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UPDATE

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RNA nucleotide methylation: 2021 update

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Abstract

Among RNA modifications, transfer of methylgroups from the typical cofactor *S*-adenosyl-L-methionine by methyltransferases (MTases) to RNA is by far the most common reaction. Since our last review about a decade ago, the field has witnessed the re-emergence of mRNA methylation as an important mechanism in gene regulation. Attention has then spread to many other RNA species; all being included into the newly coined concept of the "epitranscriptome." The focus moved from prokaryotes and single cell eukaryotes as model organisms to higher eukaryotes, in particular to mammals. The perception of the field has dramatically changed over the past decade. A previous lack of phenotypes in knockouts in single cell organisms has been replaced by the apparition of MTases in numerous disease models and clinical investigations. Major driving forces of the field include methylation mapping techniques, as well as the characterization of the various MTases, termed "writers." The latter term has spilled over from DNA modification in the neighboring epigenetics field, along with the designations "readers," applied to mediators of biological effects upon specific binding to a methylated RNA. Furthermore "eraser" enzymes effect the newly discovered oxidative removal of methylgroups. A sense of reversibility and dynamics has replaced the older perception of RNA modification as a concrete-cast, irreversible part of RNA maturation. A related concept concerns incompletely methylated residues, which, through permutation of each site, lead to inhomogeneous populations of numerous modivariants. This review recapitulates the major developments of the past decade outlined above, and attempts a prediction of upcoming trends.

This article is categorized under:
RNA Processing > RNA Editing and Modification

KEYWORDS
epitranscriptomics, methylation, RNA modification

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1 | INTRODUCTION

RNA modification in general, and RNA methylation in particular, have become a topic of intense research and publication activities during the past 10 years. This development has, in our opinion, been ignited by two seminal papers describing the distribution of N^6-methyladenosine (m^{6}A) residues in mammalian mRNA (Dominissini et al., 2012; K. D. Meyer et al., 2012), and has resulted in the reinvention of the RNA modification field as "epitranscriptomics" (K. D. Meyer & Jaffrey, 2014). While the modification field remains largely dominated by mRNA methylation, it has become clear that its biology cannot be disconnected from the remainder of modified RNA species (Ontiveros et al., 2020).

Preceding this development, our previous comprehensive review (Motorin & Helm, 2011) contained a minor section on mRNA, correspondingly larger sections on occurrence and function of RNA methylation in tRNA and rRNA, and outlooks on the topics of tRNA fragmentation and mitochondrial RNA methylation. Gratifyingly, the latter topics have grown into fields of their own right by now. While that review did indeed reflect the major developments in the preceding decade in a near-comprehensive fashion, we realize that we will be unable to repeat such an accomplishment without pointing to a number of reviews that cover important developments in the field. These include for example, modification detection techniques (Helm & Motorin, 2017; Limbach & Paulines, 2017; Linder & Jaffrey, 2019; Motorin & Marchand, 2021; Yoluc et al., 2021), complex modification machineries and their structure (Bourgeois et al., 2017; Lence et al., 2019), MTase target recognition, enzymatic mechanisms, impact of RNA methylation in numerous fields of biology and human pathology (Zaccara et al., 2019) including cancer research (Nombela et al., 2021), as well as immunology (Dalpke & Helm, 2012; Freund et al., 2019) and virology (Ruggieri et al., 2021). Structure and content of this update reflect our personal opinions on what the most important developments in the field were, and what we consider promising new angles.

A basic aspect here is the steadily climbing number of known RNA modification structures, within which methylations continue to be highly represented. Here we consider all modifications into which at least one C1 carbon unit has been incorporated either from the tetrahydrofolate cofactor, or from the more typical *S*-adenosyl-L-methionine (SAM). Most such incorporations occur directly on the nucleobase, or feature one or more linker atoms between nucleobase and C1 body. Examples for such structures are shown in (Figure 1a), and Figure 1b depicts methylations on the ribose. A particular situation is present for example, in wybutosine derivatives because the first step of its biosynthesis is a G37 methylation to m^{1}G, although after the subsequent multiple enzymatic conversions, that carbon is barely recognizable as a C1 unit any more (Figure 1c). Of note, the other (non-C1) side chains of the sulfur atom in SAM are also metabolized, such as the 3-amino-3-carboxypropyl chain in acp-type modification of uridines (B. Meyer et al., 2020) or elsewhere in wybutosine, but these are not topic of this review. An impression of the multitude of methylated positions in the various nucleotides can be obtained from Figure 2.

Also, one of the major aspects of epitranscriptome research was, and continues to be, the discovery and characterization of modification enzymes and their targets. An overview of the phylogenetic distribution in abundant RNA targets, that is, tRNA and rRNA is given in Figure 3.

While sites in other targets, especially in those of low abundance, continue to be the subject of controversial discussion, advance in the identification of the so called epitranscriptomic "writers" is steady and reproducible with few exceptions (X. Zhang et al., 2012). Consequently, we report on numerous newly identified modification enzymes acting on tRNA and rRNA. This development focusses on higher eukaryotes and has enormously benefitted from early characterization of homologs in single cell model organisms (Bjork et al., 1987; Jackman et al., 2007; Maden & Hughes, 1997; Suzuki et al., 2007). We direct attention to a number of new protein complexes that were identified in eukaryotes. Three classes of particularly sophisticated MTase complex architectures are discussed in Box 1.

The variable composition of the Trm112-centered complexes supports the relatively new notion, that tRNA and rRNA methylation may constitute a physiological response to a stimulus, rather than being an obligate maturation step that inevitably leads to full stoichiometric methylation of a given site. This notion of dynamics is a recent development in the field of RNA methylation, and it comes in different flavors. In addition to dynamic reassembly of "writer" complexes, another flavor includes modification removal by "erasers" (Ontiveros et al., 2020). Some noteworthy facts on oxidative demethylation by "erasers" are given in Box 2, and more details on mRNA demethylation are laid out in the corresponding section on coding RNA.

Of note, although oxidative removal of methylgroups by erasers is an attractive concept in gene regulation (Zaccara et al., 2019), dynamic changes of methylation stoichiometry could plausibly originate from a combination of degradation and renewed synthesis, a factor that remains remarkably under-investigated. Occurrences and

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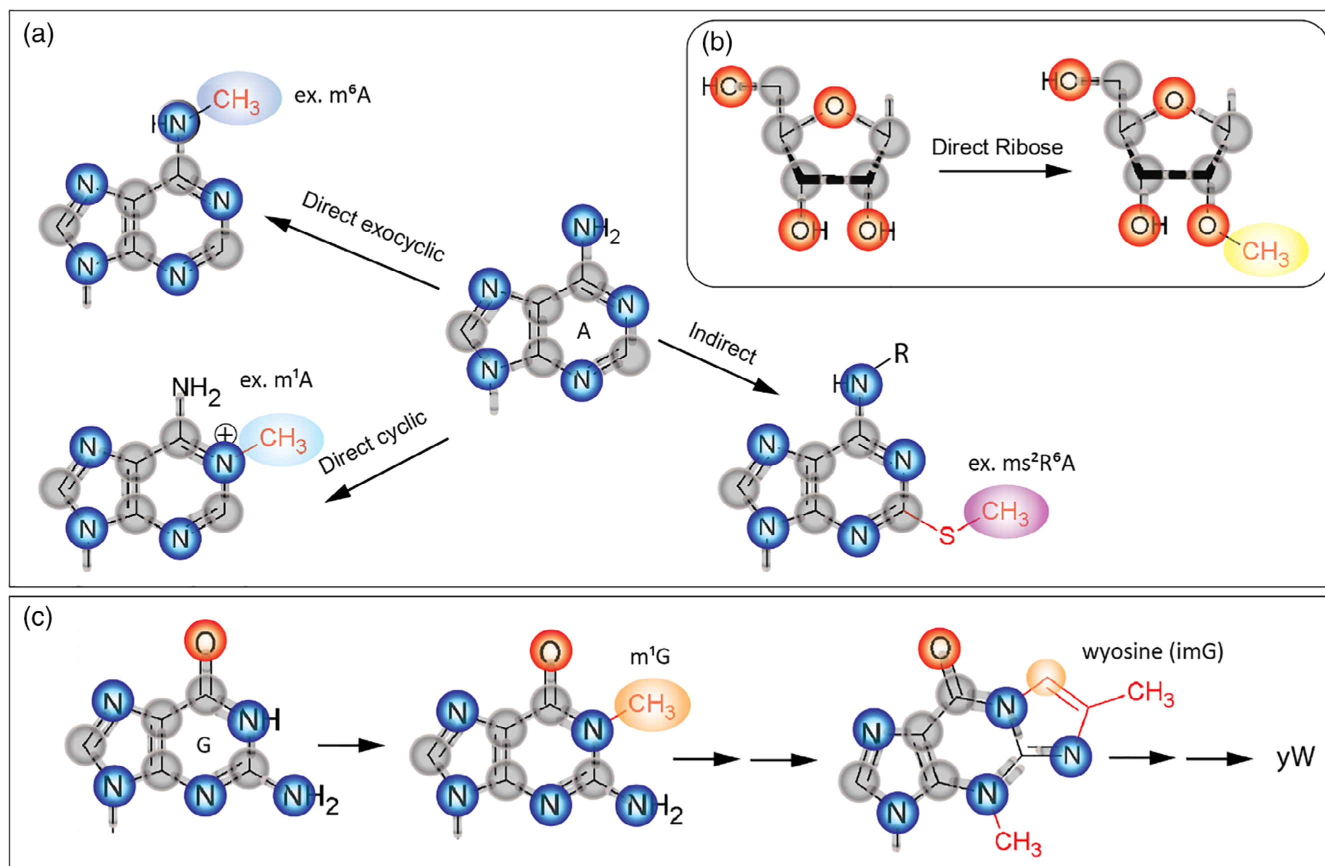

FIGURE 1 Direct and indirect methylation of nucleobases (a), of the ribose (b) and the m^{1}G37 methylation as an intermediate in the wybutosine (yW) pathway (c)

implications of partially methylated sites will be discussed in the subsequent sections, which are dedicated to tRNA, rRNA, and coding RNA. New developments in snoRNAs and other species are competently covered elsewhere (Hofler & Carlomagno, 2020).

