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# Diversity in mechanism and function of tRNA methyltransferases

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tRNA molecules undergo extensive post-transcriptional processing to generate the mature functional tRNA species that are essential for translation in all organisms. These processing steps include the introduction of numerous specific chemical modifications to nucleotide bases and sugars; among these modifications, methylation reactions are by far the most abundant. The tRNA methyltransferases comprise a diverse enzyme superfamily, including members of multiple structural classes that appear to have arisen independently during evolution. Even among closely related family members, examples of unusual substrate specificity and chemistry have been observed. Here we review recent advances in tRNA methyltransferase mechanism and function with a particular emphasis on discoveries of alternative substrate specificities and chemistry associated with some methyltransferases. Although the molecular function for a specific tRNA methylation may not always be clear, mutations in tRNA methyltransferases have been increasingly associated with human disease. The impact of tRNA methylation on human biology is also discussed.

## Introduction

RNA methyltransferases are a diverse group of post-transcriptional RNA modification enzymes responsible for the transfer of a methyl group from a methyl donor, most commonly *S*-adenosylmethionine (SAM or AdoMet), to any of several different locations on a target RNA nucleotide. Although all known major classes of cellular RNAs are subject to methylation, tRNA molecules remain the most heavily methylated molecules characterized to date and contain the most diversity in terms of types of methylation events that are observed.[1,2] While the 4 canonical RNA bases, adenosine (A), guanosine (G), cytosine (C) and uridine (U), are the most common substrates for tRNA methyltransferases, methyl groups are also added to modified nucleotides, such as pseudouridine (ψ), inosine (I) and many more complex species, as well as to the ribose 2′-hydroxyl.

tRNA methylation is an apparently ancient process; out of 18 individual modified nucleotides that occur in tRNA from all 3 domains of life, 13 are methylated nucleotides (Cm, Gm, Um, $m^5C$, $m^5U$, $m^3U$, $m^1G$, $m^7G$, $m^2G$, $m^{2,2}G$, $m^1A$, $m^6A$ and $m^{6,6}A$) (**Fig. 1**).[3,4] Consequently, characterization of the enzymes that carry out tRNA methylation can provide insight into the evolution and function of these essential macromolecules. The advent of genome sequencing and sophisticated biochemical methods facilitated an explosion in the identification of tRNA methyltransferase enzymes over the past few decades, such that the corresponding enzymes are now known for the majority of the most common tRNA methylations.[5,6] Yet, recent investigations into the molecular mechanisms and functions of these tRNA methyltransferases and their orthologs have revealed some new surprises, including unexpected diversity in chemistry and substrate specificity that suggests complex functions for these abundant tRNA modification enzymes.

The importance of tRNA methylation has often been clouded by the observation that loss of most individual modifications has little or no observable effect on cell growth. For example, only 3 tRNA modification enzymes in *S. cerevisiae* are strictly essential for cell viability (the $A_{37}$ deaminase *TAD2/TAD3*, $m^1A_{58}$ methyltransferase *TRM6/TRM61* and the $tRNA^{His}$ guanylyltransferase *THG1*), although *S. cerevisiae* strains with single deletions in many of the non-essential tRNA modification enzymes exhibit slow growth or other phenotypes, such as hypersensitivity to the presence of 5-fluorouracil.[7,8] However, the importance of some tRNA methylations (and by extension, the enzymes that catalyze them) is highlighted by several examples of convergent evolution among methyltransferase families. For example, Trm5, an archaeal and eukaryal tRNA methyltransferase, catalyzes N1 methylation at the $G_{37}$ position of tRNA.[9] Likewise, TrmD catalyzes this same methyl group addition to $G_{37}$-containing tRNAs in Bacteria.[10] While both enzymes are responsible for catalyzing essentially the same methylation reaction, the 2 are structurally unrelated and possess different recognition mechanisms, which are reminiscent of differences between the 2 classes of convergently-evolved aminoacyl tRNA synthetases.[11-16] Moreover, loss of tRNA methylation can lead to biologically significant effects on tRNA stability and function.[17-23] This review provides a current survey of the diverse landscape of known tRNA methyltransferases, with a particular emphasis on recent developments in terms of alternative functions and substrate specificities for these enzymes, and newly-revealed associations of these enzymes with human disease.

### Functional classes and conserved mechanistic features of tRNA methyltransferases

Enzymes that catalyze SAM-dependent methylation comprise at least 5 independent structural classes, with tRNA

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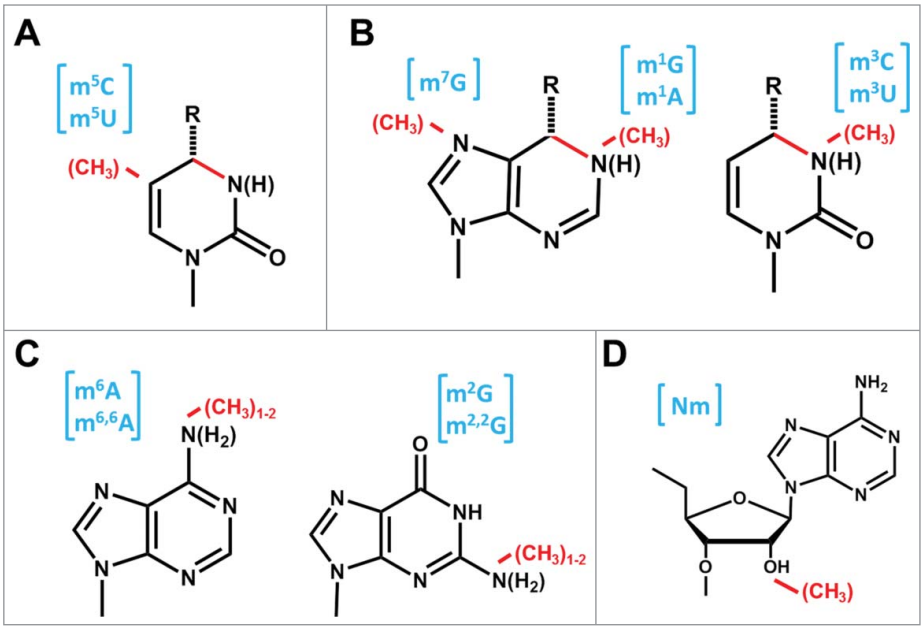


**Figure 1. Common methylated nucleotides in tRNA.** Sites of methylation are indicated by the red methyl group; abbreviations for the resulting methylated species are shown in brackets in blue. **(A)** Methylation at C5 of pyrimidines. The bond shaded in red represents a single or double bond in U or C, respectively, and the resulting presence or absence of a proton at N3 is indicated by parentheses. The group at the 4 position (carbonyl in U and amino in C) is indicated by R. **(B)** Methylation at endocyclic nitrogens of purines and pyrimidines. The red colored bond on the nucleotide base represents a single or double bond, in G/U or A/C, respectively, and the resulting presence or absence of a proton at N3 or N1 is indicated by parentheses. The methylated nucleotides $m^7G$, $m^1A$ and $m^3C$ are likely to exist as the positively charged species at physiological pH, which has also been experimentally verified for $m^7G$ and $m^1A$ in the context of an intact tRNA.[135] The group at the 6 position of purines (carbonyl in G and amino in A) or the 4 position of pyrimidines (as above) is indicated by R. **(C)** Methylation at exocyclic nitrogens of purines. Either mono- or di-methylation is observed for both purine nucleotides, as indicated, and the subsequent replacement of one or more protons on the exocyclic amino groups caused by addition of the methyl groups is indicated by parentheses. **(D)** Methylation at the 2′-oxygen of ribose. A 2′-O-methylated adenosine is shown (Am); each of the other 4 bases, as well as other modified nucleotides can also carry the 2′-O methylation.

