Two-subunit enzymes involved in eukaryotic post-transcriptional tRNA modification

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Post-transcriptional tRNA modifications occur in all domains of life, including the simplest organisms. Modifications in and around the anticodon loop are often critical for translational fidelity and efficiency, whereas modifications in the body of the tRNA often contribute to tRNA folding and stability. The importance of tRNA modifications is underscored by their high conservation in different organisms, by the frequent occurrence of a defined growth defect due to deletion of genes required for modifications, and by the increasingly frequent association of human diseases with defects in modification.

Several modifications on specific tRNA residues are known to be formed by a conserved enzyme family within all 3 domains of life. Some examples include formation of $\Psi_{38}$ and $\Psi_{39}$ (pseudouridine, standard tRNA numbering system) by the *Escherichia coli* TrmA/S. *cerevisiae* Pus3 family of pseudouridylases, formation of $\Psi_{13}$ by the *E. coli* TruD/Sc Pus7 family of pseudouridylases, and formation of $\text{t}^\text{A}_{37}$ (N$\text{N}^\text{1}$-threonylcarbamoyl-adenosine) by the core Sc Sua5/Sc YrdC and Sc Kaef/Sc Ygd families of proteins, along with other components depending on the organism. By contrast, the highly conserved $\text{m}^\text{1}G_{37}$ (1-methylguanosine) modification is catalyzed by the Sc Trm5 family of Rossman fold methyltransferases in eukaryotes and archaea, and by the unrelated *E. coli* TrmD family of SPOUT methyltransferases in prokaryotes.

Of particular interest are the modifications that are catalyzed by enzymes comprised of a catalytic subunit and a partner subunit in eukaryotes but not, apparently, in prokaryotes. In the yeast *S. cerevisiae*, there are 5 tRNA modification enzymes that are composed of 2 distinct subunits (Fig. 1, Table 1), whereas available evidence suggests only a single gene product is required in bacteria. Moreover, there are 2 additional modifications found in *S. cerevisiae* and other eukaryotes but not in bacteria that are catalyzed by 2-subunit enzymes. Here we discuss the 7 *S. cerevisiae* tRNA modification enzymes that modify cytoplasmic tRNAs and are comprised of 2 distinct protein subunits. These two-subunit modification enzymes fall into 2 classes, one of which can be further subdivided into 3 subclasses based on common themes, as described below.

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**Introduction**

The complexity of the post-transcriptional tRNA modification machinery is remarkable, with 63 genes known to be required for synthesis of the 25 chemically distinct modifications found in the cytosolic tRNA of the yeast *Saccharomyces cerevisiae*. Over the past decade it has become apparent that formation of a significant number of these modifications requires a complex comprised of 2 different subunits in eukaryotes, but where known, only one protein subunit in bacteria. Remarkably, these complexes include 2 tRNA methyltransferases that share the same scaffold (along with 2 other methyltransferases), each catalyzing formation of different modifications, as well as one methyltransferase that uses 2 different partner proteins for the same modification on different residues. Here we discuss these modifications and the corresponding complexes, to shed light on the origin and conservation of the complexes and the functions of the subunits.

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Heterodimers by Duplication and Divergence

As detailed below, there are 2 examples of eukaryotic 2-subunit enzymes that seem to have arisen by gene duplication events. For both of these complexes, it appears that the eukaryotic non-catalytic subunit has taken the place of one of the catalytic subunits of the bacterial homodimer, with the exception of wheat tRNA$^{\text{Arg(AGG)}}$. In eukaryotes, the substrate repertoire of the enzyme is increased by the non-catalytic subunit, allowing decoding of codons ending in U and C, and often A$^\text{34}$ and lack of the modification gene is lethal in S. cerevisiae, S. pombe, and E. coli.

Tad2-Tad3, the A$^\text{34}$ deaminase

Conversion of the wobble residue A$^\text{34}$ to I$^\text{34}$ (inosine) is thought to occur on the majority of tRNA species that encode an A$^\text{34}$ residue in bacteria and eukaryotes, but is not known to occur in archaea, which lack tRNA genes encoding A$^\text{34}$. In prokaryotes, I$^\text{34}$ is only known to occur on tRNA$^{\text{Arg(ACG)}}$ and all 3 bacterial tRNA$^{\text{Arg(ACG)}}$ species that have been examined contain I$^\text{34}$. All 6 (of 7) S. cerevisiae tRNA species with an encoded A$^\text{34}$ residue that have been examined have I$^\text{34}$, and all eukaryotic cytoplasmic tRNAs with an encoded A$^\text{34}$ that have been examined have the I$^\text{34}$ modification, with the exception of wheat tRNA$^{\text{Arg(ACG)}}$. I$^\text{34}$ increases the reading capacity of tRNAs, allowing decoding of codons ending in U and C, and often A.$^\text{34}$ and lack of the modification gene is lethal in S. cerevisiae, S. pombe, and E. coli.