2 | TRANSFER RNA METHYLATION

Transfer RNA (tRNA) molecules display the greatest variety of RNA modifications in general, and in consequence, also of RNA methylations. Some of those methylations are highly conserved in different kingdoms (m^{5}U54, Gm18, m^{1}G37, and m^{7}G46), while others are specific for a given branch or even group of species. Substantial efforts during the last 10 years allowed discovery of many new methylated sites in tRNA, in certain cases approaching comprehensive characterization of tRNA methylation profiles in different model organisms (Figure 4).

tRNAs from gram-negative bacterial species (e.g., *Escherichia coli*) may show methylations at seven different positions, and the same position within different tRNAs may bear different methylation types (e.g., at positions 32, 34, or 37). In comparison, Gram-positive bacteria lost Gm18 in the D-loop, but gained m^{1}A22 in the same domain. Interestingly, the same highly conserved m^{5}U54 is formed by two distinct enzymes in Gram-positive and Gram-negative bacteria. Gram-positive bacteria present the unique case of the tRNA methyltransferase TrmFO, which is folate-dependent instead of using the otherwise universal SAM cofactor (vide infra). The thermophilic bacterium *Thermus thermophilus* exhibits some extra tRNA methylations otherwise found in eukaryotes, namely m^{2}G6 and m^{1}A58, and the level of some tRNA methylations is regulated as a function of growth temperature (Kumagai et al., 1980; Ny & Bjork, 1980; Noon et al., 2003). Archaeal species show great diversity of tRNA methylation sites and many hypermethylated nucleotides (both base and 2′-*O*-methylations of the same nucleotide) are exclusively found in this living domain. Identification of the corresponding enzymatic activities is still ongoing in many instances, but essentially complete for some extensively

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FIGURE 2 Diversity of nucleotide methylation. (a) Methylation sites on the chemical structures of the four major ribonucleotides, inosine, and pseudouridine. Methylation positions at carbon atoms are in green, at the cyclic and the exocyclic nitrogens are in light and dark blue, respectively. Position of 2′-*O*-methylation of the ribose residues are in yellow. Multiple modifications may occur sequentially on a single nucleotide. Secondary methylations of other modified residues in RNA, as well various derivatives of Y-base (yW) formed by -adenosyl-L-methonine (SAM)-dependent methyltransferases (MTases) are also included (shown here in italics). Reports on the existence of $m^2{}_2Gm$, $m^6{}_2Am$, m^3Um, and m^5D remain to be confirmed (shown in gray)

studied species, like *E. coli* and *Saccharomyces cerevisiae*. Current knowledge on the tRNA methylations in different bacterial and archaeal species is summarized in Table 1.

Eukaryotic tRNA modification profiles are relatively well conserved from yeast to human (known eukaryotic activities listed in Table 2), with only a few extra sites detected in higher eukaryotic tRNAs ($m^2G5/6$, m^5C38, Gm/Ψm39, and $m^5C50/72$). In contrast, eukaryotic mitochondrial tRNAs are only sparsely modified, with only few methylations found at positions 26, 37, and 54 (*S. cerevisiae*). Mammalian mitochondrial tRNAs contain m^1A/m^1G9, m^2G10, m^3C32, transiently formed m^5C34, $m^5C48/49/50$, and m^1A58 in addition (Suzuki et al., 2020), (Suzuki & Suzuki, 2014).

The past decade has seen recurrent characteristic developments for numerous tRNA modifications: while knockouts, especially of MTase enzymes, in single cell organisms had frequently failed to display obvious phenotypes under laboratory conditions, the situation proved to be drastically different in multicellular organisms. Recent impressive progress in our understanding of molecular mechanisms, by which tRNA methylations affect physiology at the cellular and organismal level, has established numerous links to pathologies. In what follows, we will outline such a development using 5-methylcytosine (m^5C) as a well-explored example. For the subsequent discussion of other tRNA

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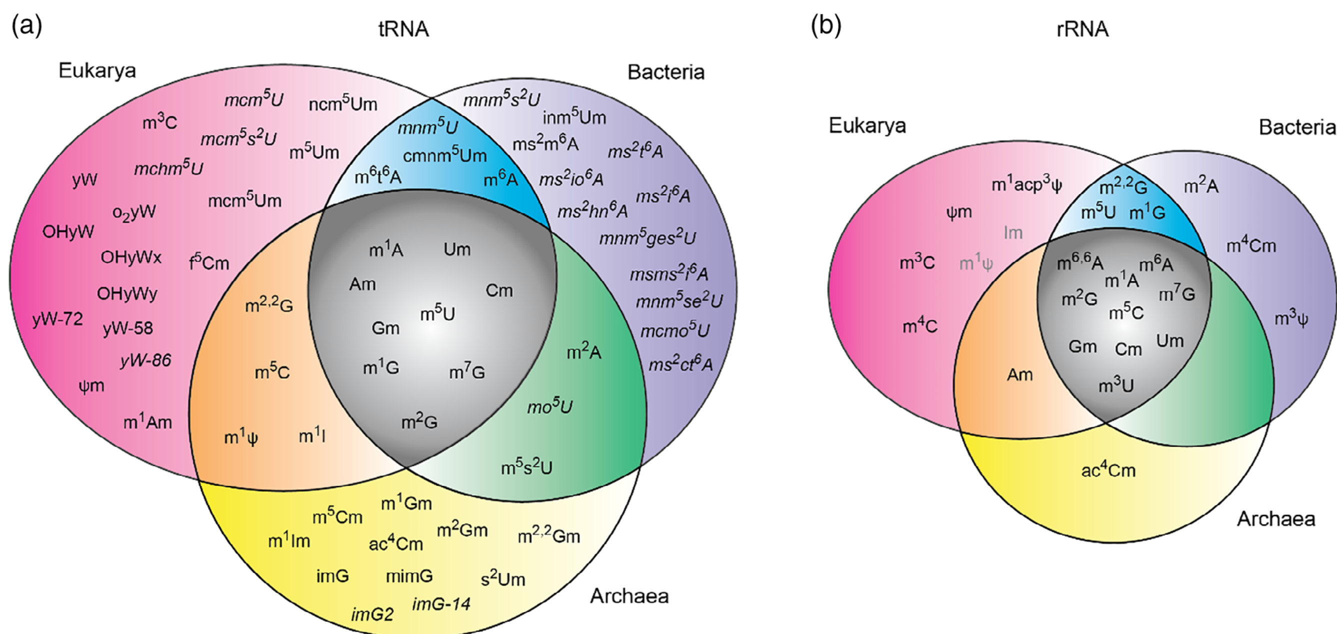

FIGURE 3 Wien diagram for phylogenetic distribution of methylated nucleotides (found in tRNA (a) and rRNA (b) species) in major life domains. Secondary methylations and derivatives of Y-base (yW) are included

BOX 1 Heterooligomeric protein MTase complexes

One of the earliest protein-only heterooligomeric MTase complexes to be characterized has emerged as the central player in mRNA methylation on the *N6*-A, yielding m^{6}A residues. Several of its newly identified protein components will be discussed in the section on mRNA methylation. Recent research on this arguably most important complex in the field has driven the development of new concepts and their interconnection in epitranscriptomics.

In tRNA methylation, a number of heterodimeric MTases are composed of one "activating," and one "catalytic" subunit, with the latter typically showing significant homology to namesakes from various MTase classes. An example was the characterization of the MTase complex Gcd10/Gcd14 (Trmt6/Trmt61A) responsible for the formation of 1-methyladenosine (m^{1}A) at position 58 in tRNA by the Hinnebusch lab (Anderson et al., 2000). Further similar complexes can be found in Table 1.

At the center of a third group of complexes of particular interest is the adapter protein Trm112. This subunit, despite its nomenclature suggesting MTase activity, acts as a noncatalytic platform that forms different complexes with variable catalytic subunits (Bourgeois et al., 2017). In yeast, these include Bud23 leading to formation of 7-methylguanosine (m^{7}G) 1575 in yeast 18S rRNA. Furthermore, complexes with Trm11 and Trm9 catalyze the formation m^{2}G at position 10 and methylation of cm^{5}U to mcm^{5}U at position 34, respectively, of certain yeast tRNAs. Interestingly, Trm112 also interacts with a MTQ2/HEMK2 to act as protein MTase on the translation termination factor eRF1. In human, two more variations are known, one including METTL5 as catalytic subunit for the formation of m^{6}A 1832 in human 18S rRNA (van Tran et al., 2019).

methylations such as 2′-OMe, m^{1}G, m^{3}C, m^{7}G, and the TΨC-loop methylations m^{1}A and m^{5}U, we will limit the discussion of physiology and pathology for reasons of space restrictions.

We will then treat a remarkable conceptual advance in the field, namely the capability of selected MTases to act on nucleosides of different chemical structure. The tRNA chapter will conclude with a section on interdependent modification events in tRNAs, so-called modification loops or circuits.

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BOX 2 Reversibility and dynamics of RNA methylation

First evidence of active enzymatic RNA demethylation was obtained in early 2000s with the discovery of a common mechanism of oxidative RNA and DNA demethylation by AlkB-family enzymes (Aas et al., 2003). Demethylation was found to affect m^1A and 3-methylcytidine (m^3C) residues, and was initially considered as a part of a hypothetical RNA repair pathway (Falnes, 2005; Falnes et al., 2004; Feyzi et al., 2007; Ougland et al., 2004; Westbye et al., 2008), but the biological relevance of demethylation remained elusive. Later, the substrate spectrum was extended to 3-methylthymine (3mT) and 1-methylguanine (1mG; Falnes, 2004).

