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Review

Roles and dynamics of 3-methylcytidine in cellular RNAs

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Modified nucleotides within cellular RNAs significantly influence their biogenesis, stability, and function. As reviewed here, 3-methylcytidine (m^3C) has recently come to the fore through the identification of the methyltransferases responsible for installing m^3C_{32} in human tRNAs. Mechanistic details of how m^3C_{32} methyltransferases recognize their substrate tRNAs have been uncovered and the biogenetic and functional relevance of interconnections between m^3C_{32} and modified adenosines at position 37 highlighted. Functional insights into the role of m^3C_{32} modifications indicate that they influence tRNA structure and, consistently, lack of m^3C_{32} modifications impairs translation. Development of quantitative, transcriptome-wide m^3C mapping approaches and the discovery of an m^3C demethylase reveal m^3C to be dynamic, raising the possibility that it contributes to fine-tuning gene expression in different conditions.

Highlights

Newly developed m^3C-detection techniques allow quantitative and/or transcriptome-wide identification of m^3C sites with single-nucleotide resolution and give new estimates of their stoichiometries.

Specific methylation targets of the known human m^3C methyltransferases METTL2A/B, METTL6, and METTL8 have recently been identified.

Features that influence substrate recognitions by m^3C methyltransferases have been uncovered, including sequence motifs within the anticodon loop, modified nucleotides at position 37, and protein cofactors.

Interplay between m^3C_{32} and t^6A_{37}/$(ms^2)i^6A_{37}$ is functionally as well as biogenetically relevant, as the presence of these two modifications influences tRNA structure.

Lack of m^3C_{32} modifications in tRNAs impairs cytoplasmic and mitochondrial translation, leading to functional consequences, such as reduced stem cell pluripotency and impaired mitochondrial function.

3-Methylcytosine: an epitranscriptomic mark coming into focus

Modified nucleotides within cellular RNAs are critical regulators of gene expression through their ability to modulate the biogenesis, function, and fate of coding and noncoding RNAs. The diverse RNA modification landscape, collectively termed the '**epitranscriptome**' (see Glossary), consists of more than 170 different types of RNA modifications across the three domains of life [1,2]. **Transfer RNA (tRNA)** is the most extensively and variably modified RNA class, but most other types of RNA are also decorated with modifications. In general, RNA modifications exert their functions by modulating the stability, structure, and/or function of RNAs. For example, modifications within tRNA cores play important roles in ensuring correct tRNA architecture, while tRNA anticodon loops are modification hotspots where the modified nucleotides expand decoding capacity and ensure the fidelity of translation [3,4]. The effects of RNA modifications can either arise directly due to the altered chemical and topological properties of the nucleotides themselves, as is the case for the tRNA modifications described earlier, or can be mediated via recognition by specific proteins. The best characterized examples of so-called modification 'reader' proteins are the YTH domain proteins that specifically bind ***N*^{6}-methyladenosine (m^6A)**-containing (pre-)mRNAs to regulate their splicing, translation, or degradation [5–10].

The epitranscriptome is not static, but rather dynamic, and dramatic changes in RNA modification profiles are observed during cellular adaptation, upon differentiation, and in numerous pathogenic conditions [11,12]. While **3-methylcytosine in DNA (3meC)** is a common and well-characterized DNA lesion, **3-methylcytosine in RNA (m^3C)** (Box 1) is a component of the eukaryotic epitranscriptome that has recently attracted much attention. In this review, we describe the current knowledge on methyltransferases installing m^3C in cellular RNAs, focusing on elements that influence their recognition/methylation of specific target RNAs. We also highlight advances in understanding the roles of m^3C modifications in regulating RNA structure and function in cellular processes as well as emphasizing links between m^3C modifications and disease.

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Box 1. Chemical and topological properties of m^3C

Cytosine is a pyrimidine base that can be converted into m^3C by covalent attachment of a methyl group to the nitrogen atom at position 3 (N^3; Figure I). Typically, 3-methylcytosine in DNA arises in single-stranded DNA as a result of alkylation, but the DNA **5-methylcytosine (5-meC)** methyltransferase DNMT3A has also been reported to install 3-meC at low levels when insertion of the target cytosine into the catalytic site is inverted [54]. In RNA, m^3C is introduced by the action of ***S*-adenosylmethionine (SAM)**-dependent methyltransferases [13,28,31,32,55]. Modeling of cytosine monophosphate into the active site of recent crystal structures of METTL6 suggests a base-flipping mechanism for the target cytidine, which is then accommodated in a positively charged cavity with the base ring pointing towards the catalytic center of the enzyme [55,56]. Methylation of N^3 endows a positive charge on the nucleobase, thereby altering both the chemical and topological properties of the nucleotide. The N^3 position of cytosine lies on the Watson-Crick interface and methylation significantly disrupts classical base-pairing interactions with guanosine nucleotides and also decreases base-pairing discrimination within C:A, C:U, and C:C interactions. Consistent with this, the presence of m^3C impedes the progress of high-fidelity reverse transcriptases and leads to G to A substitutions during cDNA synthesis by low-fidelity reverse transcriptases [57]. Interestingly, a hypermodified form of m^3C, 3,2′-*O*-dimethylcytidine (m^3Cm) has recently been detected in mammalian small RNAs (<200 nucleotides) [58].