In S. cerevisiae, I$^\text{34}$ is formed by the Sc Tad2-Tad3 protein pair. Sc TAD2 was identified as being homologous to adenosine deaminases acting on RNA, the gene was determined to be essential, a temperature sensitive mutant was generated, extracts from the temperature sensitive mutant were shown to lack A$^\text{34}$ deaminase activity, and the activity was complemented by additional recombinant Sc Tad2 purified from E. coli. However, purified recombinant Sc Tad2 itself lacked deaminase activity, and purification of Sc Tad2 from yeast cells resulted in co-purification of an additional polypeptide, called Sc Tad3. A$^\text{34}$ deaminase was concluded to be comprised of an Sc Tad2-Tad3 heterodimer, since Sc Tad2 and Sc Tad3 co-purified in stoichiometric amounts, and biochemical fractions containing both Sc Tad2 and Sc Tad3, but neither alone, could convert A$^\text{34}$ to I on a synthetic tRNA Ala construct.

It is likely that TAD2 and TAD3 arose by a gene duplication event, followed by subsequent sequence divergence, since the 120 amino acid C-terminus of Sc Tad3 is 26% identical and 45% similar to Sc Tad2, and since both Sc Tad2 and Sc Tad3 have a conserved Zn$^{2+}$ coordination motif, as well as a conserved proline that is generally required for ammonium group binding.

Although both proteins are required for binding to tRNA, mutational analysis showed that Sc Tad2 is almost certainly the catalytic subunit of the deaminase. An Sc Tad2 variant with an alanine substitution in the predicted catalytic residue E$^\text{56}$ was not active, whereas Sc Tad3 has a valine (V$^\text{218}$) in this position and is presumably inactive; moreover, a complex of the Sc Tad3-V$^\text{218}$E variant and Sc Tad2 had wild type activity, but the Sc Tad3-V$^\text{218}$E variant did not restore activity to the Sc Tad2-E$^\text{56}$A variant.

Table 1. Eukaryotic tRNA modifications that require 2-subunit enzymes.

| modification | S. cerevisiae enzyme$^{ab}$ | closest E. coli homolog$^c$ | E. coli enzyme | references |
|-------------|----------------------------|-----------------------------|----------------|------------|
| I$^\text{34}$ | Tad2-Tad3                  | TadA                        | TadA           | 44,45      |
| m$^\text{1}A^\text{88}$ | Trm61-Trm6 (Gcd14-Gcd10)  | Trm1                        | Trm1           | 44,45,58,64,68 |
| Nm$^\text{32}$ | Trm7-Trm732                | FtsJ                        | TrmJ           | 9,10,88    |
| Nm$^\text{34}$ | Trm7-Trm734 (-Rtt10, Ere2) | FtsJ                        | TrmJ           | 9,10,90    |
| m$^\text{7}G^\text{46}$ | Trm8-Trm82                 | TrmB                        | TrmB           | 78,83      |
| m$^\text{cm}^\text{1}U^\text{34}$ | Trm9-Trm112                | RlmA(I)                     | n/a$^d$        | 5,6,107,110 |
| m$^\text{G}^\text{10}$ | Trm11-Trm112               | YhdJ                        | n/a$^d$        | 3          |

$^a$First protein listed is the catalytic subunit.
$^b$Names in brackets were original names prior to discovery of involvement in tRNA modification.
$^c$Homolog of the catalytic subunit of the S. cerevisiae enzyme.
$^d$Modification is not known to occur in bacteria.
Tad2 and Tad3 homologs are widely found in eukaryotes, and evidence from eukaryotes other than \textit{S. cerevisiae} further suggests that the Tad2-Tad3 complex is required for A\textsubscript{54} deaminase activity. Thus, a screen for temperature sensitive \textit{S. pombe} mutants identified a mutant encoding an \textit{Sp} Tad3 variant with greatly reduced binding of \textit{Sp} Tad2 and associated with reduced levels of inosine in tRNA.\textsuperscript{46} Moreover, the temperature sensitive phenotype of the \textit{Sp} tad3 mutant could be suppressed by additional copies of \textit{Sp} tad2\textsuperscript{4}, suggesting that overexpression drove formation of catalytically active complexes.\textsuperscript{48} Similarly, analysis of \textit{Trypanosoma brucei} A\textsubscript{34} deaminase activity suggests the requirement of a Tad2-Tad3 complex, since a \textit{Tb} Tad2-Tad3 complex has activity, but a \textit{Tb} Tad2 homodimer lacks catalytic activity.\textsuperscript{39}

By contrast, A\textsubscript{34} deamination of tRNA\textsubscript{Arg}(ACG) is catalyzed only by TadA (the homolog of Tad2 and Tad3) in prokaryotes\textsuperscript{13,40} based on the occurrence of only this homolog in bacteria,\textsuperscript{49} and the activity of the purified proteins from \textit{E. coli} and \textit{Agrobacterium tumefaciens}.\textsuperscript{38,45}