A new twist in the field was brought by the discovery of fat mass and obesity-associated protein (FTO; Jia et al., 2008, 2011) and ALKBH5-catalyzed (Zheng, Dahl, Niu, Fedorcsak, et al., 2013) demethylation of m^6A in various cellular RNA substrates, including in particular mRNA (reviewed in Zheng, Dahl, Niu, Fu, et al., 2013). This pointed to possible dynamic regulation of m^6A mRNA modification in vivo as a new mechanism in gene expression regulation (for review Alemu et al., 2016; Mauer & Jaffrey, 2018; Rajecka et al., 2019; G. L. Xu & Bochtler, 2020).

Demethylation in eukaryotes was also shown for other modifications, including m^1A and m^3C demethylation by ALKBH1 (F. Liu et al., 2016; C.J. Ma et al., 2019) and ALKBH3 (Ueda et al., 2016, 2017) in substrates also including tRNA. In addition to acting in the removal of modifications, the oxidative activity of ALKBH1 was also demonstrated to be part of the biosynthesis pathway of 5-hydroxymethyl-2′-*O*-methylcytidine (hm^5Cm) and of 5-formyl-2′-*O*-methylcytidine (f^5Cm) at position 34 in mitochondrial $tRNA^{Met}$. There, C34 is first methylated to m^5C34 by NSUN2 and then further oxidized by ALKBH1 to hm^5C and, finally, to f5C, while independent 2′-*O*-methylation is executed by the FTSJ1/WDR6 complex (Haag et al., 2016; Kawarada et al., 2017).

2.1 | m^5C in tRNA

m^5C is widespread in different RNA species reviewed in (Burgess et al., 2015; Motorin et al., 2010) and, together with the corresponding m^5C-MTAses, is rather conserved in evolution (Bohnsack et al., 2019; Jeltsch et al., 2017; Kuznetsova et al., 2019). Most enzymes belong to the NSUN family, but the eukaryotic DNMT2, originally named for its homology to DNA-MTases was shown to modify RNA, namely m^5C38 in certain tRNA (Goll et al., 2006; Jurkowski & Jeltsch, 2011). Both, the DNMT2-related proteins from different eukaryotes (Becker et al., 2012; Tovy, Hofmann, et al., 2010; Tovy, Siman Tov et al., 2010), 23,877,245, (Govindaraju et al., 2017) and the NSUN family members were extensively studied. Interestingly, methylation-active Dnmt2-proteins are also present in a few prokaryotic species, such as *Geobacter* and *Holophaga foetida* (Shanmugam et al., 2014). Human DNMT2 catalyzes RNA methylation (Jurkowski et al., 2012), but is able to recognize and methylate DNA in DNA–RNA hybrid molecules (Kaiser et al., 2017). At the cellular level, DNMT2 methylation protects tRNAs from angiogenin-mediated stress-induced cleavage (Schaefer et al., 2010; Tuorto et al., 2012) and multiple biological functions in translation have been associated to DNMT2 activity, like accurate protein synthesis during hematopoiesis (Tuorto et al., 2015) or a role in translation of poly-Asp protein sequences (Shanmugam et al., 2015). Thus, it is not surprising that DNMT2 is involved in cancer (Elhardt et al., 2015) and azacytidine treatment was shown to inhibit, and rather specifically, DNMT2 activity which is variably expressed in cancer cell lines (Popis et al., 2016; Schaefer, Hagemann, et al., 2009).

The NSUN family contains seven homologous proteins (named from NSUN1–NSUN7), all of which have by now been shown to exhibit m^5C-MTAse activity, specific to tRNA (NSUN2/NSUN3/NSUN6), rRNA (NSUN1/NSUN4/NSUN5), and enhancer RNA (eRNA by NSUN7; Bohnsack et al., 2019; Kuznetsova et al., 2019), respectively. NSUN2 was also reported to modify vault RNA and, potentially, some sites in mRNA (Trixl & Lusser, 2019), (Schumann et al., 2020). NSUN2 (hTRM4, Misu) is probably the best studied MTase from this family, shown to modify multiple sites in human cytosolic (pos34, 48, 49, and 50; Auxilien et al., 2012) and mitochondrial tRNAs (Shinoda et al., 2019). Like its yeast homolog Trm4, NSUN2 forms covalent adducts with the substrate tRNA (Moon & Redman, 2014). Mammalian NSUN2 also localizes to mitochondria where it catalyzes m^5C formation in mitochondrial tRNAs (Shinoda et al., 2019; Van Haute, Lee, et al., 2019). In fission yeast *Schizosaccharomyces pombe*, NSUN2 functions are executed by two homologous proteins, namely Trm4a, responsible for all C48 tRNA methylations, and Trm4b, acting on C49 and

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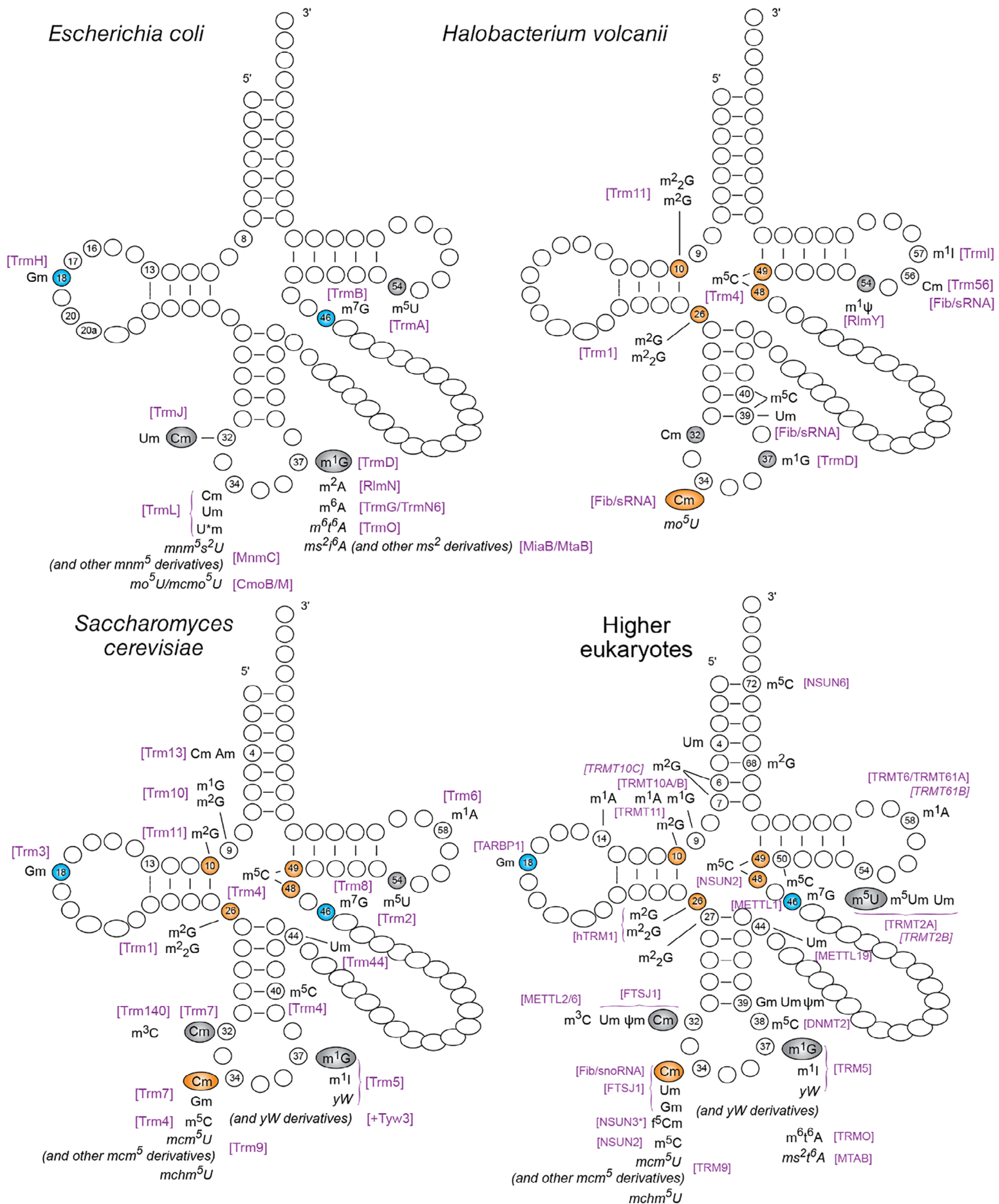

FIGURE 4 Methylated nucleotides in tRNA and their respective enzymes. Sites of nucleotide methylation on the tRNA cloverleaf structure are indicated for selected model organisms for bacteria (*Escherichia coli*), archaea (*Halobacterium volcanii*), and lower eukaryota (cytoplasmic tRNAs from *Saccharomyces cerevisiae*). For higher eukaryotes, a compilation of animal cytoplasmic tRNAs is shown. Hypermodifications that include methylation steps are also included, such as for example, various derivatives of the Y-base (yW). Universally conserved methylations are gray shaded, conserved in archaea and eukaryotes are in orange, and between bacteria and eukaryotes are in blue. m1ψ in archaeal tRNAs is considered similar to m5U

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TABLE 1 Bacterial and archaeal tRNA methylations

tRNA position	*E. coli* (de Crecy-Lagard et al., 2020) Modification	Enzyme	*B. subtilis* (de Crecy-Lagard et al., 2020) Modification	Enzyme	*T. thermophilus* (Hori, 2019) Modification	Enzyme	Archaea[a] Modification	Enzyme
6					m^2G	TrmN (TTC1157)	Cm	
9							m^2G	
9							m^1G	aTrm10
10							$m^2G/m^{2,2}G$	aTrm-m22G (Trm11)
18	Gm	TrmH			Gm	TrmH		
22			m^1A	TrmK				
26							$m^2G/m^{2,2}G$	Trm1
27							$m^2G/m^{2,2}G$	Trm1
32	Um/Cm	TrmJ					Cm	
32							m^5Cm	
34	Um/Cm/U*m	TrmL	Gm/Cm	CspR			Cm	Fib/sRNA
37	m^1G	TrmD	m^1G	TrmD			m^1G	TrmD
37	m^6A	TrmN6 (TrmG)	m^6A	TrmN6				
37	m^2A	RlmN	m^2A	RlmN				
39							Um	Fib/sRNA
39							m^5C	
40							m^5C	
46	m^7G	TrmB/ yggH	m^7G	TrmB	m^7G	TrmB		
48							m^5C	aTrm4
49							m^5C	aTrm4
49							m^7G	
51							m^5C	
54	m^5U(rT)	TrmA	m^5U(rT)	TrmFO	m^5U(rT)	TrmFO	m^5U(rT)	RlmD
54							$m^1\psi$	RlmY
56							Cm	aTrm56 and Fib/snoRNA
57							m^1A	aTrmI
57							m^1I	aTrmI
57							m^2G	
58					m^1A	TrmI		
67							m^2G	aTrm14
72							m^5C	aNSUN6

[a]Hirata et al. (2019), Hori (2014), J. Li et al. (2019), Kawamura et al. (2014), Towns and Begley (2012), and Wolff et al. (2020).

