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EDITED BY
Sunny Sharma,
The State University of New Jersey,
United States

REVIEWED BY
Hiroyuki Hori,
Ehime University, Japan
Ru-Juan Liu,
ShanghaiTech University, China

*CORRESPONDENCE
Katherine E. Bohnsack,
katherine.bohnsack@med.uni-goettingen.de

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N^2-methylguanosine and N^2, N^2-dimethylguanosine in cytosolic and mitochondrial tRNAs

Jonny Petrosyan and Katherine E. Bohnsack*

Department of Molecular Biology, University Medical Center Göttingen, Göttingen, Germany

Decoration of cellular RNAs with modified RNA nucleosides is an important layer of gene expression regulation. Throughout the transcriptome, RNA modifications influence the folding, stability and function of RNAs as well as their interactions with RNA-binding proteins. Although first detected more than 50 years ago, the modified nucleosides N^2-methylguanosine (m^2G) and N^2,N^2-dimethylguanosine ($m^2{}_2G$) have recently come to the fore through the identification and characterization of the human methyltransferases (MTases) responsible for their installation. In tRNAs, m^2G and $m^2{}_2G$ are present at the junctions between the acceptor stem and the D-arm, and the D-arm and the anticodon stem loop. Here, we review the current knowledge on the effects of mono- and di-methylation of N^2 of guanosine on base-pairing and provide an overview of $m^2{}_{(2)}G$ sites in cytosolic and mitochondrial tRNAs. We highlight key features of m^2G and $m^2{}_2G$ MTases, and describe how these enzymes specifically recognize their RNA substrates and target nucleosides. We also discuss the impact of these modifications on tRNA functions, their dynamic regulation and their implications in disease.

KEYWORDS

transfer RNA, RNA stability, translation, mitochondria, aminoacylation, RNA modification, epitranscriptome, methyltransferase

1 Introduction

Ribonucleic acids (RNAs) are multifunctional cellular molecules that serve as carriers of genetic information as well as components of ribonucleoprotein complexes and catalysts of diverse chemical reactions. To fulfill such varied functionalities, RNA molecules require greater chemical diversity than that provided by the four standard ribonucleosides (adenosine (A), cytidine (C), guanosine (G) and uridine (U)). This extended functional capability is achieved by the modification of RNA nucleosides (Shi et al., 2020). Modified nucleosides in RNAs can arise through the incoroporation of non-canonical nucleosides, such as queosine, the isomerization of canonical nucleoside, e.g. the conversion of uridine to pseudouridine (Ψ), or by the addition of chemical groups to pre-exisiting nucleosides, e.g. methylation, acetylations, etc (Ontiveros et al., 2019). Across the three domains of life, to date, around 170 different types of modified RNA nucleosides have been identified (Cappannini et al., 2023). These modified nucleosides influence the biogenesis, localization and functionality of various classes of RNAs (Shi et al., 2020).

Transfer RNAs (tRNA) are the most extensively and diversely modified class of RNAs, with approximately half of the known naturally occurring modified nucleosides being

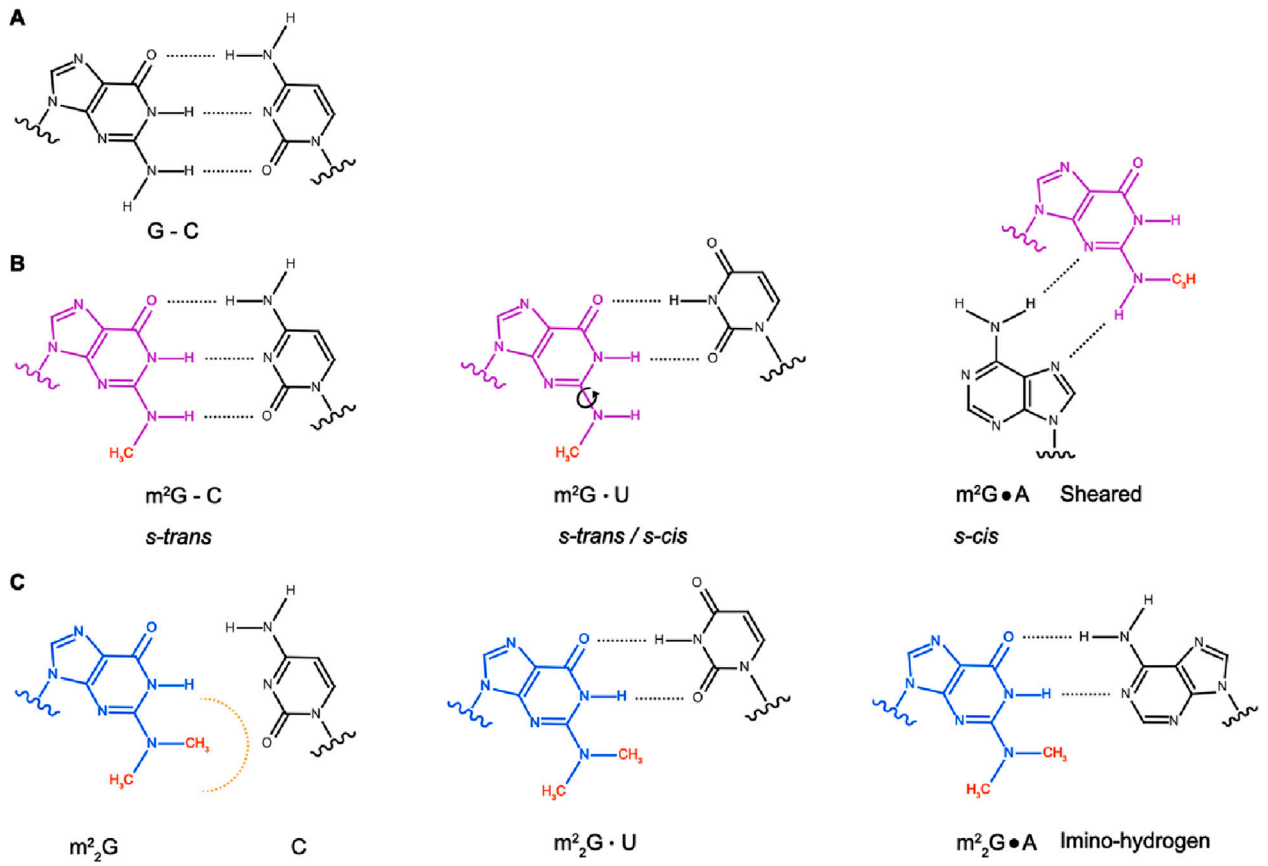

FIGURE 1
Base-pairing properties of G, m^2G and $m^2{}_2G$. **(A)** Canonical base-pairing between guanosine (G) and cytidine (C). **(B)** N^2-methylguanosine (m^2G) forms canonical base-pairing with C, with the methyl group in *s-trans* orientation. m^2G also forms non-canonical base-pairing with U, where the methyl group can adopt either *s-trans* or *s-cis* orientation, and sheared base-pairing with A, with the methyl group in *s-cis* orientation. **(C)** Conversion of G to N^2,N^2-dimethylguanosine ($m^2{}_2G$) hinders the formation of G-C canonical base-pairing but does not affect base-pairing with U. Compared to G, $m^2{}_2G$ alters the non-canonical base-pairing with A from the sheared to the imino-hydrogen form.

present within them (Cui et al., 2023; Phizicky and Hopper, 2023; Suzuki, 2021). Eukaryotic nuclear-encoded tRNAs contain, on average, 13 modified nucleosides (Pan, 2018). By contrast, five and eight RNA modifications are typically present in mitochondrial and bacterial tRNAs, respectively (Suzuki et al., 2020; Zhang et al., 2022a). This abundance of modified nucleosides in tRNAs reflects their important functions in fine-tuning tRNA structures and maintaining tRNA functionality during protein synthesis.

To fulfil their functions as adaptor molecules during translation, each tRNA is specifically charged with a cognate amino acid (Giegé and Eriani, 2023). The aminoacyl-tRNA then delivers the amino acid to the ribosome where, upon recognition of a messenger RNA (mRNA) codon complementary to the tRNA anticodon, the amino acid is added to the nascent polypeptide chain. In this context, modified nucleosides in the anticodon loop play important roles in ensuring the specificity and fidelity of anticodon–codon base-pairing and that the anticodon loop is in an optimal conformation for efficient decoding (Smith et al., 2024; Zhou et al., 2021). By contrast, modifications in other regions of tRNAs (the acceptor stem, the D- and T-arms and the connecting loops) typically contribute to tRNA folding and structure maintenance, thus regulating tRNA stability, aminoacylation and interactions with ribosomes (Yared et al., 2024; Zhang et al., 2022a). Several tRNA modifications are also implicated in regulating the cleavage of tRNAs into tRNA-derived fragments (tRFs), which fulfil cellular functions beyond those of canonical tRNAs (Kuhle et al., 2023; Muthukumar et al., 2024).

