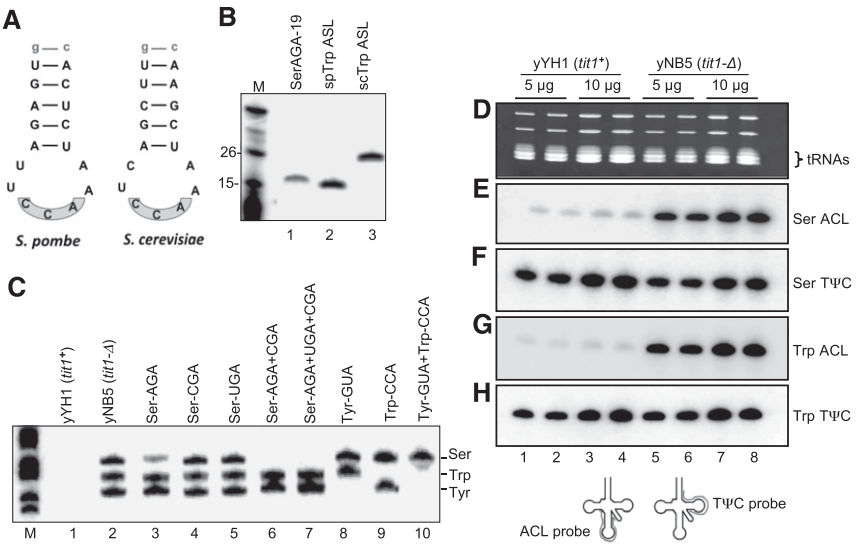

FIGURE 4. Identification of $tRNA^{Trp}_{CCA}$ as a substrate of Tit1p. (*A*) Nineteen-nucleotide ASL oligos representing $tRNA^{Trp}_{CCA}$ from *S. pombe* and *S. cerevisiae* used in *B* for in vitro modification by Tit1p; SerAGA-19 (lane *1*) from *S. pombe* is a control; Trp-19 from *S. pombe* (lane *2*) and Trp-19 from *S. cerevisiae* (lane *3*). The *S. pombe* and *S. cerevisiae* ASLs Trp-19 run differently on TBE-urea gel (see text). (*C*) In vitro modification assay after elimination of specific tRNAs by RNase H, to identify *S. pombe* substrates of Tit1p. (Lanes *1*,*2*) In vitro modification by Tit1p of cellular RNA isolated from yYH1 (*tit1*$^+$, lane *1*) and yNB5 (*tit1-Δ*, lane *2*). (Lanes *3–10*) RNA from yNB5 was pre-incubated with oligo-DNA(s) complementary to the anticodon loop of the tRNA(s) targeted for elimination indicated *above* the lanes, followed by RNase H. The RNA was then purified prior to the Tit1p modification assay. (*D–H*) Positive hybridization in the absence of i^6A37 (PHA6) assay to identify in vivo substrates of Tit1p in *S. pombe*. (*D*) The EtBr-stained gel from which the blot hybridizations *below* it were derived. Lanes *1–4* and *5–8* contain duplicate samples of 5 µg and 10 µg of RNA from yYH1 (*tit1*$^+$) and yNB5 (*tit1-Δ*) as indicated. The same blot was probed, stripped, and reprobed sequentially with the probes indicated to the *right*. (*E*,*G*) The ^{32}P-labeled probes were complementary to the anticodon loop (ACL) region of the tRNAs indicated to the *right*. (*F*,*H*) The ^{32}P-labeled probes were complementary to the TΨC region of the tRNAs indicated to the *right*. The relative positions of the ACL and TΨC probes are indicated *below* the blots.

$tRNA^{Trp}_{CCA}$ (Janner et al. 1980). To examine substrates of Tit1p in vivo, we developed a Northern blot assay that we refer to as positive hybridization in the absence of i^6A37 (PHA6) to detect i^6A37 hypomodified tRNA. The PHA6 assay works with the expectation that the bulky isopentenyl group on N^6 of A37 would interfere with stringent hybridization with a ^{32}P-oligo ACL probe that spanned the 37 position. Hybridization would be greater in the absence of i^6A37 modification as in *tit1-Δ* cells and less so in yYH1 cells in which i^6A37 was present. By this approach, for a Mod5p substrate tRNA we would expect significantly more ^{32}P-oligo ACL probe signal in *tit1-Δ* than in *tit1*$^+$ cells, after calibration to correct for loading using a ^{32}P-oligo probe complementary to the TΨC loop of the same tRNA. This was readily observed using an ACL ^{32}P-probe to $tRNA^{Ser}_{AGA}$ on the blot (Fig. 4E) derived from the gel shown in Figure 4D. The membrane was stripped and hybridized with a ^{32}P-DNA probe complementary to the TΨC loop of $tRNA^{Ser}_{AGA}$, which showed no difference between yYH1 and yNB5 (Fig. 4F). Having validated this assay for a known substrate, we next examined $tRNA^{Trp}_{CCA}$. The ACL probe hybridized with $tRNA^{Trp}_{CCA}$ from yNB5 but not yYH1 (Fig. 4G). Control hybridization with a TΨC probe revealed comparable hybridization with $tRNA^{Trp}_{CCA}$ from both strains (Fig. 4H), suggesting that $tRNA^{Trp}_{CCA}$ is modified in yYH1 but not yNB5 that has no Tit1p, and that lack of i^6A37 has no significant effect of the tRNA levels.

In an attempt to understand the apparent discrepancy in the substrate activity of $tRNA^{Trp}_{CCA}$ in *S. cerevisiae* and *S. pombe*, we first wanted to confirm (or not) that $tRNA^{Trp}_{CCA}$ is not modified in *S. cerevisiae*. The PHA6 assay did not reveal a significant difference in ACL hybridization to $tRNA^{Trp}_{CCA}$ in MOD5 (ABL8) and *mod5-Δ* cells, similar to the $tRNA^{Trp}_{CCA}$ TΨC probe (Fig. 5A–C), supporting the previous data (Keith et al. 1971; Juhling et al. 2009). We note that this approach cannot rule out the possibility that a small fraction of $tRNA^{Trp}_{CCA}$ may be modified by Mod5p in vivo.

As discussed below, tRNA introns reside between positions 37 and 38, and in such cases splicing is a prerequisite for i^6A37 modification. We considered this potentially relevant because all of the $tRNA^{Trp}_{CCA}$ genes in *S. cerevisiae* contain introns, whereas none of the $tRNA^{Trp}_{CCA}$ genes in *S. pombe* do (Lowe 2011). To see if tRNAs with introns may be precluded from i^6A37 modification, we examined *S. cerevisiae* $tRNA^{Ser}_{CGA}$, whose gene also contains an intron at the same position, using the PHA6 blot assay. The $tRNA^{Ser}_{CGA}$ ACL probe showed efficient hybridization with $tRNA^{Ser}_{CGA}$ from *mod5-Δ* but not from MOD5 cells (Fig. 5D,E), consistent with $tRNA^{Ser}_{CGA}$ containing i^6A37 (Etcheverry et al. 1979; Johansson and Bystrom 2005). Furthermore, *S. cerevisiae* $tRNA^{Tyr}$ contains i^6A37 (Madison and Kung 1967) despite introns in all of its genes (Fig. 5G; Lowe 2011). Thus, it seemed unlikely that the difference in $tRNA^{Trp}_{CCA}$ modification in *S. cerevisiae* and *S. pombe* was due to differential presence/absence of introns in their genes.