C50. Both proteins require different intron structures in pre-tRNA for substrate recognition and methylation of C34 (Muller et al., 2019). Trm4-driven $tRNA^{Leu}$(CAA) m^5C34 modification is altered under oxidative stress, which causes a translational reprogramming for more efficient translation of the TTG codon-enriched genes (C. T. Chan et al., 2012).

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TABLE 2 tRNA methylation in *Saccharomyces cerevisiae* and *Homo sapiens*

	S. cerevisiae				***H. sapiens***			
tRNA position	**Cytoplasmic modification**	**Cytoplasmic enzyme**	**Mitochondrial modification**	**Mitochondrial enzyme**	**Cytoplasmic modification**	**Cytoplasmic enzyme**	**Mitochondrial modification**	**Mitochondrial enzyme**
4	Cm/Am	Trm13						
5					m^2G			
6					m^2G			
9	m^2G							
9	m^1G	Trm10			m^1G	TRMT10A	m^1G/m^1A	TRMT10C (MRPP1)
9					m^1A	TRMT10B		
10	m^2G	Trm11			m^2G	*TRMT11*	m^2G	
14					m^1A[a]			
18	Gm	Trm3	Not present		Gm	TARBP1		
20					m^3C[b]			
26	$m^2G/m^{2,2}G$	Trm1	$m^2G/m^{2,2}G$	Trm1	$m^2G/m^{2,2}G$	hTRM1[c]		
27					$m^{2,2}G$	hTRM1		
32	Cm	Trm7/Trm732	Not present		Um/Cm/ψm	FTSJ1 or Fib/snoRNA		
32	m^3C	Trm140	Not present		m^3C	METTL2 and/or METTL6	m^3C	
34	Cm/U*m	Trm7/Trm734	Not present		Cm/f^5Cm/Um/Gm	FTSJ1		
34	m^5C	Trm4 (Ncl1)					$m^5C \rightarrow hm^5C \rightarrow f^5C$	NSUN3
37	m^1G	Trm5	m^1G	*Trm5*	m^1G	TRM5(TRMT5)		
37	m^1I	Trm5			m^1I	TRM5(TRMT5)		
38					m^5C	DNMT2 (TRDMT1)		
39					Gm/ψm			
40	m^5C	Trm4 (Ncl1)						
44	Um	Trm44			Um	*TRM44 (METTL19)*		
46	m^7G	Trm8/82	Not present		m^7G	METTL1 (WDR4)		
47d					m^3C[b]			

(Continues)

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TABLE 2 (Continued)

tRNA position	*S. cerevisiae* Cytoplasmic modification	Cytoplasmic enzyme	Mitochondrial modification	Mitochondrial enzyme	*H. sapiens* Cytoplasmic modification	Cytoplasmic enzyme	Mitochondrial modification	Mitochondrial enzyme
48	m^5C	Trm4 (Ncl1)	Not present		m^5C	NSUN2	m^5C	NSUN2[d]
49	m^5C	Trm4 (Ncl1)	Not present		m^5C	NSUN2	m^5C	NSUN2
50					m^5C	NSUN2	m^5C	NSUN2
51								
54	m^5U(rT)	Trm2	m^5U(rT)	*Trm2*	m^5U(rT)/m^5Um	TRMT2A	m^5U(rT)	TRMT2B[e]
54								
58	m^1A	Trm6/61	Not present		m^1A	TRMT6/ TRMT61A	m^1A	TRMT61B
72					m^5C	NSUN6		

Note: Italics: evidence only by sequence homology. Alternative names are given in brackets "()," heterodimeric complexes (e.g., Trm7/Trm732) are indicated by a slash "/."

[a]m1A14 was reported for tRNAPhe from human placenta (Roe et al., 1975).

[b]Cui et al. (2021).

[c]J. Liu and Straby (2000).

[d]Van Haute, Lee, et al. (2019).

[e]Laptev et al. (2020).

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The reported biological functions of NSUN2-driven RNA methylation are extremely diverse, ranging from translation (Gkatza et al., 2019), and mediation of the siRNA and miRNA pathways (Hussain, Sajini, et al., 2013; Yuan et al., 2014) to control of stress response and stem cell function (Blanco et al., 2016), epidermal and neural stem cell differentiation (Blanco et al., 2011; Flores et al., 2017; Hussain, Tuorto, et al., 2013) to stress-induced senescence (Cai et al., 2016) and control of mobile genetic element expression and genome stability (Genenncher et al., 2018). Elevated NSUN2 expression was observed in human cancers (Okamoto et al., 2012), associated with poor prognosis in head and neck squamous carcinoma (Lu et al., 2018) and determines the sensitivity of cancer cells to 5-FU treatment (Gaskin & Jacobson, 1978) as well as accelerated cell growth (Xing et al., 2015). Moreover, mutations in NSUN2 cause autosomal-recessive intellectual disability and Dubowitz-like syndrome (Abbasi-Moheb et al., 2012; Komara et al., 2015; Martinez et al., 2012) and aberrant m^{5}C tRNA methylation and accumulation of tRNA fragments contribute to neuro-developmental disorders (Blanco et al., 2014).

The other two tRNA-specific members of NSUN family are much less studied. NSUN6 modifies position C72 in human cytoplasmic tRNAs (Haag, Warda, et al., 2015), recognizing specific sequence encompassing CCA-end and three last base-paired nucleotides (Long et al., 2016), and archaeal NSUN6 homolog has similar activity for a wide range of *Pyrococcus horikoshii* tRNAs (J. Li et al., 2019). Finally, it has already been mentioned, that NSUN3 initiates the biosynthesis of 5-formyl-cytosine (f^{5}C) biosynthesis at position 34 in mitochondrial tRNA^{Met} (see Box 2). NSUN3 loss of function and thus deficient formylation of mt-tRNA^{Met} was observed in patient with impaired mitochondrial translation and thus mitochondrial respiratory chain complex deficiency (Van Haute et al., 2016).

2.2 | Ribose methylation (2′-*O*-Me, Nm) in tRNA

Enzymatic mechanisms of 2′-*O*-Me biosynthesis in tRNA were rather extensively studied (reviewed in Ayadi et al., 2019). Depending on the position and the organism, tRNA 2′-*O*-methylation is catalyzed either by stand-alone protein enzyme or by C/D snoRNP/Fib machinery in archaea (Joardar et al., 2011) and eukaryotes (Vitali & Kiss, 2019), reviewed in (Nostramo & Hopper, 2019).

Gm18 is a relatively well-conserved tRNA modification and its formation in bacteria is catalyzed by the well-studied TrmH protein, a member of the SPOUT RNA MTase family (reviewed in Hori, 2017). TrmH enzymes are divided in two classes, differing by their substrate recognition (Ochi et al., 2013). The activity of *T. thermophilus* TrmH was shown to be modulated by polyamines, allowing tRNA modification at high growth temperatures (Hori et al., 2016).

tRNA anticodon loop nucleotides are also frequently modified at positions 32 and 34. TrmL/YibK methylates wobble position 34 in bacterial tRNAs, (Benitez-Paez et al., 2010; Somme et al., 2014), and TrmJ is acting at position 32 (Jaroensuk et al., 2016). Despite substantial sequence homology, the archaeal TrmJ/Trm7-like protein differs from the bacterial homolog in its substrate specificity (Somme et al., 2014). Methylation of bacterial tRNA at positions 32 alters the oxidative stress response in *Pseudomonas aeruginosa* (Jaroensuk et al., 2016).

In eukaryotes these catalytic functions are performed by the Trm7/Trm32 and Trm7/Trm34 complexes, for positions 32 and 34, (Guy et al., 2012), (Guy & Phizicky, 2015), respectively. Compromised activity of the human Trm7-related protein FTSJ1 was shown to be involved in Nonsyndromic X-Linked Intellectual Disability (Guy et al., 2015; J. Li et al., 2020), while in yeasts the lack of Trm7-catalyzed methylations in the anticodon loop nucleotides activates the general amino acid control (GAAC) response, acting through Gcn2, which senses uncharged tRNA (Han et al., 2018). Trm7-deficient yeast mutants also show altered response to oxidative stress (Endres et al., 2020). Moreover, deficiency in Trm7/FTSJ1 activity also modulates the siRNA pathway in Drosophila (Angelova et al., 2020) and affects populations of tRNA fragments in mutant cells (Molla-Herman et al., 2020). Functional and structural roles of RNA 2′-*O*-Me as well of specific links to human diseases were recently reviewed in (Hofler & Carlomagno, 2020), (Dimitrova et al., 2019).