While Ψ is the most common modified nucleoside found in tRNAs (Fernandez-Vizarra et al., 2006), methylation is the most prominent type of tRNA modification (Höbartner et al., 2024). Nucleosides in tRNAs can carry methyl groups on the 2′-hydroxyls of ribose moieties or on any type of nucleobase. The most common methylated nucleoside in tRNAs is N^1-methyladenosine (m^1A), which is present at various positions of many tRNAs from different organisms (Oerum et al., 2017). 5-methyluridine (m^5U) in the T-loop is one of the most conserved tRNA modifications found across all three domains of life (Huang et al., 2021), whereas 3-methylcytidine (m^3C) is a eukaryotic-specific modification present at particular positions of a subset of tRNAs (Bohnsack et al., 2022). N^2-methylguanosine (m^2G) and N^2,N^2-dimethylguanosine ($m^2{}_2G$) are present in eukaryotic, bacterial, archaeal, mitochondrial, and chloroplast-derived tRNAs (Canaday et al., 1980; Hirata et al., 2016; Purushothaman et al., 2005; Stark et al., 2002; Suzuki et al., 2020),

indicating a universally conserved functional relevance of this type of modifications.

2 N^2-methylguanosine (m^2G) and N^2, N^2-dimethylguanosine (m^2_2G) in tRNAs

2.1 Topological and base-pairing properties of m^2G and m^2_2G nucleosides

Guanosine can be either mono- or di-methylated on the exocyclic nitrogen attached to carbon 2, forming m^2G or m^2_2G (Figure 1). Both types of methylation lead to non-standard conformations across the carbon 4–carbon 5 bond and affect nucleoside stacking interactions (Ginell and Parthasarathy, 1978). However, the presence of one versus two methyl groups differentially influences the base-pairing properties of the nucleoside. Mono-methylation of G to m^2G does not affect canonical Watson-Crick base-pairing (m^2G–C) and m^2G is also able to form non-canonical base pairings (m^2G·A and m^2G·U; Figures 1A, B; Rife et al., 1998). The methyl group of m^2G can adopt either s-*cis* or s-*trans* energetically stable rotamers. In canonical m^2G–C base pairing, the methyl moiety is in the s-*trans* orientation, whereas it is in the s-*cis* orientation when m^2G base-pairs with A. As a result of the free rotation around the carbon 2–nitrogen bond, both the *s-trans* and *s-cis* orientations are possible when m^2G is base-paired with U (Figure 1B; Rife et al., 1998). In contrast to the mono-methylation, di-methylation of nitrogen 2 of G to m^2_2G disturbs Watson-Crick base-pairing with C (Pallan et al., 2008). However, N^2 of G is not involved in the formation of non-canonical base pairing, so m^2_2G is still able to base-pair with A and U (Figures 1A, C; Steinberg and Cedergren, 1995). However, di-methylation changes the base-pairing mode of G with A from a sheared base-pair to a more stable imino-hydrogen bonded form (Pallan et al., 2008).

2.2 Detection methods for m^2G and m^2_2G

Quantitative detection methods with high sensitivity are essential for analyses of RNA modifications (Helm and Motorin, 2017). Several computational tools are available to predict m^2G sites in RNAs based on nucleoside chemical properties, sequence-derived features and position-specific scoring matrices (Ao et al., 2022; Chen et al., 2019; Ryvkin et al., 2013). Experimentally, similar to other modified nucleosides, m^2G and m^2_2G can be detected by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) (Wang et al., 2023; Zhang et al., 2022b). Total RNA, enriched RNA populations or specific target RNAs can be digested into nucleosides, separated by reverse-phase chromatography, then detected by MS. The retention time and mass-to-charge ratio acquired by MS determines the presence of m^2G and m^2_2G and allows its quantification in the analyte (Yoluç et al., 2021). Partial digestion of tRNAs followed by MS can identify not only the presence of these modified nucleosides but also their position within the tRNA sequence (Jones et al., 2023).

The inability of m^2_2G to form canonical base-pairing causes reverse transcriptase termination at the modification site and truncated cDNAs can be detected through the use of radioactively or fluorescently labelled primers when prior knowledge of the modification site is available (Funk et al., 2020). Alternatively, truncated cDNAs could be ligated to sequencing adaptors allowing mapping of m^2_2G modifications in a transcriptome-wide manner. By contrast, the ability of m^2G to base-pair as unmodified G makes its detection by approaches other than MS challenging. However, the high oxidizability of G has been exploited for the development of the m^2G detection approach PhOxi-Seq (Chung Kim Chung et al., 2022; Klimontova et al., 2024); oxidation of m^2G in the presence of a photoactivated catalyst leads to its conversion to 2,5-diamino-4H-imidazole-4-one, which base-pairs with G. This altered base-pairing induces clear mutational signatures during sequencing, enabling site-specific detection of m^2G either within specific RNAs or within the transcriptome. A similar mutational signature arises from the presence m^1G necessitating selective demethylation of m^1G prior to photo-oxidation for the unambiguous detection of m^2G. Importantly, m^2G is a precursor of m^2_2G (Edqvist et al., 1992), meaning that detected m^2G sites have the potential to represent intermediates during m^2_2G formation, and therefore, parallel detection of both modification types is desirable. Interestingly, antibodies against m^2_2G are commercially available, potentially offering an alternative approach for detection of the modified nucleoside in cellular RNAs.

2.3 Sites of m^2G and m^2_2G modifications in tRNAs

Bacterial and archaeal ribosomal RNAs (rRNAs) contain m^2G, m^2_2G has been detected in mRNAs from the yeast *Saccharomyces cerevisiae* (*Sc*) and the U6 small nuclear RNA (snRNA) of higher eukaryotes contains an m^2G, but m^2G and m^2_2G are predominantly found in tRNAs (De Crécy-Lagard et al., 2019; Jones et al., 2023; Noon et al., 1998; Sergiev et al., 2007; Wang et al., 2023; Yang et al., 2024).

m^2G is present at position six of cytoplasmic tRNAs from different species including the eubacterium *Thermus thermophilus (Tt)*, the thermophilic methanogenic archaeon *Methanocaldococcus jannaschii (Mj)*, *Bos taurus (Bt)* and humans (Figure 2; Purushothaman et al., 2005; Wang et al., 2023; Yang et al., 2021). Intriguingly, in specific tRNA isoacceptors from some species (for example, *Loligo bleekeri* and *Thermococcus kodakarensis* (*Tko*)), G6 is unmethylated, but m^2G is present on the opposite strand of the acceptor stem at position 67, which base-pairs with position 6 (Figure 2A; Hirata et al., 2019; Matsuo et al., 1995). Despite the high conservation of m^2G6 across mammalian tRNAs, bovine mitochondrial tRNA (mt-tRNA)^{Asp} contains m^2G6 (Suzuki and Suzuki, 2014) while this modification is lacking in the human counterpart (Suzuki et al., 2020). Furthermore, in human, bovine and chicken tRNA$^{Trp(CCA)}$, m^2G is present at position seven rather than position 6 (Yang et al., 2021).

Many archaeal and eukaryotic tRNAs also, or instead, contain N^2-methylated guanosines at position 10 (Figure 2; Bourgeois et al., 2017b; Hirata et al., 2019; Kuchino et al., 1982; Nishida et al., 2022;

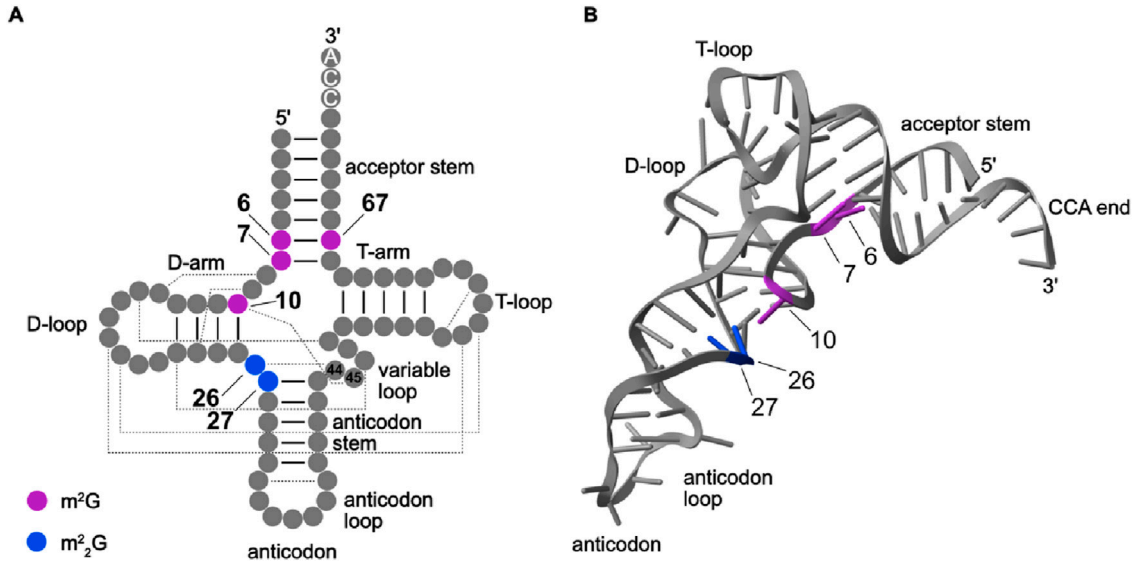

FIGURE 2
Secondary and tertiary structures indicating positions m^2G and m^2_2G in tRNAs. **(A)** The secondary structure of a canonical human tRNA with the positions of m^2G (purple) and m^2_2G (blue) modifications indicated. tRNA secondary structure and positions according to Sprinzl and Vassilenko, 2005. Base-pairings within stems are shown by black lines and base-pairings leading to tertiary structure formation are indicated by dashed lines. **(B)** The tertiary structure of $tRNA^{Asp}$ (PDB: 8OMR) is shown as a representative human tRNA. The nucleobases are shown as sticks, and m^2G and m^2_2G modifications present in human tRNAs are colored as in (A).