We next subjected RNA from *S. cerevisiae* ABL8 (MOD5) and MT8 (*mod5-Δ*) cells to in vitro modification by Tit1p. A single band from ABL8 (MOD5) was produced (Fig. 5G, lane 1) that was absent after *S. cerevisiae* $tRNA^{Trp}_{CCA}$ was targeted by antisense DNA and RNase H (Fig. 5G, lane 2). This provides evidence that *S. cerevisiae* $tRNA^{Trp}_{CCA}$ is unmodified at the N^6 of A37 in vivo since it is available for isopentenylation by Tit1p. When RNA from *mod5-Δ* cells was subjected to Tit1p, additional bands were observed as expected (Fig. 5G, lane 3). These additional bands were not observed in Figure 5G, lane 2 presumably because they were

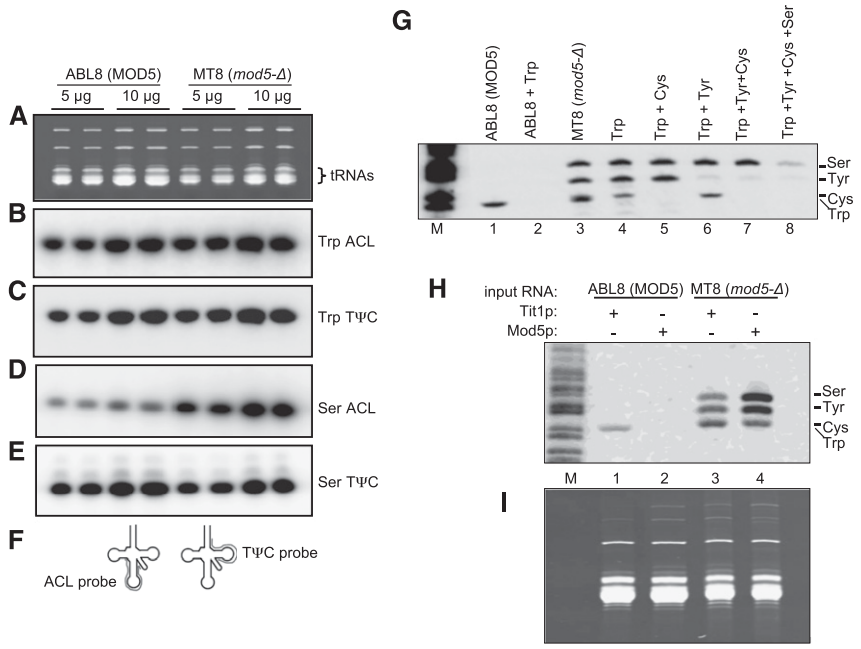

FIGURE 5. *S. cerevisiae* $tRNA^{Trp}$ is not a substrate of Mod5p in vitro or in vivo. (*A–E*) PHA6 assay of *S. cerevisiae* RNAs from MOD5 replete and *mod5-Δ* cells as described in Figure 4 but using probes complementary to the ACL and TΨC regions of the *S. cerevisiae* tRNAs schematically depicted in *F* and indicated to the *right*; the same blot was probed, stripped, and reprobed sequentially. (*G*) In vitro modification by purified recombinant Tit1p, of *S. cerevisiae* tRNAs from *mod5-Δ* cells after elimination of specific tRNAs by RNase H, to identify in vivo substrates of MOD5. In vitro modification using Tit1p of RNA from ABL8 (MOD5) cells after mock preincubation (lane *1*) or preincubation with oligo-DNA antisense to *S. cerevisiae* $tRNA^{Trp}$, both followed by RNase H treatment and purification prior to the ^{14}C-DMAPP modification assay (lane *2*). (Lane *3*) Tit1p-mediated modification of *S. cerevisiae* RNA isolated from MT8 (*mod5-Δ*) after mock preincubation. (Lanes *4–10*) RNA from MT8 (*mod5-Δ*) was preincubated with an oligo-DNA(*s*) antisense to the anticodon loop of the tRNA(s) targeted for elimination indicated *above* the lanes followed by RNase H and purification, prior to the Tit1p ^{14}C-DMAPP modification assay. The tRNAs assigned to the bands are summarized to the *right*. (*H*) Comparison of Tit1p and Mod5p for in vitro modification of tRNAs from *S. cerevisiae*. (Lanes *1*,*2*) ABL8 (MOD5) tRNA; (lanes *3*,*4*) MT8 (*mod5-Δ*) tRNA modification by Tit1p and Mod5p. The tRNAs assigned to the bands are summarized to the *right*. (*I*) Ethidium-stained gel of the assay in *H*.

already modified in the MOD5 replete cells, ABL8. Examination by antisense DNA and RNase H treatment identified these as $tRNA^{Trp}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, and $tRNAs^{Ser}$ (Fig. 5G, lanes 4–8). The data indicate that the fastest band from *mod5-Δ* cells is a combination of $tRNA^{Trp}_{CCA}$ and $tRNA^{Cys}_{GCA}$, both at 72 nt in length. *S. cerevisiae* $tRNA^{Ser}$ and $tRNA^{Tyr}$ are 82 and 75 nt, respectively.

Purified Mod5p and Tit1p differ in specificity for $tRNA^{Trp}_{CCA}$

We used purified recombinant Mod5p for in vitro modification in parallel with Tit1p (Fig. 5H,I). Tit1p but not Mod5p modified the lowest band corresponding to $tRNA^{Trp}_{CCA}$ in RNA from MOD5 replete cells (Fig. 5H, lanes 1,2). Mod5p produced three bands from *mod5-Δ* RNA (Fig. 5H, lane 4), although with preference for the upper two, as compared to Tit1p (Fig. 5H, lane 3), consistent with selective inactivity for $tRNA^{Trp}_{CCA}$. The data in Figure 5G,H suggests that Mod5p does not recognize $tRNA^{Trp}_{CCA}$ as a substrate while it does recognize $tRNA^{Cys}$, $tRNA^{Tyr}$, and $tRNAs^{Ser}$.

Inactivity of $tRNA^{Trp}_{CCA}$ for Mod5p can be reversed by a C34G substitution

To gain insight into the determinants of recognition by Mod5p, we compared the ASL regions of its five natural substrates with the $tRNA^{Trp}_{CCA}$ ASL (Fig. 6A). We examined Mod5p activity on the ASL minihelices and their substituted derivatives. Mod5p modified Ser 19 (Fig. 6B, lane 1) but not the ASL derived from *S. cerevisiae* $tRNA^{Trp}$ (Fig. 6B, lane 2, scTrp-19) or *S. pombe* $tRNA^{Trp}$ (spTrp-19) (data not shown). Our approach was to make substitutions in scTrp-19 that would convert it to a Mod5p substrate, guided by Ser 19 as an end point. We first examined the stem. Changing the C29-G41 base pair in scTrp-19 to A-U to match all other Mod5p substrates did not produce activity (Fig. 6B, lane 3), nor did other base-pair changes in the stem (data not shown). Neither did a triple-base-pair substitution at 27-43, 28-42, and 29-41 in scTrp-19 that made it identical to the Ser 19 stem produce activity (Fig. 6B, lane 4). We then focused on the anticodon.