In bacterial tRNAs, 2′-*O*-methylation in the anticodon loop was also shown to protect tRNAs against cleavage by "ribotoxic" endonucleases. This methylation is performed by the bacterial polynucleotide kinase-phosphatase (Pnkp)/hua enhancer 1 (Hen1) complex (C. M. Chan et al., 2009) and is in fact a compoinent of an RNA repair machinery (R. Jain & Shuman, 2010). Bacterial Pnkp/Hen1 performs 3′-terminal 2′-*O*-methylation during RNA repair followed by ligation of the cleaved RNA chain (P. Wang et al., 2012). Despite sequence homology with its bacterial counterpart, the mammalian Hen1 specifically methylates sncRNAs called P-element-induced wimpy testis-interacting RNAs (piRNAs) and tRNA-derived sncRNA (Peng et al., 2018).

2.3 | 1-Methylguanosine in tRNA

The major 1-methylguanosine (m^1G) sites in tRNA are located in the junction between acceptor and D-stem (positions 9/10), and in the anticodon loop (position 37). The anticodon loop methylation is catalyzed by enzymes from the TrmD/Trm5 family (Christian et al., 2013; Goto-Ito et al., 2017; Guo et al., 2019; Hori, 2017; Hou et al., 2017), while the TRM10 group is responsible for formation of $m^1G9/10$ (Howell et al., 2019; Vilardo et al., 2020). The m^1G37 is also a first intermediate in the multistep biosynthesis pathway for hypermodified residues wybutosine and wyosine derivatives found in eukaryotic and archaeal tRNAs (Urbonavicius et al., 2016), reviewed in (de Crecy-Lagard et al., 2010; Urbonavicius et al., 2014). The mechanism of m^1G37 synthesis by both, bacterial TrmD and eukaryotic Trm5 was extensively studied (Christian et al., 2010; Masuda et al., 2013; Paris et al., 2013). Despite essentially identical enzymatic reactions and products, these two proteins differ in subtle details of catalysis and tRNA recognition (Lahoud et al., 2011), making bacterial TrmD a potential antibiotic target (Hill et al., 2013; Y. Zhang et al., 2017). The methylated nucleoside m^1G37 is well known to affect ribosome frameshifting (Bjork et al., 1999; Hong et al., 2018) and to allow codon-specific regulation of translation (Hou et al., 2018). Deficiency of either of the TRM5 or TRM10 enzymatic machineries is associated with disease (Gillis et al., 2014; Metodiev et al., 2016; Oerum, Roovers, et al., 2017; Zhou et al., 2018).

2.4 | m^7G in tRNA

The methylation m^7G is frequent in tRNA (reviewed in Tomikawa, 2018) and rRNA (see rRNA modification chapter). The tRNA m^7G46 modification is mediated by TrmB in bacteria and the heterodimeric complexes Trm8/Trm82 (METTL1/WDR4) in yeast and human, respectively. Correct m^7G46 methylation is required for maintenance of mRNA translation and embryonic stem cell self-renewal and differentiation (Deng et al., 2020; Lin et al., 2018). Deficient tRNA m^7G46 modification is a cause of microcephalic primordial dwarfism (Filonava et al., 2015; Shaheen et al., 2015) and is also involved in sensitivity of cancer cells to 5-fluorouracil (Okamoto et al., 2014).

2.5 | m^3C in tRNA

Methylation of the N3 in cytosines occurs at position 32 in the tRNA anticodon loop as well as at some rare positions in the D and variable loops (Cui et al., 2021). In Eukaryotes, methylation is catalyzed by METTL2 and METTL6 (L. Xu et al., 2017), the latter being involved in regulation of pluripotency and tumor cell growth (Ignatova et al., 2020). METTL2 forms a complex with DALRD3 protein, which regulates its tRNA recognition specificity (Lentini et al., 2020).

2.6 | Namesake of the TΨC-loop methylations: 5-methyluridine (m^5U)

The TΨC-loop (or T-loop) of tRNA contains the highly conserved methylated uridine (ribothymidine, rT, and m5U) at position 54. Interestingly, this namesake of the T-loop is not found anywhere else in tRNAs. Formation of m^5U54 is catalyzed by TrmA in bacteria, and this protein belongs to a larger family of m^5U-MTases acting on tRNA and rRNA (Auxilien et al., 2011). TrmA also modifies the TΨC-like loop of the bacterial tmRNA, due to its near-perfect structural mimicry with the homologous tRNA domain (Ranaei-Siadat et al., 2013). TRMT2A is the eukaryotic pendant in cytosolic tRNA-m^5U54 methylation (Chang et al., 2019), while its close homolog TRMT2B is targeted to mitochondria, where it modifies both U54 in tRNA and m^5U429 in 12S rRNA (Laptev et al., 2020), (Powell & Minczuk, 2020). A specific mapping method found m^5U outside tRNA and rRNA in eukaryotes (Carter et al., 2019). tRNA methylation by bacterial TrmA is efficient on unmodified tRNA transcript, suggesting this methylation to be an early event of tRNA maturation (Schultz & Kothe, 2020).

While the vast majority of RNA MTases are SAM-dependent enzymes, an alternative methyl donor was found to be required for m^5U-methylation in Gram-positive bacteria (Urbonavicius et al., 2005), reviewed in (Hamdane et al., 2012, 2016). The tRNA MTase TrmFO recognizes the typical sequence consensus of the tRNA TΨC-loop (Yamagami et al., 2012) and methylates U54 in a folate/FAD-dependent manner. Rather than a direct transfer of a methylgroup as in SAM dependent MTase, the enzymatic mechanism features a C1 body at an oxidation state corresponding to formaldehyde. This C1 is activated as imine-derivative, which is then transferred from the N5 of the FAD's isoalloxazine to

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the C5 in Mannich-type reaction (Hamdane et al., 2011, 2013), and subsequently reduced to methylgroup level. Most interestingly, this mechanism bears strong resemblance to that of thymidylate synthase, the enzyme that methlyates UMP to TMP, with the remarkable difference that the analogous imine-derivative is generated on, and transferred from, the folate cofactor. Since the enyzmes are also phylogenetically different, one may conclude, that a catalytic strategy for synthesis of a 5 mU nucleobase has been "invented" *three* times in convergent evolution. In *Mycoplasma capricolum*, another flavoprotein (RlmFO) methylates m^5U1939 in 23S rRNA (Lartigue et al., 2014). Altogether, four distinct enzyme families converged in evolution to catalyze m^5U formation in different classes of RNAs (Sirand-Pugnet et al., 2020).

2.7 | m^1A in tRNA

In addition to being frequent at its typical position 58 in the T-loop, m^1A, and its deaminated derivative m1I occasionally appear at position 57 in Archaea, and m^1A also occurs at positions 9, 14, and 22 in Gram-positive bacterial species (Degut et al., 2016; Oerum, Degut, et al., 2017). Archaeal tRNAs are modified at positions 57 and 58 by the same region-specific MTase TrmI (Guelorget et al., 2010; Hamdane et al., 2014), while in human, the heterodimer TRMT6/TRMT61A is responsible for m^1A58 synthesis in cytoplasmic tRNA (Ozanick et al., 2005).

2.8 | Dual chemical specificity of modification enzymes

Yeast Trm10p catalyzes the methylation of guanosine at position 9 in tRNAs (Jackman et al., 2003) to yield m^1G9. It exhibits promiscuous methylation of nonphysiological tRNA substrates upon overexpression (Swinehart et al., 2013), while its archaeal homolog from the crenarchaeon *Sulfolobus acidocaldarius* methylates adenosine 9 to m^1A9. A similar enzyme from the euryarchaeon *Thermococcus kodakaraensis* forms both m^1G9 and m^1A9 residues in different tRNAs (Kempenaers et al., 2010). Of note, this was the first demonstration of an extended nucleotide specificity in RNA methylation (and modification in general), involving the turnover of different canonical nucleotides.

Extensive analysis of the human mitochondrial RNAse P complex identified one of its components, RNase P protein 1 (MRPP1), as TRMT10C, the functional human homolog of yeast Trm10. TRMT10C also catalyzes similar methylation reactions (m^1A9/m^1G9) in human mitochondrial tRNAs (Vilardo et al., 2012). Mutations in the TRMT10C gene are associated with multiple respiratory chain deficiencies (Metodiev et al., 2016) and mutations in the associated mitochondrial protein MRPP2 (also known as HSD10/SDR5C1, encoded by the HSD17B10 gene) also affect tRNA modification efficiency of the complex (Oerum, Roovers, et al., 2017), reviewed in Karasik et al. (2019).

In contrast, methylation of human cytoplasmic tRNAs at position 9 is ensured by a pair of homologous proteins, TRMT10A (m^1G9), and TRMT10B (both m^1A9 and m^1G9) (Howell et al., 2019; Vilardo et al., 2020). TRMT10A is linked to various pathologies, and its deficiency is not compensated by TRMT10B (Gillis et al., 2014; Igoillo-Esteve et al., 2013).

2.9 | Interdependence of tRNA modifications

Interdependence (or at least a certain temporal order) in the appearance of the tRNA modifications was already observed a while ago (Nishikura & De Robertis, 1981). However, only recently such cases have been investigated and documented in more detail. All well documented cases concern tRNA anticodon loop modifications, and most frequently modified positions 32, 34, and 37 (Figure 5).

The nucleobase queuine is a micronutrient and a precursor for the Q34 tRNA modification in most eukaryotes, with the remarkable exception of *S. cerevisiae*. Q34 was shown to control m^5C38 $tRNA^{Asp}$ methylation by Dnmt2 (Muller et al., 2015). Unmodified $tRNA^{Asp}$ is only weakly methylated and structural analysis revealed that Q34 ensures optimal positioning of the tRNA anticodon loop in the Dnmt2 active site (Johannsson et al., 2018), reviewed in (Ehrenhofer-Murray, 2017).

Other cases of modification interdependency in the tRNA anticodon include the stimulation of Trm7/Ftsj1-dependent 2′-*O*-methylation (Cm/Um) at position 34 by i^6A37. i^6A37 or t^6A37 are also required for efficient formation of m^3C32 by Trm140 (Arimbasseri et al., 2016; Han et al., 2017), reviewed in (Han & Phizicky, 2018; Maraia & Arimbasseri, 2017).