Purushothaman et al., 2005; RajBhandary et al., 1968; Suzuki et al., 2020; Wang et al., 2020). While structural studies have suggested the presence of the m^2G10 at bacterial tRNAs (Stark et al., 2002), it remains unclear if this modification is indeed present. Another example of species-specific guanosine methylation patterns in tRNAs is that while eukaryotic tRNAs typically contain m^2G10, in many archaeal tRNAs, G10 is di-methylated to form m^2_2G10 (Edqvist et al., 1995).

m^2G is also installed at position 26 of many tRNAs (Figure 2; Edqvist et al., 1992). In most cytosolic tRNAs, m^2G26 is a precursor to the m^2_2G modifications present at this position (Cappannini et al., 2023; Holley et al., 1965). Only in very few cases, such as $tRNA_i^{Met}$, is the m^2G26 modification (partially) retained (Kawamura et al., 2014; Walker, 1983; Xiong et al., 2023). By contrast, only one of the four human mt-tRNAs in which m^2G26 is installed is further methylated to form m^2_2G (Suzuki et al., 2020). The tandem occurrence of adjacent m^2_2Gs at positions 26 and 27 has been reported in tRNAs from the eubacterium *Aquifex aeolicus* ($tRNA^{Cys}$; Awai et al., 2009), and bovine and human cytosolic $tRNA^{Tyr}$ (Johnson et al., 1985; Edqvist et al., 1995; Hwang et al., 2024; Zhang et al., 2024a). Interestingly, in the archaeon *Sulfolobus acidocalclarius,* $tRNA^{Met}$ contains N^2, N^2, 2′-*O*-trimethylguanosine (m^2_2Gm26) where the ribose methylation is mediated by an RNA-guided 2′-*O*-methyltransferase (Kuchino et al., 1982).

Despite organism-specific variations in the extent of methylation (mono- versus di-methylation), guanosine N^2 modifications are present within specific tRNA regions. m^2Gs at positions 6/7 base-pair with nucleosides 67/66 forming the base of the acceptor stem, while m^2Gs at position 10 base-pair with nucleosides at position 25 terminating the D-arm as well as forming tertiary interactions with nucleosides at position 45 (Figure 2A). By contrast, position 26 of eukaryotic tRNAs is in the hinge region between the D-arm and the anticodon stem (Figure 2B). The presence of m^2G at positions 6, 7 and 67 within the acceptor stem is consistent with retention of the Watson-Crick base-pairing capacity of the mono-methylated form of G. The long-range tertiary interactions formed by $m^2_{(2)}G10$ and $m^2_{(2)}G26$ within tRNAs rationalize the presence of either N^2 mono- and di-methylated G at this position (see Section 2.5.1). Although m^2_2G prevents Watson-Crick base-pairing, in $tRNA^{Tyr}$, m^2_2G27 associates with U/A44 via non-canonical base-pairing, thus avoiding disruption of the anticodon stem (Johnson et al., 1985; van Tol et al., 1987).

2.4 m^2G and m^2_2G methyltransferases

2.4.1 Catalytic mechanism of m^2G and m^2_2G formation

The deposition of m^2G and m^2_2G modifications is catalyzed by Rossmann fold methyltransferases (MTases) that use *S*-adenosyl-L-methionine (SAM) as a methyl group donor. Similar to the methylation of amino groups on DNA and proteins, the methylation reactions catalyzed by m^2G and m^2_2G RNA MTases follow an S_N2-like substitution mechanism (Figure 3A; Abdelraheem et al., 2022; Awai et al., 2011; Hirata et al., 2016; Wang et al., 2020). The nitrogen atom of the exocyclic amino group of the guanine base initiates a nucleophilic attack onto the methyl group of SAM and when the nitrogen 2–carbon bond is formed, the carbon–sulfur bond within SAM is cleaved. The target base is thereby methylated and *S*-adenosyl-L-homocysteine (SAH), the derivate of SAM lacking the transferred methyl group, is released. This leaves the methylated nucleoside positively charged and a basic amino acid side chain within the MTase active site abstracts a proton from the former amino group so that the methylated nucleoside becomes uncharged.

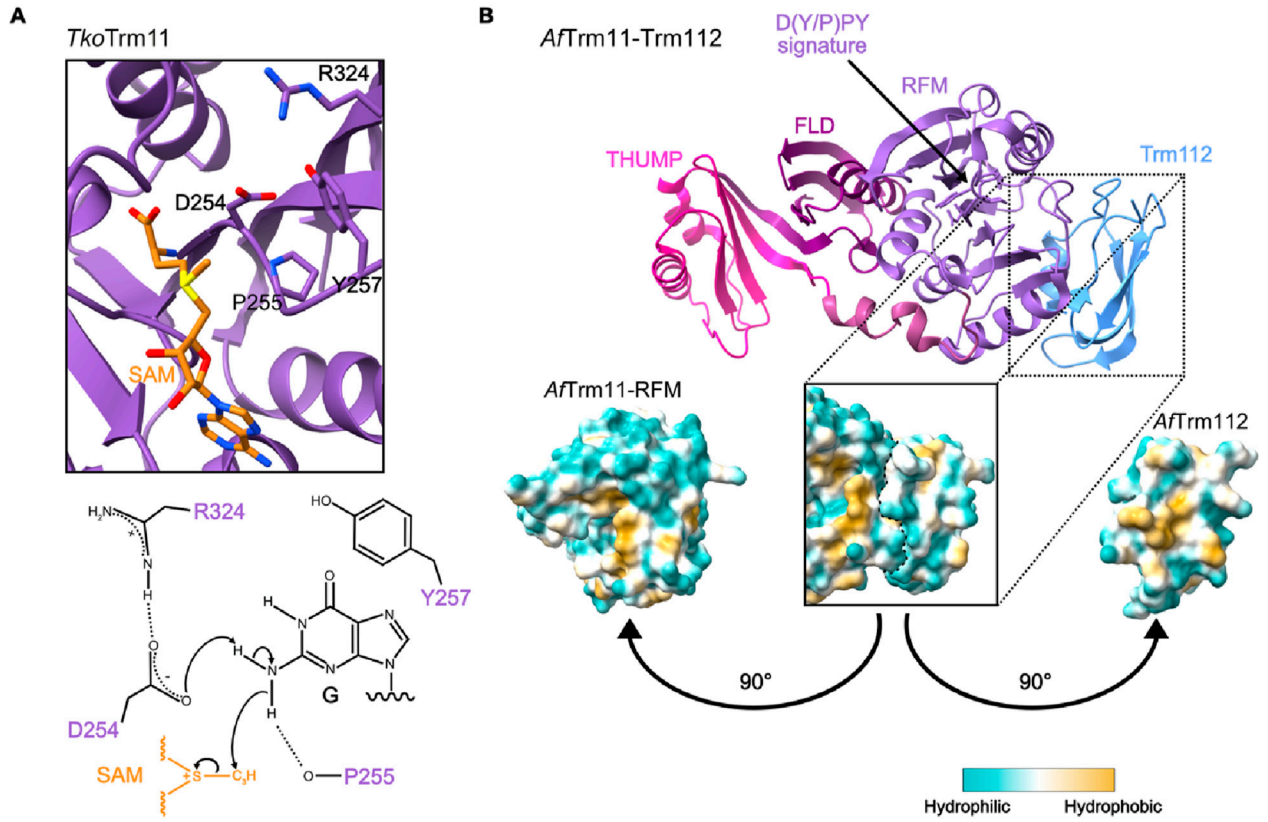

FIGURE 3
Catalytic mechanism and structure of m^{2}G10 MTase Trm11. **(A)** The catalytic center of the tRNA m$^2_{(2)}$G10 methyltransferase Trm11 from *Thermococcus kodakarensis* (*Tko*) (PDB: 5E72; top) and the proposed mechanism of the S_N2-like substitution reaction (bottom). R324 promotes the base-like activity of aspartic acid 254 (D254) to deprotonate the exocyclic amino group of guanosine (G), making it a nucleophile. This intermediate is stabilized by stacking interaction with tyrosine 257 (Y257) and able to attack the methyl group of *S*-adenosylmethionine (SAM). **(B)** Representative structure of a Trm11-Trm112 complex from *Archaeoglobus fulgidus* (*Af*; PDB: 6ZXW) with different domains of Trm11 and Trm112 highlighted (THUMP - THioUridine synthases, RNA Methylases and Pseudouridine synthases; FLD–ferredoxin-like domain; RFM–Rossmann-fold MTase. The D(Y/P)PY signature is indicated with an arrow. The lipophilicity potential map (bottom) shows the hydrophobic patches on the interacting surfaces of the Trm11 RFM domain and Trm112.