TadA was shown to be a homodimer based on its crystal structure from \textit{Staphylococcus aureus}, \textit{A. aeolicus}, and \textit{A. tumefaciens}.\textsuperscript{36,38,49} The co-crystal structure of the \textit{S. aureus} TadA homodimer bound to the 15-mer tRNA\textsuperscript{Arg(ACG)}

antiocodon stem-loop suggests that substrate binding occurs via an induced fit of the anticodon to the rigid interface between the homodimer via specific contacts predominantly with the 5 nucleotides of the anticodon loop and the C\textsubscript{32}-A\textsubscript{38} pair at the top of the loop.\textsuperscript{36}

Many residues shown to be important for tRNA binding in bacterial TadA are not conserved in \textit{Tb} Tad2, suggesting a role for \textit{Tb} Tad3 in substrate binding.\textsuperscript{50} Indeed, some findings suggest that the eukaryotic heterodimer is important for recognition of a larger region on substrate tRNAs to catalyze modification of the multiple different tRNA targets, as opposed to local structural elements of the single tRNA recognized by homodimeric TadA. Thus, although bacterial TadA has activity toward only a stem-loop RNA construct, \textit{Sc} Tad2-Tad3 requires a full tRNA construct and a proper 3-dimensional structure for activity.\textsuperscript{38,44,51} Consistent with these findings, TadA can deaminate eukaryotic tRNA\textsuperscript{Arg(ACG)}

but cannot deaminate other eukaryotic Tad2-Tad3 tRNA substrates.\textsuperscript{45} Remarkably, the \textit{Tb} Tad2-Tad3 enzyme has C to U ssDNA deaminase activity both in vivo and in vitro, further demonstrating the increased substrate repertoire of eukaryotic Tad2-Tad3 as compared to bacterial TadA.\textsuperscript{52} Alfonzo and colleagues have proposed a model wherein binding of the Zn\textsuperscript{2+} ion occurs intermolecularly, possibly granting increased ability to diversify substrates (as needed for the 7 substrate tRNAs in \textit{S. cerevisiae}) while still maintaining specificity for A\textsubscript{34}.\textsuperscript{39}

\textbf{Trm6-Trm61, the m\textsuperscript{1}A\textsubscript{58} methyltransferase}

The m\textsuperscript{1}A\textsubscript{58} (1-methyladenosine) modification is commonly found in eukaryotic tRNAs, including 21 cytoplasmic \textit{S. cerevisiae} tRNAs. This modification is also found less frequently in bacterial and archaeal tRNAs, with a greater frequency of occurrence in tRNA from thermophilic organisms.\textsuperscript{13} m\textsuperscript{1}A\textsubscript{58} likely contributes to tRNA stability,\textsuperscript{53} and \textit{S. cerevisiae} mutants lacking this modification are inviable due to degradation of hypomodified tRNA\textsubscript{Met} by the TRAMP complex (Trr4l/Air2/Mtr4p polyadenylation complex) and the nuclear exosome.\textsuperscript{16,54-57} This specific degradation of only tRNA\textsubscript{Met} is likely due to loss of stability in an important substructure unique to eukaryotic initiator tRNAs, wherein m\textsuperscript{1}A\textsubscript{58} is involved in hydrogen bonding interactions with residues A\textsubscript{20}, A\textsubscript{44}, and A\textsubscript{60}.\textsuperscript{55,58} The requirement of m\textsuperscript{1}A\textsubscript{58} for viability does not extend to all eukaryotes, since \textit{S. pombe} mutants lacking this modification are viable,\textsuperscript{46} albeit with a slow growth defect.\textsuperscript{59}

In \textit{S. cerevisiae}, m\textsuperscript{1}A\textsubscript{58} is formed by Trm6/Trm61.\textsuperscript{58} \textit{Sc} TRM6 (also named GCD10) and \textit{Sc} TRM61 (GCD14) were first identified in screens selecting for mutations that increased GCN4 expression in an \textit{Sc} gen2–101 gen3–101 mutant.\textsuperscript{60-62} GCN4 expression is higher when there is less functional elf2-GTP-tRNA\textsubscript{Met} (elf2: eukaryotic initiation factor 2) initiation ternary complex, and it was found that high copy tRNA\textsubscript{Met} suppressed the temperature sensitive phenotype of \textit{Sc} gcd10–504 (trm6) mutants and the lethality of \textit{Sc} trm6\Delta and \textit{Sc} trm61A mutants by restoring levels of the initiator tRNA.\textsuperscript{58,63} It was also shown that tRNA from \textit{Sc} trm6\Delta and \textit{Sc} trm61A mutants lacked m\textsuperscript{1}A\textsubscript{58} modification,\textsuperscript{58,64} that \textit{Sc} Trm6 and \textit{Sc} Trm61 form a complex with m\textsuperscript{1}A\textsubscript{58} catalytic activity, and that activity was dependent on the S-adenosyl methionine (AdoMet) binding domain of \textit{Sc} Trm6 and \textit{Sc} Trm61.\textsuperscript{64} The \textit{Sc} Trm6-Trm61 complex appears to be a dimer of heterodimers based on size exclusion chromatography.\textsuperscript{65}