methyltransferases found overwhelmingly in 2 of these: Class I and IV.[24] Class I, which also contains most DNA methyltransferases, is the largest class identified to date and is characterized by the presence of a Rossmann-fold domain that accommodates the SAM cofactor. Class IV methyltransferases, or so-called SPOUT (named for its members SpoU and TrmD) methytransferases, are characterized by 3 β strands folded into a deep trefoil knot.[25,26] Interestingly, the recent discovery of the $m^6A$ methyltransferase that catalyzes formation of $m^6t^6A_{37}$ in *E. coli* (TrmO) reveals that enzymes belonging to additional structural classes are possible, since this "Class VIII" enzyme comprises a novel protein fold.[27] Indeed, even among the relatively well-studied Class I and Class IV enzymes, some aspects of the overall structure and mechanism of distantly related family members is not necessarily obvious from sequence comparison alone.[26] For example, SPOUT methyltransferases generally assemble as homodimers in which each monomer contributes to the binding of SAM, but the recent identification of a monomeric SPOUT family enzyme (Trm10) suggests that alternative types of interactions with the methyl donor are possible.[28,29] A comprehensive survey of currently available tRNA methyltransferase structures and their corresponding properties has been reviewed in detail.[6]

In terms of overall catalytic features associated with SAM-dependent methyltransferases, some common themes have emerged. The chemistry of nearly all tRNA methylation reactions formally requires removal of at least one proton from the substrate nucleotide, which suggests that acid-base catalysis likely plays a role. The only exception to this theme is for methylation at any of several endocyclic nitrogen positions (including guanine N7, adenine N1 and cytosine N3), in which a nucleotide with an overall +1 charge results from addition of the methyl group (see **Fig. 1**). Indeed, for the relatively few tRNA methyltransferases for which detailed mechanistic information is known, protein residues that participate as acid-base catalysts have been identified, although the relative impact of these residues on the overall rates of reaction varies considerably. Detailed information about specific stereochemical mechanisms employed by diverse methyltransferases has been thoroughly reviewed elsewhere,[30] and here we focus on some general catalytic themes that have emerged.

Kinetic and mutational analyses identified important protein residues for several of the major classes of SAM-dependent tRNA methyltransferases, including amino acids that act as general base residues to facilitate proton removal. Not coincidentally, some of the best-characterized systems in terms of molecular mechanism are the 2 systems (bacterial TrmA and archaeal Trm5) for which co-crystal structures of the methyltransferase with tRNA or tRNA fragments have been obtained.[31,32] Nonetheless, proposed mechanisms have been indicated for many more enzymes by the numerous methyltransferase structures that are now available in complex with the critical cofactor SAM or SAH and by comparison to related enzymes.[6]

*Methylation at carbon ($m^5C$ and $m^5U$)*

Methylation at C5 of pyrimidines is a common modification found throughout life (**Fig. 1A**). $m^5U$ has been identified in tRNA and rRNA in all 3 domains, while $m^5C$ has been identified in mRNA, tRNA and rRNA in Archaea and Eukarya[2]. tRNA methyltransferases responsible for $m^5U$ or $m^5C$ formation are predominantly Class I methyltransferases utilizing SAM as the methyl donor, although an alternative methyltransferase (TrmFO) that utilizes methylene-tetrahydrofolate as the carbon donor has been observed in some bacteria. In this case, the

change in oxidation state required for the methylene group also necessitates participation of a flavin cofactor.[33-35] The crystal structure of *E. coli* TrmA, which catalyzes $m^5U_{54}$ formation, bound to a T-stem tRNA analog has been solved and this enzyme has been extensively studied, revealing the participation of 2 key residues in the methylation reaction (**Fig. 2**).[31] One of these, a conserved cysteine, acts as a nucleophile to covalently bind C6 via Michael addition, which promotes the attack of C5 on the SAM methyl group, and in turn allows for abstraction of the C5 proton by a glutamate general base.[31,36-38] Interestingly, 2 other $m^5U/m^5C$ tRNA methyltransferases, TrmFO (catalyzing $m^5U_{54}$) and Trm4 (catalyzing $m^5C$ at multiple positions), also utilize a conserved cysteine nucleophile, although other aspects of their mechanisms differ, as described above for TrmFO and in terms of the identity of the putative general base for Trm4, which is likely an aspartate (**Fig. 2**).[33,39,40]

*Methylation at endocyclic nitrogen ($m^1A$, $m^1G$, $m^3U$, $m^3C$, $m^1\Psi$ and $m^7G$)*

As with $m^5C/m^5U$, methylation occurring on endocyclic nitrogen atoms is abundant in all domains of life, with $m^3C$ and $m^1\Psi$ absent so far only from Archaea and Bacteria, respectively (**Fig. 1B**)[2]. General features of mechanisms employed by these enzymes have been revealed from comprehensive biochemical and structural studies carried out with 2 representative enzymes that catalyze the universal N1 methylation of $G_{37}$, which are a Class I methyltransferase (Trm5) in Eukarya and Archaea and a SPOUT methyltransferase (TrmD) in Bacteria. Archaeal Trm5 is the only example of a tRNA methyltransferase that has been co-crystallized in the presence of a complete tRNA and this, combined with extensive kinetic characterization and mutagenesis has resulted in a relatively good understanding of the overall catalytic mechanism.[32,41,42] As expected, a glutamate general base (proposed to remove the N1 proton prior to attack of N1 on the SAM methyl group) was recently identified that appears to function in both archaeal and human Trm5 (**Fig. 3A**).[41] However, acid-base catalysis in the Trm5 reaction is not rate-limiting for the overall reaction, and instead, the removal of the N1 proton appears to occur during a rate-limiting induced fit step, which is consistent with isotope effect experiments.[41] Class I methyltransferases also catalyze the ubiquitous N1 methylation of $A_{58}$, including the enzymes Trm6/Trm61 in Eukarya (Trm61 is the catalytic subunit), which is known as TrmI in Bacteria.[6] Structures are available for representatives of both families, albeit in the absence of tRNA but with a bound SAM or SAH cofactor, and models suggest that an aspartate residue is positioned to function as a general base (**Fig. 3B**).[43-46] For $m^1A$ formation, however, the proton appears to be removed from N6, thus promoting attack of the adjacent N1 on the SAM methyl group.

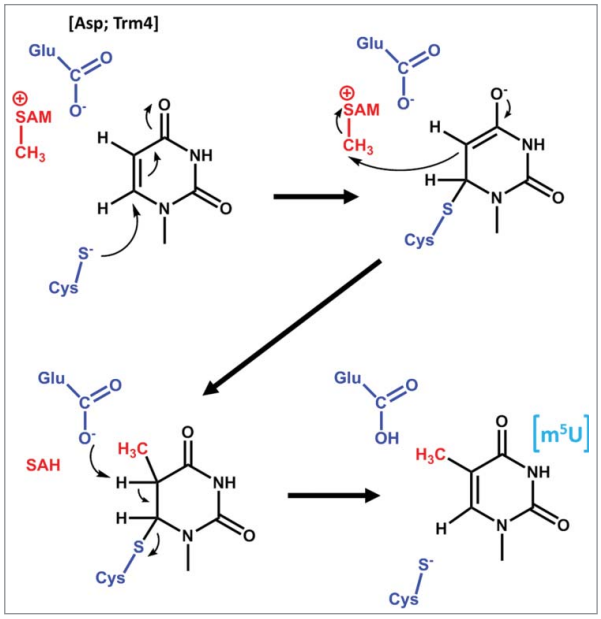


**Figure 2. Proposed mechanism for $m^5U$ methylation catalyzed by TrmA.** Two active site residues (shown in blue) are proposed to act as a nucleophile to attack C5 and create a covalent enzyme-nucleotide intermediate and a general base to remove the proton from C6 following methylation. The methyl donor (SAM) and resulting SAH are shown in red. Although other C5 methyltransferases maintain the cysteine nucleophile, the identity of the general base is not the same for other enzymes; i.e. for Trm4, an Asp is proposed to catalyze this function.