2.4.2 Guanosine N^2 MTases targeting the acceptor stem and D-arm

*Mj*Trm14 was the first m^{2}G6 MTase identified when it was discovered to target tRNA^{Cys} (Menezes et al., 2011). Subsequently, m^{2}G6 in *Tt*tRNA^{Phe} (the only bacterial tRNA that contains this modification) was shown to be introduced by *Tt*TrmN, a eubacterial ortholog of *Mj*Trm14 (Roovers et al., 2012). Despite the low sequence similarity between *Mj*Trm14 and *Tt*TrmN, crystallographic analyses revealed similar tertiary structures of these two MTases (Fislage et al., 2012). In human cells, the writer enzyme responsible for the m^{2}G6/7 modifications in cytosolic tRNAs is THUMPD3 (Yang et al., 2021). THUMPD3 has a domain architecture similar to *Mj*Trm14/*Tt*TrmN, suggesting evolutionary conservation of the characteristic features of these MTases. This fundamental domain architecture of m^{2}G6 MTases extends to Trm11/TRMT11, an enzyme conserved from archaea through eukaryotes that methylates N^2 of guanosines at position 10 of tRNAs. Trm11 from archaea such as *Pyrococcus abyssi* (*Pa*) and *Tko* not only catalyzes the installation of m^{2}G10 but can also further methylate it to m$^2_2$G10 (Armengaud et al., 2004; Hirata et al., 2016). However, *Sc*Trm11 and human TRMT11 exclusively install m^{2}G10 (Purushothaman et al., 2005; Wang et al., 2023).

The catalytic domains of these m^{2}G MTases are characterized by the sequence motif [D/N/S]-P-[P/I]-[F/Y/W/H] and this highly conserved signature specifies them as m^{2}G methyltransferases (Figure 3B; Bujnicki, 2000; Hirata et al., 2016; Wang et al., 2020). To initiate methyl group transfer from SAM to the substrate, the [D/N/S]-P residues interact with the target exocyclic amino group of the nucleobase via hydrogen bonds. From its planar position, the lone pair of electrons of the amino group is orientated towards the methyl group of the bound SAM to elicit the nucleophilic attack. The [F/Y/W/H] residue forms π–π stacking interactions with the nucleic acid that are essential to stabilize the reaction intermediate. As the m^{2}G MTases target base-paired purines, it is assumed that the base is flipped out for methylation.

Another striking feature of these m^{2}G MTases is that the catalytic domain is typically accompanied by an N-terminal ferredoxin-like domain (FLD) and an associated thiouridine synthases, RNA methylases and pseudouridine synthases (THUMP) domain (Figure 3B). The THUMP fold is an ancient RNA-binding domain that has been shown to assist diverse RNA

modification enzymes in binding their targets (Aravind and Koonin, 2001). In the context of different tRNA modification enzymes, the THUMP domains bind the 3' -CCA tails that are added to tRNAs post-transcriptionally and ultimately act as docking sites for conjugated amino acids (Fislage et al., 2012; Hirata et al., 2016; Hori, 2023; McCleverty et al., 2007; Neumann et al., 2014; Nishida et al., 2022; Randau et al., 2009; Waterman et al., 2006). Consistent with the importance of such interactions for substrate binding, the presence of a 3' -CCA end is strictly required for the association of THUMPD3 with substrate tRNAs (Yang et al., 2021). Likewise, structural analysis revealed that the THUMP domain of Trm11 from the *Archaeoglobus fulgidus* (*Af*) archaeon recognizes the 3' -CCA end of tRNAs (Bourgeois et al., 2017b). Lack of the 3' -CCA and/or destabilization of the acceptor stem significantly reduces substrate methylation by *Af*Trm11 and human THUMPD3 (Bourgeois et al., 2017b; Yang et al., 2021), indicating coordinated action of the THUMP and MTase domains. It is proposed that the FLD and following linker region adjust the distance between the THUMP fold and the catalytic domain, enabling capture of the tRNA's 3′-CCA end by the THUMPD fold to be coordinated with correct positioning of particular nucleosides in the enzyme's active site for modification (see, for example, Bourgeois et al., 2017b; Hirata et al., 2016; Neumann et al., 2014). Thus, the N-terminal non-catalytic regions of m^2G MTases fulfil dual functions in i) recognizing particular features of target RNAs to ensure recruitment to appropriate substrates and ii) acting as molecular rulers to direct methylation of the specific target nucleosides. This mode of structure-based target recognition contrasts with the sequence motif-based recognition of some modification enzymes (see, for example, Aoyama et al., 2020; Kleiber et al., 2022; Linder et al., 2015). However, it is ideally suited for MTases, such as Trm11/TRMT11 and THUMPD3, that methylate a broad spectrum of similarly folded RNAs; the diverse sequences contexts of m^2G6 and $m^2_{(2)}G10$ in tRNAs (Sajek et al., 2019) are only compatible with structure-based target recognition by the cognate MTases. Beyond the acceptor stem and 3' -CCA, tertiary interactions between the D- and T-arms are also important recognition elements for these m^2G MTases; nucleoside substitutions in the T- and variable loops that disrupt these connections perturb methylation of G10 by *Sc*Trm11 (Purushothaman et al., 2005). Furthermore, it has been shown that *Sc*Trm11-mediated methylation of G10 is sensitive to both the length of the variable loop and nucleoside substitutions at position 38 within the anticodon loop (Nishida et al., 2022), suggesting that diverse tRNA features influence substrate methylation by m^2G MTases.

2.4.3 Cofactor-mediated regulation of $m^2G6/7/10$ MTases

The prokaryotic m^2G6 MTases (*Tt*TrmN and *Mj*Trm14) and the m^2G10 MTases of some archaea (e.g., *Pa/Tko*Trm11) function independently to catalyze their target modifications (Menezes et al., 2011; Roovers et al., 2012). By contrast, the human m^2G6 and m^2G10 tRNA MTases (THUMPD3 and TRMT11, respectively), *Sc*Trm11 and the archaeal m^2G10 MTases (e.g., *Af*Trm11 and *Halloferax volcanii* (*Hv*) Trm11) associate with Trm112/TRMT112 to form MTase complexes (Figure 3B; Wang et al., 2023; Yang et al., 2021). Trm112/TRMT112 is a hub MTase cofactor protein composed of a zinc-binding domain and a four-stranded β-sheet (Heurgué-Hamard et al., 2006). Human TRMT112 interacts with at least seven MTases, increasing their stabilities and substrate affinities as well as modulating their catalytic activities (Bourgeois et al., 2017a; 2017b; Brūmele et al., 2021; Gao et al., 2020; Lacoux et al., 2020; Leetsi et al., 2019; Õunap et al., 2015; Van Tran et al., 2019; Wang et al., 2023; Yang et al., 2021). Interactions of THUMPD3, human TRMT11 and Trm11 from some archaea with Trm112/TRMT112 have been demonstrated by proximity labelling and co-immunoprecipitation experiments, and *in vitro* co-expression approaches have confirmed direct cofactor–MTase interactions (Brūmele et al., 2021; Van Tran et al., 2018; Wang et al., 2023; Yang et al., 2021) Structural analysis of *Af*Trm11 revealed that, similar to other Trm112–MTase complexes (Létoquart et al., 2014; Van Tran et al., 2019), Trm112 binds to the catalytic domain on the face opposite to the SAM binding pocket (Wang et al., 2020). While experimentally derived structures of the human TRMT11-TRMT112 and THUMPD3-TRMT112 complexes are lacking, modelling of the THUMPD3-TRMT112 complex strongly supports analogous interactions (Yang et al., 2021). Stabilization of the binding interface relies on assembly of a β-zipper formed by hydrogen bonds between specific β-strands in the MTase and cofactor. There can be several consequences of Trm112/TRMT112 binding, which include the shielding of hydrophobic patches on the surface of cognate MTases (Figure 3B; Hirata et al., 2016; Wang et al., 2020). For example, the anticipated TRMT112 binding site of THUMPD3 is strongly hydrophobic and recombinant THUMPD3 alone forms aggregates *in vitro*, while THUMPD3 co-purified with TRMT112 is soluble (Yang et al., 2021). *Af*Trm112 prevents the thermal denaturation of *Af*Trm11 *in vitro*, suggesting the importance of this cofactor in an archaeon with an optimal growth temperature of 83°C (Wang et al., 2020). Lack of TRMT112 has been shown to reduce TRMT11 and THUMPD3 levels in human cells (Brūmele et al., 2021), highlighting the role that cofactor association plays in maintaining the solubility and stability of non-bacterial m^2G MTases. Beyond regulating MTase stability, interaction with Trm112/TRMT112 can influence the catalytic activity of m^2G MTases. Although *Af*Trm11 is an active MTase alone, in the presence of Trm112, its catalytic activity is strongly enhanced (Wang et al., 2020). By contrast, *Sc*Trm11 and human TRMT11 only display methylation activity in the presence of *Sc*Trm112/TRMT112. Interestingly, the mechanistic basis of stimulation of Trm11/TRMT11 by Trm112/TRMT112 differs between species; while SAM binding by *Sc*Trm11 is enhanced by *Sc*Trm112, *Af*Trm112 does not influence the affinity of *Af*Trm11 for SAM but rather accelerates the catalytic step of the methylation reaction (Bourgeois et al., 2017b; Wang et al., 2020). Similar to eukaryotic Trm11/TRMT11, THUMPD3 alone lacks *in vitro* methylation activity as it fails to bind either substrate tRNAs or SAM, properties that are restored upon formation of the THUMPD3–TRMT112 complex (Brūmele et al., 2021; Wang et al., 2023; Yang et al., 2021).