The natural substrates can be arranged into two groups with distinct characteristics. That is, the $tRNAs^{Ser}$ (Fig. 6A, upper row) all have a G in position 35; an A, C, or U in position 34; and a long variable arm common to type 2 (or class II) tRNAs, whereas the other group members, $tRNA^{Tyr}_{GUA}$ and $tRNA^{Cys}_{GCA}$, have pyrimidine in position 35, G at 34, and a short variable arm common to type 1 (class I) tRNAs. In contrast, the nonsubstrate $tRNA^{Trp}_{CCA}$ has pyrimidines in both the 34 and 35 positions and a short variable arm. Indeed, a single substitution of scTrp-19 ASL that replaced C34 with G34 activated it as a substrate for Mod5p (Fig. 6C, lane 4). Substituting C34 with A, or C35 with G, did not convert the scTrp-19 ASL to a substrate (Fig. 6C, lanes 3,5). Thus, the scTrp-19 ASL minihelix with a single substitution converting it to the GCA anticodon (normally found on $tRNA^{Cys}_{GCA}$) was active for modification by Mod5p. This seems significant since the crystal structures of Mod5p-$tRNA^{Cys}_{GCA}$ (Zhou and Huang 2008) show three side chains of Mod5p (H86, K127, K181) in close contact with the N^6 oxy group of G34, and one side

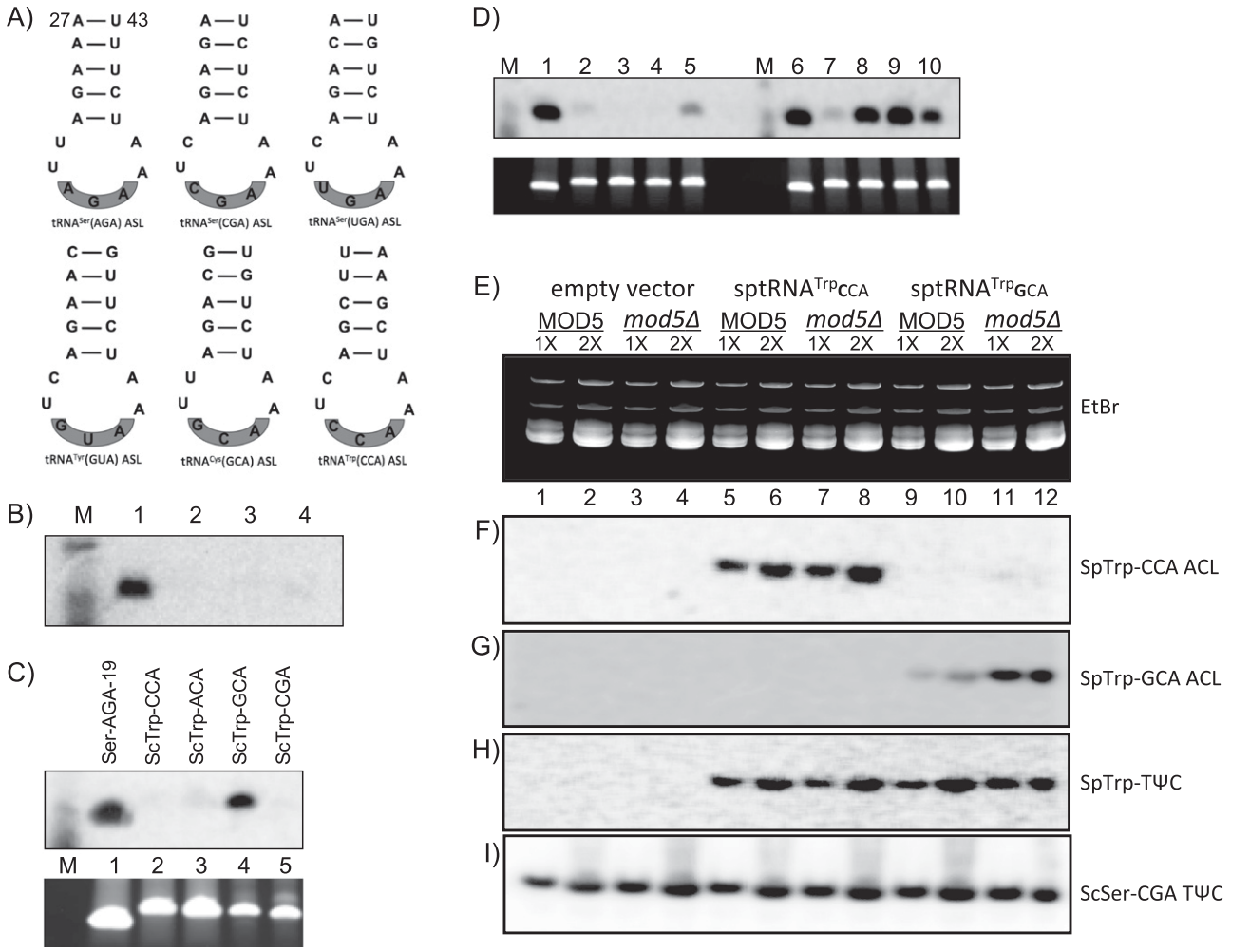

FIGURE 6. C34G substitution activates tRNA$^{Trp}_{CCA}$ as a Mod5p substrate. (*A*) Comparison of the ASL regions of the two groups of natural Mod5p substrates and the nonsubstrate tRNA$^{Trp}_{CCA}$, all of which contain A36A37A38. (*Upper* row) The three tRNAs^{Ser} substrates that comprise the non-G34 group and contain G35; (*lower* row) the first two substrates represent the G34 group, composed of tRNA$^{Tyr}_{GUA}$ and tRNA$^{Cys}_{GCA}$; (*lower right*) the nonsubstrate tRNA$^{Trp}_{CCA}$. (*B–D*) In vitro modification of ASLs: (*B*) Mutations to the stem of ASLs representing tRNA$^{Trp}_{CCA}$ do not activate it as a Mod5p substrate. (Lane *1*) Ser-AGA-19; (lane *2*) scTrp-19; (lane *3*) scTrp-19-mut2-C29A•G41U; (lane *4*) scTrp-19-mut6: U27A•A43U, U28C•A42G, C29A•G41U; the stem is identical to the Ser-AGA-19 stem in lane *1*. (*C*) C34G substitution activates ASL representing tRNA$^{Trp}_{CCA}$ as a Mod5p substrate. Mod5p modification of Ser-AGA-19 (lane *1*) or the scTrp-19 ASLs with the anticodon sequences indicated *above* the lanes (*upper* panel). (*Lower* panel) The ASLs gel in *upper* panel after staining with EtBr. (*D*) Parallel in vitro modification of the same set of ASLs by Mod5p (lanes *1–5*) and Tit1p (lanes *6–10*). (Lanes *1*,*6*) Ser-AGA-19; (lanes *2*,*7*) scTrp-ICA; (lanes *3*,*8*) scTrp-CAA; (lanes *4*,*9*) scTrp-CUA; (lanes *5*,*10*) scTrp-UCA. (*E–I*) C34G substitution activates tRNA$^{Trp}_{CCA}$ as a Mod5p substrate in vivo. MOD5 and *mod5-Δ* cells were transformed with empty vector (lanes *1–4*) or vector expressing an *S. pombe* gene encoding tRNA$^{Trp}_{CCA}$ (lanes *5–8*) or the point mutated tRNA$^{Trp}_{GCA}$ (lanes *9–12*) as indicated *above* the lanes. RNA from the transformed cells was fractionated and blotted, and the membrane was sequentially hybridized, stripped, and rehybridized with probes indicated to the *right* of the panels according to the PHA6 Northern blot assay.