The precise nature of the enzymatic mechanism employed for other endocyclic nitrogen methyltransferases is less clear. Although there are crystal structures reported for the SPOUT methyltransferases TrmD ($m^1G_{37}$), Trm10 ($m^1G_9$) and TrmY ($m^1\Psi_{54}$), only some details of catalysis have been identified.[14-16,28,47,48] Crystal structures of Trm10 ($m^1G_9$) and TrmD ($m^1G_{37}$) suggest that for each enzyme, a conserved aspartate residue may act as the general base to abstract a proton from N1 and promote methylation,[14,28] but general base residue(s) that function in TrmY have not been identified. Likewise, for $m^7G$ formation, crystal structures of eukaryal Trm8/Trm82 (Trm8 is catalytic) and bacterial TrmB (both Class I enzymes) in complex with SAM have been determined.[49,50] From these structures, highly conserved glutamate and aspartate residues are suggested to participate in neutralizing the positive charge of the solfonium ion and other important residues involved in catalysis by TrmB have been described.[51] Nonetheless, the catalytic mechanism for these representative $m^7G$ methyltransferases has not been conclusively shown. A recent addition to the endocyclic nitrogen methyltransferase family was the identification of the first family members that catalyze methylation of pyrimidines to form $m^3C$ and $m^3U$. The $m^3C$ methyltransferase Trm140 is a distantly-related member of the SPOUT enzyme family, but the basis for catalysis awaits further structural and mechanistic characterization.[52,53] Interestingly, an orthologous enzyme from *T. brucei* has also been characterized and may exhibit both $m^3C$ and $m^3U$ methylation activities, raising the possibility of further complexity in the reactions characterized by this branch of the methyltransferase family (I. Fleming and J. Alfonzo, personal communication).

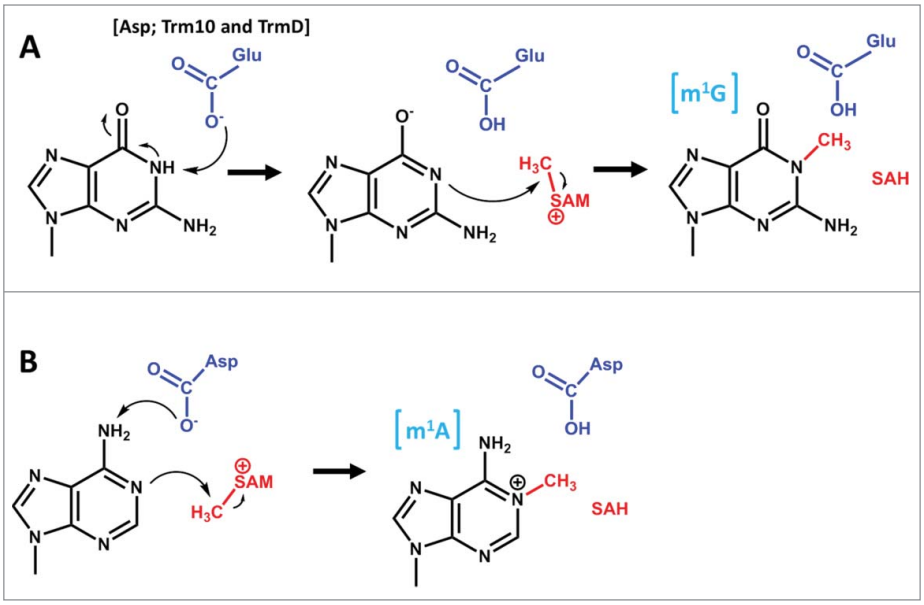


**Figure 3. Proposed mechanisms for $m^1G$ and $m^1A$ formation. (A)** Mechanism of $m^1G_{37}$ formation catalyzed by Trm5, with the active site glutamate that serves as the general base indicated in blue. For other $m^1G$ methyltransferases, the general base is proposed to be an aspartate, as indicated. **(B)** Mechanism of $m^1A_{58}$ formation catalyzed by TrmI. The suggested mechanism (based on modeled structures with the target adenosine) involves the action of the aspartate general base (blue) as shown, which may occur during the same elemental step as the attack of N1 on the SAM methyl donor (proposed based on the lower pKa for deprotonation of $m^1A$ than for deprotonation of adenosine).

*Methylation at exocyclic nitrogen ($m^6A$, $m^{6,6}A$, $m^2G$ and $m^{2,2}G$)*

The enzymes that methylate the exocyclic amines found at N6 of adenosine and N2 of guanosine have so far all been classified as members of the Class I family, and between these 2 types of modification, N2 methylation is by far more common in tRNA (**Fig. 1C**).[1,2] Although other sites of modification are known, N2 methylation of guanosines is typically found at position 10 (catalyzed by Trm11/Trm112 in eukaryotes where Trm11 is catalytic, and by TrmG10 in Archaea) and/or positions 26 and 27 (catalyzed by Trm1) and can consist of either monomethylation ($m^2G$) or dimethylation ($m^{2,2}G$), depending on the tRNA and organism that is modified.[54-59] Archaeal Trm1 from *P. horikoshii* and bacterial Trm1 from *A. aeolicus* have been characterized structurally and share similar N-terminal domains that contain the methyltransferase active site, including a characteristic DPFG/DPPY sequence motif that contains the aspartate that is proposed to act as a general base to remove a proton from the target N2 amino group, according to structural and mutational studies (**Fig. 4**).[60,61] Recently, the first tRNA methyltransferase that catalyzes exocyclic N6 methylation of adenosine was identified and, as predicted bioinformatically, was shown to correspond to *E.coli* YfiC, which catalyzes $m^6A_{37}$ modification of $tRNA^{Val}$.[62,63] This modification is more commonly found in mRNA, rRNA and DNA, and the details of the catalytic mechanism await further characterization.

*Methylation at ribose oxygen (Gm, Am, Um, Cm)*

One of the founding members of the SPOUT methyltransferase family is the highly conserved TrmH (originally SpoU) enzyme that catalyzes 2′-O methylation of $G_{18}$ (**Fig. 1D**).[25] This enzyme (known as Trm3 in eukaryotes) has been extensively characterized structurally and biochemically, and these data were the basis for the proposed mechanism in which a conserved arginine residue (attracted by the phosphate group of the tRNA substrate) acts as a general base to abstract the proton from the 2′-hydroxyl, thus activating the oxygen for attack on the SAM methyl group (**Fig. 5**).[64,65] There is strong mutational evidence in support of this proposed mechanism, although again, additional structures obtained in the presence of bound tRNA will be important for complete evaluation of the reaction mechanism.

## Lessons from tRNA methyltransferase homologs: alternative substrate recognition and catalytic features

Enzymes that catalyze known tRNA methylations have nearly all been identified, and sequence and structural similarity between family members have been extensively relied upon to suggest common mechanistic features and functions for related enzymes. In general, tRNA modification is thought to be a precise process, with a specific type of modification occurring predictably at certain sites on a given set of tRNAs. However, as diverse members of some tRNA methyltransferase enzyme families have been identified and characterized, several instances of unexpected catalytic properties have been described, yielding differences in either RNA substrate recognition and/or the chemistry of the methylation reaction itself. Several recent examples of alternative recognition and chemistry are highlighted below.