2.4.4 MTases installing $m^2_2G26/27$ modifications

Distinct from the evolutionarily-conserved and architecturally-related m^2G MTases targeting the acceptor stem and D-arm, the

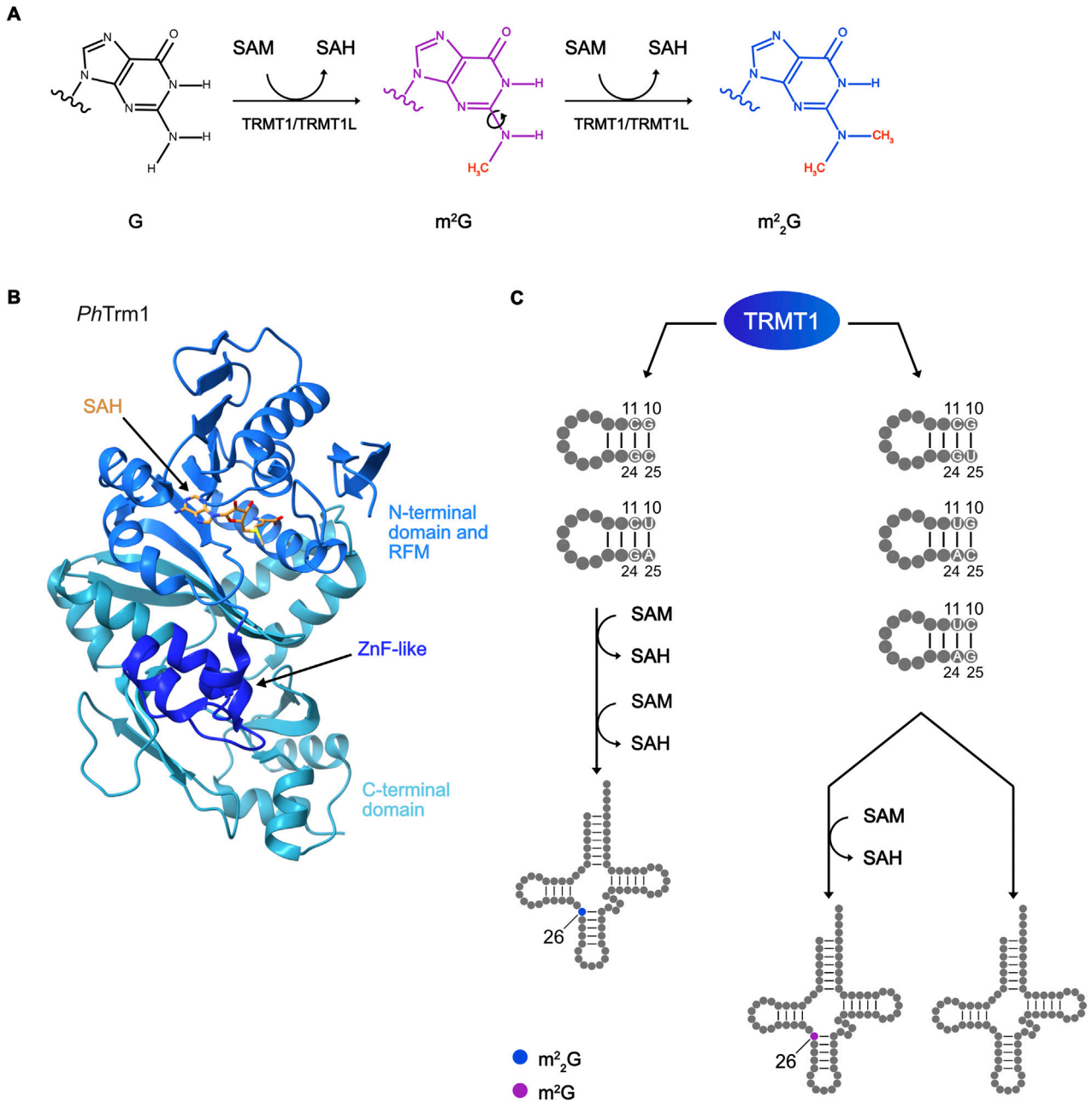

FIGURE 4
Methylation scheme of $m^2{}_2G$ installation, structure of an $m^2{}_2G$ MTase, and its substrate specificity. **(A)** Reaction scheme of $m^2{}_2G$ deposition, showing the two subsequent methylation reactions using *S*-adenosylmethionine (SAM) as a methyl group donor and releasing *S*-adenosylhomocysteine (SAH). **(B)** Representative structure of Trm1 $m^2{}_2G$ MTase from *Pyrococcus horikoshii* (*Ph*; PDB: 2YTZ) with N-terminal, Rossmann-fold MTase (RFM), and C-terminal domains highlighted. The zinc finger (ZNF)-like motif and bound SAH are indicated with arrows. **(C)** Diagram showing the mechanism of TRMT1 substrate specificity for m^2G or $m^2{}_2G$ deposition depending on the sequence of the target tRNA D-arm.

writer enzymes responsible for m^2G26, $m^2{}_2G26$ and $m^2{}_2G27$ modifications in tRNAs are Trm1/TRMT1 and TRMT1L (Figure 4A; Dewe et al., 2017; Ellis et al., 1986; Hwang et al., 2024; Zhang et al., 2024a; Constantinesco et al., 1999; 1998). On the structural level, these enzymes both contain Rossmann-fold MTase domains and zinc fingers (ZnF) of the CCCH type (Figure 4B; Awai et al., 2011; Bujnicki et al., 2002; D'Oliviera et al., 2022; Ihsanawati et al., 2008). Interestingly, despite TRMT1 and TRMT1L sharing the same two domains, they are arranged differently with the ZnF of TRMT1 C-terminal of the MTase domain and the MTase domain of TRMT1L being preceded by the ZnF. CCCH-type ZnF domains are associated with RNA binding (Fu and Blackshear, 2017), suggesting that this region of TRMT1 and TRMT1L may contribute to substrate interactions. Consistent with this, removal of the TRMT1 ZnF ablates substrate methylation (Dewe et al., 2017; Zhang et al., 2024a). It is interesting to note that the

Trm112/TRMT112 cofactor of the $m^2_{(2)}$G6/7/10 MTases, which do not themselves contain a ZnF, is also a ZnF-containing protein.

A distinguishing architectural feature of Trm1/TRMT1 and TRMT1L is the presence of a mitochondrial targeting sequence (MTS) at the N-terminal end of Trm1/TRMT1 that is lacking from TRMT1L. Cellular fractionation and immunofluorescence microscopy demonstrate the presence of Trm1/TRMT1 in the cytosol/mitochondria and nucleus, while TRMT1L is enriched in nuclei/nucleoli (Dewe et al., 2017; Hwang et al., 2024; Li et al., 1989). These sub-cellular localizations are in line with the dual functions of Trm1/TRMT1 in methylating G26 in cytosolic and mitochondrial tRNAs and the identification of the nuclear-encoded $tRNA^{Tyr}$ as the sole target of TRMT1L (Hwang et al., 2024; Xiong et al., 2023). Although TRMT1 is detected in the cytosol (Dewe et al., 2017), it is likely that m^2_2G26 modifications of cytosolic tRNAs take place prior to their nuclear export. This notion is supported by the finding that *Sc*Trm1 is enriched at the inner nuclear membrane, to which it is targeted by a specific sequence motif (Diaz-Muñoz et al., 2014; Lai et al., 2009). As the human m^2G MTases THUMPD3 and TRMT11 lack nuclear localization signals and are present exclusively in the cytosol (Brūmele et al., 2021), this suggests that m^2_2G26/27 tRNA modifications are installed prior to m^2G6/7/10 modifications. Lack of the MTS of TRMT1 obliterates methylation of mt-tRNAs while installation of m^2G26/m^2_2G26 modifications in cytosolic tRNAs is unaffected (Dewe et al., 2017). The expressed Trm1/TRMT1 protein contains an MTS and it is unclear how sufficient levels of cytosolic Trm1/TRMT1 are maintained to enable efficient tRNA methylation in this compartment.

While the methylation reactions catalyzed by Trm1/TRMT1 and TRMT1L follow the S_N2-like mechanism described for the m^2G MTases, details of how the sequential methylation reactions take place remain unclear. To accomplish di-methylation, following the first methylation reaction, release of SAH is required and the MTase must re-bind SAM to enable the second methylation reaction to occur (Figure 4A). Formation of m^2_2G is a thus two-step reaction, but it remains unknown if the MTases dissociates from the substrate after the first methylation step and re-binds, or whether SAH release and recharging with SAM take place on target-bound MTases. While *Sc*Trm1 catalyzes m^2_2G26 formation with high efficiency on some tRNA substrates, the mono-methylated m^2G intermediate is detected in in vitro methylation reactions taking place on mutated or non-endogenous tRNAs (Edqvist et al., 1994). This suggests that specific features of the tRNA substrates determine whether they are mono- or di-methylated. Strikingly, while cytosolic TRMT1 predominantly introduces m^2_2G26, within mitochondria, only mt-$tRNA^{Ile}$ contains m^2_2G26 and all other TRMT1 targets contain m^2G26. It has been shown that *Sc*Trm1 recognizes the G10:C25 and C11:G24 base-pairs in the D-stem along with nucleosides in the variable loop to specifically deposit m^2_2G26 modification (Edqvist et al., 1995; 1992). The human ortholog TRMT1 is able to catalyze m^2_2G26 formation in mt-$tRNA^{Ile}$, which contains a U10:A25 base pair and a deeper investigation of human TRMT1 recognition elements revealed that it requires C11:G24 and G10:C25 or U10:A25 base pairs for m^2_2G26 modification (Figure 4C; Xiong et al., 2023). tRNAs not satisfying these criteria (i.e., cytosolic $tRNA_i^{Met}$ and the mt-tRNAs with modified G26, except mt-$tRNA^{Ile}$) are only mono-methylated by TRMT1 to carry m^2G26. It is possible that tRNAs not satisfying the criteria for efficient m^2_2G26 installation have a lower affinity for Trm1/TRMT1 and dissociate from the enzyme after the first methylation step, but such mechanistic details remain to be clarified. Notably, both TRMT1 and archaeal Trm11 display dual activities as m^2G and m^2_2G MTases whereas eukaryotic TRMT11 and the G6/7 MTases *Mj*Trm14/*Tt*TrmN/THUMPD3 only install m^2G (Armengaud et al., 2004; Hirata et al., 2016; Menezes et al., 2011; Roovers et al., 2012; Wang et al., 2023; Xiong et al., 2023; Yang et al., 2021). The identification of tRNA elements influencing mono- versus di-methylation by TRMT1 (Xiong et al., 2023) raises the intriguing question whether features of the substrate tRNAs of *Mj*Trm14/*Tt*TrmN/THUMPD3 and eukaryotic TRMT11 dictate their action as m^2G, rather than m^2_2G, MTases or whether the nature of the methylation reaction catalyzed is inherent to the MTases themselves.