chain (Y84) in close contact to N^2 of G34 (Supplemental Fig. S2; see PDB files 3EPH, 3EPJ, 3EPK, and 3EPL).

We compared the ASL minihelices for Tit1p and Mod5p in parallel (Fig. 6D). Since the only Mod5p substrate whose gene encodes A in position 34, tRNA$^{Ser}_{AGA}$, contains inosine (I) at this position (Zachau et al. 1966; in Johansson and Bystrom 2005), we also examined ASLs with I34. The ASL with I34 was relatively inactive for both proteins (Fig. 6D, lanes 2,7). While the scTrp-19 CAA and CUA ASLs showed little if any substrate activity for Mod5p, these were more active as Tit1p substrates (Fig. 6D). scTrp-19 UCA showed significant substrate activity for Mod5p and relatively more for Tit1p although less than Ser 19 (Fig. 6D). Relative substrate activities of the ASLs are summarized in Table 2.

Activation of tRNA$^{Trp}_{CCA}$ as a Mod5p substrate by C34G substitution in vivo

We next asked if a single C-to-G substitution at position 34 would be sufficient to convert tRNA$^{Trp}_{CCA}$ from an inactive to an active Mod5p substrate in vivo. For this we cloned the wild-type *S. pombe* tRNA$^{Trp}_{CCA}$ gene and a version containing

TABLE 2. Relative activities (0–5) of Mod5p and Tit1p toward different ACL analogs of $tRNA^{Trp}$ and $tRNA^{Ser}$

	Mod5p activity	Tit1p activity
Sp-Ser AGA	5	5
Sc-Trp CCA	0	5
Sc-Trp ACA	1	1
Sc-Trp ICA	1	1
Sc-Trp GCA	5	5
Sc-Trp CAA	0	5
Sc-Trp CGA	0	3
Sc-Trp UCA	2	4
Sc-Trp CUA	0	5
Sc-Trp mut 2	0	5
Sc-Trp mut 6	1	4
Sc-Ser CGA	5	ND
Sc-Ser UGA	5	ND

Activity of Mod5p toward one of its best substrates. Sp-Ser-AGA-19 is considered as 100% and scored 5; the others are relative to this. Quantification was by Fuji PhosphorImager. Equimolar concentrations of Mod5p and Tit1p were used and their activities toward Sp-Ser AGA-19 compared in the same gel (data not shown).

a single C-to-G substitution at position 34, used them to transform *S. cerevisiae* ABL8 (MOD5) and MT8 (*mod5-Δ*) cells, and performed the PHA6 Northern blot assay (Fig. 6E–I). Six samples were examined, each with 1× and 2× concentrations of RNA, as indicated above the lanes of Figure 6E, the EtBr-stained gel from which the blot for Figure 6F–I was derived. Transformation with the empty vector showed no hybridization with any of the sptRNA$^{Trp}_{CCA}$-specific probes (Fig. 6F–H, lanes 1–4) as expected, demonstrating specificity of hybridization. sptRNA$^{Trp}_{CCA}$ isolated from MOD5 and *mod5-Δ* showed comparable hybridization with the ACL probe although with slightly more signal in lane 8 than in lane 6 (Fig. 6F, lanes 5–8). In contrast, the sptRNA$^{Trp}_{GCA}$ bearing the C34-to-G34 substitution showed significantly more hybridization with the ACL probe in *mod5-Δ* than in MOD5 (Fig. 6F, lanes 9–12), providing evidence that tRNA$^{Trp}_{GCA}$ was modified by MOD5. The sptRNA$^{Trp}_{CCA}$ TΨC probe detected comparable amounts of sptRNA$^{Trp}_{CCA}$ and sptRNA$^{Trp}_{GCA}$ (Fig. 6H, lanes 5–12). As a further control, we also probed for endogenous sctRNA$^{Ser}_{CGA}$ using a TΨC probe (Fig. 6I). Quantitation with calibration using the sptRNA$^{Trp}_{\underline{C}CA}$ TΨC probe suggested that ~10%–15% of the sptRNA$^{Trp}_{\underline{C}CA}$ may be modified by Mod5p; in contrast, ~85% of the sptRNA$^{Trp}_{\underline{G}CA}$ was modified by Mod5p (data not shown). The data in Figure 6E–I provided evidence that a C34G substitution was sufficient to activate tRNA$^{Trp}_{CCA}$ as a Mod5p substrate in vivo. The data obtained with ACLs in vitro and tRNA substrates in vivo were largely in agreement.

Mutations to the anticodon binding loop of Mod5p alter tRNA substrate preference

As alluded above, the Mod5p side chains of H86, K127, K181, and Y84 are in close contact to the oxy group at position 6 and N^2 amine groups of G34 in the Mod5p-tRNA$^{Cys}_{GCA}$ structure (PDBs 3EPH, 3EPJ, 3EPK, and 3EPL) (Zhou and Huang 2008). Sequence alignment (Supplemental Fig. S1) reveals that these are not conserved by Tit1p and furthermore that K127 follows a loop in the Mod5p-tRNA$^{Cys}_{GCA}$ structure (colored white in Supplemental Fig. S2) that helps shape the G34 binding pocket. Notably, this loop is foreshortened by three or more residues in *S. cerevisiae* and related yeasts *Zygosaccharyomyces* and *Kluyveromyces*, relative to *S. pombe*, *Schizosaccharomyces japonicus*, and other species (Supplemental Fig. S3), suggesting that it may contribute to differential substrate recognition. We mutated this region of Mod5p and examined activities on RNA isolated from MOD5 and *mod5-Δ* cells (Fig. 7A,B).