*Organism-specific features of RNA recognition*

Each tRNA species carries its own unique complement of tRNA modifications, including methylation, and therefore tRNA methyltransferases exhibit distinct tRNA substrate specificities.[1,2,66] Thus, the molecular basis by which individual tRNA methyltransferases recognize and act on specific tRNA species from among the total tRNA pool has long been an area of interest.[67] In many cases, the determinants that allow recognition of certain tRNAs are relatively straightforward to deduce, since the corresponding enzyme will modify essentially any species that encodes the correct target nucleotide at the position to be modified.[68,69] For example, $m^1G_{37}$ methylation occurs on all of the tRNA species that encode a G at position 37. Although the 2

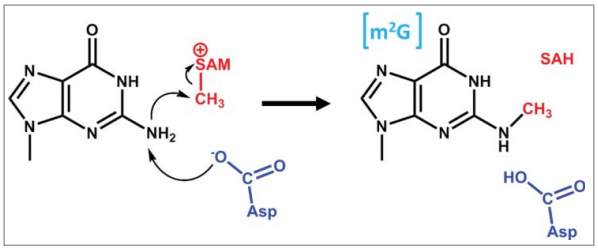


**Figure 4. Proposed mechanism for $m^2G$ formation by Trm1.** The active site aspartate (part of the DPFG motif), which is proposed to serve as a general base to abstract the proton from N2 is shown in blue. An active site tyrosine (not shown) is also proposed to stabilize the activated N2.

unrelated $m^1G_{37}$ methyltransferases (Trm5 and TrmD) each interact with tRNA substrates and recognize $G_{37}$ in slightly different ways, in both cases the enzymes appear to directly recognize the $G_{37}$ nucleotide in a specific sequence context for efficient catalysis.[11,12,70]

In contrast, there are cases of methylations that are highly tRNA species-specific, such as the $m^3C_{32}$ methylation that is a characteristic feature of $tRNA^{Thr}$, or $m^5C_{38}$ that was first associated with $tRNA^{Asp}$ in eukaryotes.[52,53,71,72] However, as additional homologs of the enzyme that catalyzes $m^5C_{38}$ modification (Dnmt2) have been characterized, considerable expansion or alteration of tRNA substrate specificity for some members of this enzyme family has been suggested. Initial reports centered on the identification of additional weakly recognized tRNA substrates for Dnmt2 and its (mainly eukaryotic) homologs, including $tRNA^{Glu}$, $tRNA^{Val}$ and $tRNA^{Gly}$, and suggested that the additional modification of minor tRNA substrates might be protective against various types of stress.[73-75] However, a more complex picture has emerged from the recent investigation of a bacterial homolog of Dnmt2 (*Geobacter sulfurreducens* Dnmt2) in which the inherent preference for *Geobacter* $tRNA^{Asp}$ has been usurped by a preference for methylation of $tRNA^{Glu}$, and indeed $tRNA^{Glu}$ appears to be the predominant $m^5C_{38}$-containing tRNA in *Geobacter* (**Fig. 6A**).[76] Precise elements that are responsible for this completely swapped tRNA substrate specificity are not known, but the nucleotides and size of the variable loop appear to play some role.[76] Moreover, even in eukaryotes where the preference for $tRNA^{Asp}$ has not yet been observed to be altered, the identification of non-substrate tRNAs bound to *Dictyostelium discoideum* Dnmt2 in vivo suggested that there may be additional roles for these enzymes that do not depend on methylation activity.[75] Therefore, even in the case of a well-characterized tRNA methyltransferase with demonstrated tRNA substrate specificity, diverse homologs can stray from these well-defined roles.

A third class of tRNA substrate specificity exists that falls somewhere in between the previous 2 classes of

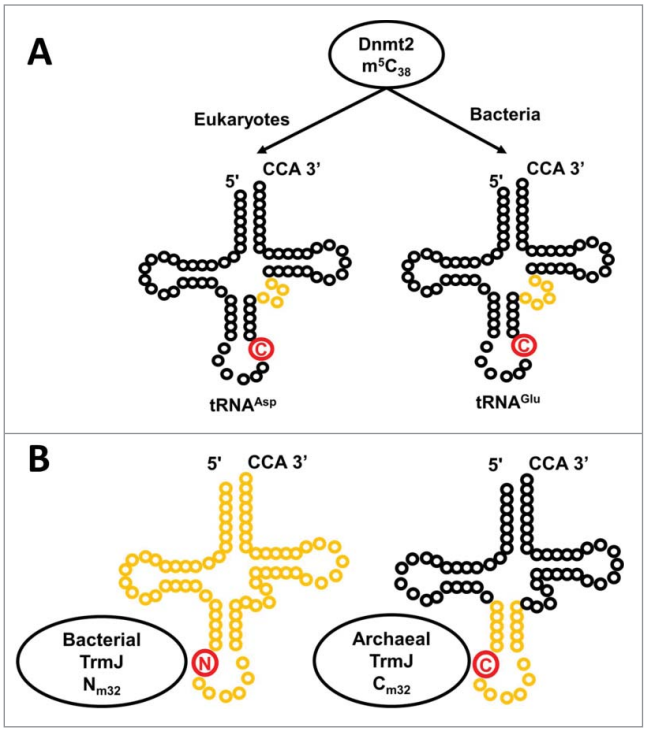


**Figure 6. Alternative substrate specificities exhibited by homologous methyltransferases. (A)** Dnmt2 catalyzes $m^5C_{38}$ methylation (the $C_{38}$ target nucleotide is indicated in red), with $tRNA^{Asp}$ thought to be the major substrate for eukaryotic Dnmt2, while $tRNA^{Glu}$ appears to be the predominant substrate for the *G. sulfurreducens* enzyme. Differences in the size and identity of nucleotides in the variable loop (highlighted in yellow) are thought to play a role in dictating alternative substrate specificity. **(B)** 2′-O methylation at position 32 is catalyzed by TrmJ. In Bacteria, TrmJ is capable of methylating any nucleotide at position 32 (indicated in red on each tRNA), whereas the archaeal enzyme is restricted to 2′-O methylation of cytosine. Moreover, the different structures required for tRNA recognition by the 2 enzymes (archaeal TrmJ requires only the anticodon stem-loop structure, while bacterial TrmJ requires a full-length tRNA) are indicated by yellow highlighting.