The m\textsuperscript{1}A\textsubscript{58} modification enzyme also appears to consist of a Trm6-Trm61 complex in other eukaryotes. Trm6 and Trm61 family proteins are found in yeast, plants, and animals,\textsuperscript{66,67} and the human methyltransferase appears to require both Trm6 and Trm61. Thus, co-expression of human \textit{TRM6} and human \textit{TRM61} suppressed the temperature sensitive growth of \textit{Sc} trm6–504 and \textit{Sc} trm61–2 mutants, restored levels of m\textsuperscript{1}A\textsubscript{58} on tRNA in these mutants, and led to m\textsuperscript{1}A\textsubscript{58} formation on human tRNA\textsubscript{Met} introduced on a plasmid, as measured by an altered electrophoretic mobility of the tRNA.\textsuperscript{67} Furthermore, expression of only human \textit{TRM6} or human \textit{TRM61} did not lead to a substantial increase in m\textsuperscript{1}A\textsubscript{58} modification in mutants, and a complex of human Trm6-Trm61 purified from yeast was able to specifically methylate yeast tRNA\textsubscript{Met}.\textsuperscript{67}

It is likely that \textit{TRM6} and \textit{TRM61} arose from a gene duplication event followed by sequence divergence, based on the sequence similarity between predicted bacterial Trm6 homologs and eukaryotic Trm6, as well as the conservation of predicted secondary structural elements in eukaryotic Trm6, eukaryotic Trm61, and predicted bacterial Trm6 homologs.\textsuperscript{66} This argument is further strengthened by the finding that many of the residues involved in \textit{Mycobacterium tuberculosis} Trm1 (a bacterial Trm6 homolog) homotetramer formation are also involved in the interaction between \textit{Sc} Trm6 and \textit{Sc} Trm61.\textsuperscript{55,65}

By contrast, m\textsuperscript{1}A\textsubscript{58} modification of bacterial and archaeal tRNA is formed by only Trm1 (the homolog of Trm61), based on the occurrence of only one Trm61 homolog and no obvious Trm6 homolog in bacterial and archaeal species,\textsuperscript{66,68,69} and the activity of the purified \textit{Thermus thermophilus}, \textit{M. tuberculosis}, and
**Pyrococcus abyssi** proteins. Based on electrospray ionization mass spectrometry of the native complex, **Tr** Trm1 is a homotrimer, and it is also likely that the **M. tuberculosis** protein is homotrimeric, based on gel filtration analysis.

One of the major functions of Trm6 in the Trm6-Trm61 complex appears to be tRNA binding. Thus, Trm6 contains an RNA recognition motif, and wild type Sc Trm6-Trm61 binds tRNA, as does a complex containing Sc Trm6 and an Sc Trm61 variant with mutations in the AdoMet binding domain, whereas Sc Trm61 by itself does not. Moreover, mutations of conserved residues predicted to be involved in the interface between Sc Trm6 and Sc Trm61 abrogate tRNA binding and m$^7$A$^58$ activity (based on the Trm1 crystal structure), but do not appear to disrupt the heterotetrameric complex or AdoMet binding. These results have led to the speculation that these conserved residues are required to form a Trm66/Trm61 interface that is required for tRNA binding, rather than for complex formation of the proteins themselves.

### Acquisition of an Unrelated Partner Protein

As detailed below, there are 5 eukaryotic methyltransferases that have acquired partner proteins unrelated to the catalytic subunit. We further divide these methyltransferases into 3 distinct subclasses.

### Acquisition of an unrelated subunit by eukaryotes for the same bacterial modification

This class of modification enzymes catalyzes conversion of G$^46$ to m$^7$G$^46$ (7-methylguanosine). The m$^7$G$^46$ modification occurs in bacterial and eukaryotic tRNAs, but to date has not been found in tRNA from archaea, although m$^7$G$^49$ is found on tRNA$^{Glu}$(AAG) from Thermoplasma acidophilum. m$^7$G$^46$ plays a role in stabilizing the tertiary fold of the tRNA, and is part of a commonly occurring base triple with N$^{13}$ and N$^{22}$. S. cerevisiae strains lacking m$^7$G$^46$ have a mild growth defect when grown at 38°C on synthetic media containing glycercol, and mutants lacking m$^7$G$^46$ and m$^7$C (5-methylcytidine) are temperature-sensitive due to degradation of tRNAVal(AAC) by the rapid tRNA decay pathway, which also affects strains lacking m$^7$G$^46$ in combination with lack of any of several other modifications in the body of the tRNA. Additionally, m$^7$G$^46$ was shown to be required for growth of *T. thermophila* at high temperature, and to be required for subsequent Gm$^{18}$ (2′-O-methylguanosine) and m$^1$G$^{37}$ modification on tRNA$^{Phe}$ as part of a tRNA modification network.