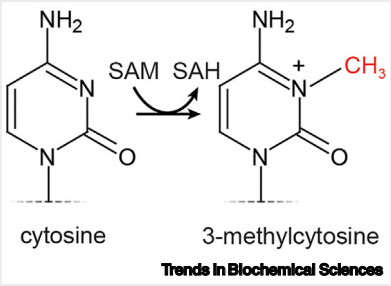


Figure I. Atomic structure of cytosine and 3-methylcytosine. The methyl group added to cytidine is highlighted in red. Abbreviations: SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.

The m^3C methylome

Newly developed m^3C detection and mapping approaches (Box 2) now provide quantitative and transcriptome-wide perspectives on m^3C modifications in cellular RNAs. Position 32 of several cytoplasmic and mitochondrial **tRNA isoacceptors** (Table 1 and Figure 1, Key figure) are prominent sites where m^3C can be detected with high stoichiometry [13–18]. m^3C has also reproducibly been detected outside tRNA anticodon loops, being present at position 47:3 within the variable loop and position 20 in the D loop of specific tRNA isoacceptors (Table 1 and Figure 1) [16,17,19,20].

The potential presence of m^3C in mRNAs is currently controversially discussed. Mass spectrometric analysis of size selected, poly(A)-enriched RNAs resulted in the detection of m^3C, suggesting its presence in mRNAs [13,21]. However, subsequent transcriptome-wide mapping approaches involving chemical cleavage and next-generation sequencing (Box 2) [14,16] did not support the presence of m^3C in mRNAs and no specific m^3C sites were pinpointed. These opposing conclusions could potentially be reconciled if m^3C is found at substoichiometric levels in a small number of mRNAs, as has been suggested for other RNA modifications, like ***N*^{1}-methyladenosine (m^1A)** [22], but further experimentation will be required to discover if this is the case.

m^3C tRNA methyltransferases

The first m^3C methyltransferase identified was *Sc*Trm140 of the budding yeast *Saccharomyces cerevisiae*, which installs m^3C_{32} in *Sc*tRNA$^{Thr(AGU/CGU/UGU)}$ (*Sc*tRNA^{Thr}) and *Sc*tRNA$^{Ser(CGA/UGA/GCU)}$ (*Sc*tRNA^{Ser}) [23,24] (Figure 1, upper left). Interestingly, *Sc*Trm140 is a fusion protein where the Rossman-fold class I methyltransferase domain is fused by programmed +1 frameshifting to an upstream open reading frame encoding an actin-binding protein [23–25]. However, the

Box 2. m^3C detection and mapping techniques

Transcriptome-wide approaches allow analysis of m^3C levels at multiple sites in parallel and enable identification of previously unmapped m^3Cs.

AlkAniline-seq: alkaline treatment and aniline cleavage of RNAs is followed by mapping of m^7G and m^3C modifications based on reverse transcriptase stalling [14,59]. Precisely how partial alkaline hydrolysis primes m^3C for aniline cleavage remains unclear.

Hydrazine-aniline cleavage sequencing (HAC-seq): treatment of small RNAs with hydrazine and aniline enables specific detection of m^3C sites, which are validated using AlkB-demethylated controls [16]. m^3C stoichiometry is estimated via cleavage ratios but low-read coverage areas can generate false positives.

AlkB-facilitated RNA methylation sequencing (ARM-seq): detection of m^1A, m^3C, and m^1G sites in tRNA fragments is accomplished by comparing the abundance of reads between AlkB-demethylated and untreated samples but this method cannot distinguish the nature of the modified residue [60].

Demethylase tRNA sequencing (DM-tRNA-seq)/demethylation-assisted multiple methylation sequencing (DAMM-seq): a modification index for m^1A, m^1G, m^2_2G, and m^3C sites is calculated by combining information from reverse transcriptase stops and mismatches and identified modification sites are validated by comparison with demethylase-treated samples [19,61,62].

Modification-induced misincorporation tRNA sequencing (mim-tRNA-seq): semi-quantitative information on the stoichiometry of several modifications, including m^3C, is obtained by analysis of misincorporations in full-length cDNAs. [17].