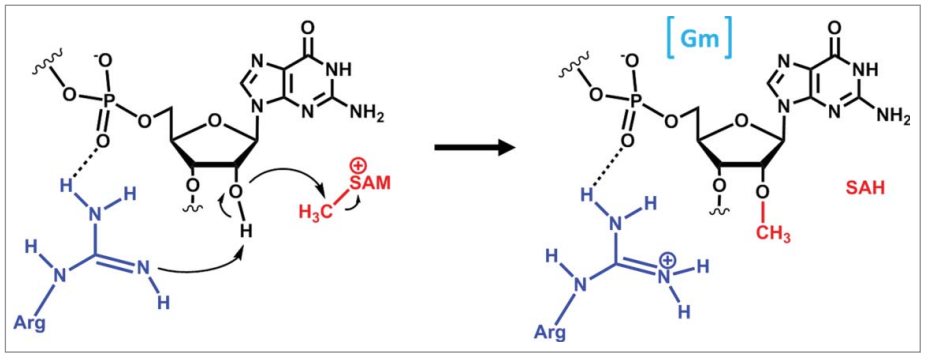


**Figure 5. Proposed mechanism for 2′-O methylation of $G_{18}$ by TrmH.** The active site arginine (shown in blue) is proposed both to stabilize the 5′-phosphodiester bond of the target nucleotide, as well as to deprotonate the 2′-OH to promote nucleophilic attack of the 2′-oxygen on the SAM methyl donor.

enzymes that are relatively non-tRNA specific (modifying any tRNA that contains a target nucleotide) or highly tRNA specific (modifying only one or 2 target tRNAs). These enzymes recognize only a subset of the possible tRNA targets that are expressed in a cell, and therefore must utilize other criteria for selection of substrates. Several examples of tRNA methyltransferases of this type have been described, including some of the enzymes that catalyze $Gm_{18}$ (Trm3 in *S. cerevisiae* and TrmH in *E. coli* and *Aquifex aeolicus*) and the enzyme that catalyzes $m^1G_9$ in *S. cerevisiae* (Trm10). The tRNA recognition problem faced by these enzymes is somewhat more complex, since it is often difficult to find specific sequence elements that are universally shared by all substrate or non-substrate tRNAs that might explain the observed patterns of substrate specificity.

For Trm3/TrmH, the sequence context of the $G_{18}$ target nucleotide, as well as the length and composition of the D-stem and loop were all found to play a role in tRNA recognition and the contributions of various important base pairs in specific context could be used to explain most of the observed specificity of *A. aeolicus* TrmH.[69,77,78] However, analysis of the pattern of tRNA substrates that contain $Gm_{18}$ in *E. coli* indicated that these rules are not necessarily universal, underscoring the need to investigate the properties of distinct family members to understand their substrate specificities. Kinetic and structural characterization of TrmH suggested that tRNA recognition occurs through an induced fit process that allows an initial non-specific interaction with tRNA followed by conformational changes that are only allowed with substrate tRNAs.[77,79] The same type of alternative substrate recognition among related SPOUT methyltransferases was also recently noted for bacterial and archaeal members of the TrmJ family (which each catalyze 2′-O methylation at position 32 of tRNA). In this case, each enzyme utilizes different elements of the tRNA for proper recognition, and even different specificities for the nucleotide target of modification, with archaeal TrmJ only catalyzing efficient Cm formation, while *E. coli* TrmJ methylates all 4 possible nucleosides (**Fig. 6B**).[80] Interestingly, structural comparison of archaeal vs. bacterial TrmJ revealed that the difference in nucleotide specificity may be related to different conformations of the methyl-cofactor in the active sites. However, many questions remain regarding the precise mechanisms by which substrate recognition occurs for either of these examples and further structural characterization of homologs with different substrate specificities will be required to completely address this question.

The tRNA $m^1G_9$ methyltransferase Trm10 also modifies only a subset of its potential substrates, since only 13 out of the 24 $G_9$-containing tRNAs in *S. cerevisiae* whose sequences have been determined are modified by Trm10 under normal growth conditions (**Fig. 7**).[81,82] The substrate specificity of Trm10 has been investigated in vitro and shown to depend somewhat on the presence of other modifications on the tRNA in order to enhance methylation of target substrates, but the proficient in vitro methylation activity of *S. cerevisiae* Trm10 with tRNAs that are not detectably modified to any level in vivo, combined with the ability of some non-substrate tRNAs to acquire methylation upon overexpression of Trm10, suggests that the recognition of Trm10 can also be more flexible than would be expected from the restricted subset of tRNAs that carry the $m^1G_9$ modification.[81] As with TrmH, the properties that determine substrate recognition in one organism may not necessarily be the same as those that dictate specificity in another; for example, $tRNA^{Gly}_{GCC}$ is a robust substrate for methylation by *S. cerevisiae* Trm10, both in vitro and in vivo, but in humans, $tRNA^{Gly}_{GCC}$ contains an unmethylated $G_9$.[1,2] Although distinct sequence determinants between the yeast and human tRNA that explain this difference in substrate specificity may yet be identified, the sequence conservation between eukaryotic tRNAs and the need for each enzyme to act on multiple substrates diminishes the possibilities for recognition based entirely upon sequence. Interestingly, also like the TrmH/Trm3 family where the *T. thermophilus* enzyme is non-tRNA specific,[69] some members of the Trm10 family appear to exhibit completely promiscuous tRNA methylation activity. Most well-studied is the tRNA methylation activity of one of the 3 human

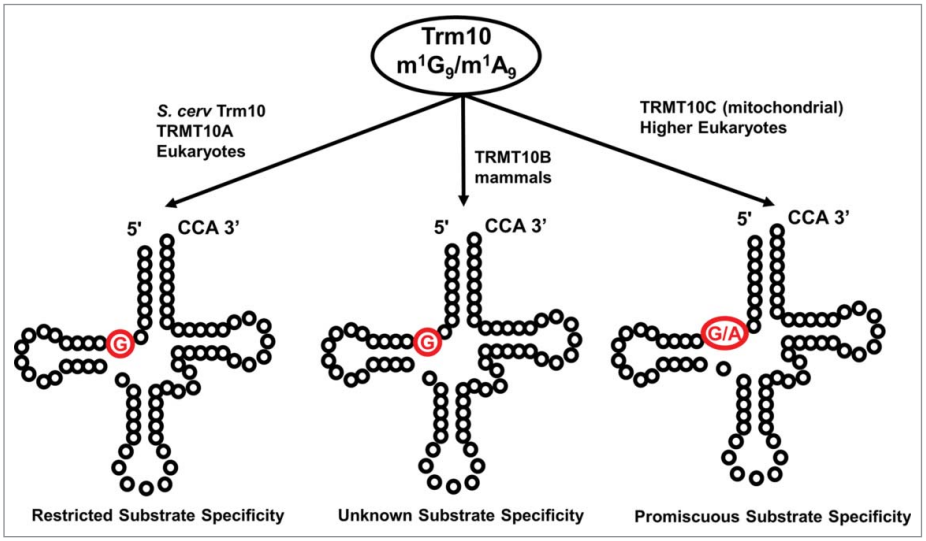


**Figure 7. The Trm10 family of methyltransferases exhibit differences in chemistry and substrate recognition.** Trm10 enzymes were originally identified based on their ability to catalyze $m^1G_9$ methylation (the $G_9$ target nucleotide is shown in red), which only occurs on a subset of tRNAs in *S. cerevisiae* (left panel). Subsequent investigation revealed that higher eukaryotes encode up to 3 Trm10 paralogs (TRMT10A, B and C, as indicated) and that the methylation activity and substrate specificities of these enzymes are distinct. TRMT10A (left panel) is most similar to *S. cerevisiae* Trm10 and exhibits similar biochemical properties. TRMT10B (middle panel), like TRMT10A, is a $G_9$ methyltransferase but substrate specificity has not been well characterized, while TRMT10C (right panel) is a strictly mitochondrial methyltransferase with much broader tRNA substrate specificity and the unusual ability to catalyze both $G_9$ and $A_9$ methylation.

paralogs of Trm10 (TRMT10C). This enzyme, which is part of the unique entirely proteinaceous ribonuclease P complex in mammalian mitochondria, exhibits a broad tRNA specificity that is much less restrictive than its cytosolic counterparts.[83,84] The recent crystal structure of fungal Trm10 suggests that the tRNA is recognized by a positively charged surface on the monomeric enzyme, but no co-crystal structure with tRNA is available.[28]