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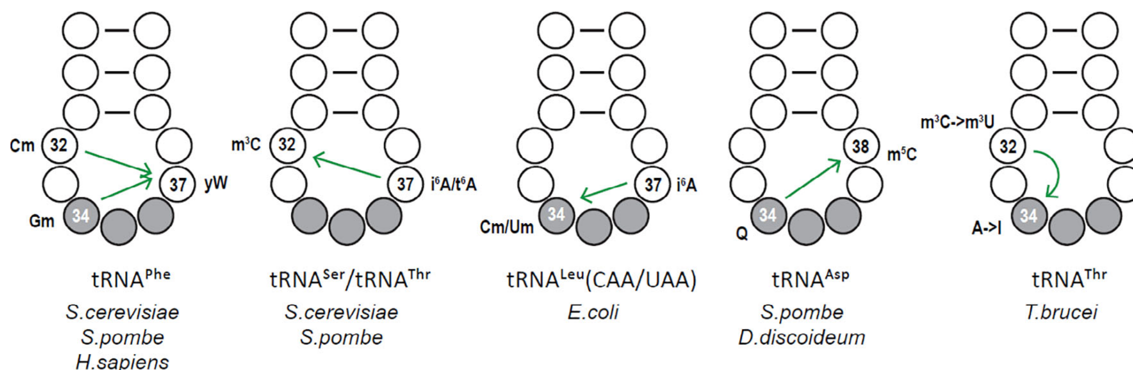

FIGURE 5 Schematic representation of tRNA modification dependency circuits in the anticodon loop. From left to right: Cm32 and Gm34 drive yW37 formation in $tRNA^{Phe}$ of *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and humans, i^6A37 (in $tRNA^{Ser}$ of *S. pombe* and *S. cerevisiae*) or t^6A37 (in $tRNA^{Thr}$ of *S. cerevisiae*) stimulate m^3C32 formation, i^6A37 drives Cm34 and Um34 formation in $tRNA^{Leu}$(CAA) and $tRNA^{Leu}$(UAA) of *Escherichia coli*, Q34 is required for m^5C38 formation in $tRNA^{Asp}$ of *S. pombe* and *Dictyostelium discoideum* and conversion of C32 to m^3C32 followed by deamination to m^3U32 drives A34 → I34 deamination in $tRNA^{Thr}$(AGU) of *Trypanosoma brucei*. (Figure adapted from Han & Phizicky, 2018)

Inversely, tRNA methylations at positions 32 and 34 greatly stimulate the synthesis of anticodon-adjacent m^1G37 in *S. cerevisiae*, (which constitutes as a first step in yW37 pathway (Guy & Phizicky, 2015). Within the anticodon, m^3C/ m^3U32 modifications in trypanosomal tRNA drive A34 → I deamination (McKenney et al., 2018; Rubio et al., 2017), reviewed in S. Dixit et al. (2019).

3 | RIBOSOMAL RNA METHYLATION

During the past decade, substantial new insight into rRNA methylation derived mostly from eukaryotes. The complete modification content of rRNAs was reported from *cerevisiae*, *falciparum*, and human, providing model data for yeasts, protists, and mammals. Following a general trend in RNA modification, several reports investigated dynamics and differentially modified subpopulations of rRNA. Also, a respectable number of enzymes responsible for rRNA methylation, were identified. Finally, structural biology has revealed interesting, though occasionally disputed features of rRNA modification.

3.1 | Mapping of rRNA methylations

Several newly developed high throughput methods for the mapping of RNA modifications, for example, for m^3C, m^7G, and Nm (Marchand et al., 2016, 2018), have been calibrated with known methylation sites in rRNA as a reference, only rarely coming up with a de novo detection of rRNA modifications. Modifications in rRNA have also become a playground for the development of modification-aware sequencing approaches using nanopore devices, because comparison of native modified and in vitro transcribed unmodified rRNA is technically straightforward (Smith et al., 2019).

For baker's yeast *S. cerevisiae*, a nucleoside analysis of modifications (J. Yang et al., 2015, 2016, 2017) as well a fragment-based LC–MS analysis (Taoka et al., 2016), allowed the establishment of a high-confidence reference. Similar maps were also generated for fission yeast (Taoka et al., 2015), *Leishmania* (Nakayama et al., 2019) and, more recently, for human rRNA (Taoka et al., 2018), providing valuable resources for in depth biological investigations.

3.2 | Modification heterogeneity in rRNA

The above cited maps of rRNA modifications may be considered complete, with the possible exception of incompletely (Buchhaupt et al., 2014) or even alternatively modified sites (Taoka et al., 2016), which were, again, first described in yeast. The possibility of a partial or alternative modification state, if applied separately to each rRNA modification site,

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implies an enormous number of possible permutations. Ribosome heterogeneity has become a common concept with respect to the protein composition (Norris et al., 2021), and indeed, ribosome modification heterogeneity and dedicated ribosome modivariants have subsequently emerged as concepts of biological significance for example, in the context of pathologies (Janin et al., 2019) and stress-related alterations (Heissenberger et al., 2019).

3.3 | Eukaryotic modification enzymes

Similar to the general development in the tRNA field, RNA methyltransferases were typically first identified and characterized in *S. cerevisiae*, and homologs in higher eukaryotes then identified and validated for example, in *C. elegans* (Navarro et al., 2021), fruit fly, and mammals. Table 3 gives an overview of eukaryotic protein-only RNA MTases identified for rRNA. For methylations guided by small nucleolar RNAs refer to Sloan et al. (2017), and for prokaryotic rRNA modifications refer to our previous review Motorin and Helm (2011). Given the very fundamental involvement of rRNA methyltransferases in ribosome biosynthesis and maturation, corresponding knockout organisms and cell lines were reported with a variety of molecular phenotypes. These pertain mostly to rRNA assembly, and thus affect protein biosynthesis and, consequently, cell growth.

3.4 | Visualization of rRNA methylations by structural biology

With ever-increasing resolution of ribosome structures allowing the visualization of known rRNA modifications in electron density maps, recent advances in CryoEM have induced reports on the de novo discovery of rRNA modifications (Natchiar et al., 2017), claiming modifications of hitherto unknown chemical structures. So far, these modifications have yet to be confirmed by the authors (Natchiar et al., 2018) or by other groups looking at rRNA modifications by CryoEM. Other modifications such as cytidine acetylations, where also observed by CryoEM, this time validated by chemical mapping and mass spec approaches (Sas-Chen et al., 2020). However, given the current lack of universally acknowledged quality control routines, as known from X-ray crystallography, it may take some time before CryoEM can be validated as a means for the faithful detection of RNA methylations without additional biochemical evidence.

3.5 | RNA methylation in antibiotic resistance

The link between rRNA modifications and sensitivity of bacterial ribosomes to various aminoglycoside antibiotics were known for a long time (reviewed in Doi et al., 2016; Schaenzer & Wright, 2020). In general, antibiotic resistance is not related to housekeeping rRNA modification enzymes, but is rather provided by plasmid-born rRNA-MTases of different families. Resistance is typically mediated by introduction of additional rRNA modifications that may interfere with antibiotic binding (Atkinson et al., 2013). More recently, certain tRNA modifications have been also highlighted as potential targets for antibiotics. This concerns n particular m^1G37, which contributes to integrity of the Gram-negative cell envelope (Hou et al., 2020).

Considering that bacterial ribosome is a central target of multiple antibiotics, it is not surprising that more and more rRNA modifications have been shown to modulate antibiotic resistance. This is the case for resistance to various aminoglycosides conferred by SSU MTases from the ArmA/Rmt family, which methylate N7 of G1405 (Zarubica et al., 2011), by the 16S rRNA m^5C1404-specific MTase EfmM in *Enterococcus faecium* (Galimand et al., 2011) and by NpmA, which modifies 16S-A1408 to m^1A (Dunkle et al., 2014), (Kanazawa et al., 2017). NpmA may also modify G1408 to m^1G in an additional case of dual specificity (Zelinskaya et al., 2015). Resistance to the macrolide antibiotic tylosin is conferred by two m^1G residues in 23S rRNA formed by TlrD and $RlmA^I$ (Yakhnin et al., 2019). In further instances, rRNA methylation was postulated as a resistance mechanism, but the exact nature or/and position of RNA methylation remains unknown (Qin et al., 2013).

It is noteworthy that the presence of rRNA modifications may not only confer antibiotic resistance, but also increase antibiotic sensitivity (Poldermans et al., 1979). This was demonstrated for TlyA-directed 2′-*O*-methylation of both ribosomal subunits, which increases sensitivity to capreomycin (Monshupanee et al., 2012).