In *S. cerevisiae*, m$^7$G$^46$ is formed by the Sc Trm8-Trm82 protein pair. Sc Trm8-Trm82 was discovered using a biochemical genomics approach when it was found that protein purified from *S. cerevisiae* strains expressing tagged open reading frames for either Sc Trm8 or Sc Trm82 yielded m$^7$G formation activity on pre-tRNA$^{Phe}$. Evidence indicating that the enzyme is composed of the Sc Trm8-Trm82 complex includes the observation that deletion of either gene results in lack of m$^7$G modification on tRNA, that the 2 proteins form a stoichiometric complex, that recombinant Sc Trm8 purified from *E. coli* has low in vitro m$^7$G formation activity that is increased 250-fold when co-expressed with Sc Trm82, and that co-translation is required for an active complex. Sc Trm8 contains a methyltransferase domain and is the catalytic subunit of the enzyme, whereas Sc Trm82 is a WD-repeat protein.

In other eukaryotes, the m$^7$G$^46$ modification enzyme also appears to consist of the Trm8-Trm82 protein pair. Trm8 and Trm82 homologs are found in yeast, plants, and animals, and co-expression of human METTL1 (human TRM8) and WDR4 (human TRM82) complemented the m$^7$G$^46$ modification defect in *trm8Δ* or *trm82Δ* mutant cells, whereas expression of only human METTL1 or human WDR4 did not.

In humans, it was also recently reported that HeLa cells with reduced levels of human METTL1 and human NSUN2 (required for m$^7$C) are sensitive to 5-fluorouracil, resulting in a decrease in tRNA$^{Glu}$(AAG) levels. Interestingly, human METTL1 was found to be inactivated by phosphorylation at Ser27 by protein kinase B, suggesting a possible mechanism to regulate m$^7$G modification levels in the cell.

By contrast, in bacteria m$^7$G$^46$ is formed by the Trm8 homolog TrmB alone, based on the apparent absence of Trm82 homologs, the activity of purified *E. coli* TrmB, and the ability of *E. coli* TrmB to complement the lack of Sc Trm8-Trm82 in S. cerevisiae *trm8Δ* double mutants. *E. coli* TrmB is monomeric, whereas *Bacillus subtilis* TrmB is homodimeric in solution and in its crystal structure, which has been proposed as a first evolutionary step in the requirement for a dimeric enzyme.

It is clear that part of the role of Sc Trm82 is to maintain levels of Sc Trm8 in yeast, and that Sc Trm82 is also required for other reasons, since Sc Trm8 levels are greatly reduced in Sc *trm82Δ* mutants, but restoration of levels in *S. cerevisiae* through Sc Trm8 overexpression only marginally restores m$^7$G$^46$ activity. In the crystal structures of members of the *E. coli* TrmB/Sc Trm8 family, the B4-α-D loop region of unbound Sc Trm8 is in a much different conformation than that of the Sc Trm8-Trm82 complex and that of the *B. subtilis* TrmB, which have a similar conformation to one another. In *S. cerevisiae*, the distinct conformation of the unbound form is stabilized by a salt bridge between R195 and E204, which is unable to form in the Sc Trm8-Trm82 dimer due to steric constraints imposed by Sc Trm82. The equivalent residue to Sc Trm8 R195 (R129) in *B. subtilis* TrmB points in the opposite direction compared to unbound R195 in Sc Trm8, and alanine substitution of the equivalent arginine residue in *E. coli* TrmB results in loss of more than 90% of its methyltransferase activity, suggesting that this residue is important for Trm8/TrmB activity.

Sc Trm82 does not appear to be involved in tRNA binding since tRNA$^{Phe}$ cross-links only to Sc Trm8, and not to Sc Trm82, and since the best fit small-angle X-ray scattering (SAXS) model of the Sc Trm8-Trm82 complex bound to tRNA suggests that only Sc Trm8 is involved in tRNA binding. This SAXS model also suggests that Sc Trm8 binds the tRNA through the local structure around the variable region, especially the D-stem and T-stem, which is consistent with the finding that deletion of these stems leads to complete loss of methylation.
Acquisition of 2 different unrelated subunits by eukaryotes for the same modification at different locations

The Trm7 methyltransferase is an example of this subclass, wherein a catalytic subunit engages 2 distinct partner proteins to direct the same 2'-O-methyltransferase activity to different residues: to C32 and N34 to form Cm32 andNm34. Nm32 andNm34 modifications are found in tRNAs from eukaryotes, bacteria, and archaea. Cm32 andNm34 occur in tandem on 3 tRNA species from S. cerevisiae, andCm32 andNm34 modification of tRNAphe appears to be highly conserved in eukaryotes, occurring in 16 of 17 eukaryotic tRNA Phe species that have been examined. Although Cm32 andNm34 are found in tandem on tRNA Phe, they are in chemically distinct environments from each other. Other Nm32 andNm34 modifications are found in mammalian, insect, plant, bacterial, andarchaeal tRNAs, although not always in tandem. Cm32 andNm34 modifications appear to be important in eukaryotes, although their roles are not well understood. Thus, S. cerevisiae and S. pombe mutants lacking Cm32 andNm34, and S. pombe mutants lacking Gm34, have a severe growth defect due to reduced function of tRNA Phe, whereas S. cerevisiae or S. pombe mutations lacking only Cm32 are healthy. Furthermore, Cm32 andNm34 on tRNA Phe are also important for the formation of wybutosine (yW37) from m1G37 in S. cerevisiae and S. pombe (Fig. 2). In bacteria and archaea, there are no reported deleterious phenotypes associated with lack of Nm32, but lack ofNm34 in E. coli causes a defect in amber (UAG) suppression by tRNA Leu(CUA), which was suggested to implicate Nm34 in wobble codon:anticodon pairing.