The question of RNA substrate specificity has recently been extended beyond tRNA itself by the discovery of methyltransferases with activities on other substrates apart from those originally associated with the enzyme family. For example, one of the earliest identified tRNA methyltransferases, TrmA, is a well-studied enzyme that catalyzes the highly conserved $m^5U_{54}$ modification in the T-loop; however, the *E. coli* enzyme was also recently implicated in an $m^5U$ modification that occurs in the tRNA-like domain of tmRNA.[85] Indeed, a large family of $m^5U$ methyltransferases has been identified in which an ancestral bacterial enzyme is thought to have duplicated into multiple groups of related enzymes with distinct substrate specificities, with the TrmA enzymes acting on tRNA (and tRNA-like molecules) and other homologs (RlmC and RlmD family members) acting on rRNA substrates.[86,87] But, even among these classes the lines can become blurred, since a recently described homolog of RlmD from *Pyrococcus abyssi* catalyzes a TrmA-like tRNA methylation, instead of acting on rRNA.[88,89] Structural characterization of several $m^5U_{54}$ enzymes from all 3 families suggests that there may be conserved conformational changes that cause refolding of the tRNA target, thus explaining the tRNA specificity of some of these enzymes.[31,88,90] Another case of a dual-specificity methyltransferase was recently reported with the identification of the $m^2A_{37}$ methyltransferase from *E. coli*. In this case, the RlmN enzyme previously associated with catalyzing the $m^2A$ modification in rRNA similarly catalyzes the same modification in tRNA, both in vitro and in vivo.[91] Finally, there are cases in which the practical substrate pool for a given methyltransferase is defined by its localization or expression patterns in the cell. For example, in *Trypanosoma brucei*, a paralog of the well-characterized Trm5 enzyme that catalyzes $m^1G_{37}$ formation localizes to the mitochondrion, which was surprising since *T. brucei* does not encode any tRNAs in its mitochondrial genome and would be presumed to import $m^1G_{37}$-containing tRNA generated by nuclear Trm5-catalyzed modification.[92] In this case, the mitochondrial Trm5 appears to function to protect translational fidelity in the mitochondria by methylating any unmethylated tRNA that is imported into the organelle by the apparently non-discriminating import machinery. In sum, the inherent RNA substrate flexibility associated with many tRNA methyltransferases suggests that additional substrates remain to be identified for these enzymes, and elucidation of these will be important for a complete understanding of the biological functions of these enzymes.

*Alternative chemistry associated with tRNA methyltransferases*

In the examples described above, although the tRNA target for a given methylation might differ, the chemical nature of the methylation reaction is not changed between closely related family members. However, again recent observations have challenged the universality of even this feature of tRNA methyltransferase activity. The Trm10 family is widely distributed among Eukarya and Archaea, reflecting the highly conserved nature of the $m^1G_9$ modification in tRNA.[82] However, in addition to the questions about RNA substrate specificity described above, recent characterization of several Trm10 homologs demonstrated that some family members are capable of catalyzing $m^1A_9$ formation, either in addition to or instead of the $m^1G_9$ modification activity.[84,93] Although the methylation in this case occurs on the same position (N1) of the purine ring, the difference in the expected protonation state of the target nitrogen for these 2 bases (protonated $G_9$ and deprotonated $A_9$) raises questions about the mechanism, and specifically about the role of acid-base catalysis, in an enzyme that is capable of catalyzing both of these reactions presumably using the same active site (**Fig. 3**). It is worth noting that residues characterized so far to act as a general base for other $m^1G$ vs. $m^1A$ methyltransferases (**Fig. 3**) are proposed to remove the proton from different atoms (N1 in the case of guanosine and N6 in the case of adenosine), and the possibility of alternative targets for the putative general base in Trm10 has not been evaluated. Alternatively, a mechanism involving the pre-dissociation of the N1 proton prior to the rate-determining step for chemistry, as has been observed with Trm5 could also explain this dual specificity.

In another example of unusual chemistry, also involving an $m^1G$ methyltransferase, an ortholog of the $m^1G_{37}$ enzyme Trm5 from *P. abyssi* is suggested to catalyze 2 distinct methylation reactions during the production of the hypermodified wyosine derivative mimG.[94,95] One of these is the familiar methylation at N1 to produce $m^1G_{37}$, but the second reaction involves a subsequent methylation at N7 of the 4-demethylwyosine intermediate (imG-14) to yield imG2 (**Fig. 8A**). Although the imG-14 methylation reaction involves an entirely different target atom (N7) on the nucleotide base from the well-characterized $m^1G$ chemistry, some similarities in terms of the chemical environment of these 2 nitrogens have been noted and future structural and functional characterization will be required to determine the precise mechanism of this dual functional enzyme.[94] Finally, the lack of evolutionary relatedness between Trm5 and TrmD $m^1G_{37}$ methyltransferases is underscored by the recent observation that *E. coli* TrmD, but not Trm5 (or any other known SAM-dependent methyltransferase for that matter) depends on the participation of a divalent metal ion, presumably $Mg^{2+}$, for catalytic activity. The precise role of this $Mg^{2+}$ ion remains to be fully determined, but appears to both stabilize the developing negative charge on O6 during methyltransfer and to assist in deprotonation of N1 by the aspartate general base (**Fig. 3**).[96]

Differences in the chemical nature of the reaction catalyzed by tRNA methyltransferases have been extended to the cofactor molecule itself. The SAM cofactor has seemingly been selected as the preferred methyl donor for most tRNA methyltransferases, likely due to the high reactivity of the methyl group bound to the positively-charged destabilized sulfonium ion, and only a small number of enzymes (bacterial members of the TrmFO family) utilize the alternative methylene-tetrahydrofolate (THF) cofactor for as a methyl donor.[33,35,97] However, the recent discovery of a

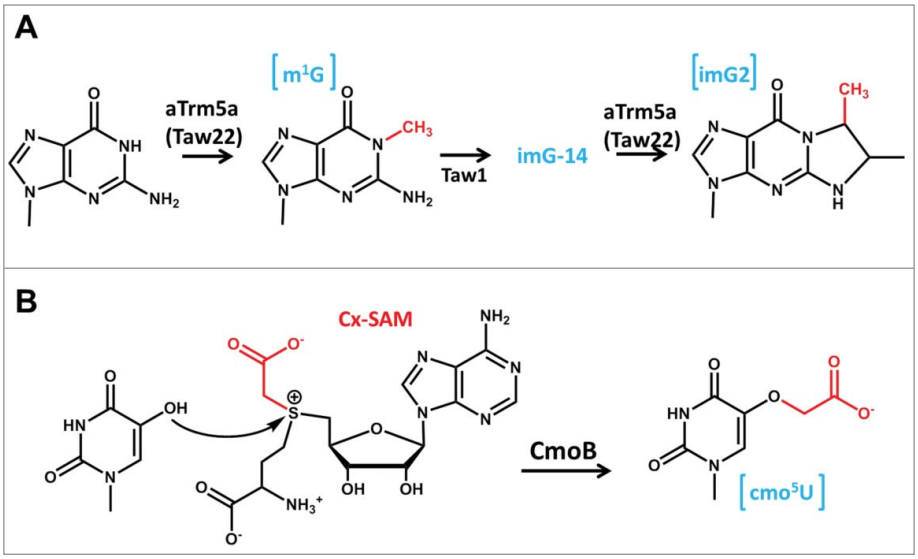


**Figure 8. Unusual chemistry associated with tRNA methyltransferase homologs. (A)** An archaeal homolog of Trm5 (aTrm5a, also known as Taw22) catalyzes 2 distinct methylation reactions during the formation of the wyosine derivative, mimG. The first reaction, formation of $m^1G$ (added methyl group is highlighted in red) is the same reaction catalyzed by other studied Trm5 enzymes, but $m^1G$ is subsequently converted to imG-14 (by the action of Taw1) and this modified nucleotide (not shown) is the substrate for the second methylation reaction catalyzed by aTrm5 to form imG2 (the second methyl group added by aTrm5a is shown in red). **(B)** An unusual SAM-derivative. The metabolite Cx-SAM, with the carboxymethyl group highlighted in red, is attacked by the hydroxyl oxygen at the 5 position of $ho^5U$ to generate $cmo^5U$.

new metabolite, carboxy-S-adenosyl methionine (Cx-SAM) has increased the diversity of cofactors that are associated with even SAM-dependent methyltransferases (**Fig. 8B**).[98] In *E. coli*, 2 members of the SAM methyltransferase superfamily, CmoA and CmoB, act together to synthesize the $cmo^5U_{34}$ modification, with CmoA catalyzing the biosynthesis of Cx-SAM and CmoB acting as the carboxymethyl transferase to generate the modified nucleotide (**Fig. 8B**). This expansion of chemistry raises the possibility of additional versatility associated with the SAM cofactor that remains to be explored.