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TABLE 3 Eukaryotic rRNA methylation by protein-only enzymes

Modification	Organism	Compartment	Position	Enzyme	References
SSU					
$m^1acp^3\psi$	*S. cerevisiae*	cyto	1191	Nep1(Emg1)	B. Meyer et al. (2011), B. Meyer et al. (2016)
$m^1acp^3\psi$	*H. sapiens*	cyto	1248	EMG1/TSR3	Wurm et al. (2010)
m^7G	*S. cerevisiae*	cyto	1575	Bud23/Trm112	Figaro et al. (2012)
m^7G	*H. sapiens*	cyto	1639	WBSCR22(Merm1)/ Trm112	Zorbas et al. (2015)
m^6A	*H. sapiens*	cyto	1832	METTL5/Trm112	van Tran et al. (2019)
m^6A	*D. melanogaster*	cyto	ND	Mettl5/dmTrm112 (CG12975)	Leismann et al. (2020)
$m^6{}_2A$	*S. cerevisiae*	cyto	1781/1782	Dim1	Lafontaine et al. (1994), Lafontaine et al. (1995)
$m^6{}_2A$	*H. sapiens*	cyto	1850/1851	DIMT1L	Zorbas et al. (2015), Zorbas et al. (2015)
$m^6{}_2A$	*H. sapiens*	mito	936/937	TFB1M	McCulloch et al. (2002)
m^5U	*H. sapiens*	mito	429	TRMT2B	Powell and Minczuk (2020)
m^4C	*H. sapiens*	mito	839	METTL15	Van Haute, Hendrick, et al. (2019)
m^5C	*H. sapiens*	mito	841	NSUN4	Metodiev et al. (2014)
LSU					
m^1A	*S. cerevisiae*	cyto	645	RRP8(Bmt1)	Peifer et al. (2013), Bousquet-Antonelli et al. (2000)
m^1A	*H. sapiens*	cyto	1309	NML	Taoka et al. (2018) Waku et al. (2016) Sharma et al. (2018)
m^1A	*C. elegans*	cyto	674	T07A9.8 (rram-1)	Yokoyama et al. (2018)
m^1A	*S. cerevisiae*	cyto	2141	Bmt2	Sharma, Watzinger, et al. (2013)
m^1A	*H. sapiens*	cyto	3749		Taoka et al. (2018)
m^5C	*S. cerevisiae*	cyto	2278	Rcm1	Sharma, Yang, et al. (2013), Gigova et al. (2014)
m^5C	*H. sapiens*	cyto	3782	NSUN5	Janin et al. (2019), Heissenberger et al. (2019)
m^5C	*M. musculus*	cyto	3438	NSUN5	Heissenberger et al. (2019)
m^5C	*C. elegans*	cyto	3381	NSUN5	Schosserer et al. (2015)
m^5C	*D. melanogaster*	cyto	3620	NSUN5	Schosserer et al. (2015)
m^6A	*H. sapiens*	cyto	4190 (4220)	ZCCHC4	Ren et al. (2019), Pinto et al. (2020), H. Ma et al. (2019)
m^3U	*S. cerevisiae*	cyto	2634	Bmt5	Sharma et al. (2014)
m^3U	*H. sapiens*	cyto	4470	ND	Taoka et al. (2018)
m^3U	*S. cerevisiae*	cyto	2842	Bmt6	Sharma et al. (2014)
m^3U	*H. sapiens*	cyto	4500	ND	Taoka et al. (2018)
m^5C	*S. cerevisiae*	cyto	2870	Nop2	Sharma, Yang, et al. (2013), Bourgeois et al. (2015)
m^5C	*H. sapiens*	cyto	4387	P120 (NSUN1)	Bourgeois et al. (2015)
m^1A	*H. sapiens*	mito	947	TRMT61B	Bar-Yaacov et al. (2016)
Gm	*H. sapiens*	mito	1145	MRM1	Rebelo-Guiomar et al. (2019)
Um	*S. cerevisiae*	mito	2791	Mrm2	Pintard et al. (2002)

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TABLE 3 (Continued)

Modification	Organism	Compartment	Position	Enzyme	References
Um	*H. sapiens*	mito	1369	MRM2(FTSJ2)	Garone et al. (2017)
Gm	*H. sapiens*	mito	1370	MRM3(RNMTL1)	Rebelo-Guiomar et al. (2019)

Note: Alternative names for proteins are in (), subunits of multiproteins complexes are separated by "/." Numbers in brackets denote numbering inconsistencies within the rRNA.
Abbreviation: ND, not determined.

Equally interesting, the absence or inhibition of endogenous RlmN-dependent C2 methylation of A2503 to m^{2}A confers linezolid resistance to *Staphylococcus aureus* (LaMarre et al., 2011) and tiamulin resistance to clinical strains of *Firmicutes* phylum (Stojkovic et al., 2016). An even more complex regulatory circuit was demonstrated for telithromycin susceptibility in *Streptococcus pneumonia*. Here, RlmCD-mediated m^{5}U747 methylation promotes efficient m^{1}G748 methylation by RlmAII MTase and thus facilitates telithromycin binding to the ribosome (Shoji et al., 2015).

4 | CODING RNA METHYLATION

The occurrence of m^{6}A in mRNA was established and even "mapped" early on (Horowitz et al., 1984). Other reported mRNA modifications from that time included ribose methylations and m^{5}C, (Dubin & Stollar, 1975; Dubin et al., 1977), albeit without localization data. Over the last decade, diverse studies reported mapping of further methylated nucleotides in mRNA, including m^{1}A, (Dominissini et al., 2016; X. Li et al., 2016; Safra et al., 2017), m^{6}A (Zaccara et al., 2019), m^{7}G (L. S. Zhang et al., 2019), m^{5}C (Squires et al., 2012; Khoddami & Cairns, 2013; Hussain, Aleksic, et al., 2013), m^{6}A$_m$ (Linder et al., 2015), and other N$_m$ modifications (Dai et al., 2017). Modified nucleotides were reported from all different transcript regions, albeit with biases depending on the type of methylation. Occurrence, quantity, and positioning of many modifications in mRNA are all grounds for controversial discussion in the community.

4.1 | m^{6}A in mRNA

The most abundant internal modification in mRNA is clearly m^{6}A, detected in the 5′UTR, coding region and 3′UTR (Zaccara et al., 2019). The m^{6}A modification is introduced co-transcriptionally by a MTase (writer) complex at the meanwhile famous DRACH motif (D = G/A/U, R = G/A, H = A/U/C). This complex was among the earliest protein-only heterooligomeric MTase complexes to have been characterized in some depth, and has emerged as the central player in mRNA methylation on the *N6*-A. Because of this, it has seen a renaissance of attention from various disciplines including biochemistry, cell biology, genetics in various organisms, and structural biology (Sledz & Jinek, 2016; P. Wang et al., 2016; X. Wang et al., 2016). Work in the 1980s by Rottmann's group had identified a large catalytic complex of ~1 MDa, which could be divided into at least two subcomplexes (Rottman et al., 1994). More recently, several protein components of these two complexes, now named MAC and MACOM, have been identified (Knuckles et al., 2018; Lence et al., 2019). MAC being the smaller subcomplex is essentially composed of catalytic MTase now named METTL3, and a noncatalytic paralog METTL14.

MACOM contains at least four proteins, namely VIRMA, RBM15, ZC3H13, and HAKAI. An additional protein, WTAP, may mediate an interaction between MAC and MACOM, but the final composition of these complexes awaits confirmation, ideally including structural biology.

Identification and characterization of these protein components and their contribution to the regulation of the methylation process has received much attention in the field. Wilms' tumor-associated protein (WTAP) is the key METTL3 adaptor, essential for m^{6}A formation (Agarwala et al., 2012). Further important interactors of the complex are Virma, main interactor of WTAP, and RBM15/15B, two paralogous RNA-binding proteins (Horiuchi et al., 2013; Patil et al., 2016). Other involved and known WTAP interactors are Zc3h13, binding RBM15/15B and finally linking it to WTAP, and CBLL1 (Patil et al., 2016), also known as Hakai. Besides the m^{6}A mRNA pathway

in eukaryotes, the methylation complex with the corresponding homologs can also be found in *Drosophila melanogaster* (Lence et al., 2017).

Once introduced, m^6A mediates its function through m^6A-binding proteins, the so-called "readers." The first and best-studied m^6A readers are the YTH domain-containing proteins (Dominissini et al., 2012; F. Li et al., 2014; Zhu et al., 2014). In mammalians, YTH domain-containing proteins can be located in the nucleus (YTHDC1; Hartmann et al., 1999), in the cytoplasm (YTHDF1-3; A. Li et al., 2017; X. Wang et al., 2014), as well as in both (YTHDC2; Wojtas et al., 2017). Thereby, the nuclear YTHDC1 was reported to affect mRNA splicing (Xiao et al., 2016) and export (Roundtree et al., 2017). Furthermore, YTHDC2 (Bailey et al., 2017; D. Jain et al., 2018), plays a role in mRNA degradation (Kretschmer et al., 2018; Wojtas et al., 2017) and translation initiation regulation (Dhote et al., 2012; Hsu et al., 2017). The molecular mechanisms of YTHDF protein functions remain controversial. In contrast to recent studies, reporting the binding of the same m^6A-modified mRNAs for YTHDF1-3 and mediation of mRNA degradation and cellular differentiation (Du et al., 2016; Kennedy et al., 2016; Zaccara & Jaffrey, 2020), earlier studies had reported different and more specialized effects for the YTHDF proteins, including also enhanced mRNA translation (Shi et al., 2017; X. Wang et al., 2014; X. Wang et al., 2015).

As already mentioned in Box 2, the regulatory processing of m^6A in mRNA is further complicated by the so-called "eraser" proteins, demethylases that oxidatively remove methylation. ALKBH5 is known to demethylate mRNA in the nucleus (Zheng, Dahl, Niu, Fedorcsak, et al., 2013). Furthermore, upregulation of ALKBH5 seems to play a role in certain cancer cell lines (D. Dixit et al., 2017; C. Zhang et al., 2016; S. Zhang et al., 2017). A protein whose demethylation activity toward m^6A is controversial in different studies is the FTO. The initially reported demethylase activity toward m^6A in mRNA (Jia et al., 2011) was questioned (Garcia-Campos et al., 2019; Hess et al., 2013; S. Zou et al., 2016) by experiments indicating m^6Am as the major or even exclusive substrate of FTO (Mauer et al., 2017), (Relier et al., 2021). Then again, there is evidence that FTO rather acts on m^6Am sites in snRNA than on mRNA transcripts (Mauer et al., 2019). Additional studies are required to further elaborate the regulatory interactors of the m^6A methylation and demethylation mechanism.