2.5 Functions of m^2G and m^2_2G tRNA modifications

2.5.1 Influence of $m^2_{(2)}$G modifications and MTases on tRNA folding and structure

Modified nucleosides can influence RNAs directly by modulating RNA folding and/or RNA–RNA interactions. Alternatively, recognition of specific modified RNA nucleosides by proteins termed "readers" can have functional consequences, such as destabilization or altered translation (Lewis et al., 2017; Roundtree et al., 2017). To date, no proteins specifically recognizing m^2G or m^2_2G have been identified, implying that these modifications predominantly exert direct effects on the RNAs that contain them. At the level of base-pairing, chemical and molecular dynamics studies show that m^2G:C and G:C base-pairs in RNA duplexes and GNRA tetraloops are isoenergetic, indicating that the m^2G modification does not directly contribute to RNA structural stability (Bavi et al., 2013; Rife et al., 1998). The presence of a methyl group may weakly stabilize RNA structures due to improved hydrophobic interactions, but this effect is canceled out by mild destabilization due to the loss of rotational entropy of the methyl group (Rife et al., 1998).

Modifications within the core of tRNAs are typically thought to have stabilizing effects, and although the precise molecular function(s) of the $m^2_{(2)}$G modifications remain to be clarified, these methylations likely contribute to tRNA structure by regulating the formation of tertiary interactions within the tRNA core. Interactions between the G10-C25 base-pair and nucleoside 45 in the variable loop as well as between m^2_2G26 and nucleoside 44 are important for maintaining the L-shaped tRNA architecture (Figure 5A; Shi and Moore, 2000). In human cells, when m^2G10 is lacking, the global structures of tRNAs determined by native gel electrophoresis are not noticeably affected (Wang et al., 2023), suggesting that the impact of this modification on tRNA architecture is subtle. This notion is supported by the observation that m^2G10 alone does not influence the folding of mt-$tRNA^{Lys}$, but that it augments the stabilizing effect of m^1A9 modifications (Kobitski et al., 2011).

When m^2_2G is present in eukaryotic tRNAs, it is primarily found linking the D-arm and anticodon stem (Edqvist et al., 1992) where it

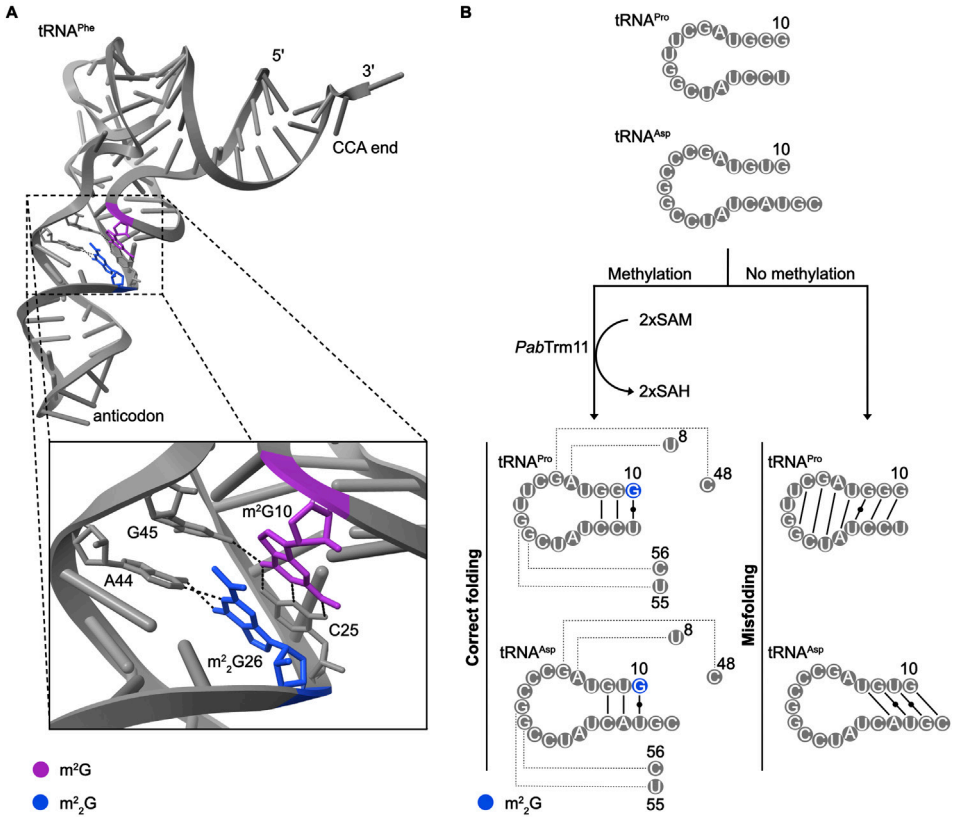

FIGURE 5
Influence of $m^2_{(2)}G$ modifications on tRNA folding and structure. **(A)** Interactions of the m^2G10-C25 base-pair with G45 and m^2_2G26 with A44 in *S. cerevisiae* $tRNA^{Phe}$ (PBD: 1EHZ). **(B)** Alternative base-pairing interactions within *Pyrococcus abyssi* (*Pab*) $tRNA^{Pro}$ and $tRNA^{Asp}$ depending on the presence of m^2_2G10.

has been suggested to act as a molecular hinge (Holbrook et al., 1978). As di-methylation of G strongly impacts its base-pairing properties, it is likely that the presence of m^2_2G26 limits the formation of alternative, sub-optimal tRNAs conformations that then represent an energetic hurdle requiring to be overcome to allow correct folding (Steinberg and Cedergren, 1995; Urbonavičius et al., 2006). m^2_2G26 is much more prominent within cytosolic tRNAs than mitochondrial tRNAs, which may reflect the ability of many mt-tRNAs to adopt non-canonical architectures. Consistent with the notion that m^2_2G26 promotes correct tRNA folding, the presence of m^2_2G generally shift the equilibrium of duplex-hairpin structures toward the hairpin form (Pallan et al., 2008). The importance of m^2_2G in ensuring proper tRNA folding is emphasized by the presence of this modification at position 10 in some hyperthermophilic archaea; in this context, the di-methylation of G10, rather than the mono-methylation typically observed in eukaryotes, may play an important role in ensuring the establishment of correctly folded tRNAs at high temperatures (Hirata et al., 2019). For example, in the archaeon *P. abyssi*, N^2 dimethylation of G10 has been proposed to impede the formation of aberrant base-pairings in $tRNA^{Pro}$ and $tRNA^{Asp}$, thus shifting the equilibrium towards correctly folded tRNAs (Figure 5B; Urbonavičius et al., 2006). Potentially linked to defective folding, lack of m^2_2G26 modifications is associated with decreased tRNA stability (Porat et al., 2023; Zhang et al., 2024a). Interestingly, the levels of acp^3U and dihydrouridine modifications in several tRNAs depends on the m^2_2G27 MTase TRMT1L (Hwang et al., 2024; Zhang et al., 2024b); within this modification circuit, it remains to be determined if m^2_2G27 is a pre-requisite for the other modifications because of changes in tRNA architecture that it induces or whether its presence is directly sensed by the corresponding modification enzymes.

Beyond the direct effects of m^2_2G26 modifications on tRNA structure, a non-catalytic role for the *Schizosaccharomyces pombe* (*Sp*) m^2_2G26 MTase Trm1 in chaperoning pre-tRNA folding has also been proposed (Porat et al., 2023; Vakiloroayaei et al., 2017). *In vitro*, both wild-type and catalytically inactive Trm1 display RNA strand annealing and dissociation activities that are characteristic of RNA chaperones (Porat et al., 2023). Supporting a functional relevance of this additional activity of *Sp*Trm1, the comined lack of *Sp*Trm1 and the well-established RNA chaperone La is synthetically lethal (Vakiloroayaei et al., 2017).