The *S. cerevisiae* tRNAs^{Ser} type 2 substrates of Mod5p migrate as one band with the slowest gel mobility due to their large variable arm, while the G34 group, comprised of tRNA$^{Tyr}_{GUA}$ and tRNA$^{Cys}_{GCA}$, are shorter and each exhibits distinct faster mobilities (Fig. 5G,H). In *S. cerevisiae* there are 16 genes for the tRNAs^{Ser} substrates, eight genes for tRNA$^{Tyr}_{GUA}$, and four genes for tRNA$^{Cys}_{GCA}$. Thus, the relative intensities of the three bands observed after Mod5p-mediated in vitro modification should reflect the net effect of their differential abundances and efficiencies as substrates.

Wild-type Mod5p produced three bands comprising both groups of substrates with the pattern observed in Figure 7A, lane 1, in which the middle band is the most intense. In contrast, the mutated Mod5p-loop-K127D protein exhibited selective decrease in the lower two bands, tRNA$^{Cys}_{GCA}$ and tRNA$^{Tyr}_{GUA}$, relative to the upper tRNAs^{Ser} group band (Fig. 7A, lane 2). The single point mutated proteins Mod5p-K127D and Mod5p-Y84S produced patterns nearly indistinguishable from wild-type Mod5p (Fig. 7A, lanes 1,3,4) and thereby serve as controls for the loop-mutated Mod5p (Fig. 7A, lane 2). Mod5p-K181H consistently showed slight reduction of the two lower bands relative to the upper band (see legend for Fig. 7B; Supplemental Fig. S4). Quantitative scanning of each of the lanes of the gel in Figure 7A is shown in Figure 7B. The same differential patterns were reproducibly observed for the loop-mutated Mod5p and -K181H proteins in multiple experiments including when different preparations of the *mod5-Δ* RNA were used (Supplemental Fig. S3; data not shown). The Mod5p-loop mutated protein also exhibited selective disproportionate decrease in activity toward a Tyr-19-GUA relative to Ser-AGA-19 and the wild-type Mod5p (Fig. 7C). These data support the idea that for the G34 group of Mod5p substrates (G34, Y35, and short variable arm; tRNA$^{Cys}_{GCA}$ and tRNA$^{Tyr}_{GUA}$), G34 is a determinant of activity. Mutation of the G34 binding loop of Mod5p selectively inhibits activity toward these substrates more than the non-G34 substrates, tRNAs^{Ser}. Although we hoped that point mutations to Mod5p making it more similar to

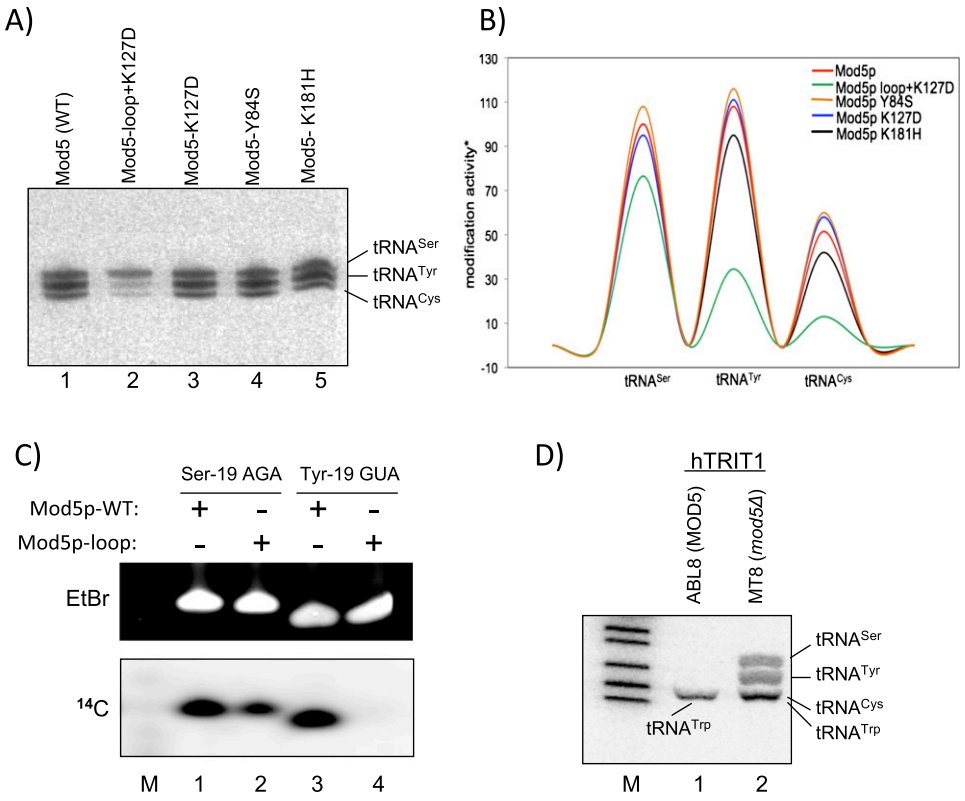

FIGURE 7. The anticodon binding loop of Mod5p alters tRNA substrate preference. (*A*) In vitro modification of *S. cerevisiae mod5-Δ* RNA by wild-type Mod5p (lane *1*) and various mutated Mod5 proteins (lanes *2–5*). Mod5p-loop+K127D (lane *2*) contains an 8-amino-acid insertion after position 120 as well as the K127D mutation; the Mod5 proteins used in lanes *3–5* are single point mutations as described *above* the lanes. (*B*) Quantitative scanning of each lane of the gel shown in *A* using a Fuji PhosphorImager. For normalization, the total counts observed in $tRNAs^{Ser}$ in Mod5p (wild-type, lane *1*) were considered as 100%, and the others were compared accordingly. Note that the black tracing (Mod5p-K127D) for $tRNAs^{Ser}$ is not visible because it was completely masked (overlaid) by the red tracing (Mod5p wild type). (*C*) In vitro modification of ASL substrates indicated *above* the lanes by Mod5p wild type and Mod5p-loop+K127D; (*upper* panel) EtBr staining of gel autoradiogram shown in *lower* panel. (*D*) In vitro modification of *S. cerevisiae* MOD5 (ABL8) and *mod5-Δ* (MT8) RNA by purified recombinant human TRIT1.

Tit1p would activate it for recognition of $tRNA^{Trp}_{CCA}$, this was not observed.

Human TRIT1 appears similar to Tit1p in its anticodon binding loop length and exhibits analogous substrate specificity for $tRNA^{Trp}_{CCA}$

According to multiple sequence alignment, the loop in Mod5p containing K127 appears to be foreshortened by internal deletions in the Mod5 proteins from *S. cerevisiae* and its close homologs in *Kluyveromyces lactis* and *Zygosaccharyomyces rouxii*, relative to *S. pombe* and *S. japonicus* and other species (Supplemental Fig. S3). Similar loop length and lack of conservation of other residues of Tit1p and TRIT1 relative to Mod5p prompted us to examine TRIT1 substrate specificity for $tRNA^{Trp}_{CCA}$. Figure 7D shows that purified recombinant human TRIT1 can indeed modify $tRNA^{Trp}_{CCA}$. We conclude that hTRIT1 and Tit1p exhibit an apparently similar substrate recognition that enables $tRNA^{Trp}_{CCA}$ modification, but differ from Mod5p, which is more restricted.