*Methylation reactions influenced by partner proteins*

An interesting feature of some tRNA methyltransferases is their requirement for a partner protein for efficient catalysis. To date, partner proteins have been shown to interact with a catalytic subunit of several (so far, strictly eukaryotic) tRNA methyltransferases to organize the methyltransferase active site and/or participate in substrate recognition (**Table 1**). The *S. cerevisiae* $m^7G_{46}$ methyltransferase complex Trm8/Trm82 is one such example in which the catalytic subunit (Trm8) requires Trm82 for function.[50,99] Structural characterization, mutational analyses and domain deletions demonstrated that recognition of the substrate tRNA by Trm8/82 and its bacterial homolog, TrmB are similar in that both enzymes recognize their substrate base from the T-stem side.[49-51,100] Yet, while the bacterial and eukaryotic enzymes are similar in terms of tRNA recognition, they differ in that the eukaryotic enzyme requires Trm82 for catalysis, both in vitro and in vivo.[101] Trm82 binds to Trm8 and induces a conformational change resulting in formation of the optimal active site.[50]

The Trm6/Trm61 complex responsible for $m^1A_{58}$ formation in *S. cerevisiae* (also known as Gcd10/Gcd14) is similar to Trm8/Trm82 in that only one of the subunits (Trm61) contains a functional methyltransferase active site.[102,103] However, the role of the partner subunit in this complex is slightly different. Initial characterization revealed that Trm6 is primarily responsible for the functional interaction with tRNA, although more recent work demonstrated that residues from both subunits participate in tRNA binding.[103,104] This contrasts with Trm82, which does not have inherent tRNA affinity on its own. Interestingly, the participation of a separate partner protein is not necessarily conserved throughout evolution, since the TrmI enzyme (a homolog of Trm6/Trm61 that catalyzes $m^1A_{58}$ modification in Archaea and Bacteria) exists as a homotetramer, although TrmI similarly utilizes 2 subunits to interact with tRNA.[43-45] Furthermore, using the TrmI structure, attempts to abolish Trm6/Trm61 formation and enzyme activity through mutational analysis were unsuccessful, further underscoring differences between the Trm6/Trm61 and its bacterial/archaeal counterparts.[104]

Another protein partnership discovered to be involved in tRNA methylation was Trm11/Trm112, which catalyzes $m^2G_{10}$ formation in eukaryotes.[54] It was determined, as with Trm8 and Trm61, that Trm11 is the catalytic subunit and requires the Trm112 protein subunit to efficiently catalyze $m^2G_{10}$ formation in vivo, although for this enzyme, additional partner proteins may participate in the reaction, since efficient in vitro activity of the Trm11/Trm112 complex has not yet been detected.[54] Moreover, Trm112 is a promiscuous methyltransferase partner that is also required for the activities of Trm9 and Mtq2p, which catalyze the last step of $mcm^5U_{34}$ and methylation of eRF1, respectively.[54,105-107] The discovery of the non-tRNA related Mtq2p/Trm112 partnership is particularly striking and underscores the involvement of Trm112 in other biological activities such as cell division in *Arabidopsis*, methylation of 18S rRNA, ribosome biogenesis and translation.[108-110]

In contrast to Trm11/Trm112 where the partner protein interacts with multiple different methyltransferases, the Trm7 methyltransferase represents an alternative example where the catalytic subunit interacts with multiple different partner proteins. Trm7, a homolog of human FtsJ, is responsible for 2′O methylation of $C_{32}$ and $C_{34}$ in the anticodon loop of $tRNA^{Phe}$.[111,112] Loss of Trm7 results in a slow growth phenotype due to a nonfunctional $tRNA^{Phe}$.[111,113] What is

**Table 1.** Eukaryotic tRNA methyltransferases requiring a partner for efficient catalysis

| Methylation | Catalytic Component | Partner | Function of Partner |
|---|---|---|---|
| $m^7G_{46}$ | Trm8 | Trm82 | Trm82 required for $m^7G_{46}$ catalysis by Trm8 by inducing a conformational change in the catalytic component to an active conformation |
| $m^1A_{58}$ | Trm61 | Trm6 | Trm6 essential for functional interaction with the substrate tRNA although residues from Trm61 also contribute to tRNA binding |
| $m^2G_{10}$ | Trm11 | Trm112 | Trm112 required for $m^2G_{10}$ formation by Trm11. Trm112 also participates in methylation during $mcm^5U_{34}$ modification by Trm9, methylation of eRF1, methylation of 18S rRNA, ribosome biogenesis |
| $Cm_{32}$ | Trm7 | Trm732 | Trm732 directs Trm7 to the $C_{32}$ position of $tRNA^{Phe}$ |
| $Cm_{34}$ | Trm7 | Trm734 | Trm734 directs Trm7 to the $C_{34}$ position of $tRNA^{Phe}$ |

striking is that this methyltransferase is directed to each of these positions by either of 2 protein partners, Trm732 or Trm734, which are each responsible for interacting with Trm7 to target the indicated modification.[113]

The discovery of these diverse protein partnerships gives rise to the possibility that other unknown protein partners for tRNA methyltransferases may remain to be identified. For example, in the case of Trm10, organism-specific patterns of tRNA substrate specificity, as described above, have not been satisfactorily explained by the tRNA recognition properties of the purified enzyme in vitro or in vivo, and it remains possible that additional subunits are involved.[81] In the future it will be interesting to understand eukaryotic tRNA methyltranferase partners and how they differ from their homooligomeric bacterial counterparts, possibly leading to a new group of drug targets.