In S. cerevisiae, Cm32 is formed by theSc Trm7-Trm732 protein pair, andNm34 is formed by the Sc Trm7-Trm734 pair. Sc TRM7 was identified by searching for S. cerevisiae homologs of the E. coli 2'-O-methyltransferase RrmJ (Rrmf), which 2'-O-methylates U2552 residue of the 23S tRNA subunit. It was found that tRNA Phe, tRNA Leu(UAA), and tRNA Thr from S. cerevisiae trm7A mutants lacked Cm32 andNm34, and that tagged Sc Trm7 purified from yeast cells was able to form Cm32, and to a lesser extent, Gm34 on tRNA Phe. It was later shown that Sc Trm732 is required for Cm32 formation and that Sc Trm734 is required forNm34 formation by showing that all 3 Sc Trm7 tRNA substrates from Sc trm732Δ or Sc trm734Δ mutants completely lacked their respective Cm32 orNm34 modifications, that extracts from Sc trm732Δ or Sc trm734Δ mutants were unable to form their respective Cm32 orNm34 modifications on synthetic substrates, and that Sc Trm7 forms a distinct complex with Sc Trm732, and a distinct separate complex with Sc Trm734. The requirement of Sc Trm732 for Cm32 modification on tRNA Phe and of Sc Trm734 for Gm34 modification was further demonstrated by the failure of overexpressed Sc Trm7 to suppress the slow growth phenotype of Sc trm732Δ trm734Δ mutants. Sc Trm732 is an armadillo repeat protein that contains a domain of unknown function (DUF2428), and Sc Trm734 is a WD-repeat protein.

The requirement for the Trm7-Trm732 protein pair forNm32 formation, and of the Trm7-Trm734 protein pair forNm34 formation is likely conserved throughout eukaryotes. Thus, analysis of 25 eukaryotic genomes comprising all 5 eukaryotic supergroups readily identified Trm7 homologs in all 25 organisms, Trm732 homologs in 22 organisms, and Trm734 homologs in 14 organisms. In S. pombe, Sp trm732 mutants lack Cm32, Gm34, and yW37 on their tRNA Phe, Sp trm734Δ mutants lack Cm32, andSp trm734Δ mutants lack Gm34. Furthermore, expression of human FT SJ1 (the predicted TRM7 human homolog) suppressed the growth defect of Sc trm7Δ mutants by forming Cm32 on tRNA Phe, and suppression and modification by human FT SJ1 required either Sc TRM732 or human THADA (the predicted TRM732 human homolog). These findings implicate defective Nm32 andNm34 modifications in nonsyndromic X-linked intellectual disability, since mutations inFTSJ1 are strongly linked to this disease.

The formation ofNm34 in E. coli and in the archaeon Sulfolobus acidocaldarius requires members of the homodimeric TrmJ SPOUT methyltransferase family, which are not obviously related to Trm7, Trm732, or Trm734. E. coli TrmJ appears to require elements in the D-stem and loop for modification activity, whereas Sc TrmJ appears to require elements solely in the anticodon loop.

The formation ofNm34 requires distinct genes in bacteria and archaea, neither of which are related to components of the TRM7 modification machinery. Thus, in E. coli, Cm34 andNm34 on certain tRNA Leu species are formed by the SPOUT methyltransferase TrmL, which recognizes its substrates by interactions with specific residues, including the N6-(isopentenyl)-2-methylthiodioadenosine modification formed from A37 in the anticodon loop of substrate tRNAs. By contrast, in the archaeon Haloferax volcanii, Cm34 formation on tRNA Thr and on elongator tRNA Met require box C/D snoRNPs (small nucleolar ribonucleoprotein) specific to each corresponding pre-tRNA. Comparison of Trm7 to other FtsJ family proteins suggests a possible reason for the requirement of additional proteins for Trm7 activity, since the stem-loop tRNA substrates modified by Trm7 are slightly different than the tRNA stem-loop substrates modified by other proteins in this family (Fig. 2). Thus, E. coli
FtsJ (which is ~34% identical to Sc Trm7) methylates the first residue (Um2552) of the 5-base A-loop in the 23S rRNA,91,92 S. cerevisiae Mrm2 (~29% identical to Sc Trm7) methylates the first residue (Um2971) of the 5-base A-loop in the the 21S mitochondrial rRNA,101 and S. cerevisiae Spb1 (~34% identical to Sc Trm7) methylates the second residue (Gm1992) of the same 5-base A-loop of cytoplasmic 27S pre-rRNA,102 each apparently acting alone. We therefore speculate that Sc Trm7/32 may help Sc Trm7 to recognize and modify the first residue of the 7-base loop of rRNA (as opposed to the 5-base loop in rRNA), and that Sc Trm734 may help Sc Trm7 recognize the N34 residue, which is the third residue in the anticodon loop, and chemically distinct from the substrate residues of the other known FtsJ family members.