4.2 | m^5C in mRNA

First data suggestive of the occurrence of m^5C in coding (including viral) RNA date back to the 1970s (Dubin & Stollar, 1975), (Dubin et al., 1977). After a slack period of several decades, the adaptation of bisulfite sequencing of m^5C was adapted from DNA to RNA (Schaefer, Pollex, et al., 2009). This entailed a first transcriptome-wide application (Squires et al., 2012) preceding even the "m^6A-goldrush." The originally postulated occurrence of several tens of thousands of m^5C sites in mRNA appeared to be in the order of magnitude of what is commonly reported for m^6A (Squires et al., 2012). However, subsequent mRNA reports yielded highly variegated numbers of m^5C sites in various organisms (Amort et al., 2017; David et al., 2017; Huang et al., 2019; Hussain, Aleksic, et al., 2013; Khoddami et al., 2019; Selmi et al., 2021), ranging as low as single digits even in mammals (Legrand et al., 2017). Numerous improvements to mapping protocols were proposed, and the resulting increase in stringency points to overall rather low numbers. This is consistent with LC–MS data that clearly establish m^5C quantities well below that of m^6A in mRNA (Legrand et al., 2017). Several m^5C reader proteins have also been reported, including ALYREF and YBX1 (Chen et al., 2021; X. Yang et al., 2017; F. Zou et al., 2020).

4.3 | m^1A in mRNA

Like for m^5C, the history of m^1A detection in mRNA remains a controversial one. Originally posited after the application of a commercial antibody directed against the modified nucleobase 1 mA in MeRIP experiments followed by RT-arrest based mapping (Dominissini et al., 2016), (X. Li et al., 2016), m^1A residues in eukaryotic mRNA were estimated to range from thousands to tens of thousands. Diverging data suggested a much lower number with a maximum in the low double-digit range (Safra et al., 2017). Interestingly, among the few consensual sites is the mitochondrial ND5 mRNA (Grozhik et al., 2019). The ensuing technical debate on m^1A mapping methods (Schwartz, 2018; Xiong et al., 2018) took a decisive turn when a secondary specificity for said commercial antibody was reported, which was found to also bind cap structures (Grozhik et al., 2019), (Helm et al., 2019).

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4.4 | Ribose methylation (2′-*O*-Me), m^7G, and m^3C

Ribose methylations and m^7G are solidly established as part of higher cap structures in mRNA and viral RNA (vide infra). In addition, transcriptome-wide searches have uncovered evidence that hints at minor amounts of m^3C, m^7G, and ribose methylations potentially residing in coding RNA and lncRNA, by mapping methods which are, in part, controversially discussed in the community (Dai et al., 2017; Enroth et al., 2019; Malbec et al., 2019; Marchand et al., 2018; L. Xu et al., 2017; L. S. Zhang et al., 2019).

4.5 | Cap structures

Certain cellular RNAs, typically transcribed by RNA pol II, undergo specific modification at the 5′-end, consisting in formation of m^7GpppN cap by guanylyltransferase followed by single or multiple methylations of the "inversed" G to m^7G or higher methylated Gs such as $m^{227}G$ in snRNAs/snoRNAs. The bulk of cap structures are introduced co-transcriptionally to the 5′end of eukaryotic mRNAs. As previously outlined (Motorin & Helm, 2011), some cases are known of cap structure trans-splicing, resulting in transfer from their original transcript to another for example, in snRNAs. Cap structures are numbered 0–4 according to methylations on the capping guanosine and the first nucleotide transcribed at the TSS (Byszewska et al., 2014; Galloway & Cowling, 2019; Inesta-Vaquera & Cowling, 2017). The status of the latter has received particular recent attention, in particular the possibility of oxidative demethylation of the N6 methylgroup in m^6Am by FTO (Mauer et al., 2019). The CAPAM methyltransferase has been identified and structurally characterized in great detail (Akichika et al., 2019). Also, the development of comprehensive methods for cap population profiling (Culjkovic-Kraljacic et al., 2020) has advanced the biochemical understanding of cap structures, ultimately turning up no evidence for the presence of m^1A in caps (J. Wang et al., 2019). Viral RNA capping, which has also returned to the focus of attention, is discussed below.

4.6 | Viral RNA methylation

Viral RNAs, both genomic and coding, are exposed to the activity of the host cell RNA modification machinery (Ruggieri et al., 2021), since they are replicated/transcribed in eukaryotic cells. In addition, some complex viruses encode their own RNA modification enzymes, typically cap-related m^7G-and 2′-*O*-MTases, whose specific modification of viral transcripts results in modulation of viral RNA recognition by host restriction systems. Historically, the detection of cap modification and internal modifications went parallel in conventional mRNA and in viral RNA (Ruggieri et al., 2021).

Viral RNAs obtain or modify cap structures by a number of mechanisms, including "cap snatching" from cellular Pol II transcripts, as well as the use of viral encoded capping and MTase enzymes. Comprehensive reviews cover biosynthesis, mechanisms, and functions of viral RNA caps (Decroly et al., 2011; Ramanathan et al., 2016).

4.7 | Internal viral RNA methylation

Both, the historic discovery and the latest development in internal viral RNA modifications parallel that of coding RNA, not only in terms of history, but also in technology. Thus, several of the modification mapping techniques developed in the past decade (vide supra), were applied to viral RNA. Much like for mRNA, the presence of m^6A residues remains uncontested, but not their precise positions and stoichiometry. Both in cellular and viral coding RNAs, m^6A is considered the most prevalent internal modified nucleotide. Its presence was reported already in the 70's and pioneering work on Rous Sarcoma virus (Csepany et al., 1990) allowed to map some of these residues to the precursor sequence (RGACU) of what became later know as the DRACH motif (vide supra). Numerous RNA viruses have by now been mapped for m^6A, including flaviviruses (Ruggieri et al., 2021), HIV (Tsai et al., 2021), and others (Williams et al., 2019).

Similarly, cases of m^5C detection in viral RNA are increasing. For example, Dnmt2-derived m^5C residues were suggested to be present in Drosophila C virus (Durdevic et al., 2013) and experimentally detected in HIV-1 (Courtney, Tsai, et al., 2019), the catalysis apparently effect by rather the promiscuous NSUN2 m^5C-MTase. Another study by the

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same group also pointed out multiple m^{5}C residues in murine leukemia virus (MLV) genomic RNA (Courtney, Chalem, et al., 2019). Very recently, m^{5}C was also found in Epstein–Barr virus encoded RNA EBER1, which contains one single NSUN2-dependent m^{5}C residue, detected by LC–MS and further mapped by bisulfite sequencing (Henry et al., 2020).

First evidence pointing to internal 2′-*O*-methylation of viral RNA came from investigations on West Nile virus and dengue virus (DENV), since their own NS5 2′-*O*-MTase is capable of modifying not only the penultimate adenosine nucleotide, but also some internal residues (Dong et al., 2012). The MTase + CTD domain of the L protein, encoded by the Sudanebolavirus (SUDV), was similarly shown to catalyze internal 2′-*O*-methylation (Martin et al., 2018). Numerous modification types detected in flaviviral RNA by LC–MS included also 2′-*O*-methylation (Lichinchi et al., 2016; McIntyre et al., 2018).

Retroviruses such as HIV and MLV, do not encode an NS5-like 2′-*O*-MTase, in contrast to flaviviruses. A detailed analysis, including the Ribomethseq approach, turned up strong evidence for HIV-1 RNA internal methylation on least 17 Nm modification sites. This pointed to the recruitment of cellular MTases, and indeed, the human 2′-*O*-MTase FTSJ3 was found to be tightly associated with TAR HIV-1 RNA via interaction with TRBP. FTSJ3 exhibited in vitro methylation activity, catalyzing mostly Am residue formation in HIV RNA. Further validation of these HIV-1 RNA Nm sites was performed by primer extension at low concentrations of dNTP (Ringeard et al., 2019). Significant 2′-*O*-methylation as well as other modified residues were also reported from MLV RNA (Courtney, Chalem, et al., 2019).

Further analyses on RNA viruses included first rough maps of coronaviral RNA modifications obtained by nanopore sequencing (Kim et al., 2020). Progress in elucidating the function of viral RNA methylation in such diverse aspects as translation, immune-evasion, and virus assembly was recently reviewed (Ruggieri et al., 2021).

5 | CONCLUSION

RNA methylation has proven to be a very dynamic field in the past decade and is likely to remain this way for some time. We surmise, that, while stoichiometric nucleotide methylations are well covered by various analytics, substoichiometric methylation, especially in mRNA sequences and other RNA species of low abundance, will remain technically challenging, and hence subject to controversial results and discussion. Here, the field looks to nanopore sequencing for delivery. Because this technology relies on a fundamentally different principle than all other sequencing methods, it can, in principle, directly detect a modified nucleotide with an RNA chain, whereas Illumina-based techniques are all about reconstructing information from sequencing of cDNA (Helm & Motorin, 2017). However, recently published approaches to modification detection by nanopore still use an indirect feature, namely correlation of erroneous base-calling (Begik et al., 2021; Kim et al., 2020; H. Liu et al., 2019; Smith et al., 2019), similar to the misincorporation concept used with Illumina (Hauenschild et al., 2015; Werner et al., 2020). Future approaches are likely to include machine learning of modified RNA, such as to enable identification of the modifications directly from the electric signal readout generated during their passage through the pore.

Branching out from the principle developments outlined here, more and more fields and sub-disciplines in the life sciences become affected by important conclusions, pathways, and methods from our field. One such field, that seems to still be under the radar of most colleagues in epitranscriptomics, is RNA damage. Damage to RNA can be induced by a number of reactive species under physiological conditions, and result in noncanonical nucleotide species, including not only oxidized species, but also methylated ones (Yan & Zaher, 2019). The constantly growing number of newly discovered noncanonical structures may soon include some that blur the line between RNA modification and RNA damage. This may apply for example, to the recently reported msms^{2}i^{6}A modification, which arises as a combination of oxidation and methylation, potentially occurring accidentally rather than with a physiological function (Dal Magro et al., 2018). More connections between modifications and damaging alkylation (Reichle et al., 2019) are liable to appear soon, given the increased interest in enzymes that can oxidatively remove such damage (Borek et al., 2020).

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Yuri Motorin: Conceptualization (equal); data curation (equal); writing – original draft (equal); writing – review and editing (equal). **Mark Helm:** Conceptualization (equal); data curation (equal); writing – original draft (equal); writing – review and editing (equal).

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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