2.5.2 Effects of $m^2_{(2)}Gs$ in tRNAs on cellular processes

2.5.2.1 Impacts on cellular growth

Beyond the molecular level, cellular phenotypes associated with m^2_2G, and especially m^2G, tRNA modifications have proved challenging to identify. While the absence of modified nucleosides within the anticodon loop typically leads to demonstrable defects on translation, the effects of individual

RNA modifications in other regions of tRNAs are often more subtle. This likely reflects the fact that the dense network of modified nucleosides present in most tRNAs functions co-operatively to fine-tune tRNA structure and function. As has been observed for other types of modifications, such as ribosomal RNA 2′-*O*-methylations and pseudouridylations (King et al., 2003; Liang et al., 2009), the absence of an individual m^2G/m^2_2G modifications often does not induce an obvious phenotype, but the combined loss of two or more modifications can lead to significant phenotypic effects. For example, in *S. cerevisiae*, the simultaneous deletion of the *trm11* and *trm1* genes (i.e., preventing the formation of m^2G10 and m^2_2G26) increases doubling time and affects cell morphology (Purushothaman et al., 2005). Re-expression of either Trm11 or Trm1 leads to restoration of the wild-type phenotype, indicating that both m^2G10 and m^2_2G26 modifications are physiologically relevant. The mutual connection between these two modifications is rationalized by the tertiary structure of yeast $tRNA^{Phe}$, which reveals that m^2G10 and m^2_2G26 are stacked on each other with methyl groups pointed to the same face of the tRNA (Purushothaman et al., 2005). Thus, a single hydrophobic group on the surface of the tRNA provided by either of these methylation marks may be enough to ensure normal tRNA folding and function. Notably, TRMT1-deficient human cells exhibit a decreased proliferation rate (Dewe et al., 2017), perhaps suggesting that m^2_2G26 is more functionally relevant in human cells than in yeast. Similarly, while knockout of the $m^2G6/7$ writer THUMPD3 in HEK293T cells mildly reduces growth rate (Yang et al., 2021), knockout of THUMPD3 or the m^2G10 MTase TRMT11 in HCT116 cells does not significantly perturb cell growth (Wang et al., 2023). However, when both THUMPD3 and TRMT11, and their cognate $m^2G6/7$ and m^2G10 tRNA modifications, are lacking, cellular proliferation is significantly impaired (Wang et al., 2023).

2.5.2.2 Roles in translation regulation

Given the function of tRNAs as adaptor molecules during translation, defects in protein synthesis likely underpin the perturbed growth of cells lacking m^2G and/or m^2_2G in tRNAs. Generally, these modifications could modulate different aspects of translation by impacting tRNA charging or molecular interactions between tRNAs and ribosomes. Recent data demonstrate that lack of THUMPD3 and/or TRMT11 does not affect the aminoacylation status of m^2G6-, m^2G10-or m^2G6+10-containing tRNAs (Wang et al., 2023), indicating that in contrast to other tRNA modifications (Clifton et al., 2021; Gieg and Eriani, 2023), these methylation marks are not recognized by the tRNA aminoacyl synthetases. However, nascent protein synthesis is globally affected by lack of THUMPD3 in HEK293T cells (Yang et al., 2021), and the absence of both THUMPD3 and TRMT11 from HCT116 cells causes reduced monosome/polysome levels and impairs global translation in human HCT116 cells (Wang et al., 2023). As tRNA structure and charging are not substantially affected by lack of $m^2G6/7/10$, the molecular basis of these translation defects remains to be determined.

Absence of the m^2_2G26 tRNA modifications in the tRNA hinge region also affects translation. In *S. pombe*, the m^2_2G modification has been suggested to enhance the activity of a nonsense suppressor tRNA in decoding the UAA stop codon, thus contributing to the regulation of translation termination (Niederberger et al., 1999). The nascent synthesis of proteins is globally down-regulated in cells lacking TRMT1, suggesting a role for m^2_2G26 modifications in cytosolic tRNAs in maintaining efficient basal translation (Dewe et al., 2017). The effects of lack of TRMT1 on mitochondrial translation are, however, more nuanced. While the levels of CYB and ATP6 are unaffected by lack of m^2G/m^2_2G26 in mt-tRNAs, increased expression of the Complex I and IV subunits ND5/CO-I, ND4, ND2, ND1, and CO-2/CO-3 is observed upon TRMT1 knockout (Dewe et al., 2017). While the molecular basis of this phenotype is unclear, it is striking as sub-stoichiometric mt-tRNA modifications are typically associated with impaired, rather than enhanced, mitochondrial translation (Bohnsack and Sloan, 2018). Interestingly, due to sequence specificities, cytoplasmic $tRNA_i^{Met}$ and $tRNA_e^{Met}$ contain m^2G26 and m^2_2G26, respectively (Cappannini et al., 2023). It has, therefore, been suggested that the presence of the mono- and di-methylated forms fulfill distinct roles in translation initiation and translation elongation (Xiong et al., 2023). However, precisely how the differently methylated forms of G26 might optimize the alternative $tRNA^{Met}$ forms for these different functions is still unknown.

2.5.2.3 Implications in RNA processing

In mitochondria, the presence of m^2_2G26 in $mt\text{-}tRNA^{Ile}$ is not only necessary for efficient translation, but has also been shown to impact tRNA processing (Zhang et al., 2021). Transcription of the mitochondrial genome, composed of a light and heavy DNA strand, synthesizes polycistronic RNA precursors in which the mt-rRNAs and mt-mRNAs are typically separated by mt-tRNAs that are excised to release the individual RNAs (Jedynak-Slyvka et al., 2021; Ojala et al., 1981). m^2_2G26 in $mt\text{-}tRNA^{Ile}$ is detected within nascent, unprocessed mt-RNA, where it is implicated in regulating mt-tRNA processing (Zhang et al., 2021). The presence of m^2_2G26 affects folding of $mt\text{-}tRNA^{Ile}$, thus altering interactions between the heavy and light strand transcripts such that the ds mt-RNA is cleaved more efficiently when m^2_2G26 in $mt\text{-}tRNA^{Ile}$ is present than when it is lacking (Zhang et al., 2021).

m^2G and m^2_2G modifications are exclusively present in the 5′-half of eukaryotic tRNAs. When tRNAs are cleaved to produce tRFs, those arising from the 5′ half are typically the most stable and can have tRNA-independent functions in gene expression regulation (Tosar et al., 2018). The functional implications of m^2G and m^2_2G in tRNA-derived fragments are currently poorly understood, but a possible epigenetic function has been suggested (Chen et al., 2016). RNA-mediated transgenerational epigenetic inheritance may rationalize the observed connection between paternal diet and metabolic disorders in offspring (Ng et al., 2010). The high levels of m^2G in sperm tRFs of mice fed a high-fat diet therefore raise the possibility of a role for this modification related to epigenetic inheritance (Chen et al., 2016). Alongside potential roles in fine-tuning tRNA structure, such a function could increase the selective pressure to maintain m^2G within the 5′ half of tRNAs.

2.6 m^2G and m^2_2G stoichiometries in tRNAs and their dynamic regulation

The RNA modification landscape is dynamic as modifications can be differentially introduced in specific conditions and some

methylation marks can be actively removed by demethylases (Roundtree et al., 2017). Nucleosides in tRNAs are typically constitutively modified, and consistent with this, MS analyses suggest high stoichiometries of m^2G and $m^2{}_2G$ within tRNAs (Suzuki and Suzuki, 2014; Wang et al., 2023; Xiong et al., 2023; Yang et al., 2021). A potential exception is m^2G10 of *sc*tRNA$^{Val(ACC)}$, which has recently been shown to be sub-stoichiometrically methylated (Nishida et al., 2022). Interestingly, an association between the activity of RNA polymerase III, which synthesizes nuclear-encoded tRNAs, and the efficiency of $m^2{}_2G26$ modification has been suggested in *S. cerevisiae* and human cells (Arimbasseri et al., 2015). When tRNA synthesis is reduced, $m^2{}_2G$ is more abundant, and when the RNA polymerase III repressor Maf1 is lacking and tRNAs levels are elevated, the incorporation of $m^2{}_2G26$ is decreased. A model is proposed in which Trm1/TRMT1 becomes limiting when tRNAs are overexpressed, leading to sub-stoichiometric $m^2{}_2G26$.

As well as differential installation depending on tRNA abundance, $m^2{}_2G26$ can be actively erased. The mitochondria-localized α-ketogluterate- and Fe^{2+}-dependent AlkB family member, ALKBH7, has been shown to demethylate $m^2{}_2G26$ in mt-tRNA^{Ile} (Zhang et al., 2021). *In vitro*, ALKBH7 efficiently demethylates $m^2{}_2G$ to yield G, without substantial accumulation of the m^2G intermediate. As described above, the presence of $m^2{}_2G26$ in mt-tRNA^{Ile} influences processing of the polycistronic mt-RNA and demethylation of this position by ALKBH7 slows processing of the transcript, perhaps helping ensure accurate cleavage (Zhang et al., 2021). An atomic resolution structure of ALKBH7 revealed a solvent-exposed active site and negatively charged surface that was initially anticipated to repel RNAs due to their negatively charged backbone (Wang et al., 2014). However, ALKBH7 contains nucleic acid-binding loops, also present in other AlkB family members that target RNAs, and this region likely mediates interactions with the substrate RNA (Zhang et al., 2021). The ability of ALKBH7 to efficiently demethylate $m^2{}_2G26$ and the identification of key amino acids required for this activity draws parallels with the development of mutant forms of *Escherichia coli* AlkB that can be leveraged to demethylated $m^2{}_2G$ sites *in vitro*. As $m^2{}_2G$ blocks reverse transcriptase progression, its removal is a pre-requisite for tRNA sequencing approaches that rely on reverse transcription read-through, such as hydro-tRNAseq and ARM-seq (Padhiar et al., 2024). Wild-type AlkB efficiently demethylates m^1A and m^3C (Zheng et al., 2014) and an aspartic acid 135 to serine (D135S) substitution allows demethylation of N^1-methylgaunosine to G. The removal of $m^2{}_2G$ is particularly challenging due to the deeply buried position of $m^2{}_2G26$ into the tRNA structure, however, D135S and leucine 118 to valine (L118V) substitutions that mimic key residues of ALKBH7 enable selective and efficient conversion of $m^2{}_2G$ to m^2G (Dai et al., 2017). This activity differs from that of ALKBH7, which fully demethylates $m^2{}_2G$ to G, (Zhang et al., 2021). However, as m^2G maintains its canonical base-pairing ability, the removal of one methyl group from $m^2{}_2G$ is sufficient to allow reverse transcriptase progression and subsequent tRNA sequencing.