S. cerevisiae Trm734 has also been implicated in endoplasmic recycling, seemingly unrelated to tRNA modification. Sc TRM734 (ERE2) was identified in a genome-wide screen for deletion mutants with increased canavanine resistance due to defects in endoplasmic recycling.103 It was suggested that Sc Trm734 regulates the function of Sc Ere1, which was identified in the same screen, since it interacts with Trm734 in membrane bound fractions on a glycerol gradient, co-immunoprecipitates with Sc Trm734, and since some Trm734 colocalized with Sc Ere1 in endosomal compartments in ESCRT (endosomal sorting complexes required for transport) mutant cells. Thus, it may be that cytolicic Sc Trm734 functions for tRNA modification, and that membrane-bound Sc Trm734 plays a role in endoplasmic recycling.103

Sc TRM734 (RTT10) was also identified in a screen for S. cerevisiae deletion mutants that showed increased Ty1 retrotransposition.104 In retrospect, however, this defect is likely due to decreased expression of an important protein(s) involved in repression of Ty1 transposition caused by lack of Nm34 modification, since Sc TRM7 was identified in the same screen.

Acquisition of a common unrelated partner protein for different methyltransferases

The Trm9 and Trm12 methyltransferases are examples of proteins in this subclass, wherein different catalytic subunits engage the same partner protein Trm112 to direct different chemical modifications on different residues. The first tRNA methyltransferase that requires Trm112 is responsible for formation of the terminal methyl group of mccm5U,107 which is found only in eukaryotes and archaea, but not in bacteria.13 Although the precise role of this modification is not clear, it is likely involved in tRNA stability since lack of this modification in combination with lack of m2,2G26 (N2,N2’-dimethylguanosine) in S. cerevisiae results in slow growth.3

In S. cerevisiae, mccm5U is formed by the Sc Trm11-Trm112 pair.5 Sc TRM11 was identified by bioinformatics approaches as a putative methyltransferase, and it was found that extracts from S. cerevisiae cells lacking Sc TRM11 were unable to catalyze formation of m7G on synthetic tRNA3U(UAG). However, although purified Sc Trm11 from yeast yielded m7G activity, Sc Trm11 purified from E. coli did not, and it was shown that Sc trm11Δ mutants lacked m7G activity, that tagged Sc Trm112 purified from yeast exhibited m7G activity, and that Sc Trm11 and Sc Trm112 formed a complex.3 Sc Trm112 was later shown explicitly to be required for Sc Trm11 activity using a wheat germ cell free system.112

Since Sc Trm11 and Sc Trm112 homologs are found widely in eukaryotes and in archaea,3 it is likely that they are required for m7G10 activity in other organisms, although to our knowledge this has not been tested experimentally. Therefore the precise role of Sc Trm112 for Sc Trm11 activity is not known, but Sc Trm11 stability does not appear to be affected by Sc Trm112 levels.3

Trm112 has distinct roles in other methyltransferase complexes

Trm112 is also required for the function of 2 other 2-subunit methyltransferase enzymes. We will briefly discuss these enzymes.
because studies on their structure and function suggest several possible roles for Trm112 in Trm9 and Trm11 function, and because the relative amounts of each complex may affect the methylation activity of the other Trm112-containing complexes, since Trm112 appears to be limiting in *S. cerevisiae*.\(^{8,113}\)

Analysis of the Mtq2-Trm112 complex suggests that Trm112 functions to solubilize Mtq2 and to place it in an active conformation.\(^{4,114}\) In *S. cerevisiae*, Sc Mtq2-Trm112 is the enzyme responsible for N\(^\delta\) methylation of the glutamine residue of the GGQ tripeptide motif of eukaryotic release factor 1 (eRF1).\(^{4,115,116}\) The crystal structure of Mtq2-Trm112 from *Encephalitozoon cuniculi* consists of a single heterodimer, and suggests that *E. cuniculi* Trm112 may function to solubilize *E. cuniculi* Mtq2 by masking a hydrophobic patch involved in the dimer interface.\(^{114}\) Indeed, the presence of *Sc* Trm112 increases the solubility of *Sc* Mtq2 when the proteins are expressed in *E. coli*.* Analysis of the crystal structure also suggests that *E. cuniculi* Trm112 may be important for the conformation of the AdoMet binding domain of *E. cuniculi* Mtq2, since a loop motif is involved in both interaction with *E. cuniculi* Trm112 and with the AdoMet cofactor.\(^{114}\)