DISCUSSION

The major new finding reported here is that activity of the DMATase, Mod5p, of the model organism, *S. cerevisiae*, is quite sensitive to the base identity of wobble position 34. Specifically, Mod5p does not recognize $tRNA^{Trp}_{CCA}$ as a substrate despite the presence of A36A37A38. A single base substitution that changes $tRNA^{Trp}_{CCA}$ to $tRNA^{Trp}_{GCA}$ activates it for modification by Mod5p, consistent with crystal structure contacts between G34 of $tRNA^{Cys}_{GCA}$ and Mod5p. This is surprising since examination of MiaA substrates would not have predicted this (Motorin et al. 1997; Soderberg and Poulter 2000). As noted in the Introduction, tRNAs that carry i^6A in prokaryotes include the full set of those that decode all UNN codons, whereas in eukaryotes the subset of tRNAs with i^6A is more restricted. Several eukaryotic tRNAs that decode UNN codons lack the A36A37A38 motif and would therefore not be expected to be DMATase substrates. However, the work here shows that i^6A restriction extends to $tRNA^{Trp}_{CCA}$ in *S. cerevisiae* despite the presence of A36A37A38. This restriction can be overcome by substitution of $tRNA^{Trp}_{CCA}$ to $tRNA^{Trp}_{GCA}$. Why

eukaryotes restrict i^6A37 to only a subset of tRNAs is unknown.

Molecular plasticity

It was helpful to sort the natural substrates of Mod5p into two groups, the type 2 group composed of $tRNAs^{Ser}$ that contain A, U, or C at position 34, G at position 35, and a large variable arm; and the type 1 group that differs in that its members, $tRNA^{Cys}_{GCA}$ and $tRNA^{Tyr}_{GUA}$, has G at position 34, pyrimidine at 35, and a small extra arm. The cumulative data suggest that G34 is an important determinant of Mod5p activity for the $tRNA^{Cys}_{GCA}$ and $tRNA^{Tyr}_{GUA}$ of the type 1 group. Consistent with this, $tRNA^{Trp}_{CCA}$, which resembles the type 1 group with pyrimidine at position 35 and a small variable arm but lacks G at 34 and is inactive as substrate, can be converted to a Mod5p substrate by a single C-to-G substitution at position 34. However, G34 is clearly not required in the natural group 2 substrates, which all have G at position 35 and a large variable arm. The results suggest that Mod5p and other eukaryotic DMATases can recognize determinants in addition to the major one, A36A37A38, in substrate tRNAs that otherwise differ in anticodon sequence and other features. The data suggest molecular plasticity in the recognition of different types of tRNA substrates that differ, among other features, in the sequence at positions 34 and 35 of their anticodons.

Using synthetic ASLs, we found that Tit1p required a longer stem length than was reported for MiaA. The longer length requirement by Tit1p is consistent with the interpretation that the eukaryotic DMATase prefers more structure at the top of the stem because this is where the eukaryote-specific Zn-finger-containing C-terminal region interacts, as seen in the published crystal structure.

A second conclusion is that the homologous DMATases of two distant yeasts overlap in substrate specificity but do not recognize all of the same tRNAs. Specifically, *S. cerevisiae* Mod5p does not modify $tRNA^{Trp}_{CCA}$, while *S. pombe* Tit1p clearly does. Examination of the existing cocrystal structure of Mod5p-$tRNA^{Cys}_{GCA}$ provides insight into a structural basis for this since Tit1p has not conserved the amino acid side chains in Mod5p that make base-specific contacts to G34. Therefore, this study illustrates previously unappreciated biological diversity of the tRNA-isopentenyltransferase system of eukaryotes. These findings advance understanding of the functional and mechanistic basis of differential tRNA i^6A37 modification.

Multiple sequence alignment and mutagenesis of the Mod5p G34 binding loop that contains K127 and comprises a region of significant difference from Tit1p and other DMATases suggested that human TRIT1 may exhibit activity for $tRNA^{Trp}_{CCA}$, which we then demonstrated. The alignment would also suggest that the pathogenic *Candida* species may also exhibit broad specificity, although this remains to be determined.

We have shown that Tit1p catalyzes isopentenylation on tRNAs in vitro and in *S. pombe*. *Tti1-Δ* cells show loss of TMS that is complemented by ectopic Tit1p but not by a catalytic mutant. This reflects importance of i^6A37 in the function of suppressor-tRNA in codon-specific TMS. This is consistent with anticodon loop modification effects on ASL structure and activity (Agris 2008) as the isopentenyl group appears to limit anticodon loop width and increase stacking with codon:anticodon bases affecting base-pairing and codon-specific translation (Bjork 1995; Agris et al. 2007). The Tit1p constructs with deletion of the extended C terminus or substitution of two cysteines of the Zn finger greatly reduced activity in vitro and for TMS in vivo. The Mod5p Zn finger interacts with the top of the anticodon stem (Zhou and Huang 2008). We suspect that the 15-nt and 17-nt ASLs were not modified by Tit1p because of inefficient interaction with the Zn finger due to their short stem, although other interpretations are possible.

Species-specific tRNA systems

Reliable genome-scale tRNA gene prediction indicates that tRNA gene copy number and corresponding codon usage vary between related species (Lowe 2011), suggesting that dynamics in decoding systems accompanies genome evolution and speciation. Results reported here, that *S. cerevisiae* and *S. pombe* differ in their subsets of i^6A37-containing tRNAs (Table 1) and that this is in part due to differential recognition of $tRNA^{Trp}_{CCA}$ by their otherwise homologous DMATases, is consistent with the idea that alterations in the dynamics of tRNA-related decoding systems accompany genome evolution and/or speciation.

Codon-specific translation effects of anticodon loop modification

While i^6A37 is carried by $tRNAs^{Ser}_{AGA}$, UGA, and CGA, as well as $tRNA^{Tyr}$ in both *S. cerevisiae* and *S. pombe*, each has another i^6A37-containing tRNA not found in the other yeast. $tRNA^{Cys}_{GCA}$ carries i^6A37 in *S. cerevisiae* but not in *S. pombe*, in which $tRNA^{Cys}_{GCA}$ contains G37, as summarized in Table 1. Since i^6A37 affects codon-specific translation, the mRNAs that are sensitive to it may differ in organisms with different subsets of i^6A37 tRNAs, including perhaps mRNA subsets with differential synonymous codon use bias (Begley et al. 2007). Indeed, in *S. cerevisiae* the anticodon U34 base modification mediated by Trm9p appears to have been "keyed" to the translation of a specific subset of mRNAs with large bias in their use of the cognate codons (Begley et al. 2007). Somewhat similar perhaps are the effects of the N^6-threonylcarbamoyl adenosine at position 37 (t^6A37) of tRNAs decoding ANN codons (El Yacoubi et al. 2009, 2011; Srinivasan et al. 2011). Phenotypes attributable to lack of this modification are pleiotropic (El Yacoubi et al. 2011; Srinivasan et al. 2011), presumably related at least in part to

mRNA-specific effects. Remarkably, deficiency of t^6A37 promotes de-repression of specific mRNAs that bear upstream ORFs whose translation requires t^6A37-modified tRNA (Daugeron et al. 2011). Determining whether or not defects in codon-specific translation of different subsets of i^6A37-hypersensitive mRNAs can account for the phenotypic differences observed for *S. pombe tit1-Δ* mutants and *S. cerevisiae mod5-Δ* mutants will be a challenge for the future.