Alterations in m^2G and $m^2{}_2G$ stoichiometries have been observed in *S. cerevisiae* tRNAs upon exposure to cellular stress (Chan et al., 2010), suggesting that regulation of these modifications could contribute to the dynamic adaptation of gene expression. More specifically, exposure to hydrogen peroxide leads to an increase in $m^2{}_2G$, while the levels of m^2G are decreased in a dose-dependent manner. The assessment of changes in the levels of these modified nucleosides in a non-site-specific manner limits the mechanistic understanding of the observed dynamics. These alterations in m^2G and $m^2{}_2G$ levels in tRNAs may arise due to differential expression of the MTases responsible for installing these modifications in oxidizing conditions, but it is also possible that the catalytic activity of these enzymes is affected by the stress condition. To date, no $m^2{}_{(2)}G$ demethylase has been identified in yeast, but in case stress-induced changes in the $m^2{}_{(2)}G$ landscape also occur in human cells, it is possible that ALKBH7 is dynamically regulated to modulate the erasing of specific methylation marks. Post-translational modifications are an efficient way to modulate protein functions, and high-throughput phosphoproteomics identify numerous sites within functionally important regions of the m^2G and $m^2{}_2G$ MTases (Hornbeck et al., 2015). This raises the possibility that stress-induced post-translational modification dynamics may contribute to the regulation of m^2G and $m^2{}_2G$ modifications in tRNAs. Lack of TRMT1 or TRMT1L leads to hypersensitivity to oxidative stress, suggesting an important role for $m^2{}_2G26/27$ in cell survival in this condition (Dewe et al., 2017; Hwang et al., 2024). Elevated levels of reactive oxygen species (ROS) are observed in the absence of TRMT1 (Dewe et al., 2017), but it remains unclear how these enzymes and/or their cognate modifications contribute to maintaining redox homeostasis in cells.

Increasing evidence suggests co-ordination of the metabolic statuses of cells with their epitranscriptomes (Liu et al., 2024). Such connections likely arise as a result of the use of various metabolic intermediates as modification enzyme cofactors. For example, α-ketogluterate is essential for oxidative demethylation reactions taking place on RNAs (Fedeles et al., 2015), such as the demethylation of $m^2{}_2G26$ in mt-tRNA^{Ile} by ALKBH7, and the methyl group donor in most cellular methylation reactions, including that generate m^2G and $m^2{}_2G$, is SAM (Sun et al., 2021). SAM is synthesized from the sulfur-containing amino acid methionine, and it has been speculated that in the archaeon *M. jannaschii,* Trm14-dependent methylation of m^2G6 of tRNA^{Cys}, which is charged with another sulfur-containing amino acids may play a role in the regulation of protein synthesis depending on sulfur availability (Menezes et al., 2011).

2.7 Disease implications of m^2G and $m^2{}_2G$ tRNA modifications

Given the importance of the epitranscriptome for gene expression regulation and the critical roles of modifications in tRNAs for their functionality in protein synthesis, unsurprisingly, dysfunction or abnormal expression of the >50 tRNA modifying enzymes and their cofactor proteins are associated with human diseases (Chujo and Tomizawa, 2021).

Alternations in the expression levels of the m^2G MTase TRMT11, its TRMT112 cofactor and the $m^2{}_2G$ demethylase ALKBH7 are observed in various cancers. For example, overexpression of TRMT11 in high-grade gliomas is associated with increased cellular proliferation, invasion, migration and glial tumor growth (Di et al., 2013). Over-expression of TRMT112 is

observed in most cancer types (Xu et al., 2022), highlighting its potential as a prognostic predictor. In ovarian serous carcinoma, *ALKBH7* expression correlates positively with the pathological stage of the disease (Cai et al., 2021) and higher expression of *ALKBH7* is observed in lung adenocarcinoma where it is associated with shorter overall survival time in patients (Wu et al., 2021). A single-nucleotide polymorphism in *ALKBH7* that leads to an arginine 191 to glutamine (R191Q) substitution is correlated with prostate cancer, and perturbed co-substrate binding by this variant suggests that defective m^2_2G demethylation underlies this association (Walker et al., 2017). These and other studies indicate the potential of ALKBH7 demethylase as a target for anticancer therapy (Chen et al., 2022).

Aberrant tRNA modifications are generally linked to metabolic diseases and neurological disorders, while defects in mt-tRNA modifications often leads to mitochondrial encephalomyopathies (Bednářová et al., 2017; Bohnsack and Sloan, 2018; Boughanem et al., 2023; Liu et al., 2024). The high metabolic demands and complex development of the brain rationalize the profound association of defective protein synthesis with these conditions. For example, consistent with its mitochondrial localization, ALKBH7 is involved in programmed cell necrosis and fatty acid metabolism (Fu et al., 2013; Solberg et al., 2013), and mice lacking *ALKBH7* display an obesity phenotype (Solberg et al., 2013). Moreover, the m^2_2G26 and m^2G26 modifications deposited by TRMT1 in cytoplasmic and mitochondrial tRNAs are associated with microcephaly, intellectual disabilities, and epilepsy (Blaesius et al., 2018; Davarniya et al., 2015; Najmabadi et al., 2011). The autosomal recessive mutations of TRMT1 associated with these diseases often affect the enzyme's catalytic activity, cause expression of truncated proteins that cannot associate with target RNAs or lead to nonsense-mediated mRNA decay (Blaesius et al., 2018; Zhang et al., 2020). TRMT1 variants associated with intellectual disabilities, which lack m^2_2G deposition activity, cannot rescue normal cellular translation or oxidative stress sensitivity in TRMT1-deficient human cells (Dewe et al., 2017). However, as TRMT1 targets both cytoplasmic and mitochondrial tRNAs, further investigation is needed to clarify how aberrantly modified tRNAs in these two translation systems contribute to the pathogenesis. Interestingly, it was recently shown that upon neuronal activation, TRMT1 relocalizes from mitochondrial into distinct nuclear foci, suggesting that sequesteration may contribute to the dynamic regulation of m^2_2G26 modifications in the context of neuronal function (Jonkhout et al., 2021).

Interestingly, infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) reduces the levels of m^2_2G modifications and it has recently emerged that TRMT1 is a target of the SARS-CoV-2 protease Nsp5 (Lu and Zhou, 2023; Zhang et al., 2024b). Nsp5 cleaves TRMT1 within the ^{530}QANFT^{534} sequence that matches the consensus motif of SARS-CoV-2 polyprotein cleavage sites (D'Oliviera et al., 2022). Nsp5-mediated processing leads to TRMT1 cleavage fragments that contain either the catalytic MTase domain or the ZNF that is required for RNA substrate interaction, and uncoupling of these two features renders TRMT1 functionally inactive. Lack of TRMT1 reduces SARS-CoV-2 RNA replication and particle infectivity, leading to the hypothesis that the virus might self-limit its proliferation by altering the host translation machinery through TRMT1 degradation and reduced levels of m^2_2G-modified tRNAs (Zhang et al., 2024b). A role for m^2G7 in $tRNA^{Trp}$ in regulating the replication of the Avian myeloblastosis virus (AMV) has also been proposed (Keith and Heyman, 1990). During budding, retroviruses package host tRNAs, predominantly $tRNA^{Trp}$, to prime viral reverse RNA-DNA synthesis (Waters and Mullin, 1977). Unmethylated $tRNA^{Trp}$ can fulfil this function but $tRNA^{Trp}$ containing m^2G7 is not packaged into viral particles (Keith and Heyman, 1990). This suggests that the methylation mark may act as a selectivity determinant for packaging, potentially through altering interactions with retroviral proteins.

3 Discussion

While m^2G and m^2_2G modifications have long been recognized as key features of many cytosolic and mitochondrial tRNAs, recent years have seen rapid advances in the understanding of how these methylation marks are installed and how they contribute to tRNA function. The identification of MTases responsible for installing m^2G and m^2_2G tRNA modifications has revealed, on the one hand, many common features shared between these enzymes and, on the other hand, shown how each protein is specifically adapted to appropriately methylate particular tRNA nucleosides. A growing body of evidence suggests dynamic regulation of m^2G and m^2_2G in (mt-)tRNAs, linking these methylations to the adaptive response to cellular stress. The development of novel detection methods for m^2G would expedite quantitative analyses of the stoichiometries of this type of modification in different cell types and tissues, various growth and stress conditions, and during development in the future. Deeper understanding of the molecular basis of pathogenic conditions associated with defects in m^2G and m^2_2G will be driven by molecular and biochemical studies that dissect the precise roles of these modifications in regulating tRNA function.

Author contributions

JP: Visualization, Writing–original draft, Writing–review and editing. KB: Conceptualization, Funding acquisition, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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