**tRNA methylation reactions in human health and disease**

The function of a single methyl group added to RNA has in many cases been difficult to discern. This is largely due to the frequent lack of obvious consequences for the cell upon loss of the modification. However, at least 4 possible molecular functions have been associated with tRNA methylation to date. First, methyl groups can play a structural role that prevents formation of alternative secondary or tertiary structures (usually by interfering with Watson-Crick base pairing). For example, the presence of $m^1A_9$ methylation in human mitochondrial $tRNA^{Lys}$, blocks base pairing between $A_9$ and $U_{64}$, and in turn destabilizes a nonfunctional base pairing between residues 8:65 and 10:63, thus promoting the canonical secondary structure of the tRNA.[23,114] Second, the presence of methyl groups (particularly 2′-O methylation) can affect the thermodynamic stability of tRNA. This is most well-substantiated by the case of tRNA species from thermophiles, which contain significantly more 2′-O methylation than their mesophilic or psychrophilic counterparts.[115] Third, methylation of tRNA (among other modifications) contributes to the overall stability of tRNA in vivo by protecting it from degradation by various pathways, including the nuclear TRAMP complex and/or rapid tRNA decay (RTD).[20,116] RTD was initially associated with the simultaneous loss of methylation at multiple sites on the tRNA, such as at positions 46 and 49 in *trm8*Δ/*trm4*Δ strains that leads to degradation of $tRNA^{Val}_{AAC}$, but loss of even a single methylation can cause susceptibility of the tRNA to RTD.[17-19] Moreover, the new discovery that RTD is a widespread tRNA surveillance mechanism that acts on a large number of tRNA species in *S. cerevisiae* opens the door to many other connections between RTD and tRNA methylation.[117] Finally, methylation (among other modifications) in the anticodon loop is shown to exert important effects on translation. An example of this is the previously described $m^1G_{37}$ modification, which prevents frameshifting, particularly at codons that start with a C, and the slow growth phenotype associated with deletion of *TRM5* in *S. cerevisiae* is likely due to decreased translational fidelity.[9,118] Despite remaining unanswered questions regarding the molecular function(s) of tRNA methylation, defects in tRNA methyltransferases have been increasingly associated with significant biological consequences, including a number of human diseases.

*Dynamics of methylation in stress and disease*

The modification status of tRNA is often considered to be a static feature of tRNA biology, with each tRNA species in the cell carrying a uniform complement of modified nucleotides. However, the modification state of tRNA can change in response to cell growth conditions and the mol/mol quantity of a particular methylation on a single tRNA isotype can vary. Interestingly, some methyltransferases may function in a network where changes in one enzyme allow for altered activity of another. For example, deletion of *TRM82* (**Table 1**) in *S. cerevisiae* results in a detectable increase in other tRNA methylations, suggesting that Trm82 could negatively affect other methyltransferases or modification enzymes.[119]

Rearrangements of tRNA methylation in response to stress have been associated with translational control. In *S. cerevisiae*, $tRNA^{Leu}_{CAA}$ contains $m^5C$ at positions 34 and 48, which are both introduced by the methyltransferase Trm4, and the *trm4*Δ strain is hypersensitive to growth in the presence of $H_2O_2$.[119] Analysis of the methylation status of $tRNA^{Leu}_{CAA}$ derived from wild-type cells grown in the presence of $H_2O_2$ revealed a substantial increase in levels of $m^5C_{34}$ (by 70%) and a smaller (20%) decrease in the amount of $m^5C_{48}$.[120] Since $tRNA^{Leu}_{CAA}$ is the only tRNA in *S. cerevisiae* that contains the $m^5C_{34}$ modification, it was proposed that this anticodon methylation could stimulate translation of UUG-enriched mRNA. Interestingly, a protein of the 60S ribosome, Rpl22A, contains an overrepresentation of UUG codons in its mRNA transcript and growth of an *rpl22a*Δ strain is also hypersensitive to growth in the presence of $H_2O_2$.[120] Together, these results suggest that Rpl22A expression

is advantageous for survival under oxidative stress conditions, and that Trm4 plays a regulatory role in the expression of this protein under these stress conditions, due to the increase in its production of $m^5C_{34}$ in $tRNA^{Leu}_{CAA}$.

Trm4 and its corresponding $m^5C$ methylation are also involved in another example of altered levels of tRNA modification in response to stress. In a separate investigation of the $G_{-1}$ nucleotidyl transferase Thg1, it was observed that $tRNA^{His}$, the substrate of Thg1 in *S. cerevisiae*, accumulates additional $m^5C$ methylations at positions 48 and 50 when Thg1 function (and hence the $G_{-1}$ residue that is required for efficient aminoacylation) is lost due to repressed expression of the gene.[121] However, other challenging growth conditions that are not directly related to histidine or $tRNA^{His}$, such as starvation for other amino acids or glucose, also lead to accumulation of additional $m^5C$ on the same tRNA.[122] The fact that the additional $m^5C$ modification only occurs on $tRNA^{His}$ and not on other tRNAs that also contain unmodified C nucleotides at the same positions is not understood and may have to do with localization or other particular chemical features of this tRNA. Although the biological function of $m^5C$ accumulation in response to stress in *S. cerevisiae* has not been deduced, it is posited that the additional $m^5C$ could confer a protective element to the tRNA, perhaps by decreasing its susceptibility to degradation or by preventing stress-associated cleavage events.[122] More work is required to fully understand the function of altered methylation levels in biological systems, but these data suggest that tRNA methylation is much more complex and dynamic than previously understood.

The previous examples all demonstrate phenotypic responses related to loss of tRNA methylations in unicellular eukaryotes, but tRNA modification also has important effects on human biology. A growing number of human disease syndromes are associated with genetic defects in tRNA methylation enzymes, and although the specific mechanism(s) by which mutations in human tRNA methyltransferases can cause these effects are not well-understood, it appears that loss of tRNA methylation can have devastating consequences for higher eukaryotes. Multiple associations between tRNA defects (including modifications) and human mitochondrial disease are well-established (see[123]), and here we focus instead on recent developments specifically associated with mutations in individual tRNA methyltransferases.

Two independent reports recently identified human familial syndromes involving microcephaly, intellectual disability and defects in glucose metabolism with mutations in TRMT10A, which is one of the 3 paralogs of *S. cerevisiae* Trm10 that are encoded by mammals, including humans.[82] Two separate mutations (either nonsense or missense) in TRMT10A were identified in multiple members of different large families and shown to correlate with the observed patient abnormalities.[124,125] Biochemical studies of the missense mutant of TRMT10A (with an G208R substitution in the putative SAM binding site) demonstrated a complete loss of function associated with the mutation, which suggests that loss of tRNA methylation could be the direct cause of the disease symptoms.[124] Notably, TRMT10A is ubiquitously expressed, but particularly high concentrations are observed in embryonic and adult brains as well as the pancreas.[125] However, knockdown of TRMT10A in β-cells did not impair insulin secretion or levels, but instead induced apoptosis, which is perhaps connected to the observation that tRNAs prevent formation of the apoptosome by inhibiting binding of cytochrome c to Apaf-1.[126,127]

In the same vein, multiple mutations in NSun2, a human homolog of the $m^5C$ tRNA methyltransferase Trm4, are correlated with intellectual disability and a Dubowitz-like syndrome characterized by distinct facial features and other physical abnormalities.[128-130] In the case of NSun2, tRNAs lacking $m^5C$ are cleaved endonucleolytically by angiogenin causing the resulting 5′ tRNA fragments to accumulate.[131] It is noteworthy that angiogenin cleaves tRNA at the variable loop, while NSun2 also methylates at multiple positions in the variable loop.[132,133] An exciting hypothesis that connects the loss of tRNA methylation to disease postulates that, when under stress, such as the oxidative stress that has been linked to neurodevelopmental disorders, accumulation of 5′ tRNA fragments would repress translation, and the concomitant lower protein levels could then explain decreased neuronal size.[131] In support of this, the inhibition of angiogenin via RNAi inhibits cleavage of non-methylated tRNAs and rescues brain size of NSun2-/- mice.[131]

Other genetic mutations in tRNA methyltransferases that cause human disease phenotypes may yet be described, since tRNA and various associated modification enzymes have been implicated in a number of other diseases, such as cancer and metabolic syndromes.[6,134] Further characterization of the molecular basis for the specific effects of loss of methylation on human biology will be important for a complete understanding of these diseases, and therefore for developing potential therapies. The recent resurgence in interest in tRNA biology and processing events and in the enzymes that catalyze these reactions will likely yield new discoveries that could improve human health.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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