MATERIALS AND METHODS

RNA and DNA oligos

RNA and DNA oligos were purchased from IDT (Integrated DNA Technologies).

Strains

yYH1 is *h⁻ leu1-32:: [tRNAmSer7T-leu1⁺] ura4-D ade6-704*. yNB5 is *h⁻ leu1-32:: [tRNAmSer7T-leu⁺] ura4-D tit1Δ-Kan⁺ ade6-704*. yAS99 is *h⁻ ade6-704, ura4-D, leu1-32*. ABL8 and MT8 were obtained from Anita Hopper (Gillman et al. 1991). The medium used for *S. pombe* was YES (3% glucose, 0.5% yeast extract + 225 mg/L adenine, histidine, leucine, uracil, and lysine). Edinburgh Minimal Media (EMM) lacking leucine and uracil was used with adenine at 10 mg/L for tRNA-mediated suppression (TMS) as previously described (Huang et al. 2005, 2006).

Plasmids

tit1⁺ including 600 bp of upstream DNA was cloned into pREP42X from which the NMT promoter was excised so that Tit1p was expressed from its own promoter with an HA tag on the C terminus. Transformed cells were grown in EMM lacking uracil to an $O.D._{600}$ of 0.5. Mutagenesis was carried out by QuickChange XL (Stratagene). All constructs were verified by sequencing.

S. pombe $tRNA^{Trp}$ was cloned into pRS316. Transformed *S. cerevisiae* cells were grown in SC medium lacking uracil, to an $O.D._{600}$ of 0.6.

Northern blotting including PHA6 assay

S. pombe cells were grown to an $O.D._{600}$ of 0.5 in liquid media. RNA was extracted with hot acidic phenol:chloroform. Five micrograms of total RNA was electrophoresed in Novex 10% TBE-urea polyacrylamide gels and transferred to GeneScreen Nylon membranes using Invitrogen iBlot transfer apparatus, cross-linked by UV, and baked under vacuum. Blots were incubated overnight at highly stringent hybridization temperatures with ^{32}P-labeled oligo-DNA complementary to the RNA species indicated.

Mid-Western blotting

Total RNA was electrophoresed, transferred to nitrocellulose, and processed as above. The membrane was blocked for 1 h at room temperature in 5% skim milk/1× PBS/0.05% Tween 20. Anti-i^6A antibody (provided by Anita Hopper) was added at 1/50 dilution. After washing three times for 5 min each in 1× PBS/0.05% Tween 20, a secondary anti-rabbit HRP antibody was incubated for 1 h in 1% skim milk/1× PBS/0.05% Tween 20 at 1/500 dilution and then processed for chemiluminescence. The membrane was washed three times for 5 min each with 1× PBS/0.05% Tween 20 and exposed to a PhosphorImager screen.

Protein extraction and immunoblotting

Cells were grown to an $O.D._{600}$ of 0.5. Protein was extracted in 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.5% NP-40, and 0.1 mM PMSF (Phenyl Methylsulfonyl Fluoride) and added fresh, using glass beads and a BioSpec Products MiniBead Beater. Debris was pelleted, and the supernatant was adjusted to 10% glycerol and frozen at −80°C. Protein was analyzed on 4%–12% Bis-Tris PAGE gels (Novex) and stained with Simply Blue SafeStain (Invitrogen). To examine tagged proteins, the gel contents were transferred to PVDF membrane using iBlot apparatus (Invitrogen). Anti-HA antibody was used followed by secondary Ab, and the membrane was processed for chemiluminescence according to standard methods.

Protein purification

DNAs encoding Tit1p and its derivatives Mod5p and TRIT1 were cloned into pET15b (Invitrogen) and expressed in *E. coli* BL21(DE3)pLysS (Invitrogen), each with a His6 tag. Induced protein was purified by Talon metal affinity (Clontech) and the yield quantified by Bradford assay (Bio-Rad).

In vitro tRNA isopentenylation assay

Twenty micrograms of total RNA purified from yNB5 (*tit1-Δ*) or yYH1 (wild-type) or synthetic ASL at 5 μM final concentration was resuspended in 100 μL of reaction buffer: 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol, 1 nmol of ^{14}C-DMAPP (10 μM, dimethylallyl pyrophosphate), and 100 U of Superasein (Ambion AM2694). Recombinant Tit1p at 250 nM was used for each reaction. After incubation, RNA was extracted with acidic phenol–chloroform. The organic phase was re-extracted with 250 μL of TES (10 mM Tris-Cl at pH 7.5, 10 mM EDTA, 0.5% SDS). The aqueous phase (RNA) was precipitated and resuspended in formamide RNA loading buffer, dried, and electrophoresed on a 12% TBE/urea gel. After drying the gel was exposed to a PhosphorImager screen. For RNase H experiments, a 25-mer antisense oligo DNA complementary to positions 23 to 47 of the tRNA was used.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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REFERENCES

Agris PF. 2008. Bringing order to translation: the contributions of transfer RNA anticodon-domain modifications. *EMBO Rep* **9:** 629–635.

Agris PF, Vendeix FA, Graham WD. 2007. tRNA's wobble decoding of the genome: 40 years of modification. *J Mol Biol* **366:** 1–13.

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

Benko AL, Vaduva G, Martin NC, Hopper AK. 2000. Competition between a sterol biosynthetic enzyme and tRNA modification in addition to changes in the protein synthesis machinery causes altered nonsense suppression. *Proc Natl Acad Sci* **97:** 61–66.

Bjork GR. 1995. Biosynthesis and function of modified nucleosides. In *tRNA: Structure, biosynthesis, and function* (ed. D Soll, UL Rajbhandary), pp. 165–205. ASM Press, Washington, DC.

Chimnaronk S, Forouhar F, Sakai J, Yao M, Tron CM, Atta M, Fontecave M, Hunt JF, Tanaka I. 2009. Snapshots of dynamics in synthesizing N^6-isopentenyladenosine at the tRNA anticodon. *Biochemistry* **48:** 5057–5065.

Daugeron MC, Lenstra TL, Frizzarin M, El Yacoubi B, Liu X, Baudin-Baillieu A, Lijnzaad P, Decourty L, Saveanu C, Jacquier A, et al. 2011. Gcn4 misregulation reveals a direct role for the evolutionary conserved EKC/KEOPS in the t^6A modification of tRNAs. *Nucleic Acids Res* doi: 10.1093/nar/gkr178.

Dihanich ME, Najarian D, Clark R, Gillman EC, Martin NC, Hopper AK. 1987. Isolation and characterization of MOD5, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of *Saccharomyces cerevisiae*. *Mol Cell Biol* **7:** 177–184.