Analysis of the overall structure of the *E. cuniculi* Mtq2-Trm112 complex suggests that Trm112 may also function in substrate binding.\(^{114}\) Although the structure of the methyltransferase domain of *E. cuniculi* Mtq2 is most similar to that of *E. coli* PrmC (the protein that N\(^\delta\) methylates the Gln residue of the GGQ motif of bacterial release factors RF1 and RF2),\(^{117,118}\) the overall structure of the *E. cuniculi* Mtq2-Trm112 complex most closely resembles that of *E. coli* RlmA(1),\(^{119}\) which methylates 23S rRNA residue G\(_{745}\).\(^{120,121}\) The Zn\(^{2+}\) binding domain of *E. cuniculi* Trm112 superimposes well with the Zn\(^{2+}\) binding domain of *E. coli* RlmA(1), which has been implicated in rRNA binding,\(^{119}\) suggesting that Trm112 may be important for substrate recognition or binding.\(^{114}\)

In addition, analysis of RlmA(1) structure suggested a role for Trm112 in binding and activity of Trm9, since RlmA(1) is the closest *E. coli* homolog of *S. cerevisiae* Trm9, and a model of Trm112 binding to Trm9 correctly predicted residues important for both Trm9-Trm112 protein-protein interactions and activity.\(^{114}\)

Analysis of the Bud23-Trm112 complex indicates that Trm112 maintains Bud23 protein levels.\(^{7}\) In *S. cerevisiae*, the *Sc* Bud23-Trm112 complex is required for the m\(^7\)G modification of G\(_{1575}\) on the 18S subunit of rRNA in yeast.\(^{7,8,122}\) Sc trm112Δ mutants have severely reduced levels of Sc Bud23,\(^{7}\) and surprisingly, the *Sc* bud23Δ slow growth phenotype can be rescued by expression of a catalytic null *Sc* Bud23 variant,\(^{122}\) further indicating that the role of *Sc* Trm112 in this complex is not for catalysis. Moreover, the slow growth phenotype of the *Sc* trm112Δ mutant is almost completely due to Bud23 defects, since the *Sc* bud23Δ trm112Δ double mutant grew as poorly as the *Sc* bud23Δ or *Sc* trm112Δ single mutants.\(^{5,8}\)

It appears that *Sc* Trm112 may also act to integrate methylation signals between tRNA, the ribosome, and release factors (Fig. 3).\(^{114}\) Because *Sc* Trm112 is likely present in non-stoichiometric amounts compared to its methyltransferase partners,\(^{8,113}\) the activity of each methyltransferase partner is sensitive to the relative amounts of the other *Sc* Trm112 methyltransferase partners. For instance, overexpression of *Sc* Trm11 or *Sc* Mtq2 results in resistance to zymocin toxicity and reduced binding between *Sc* Trm112 and *Sc* Trm9. Furthermore, overexpression of *Sc* Mtq2 leads to cell growth defects, presumably by titrating *Sc* Trm112 away from *Sc* Bud23, and this defect is partially rescued by overexpression of *Sc* Bud23.\(^{7}\)

### Concluding Remarks

Several themes emerge upon analysis of the protein pairs involved in eukaryotic tRNA modification. As described above, tRNA modification enzymes have acquired a second subunit either by duplication of a gene or by acquisition of an unrelated partner protein, but with multiple different properties. One functional theme that has emerged is that the addition of a partner protein may allow for an expansion of the substrate repertoire for a given enzyme, as for the eukaryotic Tad2-Tad3 enzyme, which modifies an additional 6 tRNA species (in *S. cerevisiae*) in contrast to its homodimeric bacterial TadA counterpart, which appears to only modify tRNA\(^{\text{Arg}}(\text{ACG})\). A second theme is the expanded location of modifications facilitated by the acquisition of different partners for eukaryotic Trm7 activity, allowing modification of 2 different residues in chemically distinct environments in the same tRNA species. A third theme that emerges is the distinct possibility of cross-pathway regulation by modification. This is most clear for the different methyltransferases that each require association with limiting amounts of Trm112 for crucial methylations involved in translation termination, tRNA modifications, and ribosome biogenesis. The modulation of several methyltransferase activities by one protein in theory allows the integration of multiple signals to fine-tune translation.
Similarly, it is possible that Trm734 could also act to integrate different cellular functions because of the involvement of Trm734 in endoplasmic recycling and retrotransposition. While we have pointed out some of the common themes in these 2-subunit eukaryotic tRNA modification enzymes, it remains unclear precisely how the non-catalytic subunits function in the modification complexes. Although we have cited evidence describing roles of these proteins in maintaining stability, altering conformations of proteins, expanding substrate repertoire, and promoting substrate binding, in many cases the evidence is indirect and limited. Furthermore, the mechanism by which unrelated partners were acquired is not clear in most cases. Further research will undoubtedly lead to insight into these other questions regarding the roles of partner proteins in these important and conserved modification reactions.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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