Egel R, Kohli J, Thuriaux P, Wolf K. 1980. Genetics of the fission yeast *Schizosaccharomyces pombe*. *Annu Rev Genet* **14:** 77–108.

El Yacoubi B, Lyons B, Cruz Y, Reddy R, Nordin B, Agnelli F, Williamson JR, Schimmel P, Swairjo MA, de Crecy-Lagard V. 2009. The universal YrdC/Sua5 family is required for the formation of threonylcarbamoyladenosine in tRNA. *Nucleic Acids Res* **37:** 2894–2909.

El Yacoubi B, Hatin I, Deutsch C, Kahveci T, Rousset JP, Iwata-Reuyl D, Murzin AG, de Crecy-Lagard V. 2011. A role for the universal Kae1/Qri7/YgjD (COG0533) family in tRNA modification. *EMBO J* **30:** 882–893.

Ericson JU, Bjork GR. 1991. tRNA anticodons with the modified nucleoside 2-methylthio-N^6-(4-hydroxyisopentenyl)adenosine distinguish between bases 3′ of the codon. *J Mol Biol* **218:** 509–516.

Etcheverry T, Colby D, Guthrie C. 1979. A precursor to a minor species of yeast $tRNA^{Ser}$ contains an intervening sequence. *Cell* **18:** 11–26.

Gefter ML. 1969. The in vitro synthesis of 2′-omethylguanosine and 2-methylthio ^{6}N (γ,γ, dimethylallyl) adenosine in transfer RNA of *Escherichia coli*. *Biochem Biophys Res Commun* **36:** 435–441.

Gillman EC, Slusher LB, Martin NC, Hopper AK. 1991. *MOD5* translation initiation sites determine N^6-isopentenyladenosine modification of mitochondrial and cytoplasmic tRNA. *Mol Cell Biol* **11:** 2382–2390.

Holness NJ, Atfield G. 1976. The nucleotide sequence of cysteine transfer ribonucleic acid from baker's yeast. Products of complete digestion with pancreatic ribonuclease and ribonuclease T1. *Biochem J* **153:** 437–446.

Huang Y, Intine RV, Mozlin A, Hasson S, Maraia RJ. 2005. Mutations in the RNA polymerase III subunit Rpc11p that decrease RNA 3′ cleavage activity increase 3′-terminal oligo(U) length and La-dependent tRNA processing. *Mol Cell Biol* **25:** 621–636.

Huang Y, Bayfield MA, Intine RV, Maraia RJ. 2006. Separate RNA-binding surfaces on the multifunctional La protein mediate distinguishable activities in tRNA maturation. *Nat Struct Mol Biol* **13:** 611–618.

Janner F, Vogeli G, Fluri R. 1980. The antisuppressor strain sin1 of *Schizosaccharomyces pombe* lacks the modification isopentenyladenosine in transfer RNA. *J Mol Biol* **139:** 207–219.

Jenner LB, Demeshkina N, Yusupova G, Yusupov M. 2010. Structural aspects of messenger RNA reading frame maintenance by the ribosome. *Nat Struct Mol Biol* **17:** 555–560.

Johansson MJ, Bystrom AS. 2005. Transfer RNA modifications and modifying enzymes in *Saccharomyces cerevisiae*. In *Topics in current genetics: Fine-tuning of RNA functions by modification and editing* (ed. H Grosjean), pp. 88–120. Springer-Verlag, Berlin, Heidelberg.

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.

Keith G, Roy A, Ebel JP, Dirheimer G. 1971. The nucleotide sequences of two tryptophane-tRNAs from brewer's yeast. *FEBS Lett* **17:** 306–308.

Kohli J, Kwong T, Altruda F, Soll D, Wahl G. 1979. Characterization of a UGA-suppressing serine tRNA from *Schizosaccharomyces pombe* with the help of a new in vitro assay system for eukaryotic suppressor tRNAs. *J Biol Chem* **254:** 1546–1551.

Kohli J, Munz P, Soll D. 1989. Informational suppression, transfer RNA, and intergenic conversion. In *Molecular biology of the fission yeast* (ed. A Nasim et al.), pp. 75–96. Academic Press, San Diego.

Lemieux J, Lakowski B, Webb A, Meng Y, Ubach A, Bussiere F, Barnes T, Hekimi S. 2001. Regulation of physiological rates in *Caenorhabditis elegans* by a tRNA-modifying enzyme in the mitochondria. *Genetics* **159:** 147–157.

Lowe TM. 2011. A genomic tRNA database. http://gtrnadbucscedu/.

Madison JT, Kung HK. 1967. Large oligonucleotides isolated from yeast tyrosine transfer ribonucleic acid after partial digestion with ribonuclease T1. *J Biol Chem* **242:** 1324–1330.

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

Persson BC, Esberg B, Olafsson O, Bjork GR. 1994. Synthesis and function of isopentenyl adenosine derivatives in tRNA. *Biochimie* **76:** 1152–1160.

Rafalski A, Kohli J, Agris P, Soll D. 1979. The nucleotide sequence of a UGA suppressor serine tRNA from *Schizosaccharomyces pombe*. *Nucleic Acids Res* **6:** 2683–2695.

Seif E, Hallberg BM. 2009. RNA–protein mutually induced fit: Structure of *Escherichia coli* isopentenyl-tRNA transferase in complex with tRNA(Phe). *J Biol Chem* **284:** 6600–6604.

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.

Soderberg T, Poulter CD. 2001. *Escherichia coli* dimethylallyl diphosphate:tRNA dimethylallyltransferase: Site-directed mutagenesis of highly conserved residues. *Biochemistry* **40:** 1734–1740.

Spinola M, Galvan A, Pignatiello C, Conti B, Pastorino U, Nicander B, Paroni R, Dragani TA. 2005. Identification and functional characterization of the candidate tumor suppressor gene TRIT1 in human lung cancer. *Oncogene* **24:** 5502–5509.

Srinivasan M, Mehta P, Yu Y, Prugar E, Koonin EV, Karzai AW, Sternglanz R. 2011. The highly conserved KEOPS/EKC complex is essential for a universal tRNA modification, t^6A. *EMBO J* **30:** 873–881.

Thuriaux P, Minet M, Hofer F, Leupold U. 1976. Genetic analysis of antisuppressor mutants in the fission yeast *Schizosaccharomyces pombe*. *Mol Gen Genet* **142:** 251–261.

Vacher J, Grosjean H, Houssier C, Buckingham RH. 1984. The effect of point mutations affecting *Escherichia coli* tryptophan tRNA on anticodon–anticodon interactions and on UGA suppression. *J Mol Biol* **177:** 329–342.

Zachau HG, Dutting D, Feldmann H. 1966. The structures of two serine transfer ribonucleic acids. *Hoppe Seylers Z Physiol Chem* **347:** 212–235.

Zhou C, Huang RH. 2008. Crystallographic snapshots of eukaryotic dimethylallyltransferase acting on tRNA: Insight into tRNA recognition and reaction mechanism. *Proc Natl Acad Sci* **105:** 16142–16147.