Hydrolysis-based tRNA sequencing (tRNA-HySeq or hydro-tRNAseq): size-selected RNAs are subjected to partial alkaline lysis to overcome reverse transcriptase-impeding modifications or secondary structures before sequencing [20,63].

Site-specific and site-independent techniques are employed when the m^3C site of interest is known or not relevant.

Primer extension: based on detection of truncated cDNA fragments arising from interrupted reverse transcriptase progression, this approach can be readily applied to most target RNAs to detect m^3C, but also other modified nucleotides. Secondary structures or other modifications on the Watson-Crick face can be problematic.

Deoxyribozymes: DNA enzymes that differentially cleave unmodified cytidine and m^3C enable monitoring of the m^3C status of specific sites within bulk RNA or in synthetic/*in vitro* transcribed RNA [15].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS): a quantitative method to determine levels of m^3C modification in bulk RNA, but does not provide information on modification sites and cannot reliably detect modifications in individual specific RNA species without prior isolation.

Nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS): analysis of isotope-labeled nucleotides in RNAs by mass spectrometry provides a quantitative and dynamic view on modification levels in bulk RNA or purified samples [64].

Anti-m^3C antibody: commercial anti-m^3C antibodies suitable for dot blots are available, however, further validation for other applications is required.

Glossary

S-Adenosylmethionine (SAM): methyl group donor for many methylation reactions.
D and TΨC loops: loops within the tRNA cloverleaf structure containing dihydrouridine (D) and threonine-pseudouridine-cytidine (TΨC), respectively.
DRACH motif: nucleotide sequence motif recognized by *Hs*METTL3/*Hs*METTL14 (D = A, G, or T, R = A or G, H = A, C, or T).
Epitranscriptome: collective term for modified RNA nucleotides present within the transcriptome.
N^6-Isopentyladenosine (i^6A): modified version of adenosine carrying an isopentyl group on nitrogen 6.
N^1-Methyladenosine (m^1A): modified derivative of adenosine carrying a methyl group on nitrogen 1.
N^6-Methyladenosine (m^6A): modified derivate of adenosine carrying a methyl group on nitrogen 6.
3-Methylcytosine in DNA (3meC): modified derivative of cytosine carrying a methyl group on nitrogen 3.
5-Methylcytosine in DNA (5-meC): modified derivative of cytosine carrying a methyl group on carbon 5.
3-Methylcytosine in RNA (m^3C): modified derivative of cytosine carrying a methyl group on nitrogen 3.
2-Methylthiol-N^6-isopentyladenosine (ms^2i^6A): modified derivative of adenosine carrying an isopentyl group on nitrogen 6 and a methylated thiol group on carbon 2.
3-Methyluridine (m^3U): modified derivative of uridine carrying a methyl group on nitrogen 3.
N^6-Threonylcarbamoyladenosine (t^6A): modified derivative of adenosine carrying a threonylcarbamoyl group on nitrogen 6.
Transfer RNA (tRNA): adaptor molecule responsible for decoding messenger RNA codons and bringing appropriate amino acids to the ribosome for formation of polypeptides.
tRNA isoacceptors: tRNAs that are charged with the same amino acid but recognize different codons.
tRNA synthetases: family of enzymes responsible for conjugation of appropriate amino acids to specific tRNAs.

methyltransferase domain alone is sufficient for installation of m^3C_{32} in $tRNA^{Thr/Ser}$ and it remains unclear whether coexpression with the actin-binding domain has any functional relevance or consequences. Notably, m^3C is undetectable in RNA from yeast lacking *ScTRM140*, implying that *Sc*Trm140 is solely responsible for m^3C formation in *S. cerevisiae* [23]. Strikingly, a gene duplication event in *Schizosaccharomyces pombe* led to expression of two distinct m^3C methyltransferases (Figure 1, upper right). *Sp*Trm140 and *Sp*Trm141 share approximately 33% identity but are functionally distinct as *Sp*Trm140 catalyzes formation of m^3C_{32} in *Sp*$tRNA^{Thr(AGU/CGU/UGU)}$ (*Sp*$tRNA^{Thr}$) whereas *Sp*Trm141 mediates methylation of C_{32} of *Sp*$tRNA^{Ser(AGA/CGA/UGA/GCU)}$ (*Sp*$tRNA^{Ser}$) [26]. Combined deletion of *SpTrm140* and *SpTrm141* abolishes detection of m^3C, suggesting that other m^3C methyltransferases do not exist in *S. pombe*.

In *Trypanosoma brucei*, C_{32} of *Tb*$tRNA^{Thr}$ is also methylated by a Trm140 homolog *Tb*TRM140a, but intriguingly, this m^3C_{32} then undergoes partial deamination to **3-methyluridine (m^3U)**

Table 1. tRNA m^3C writers, stoichiometries, and elements required for modification installation

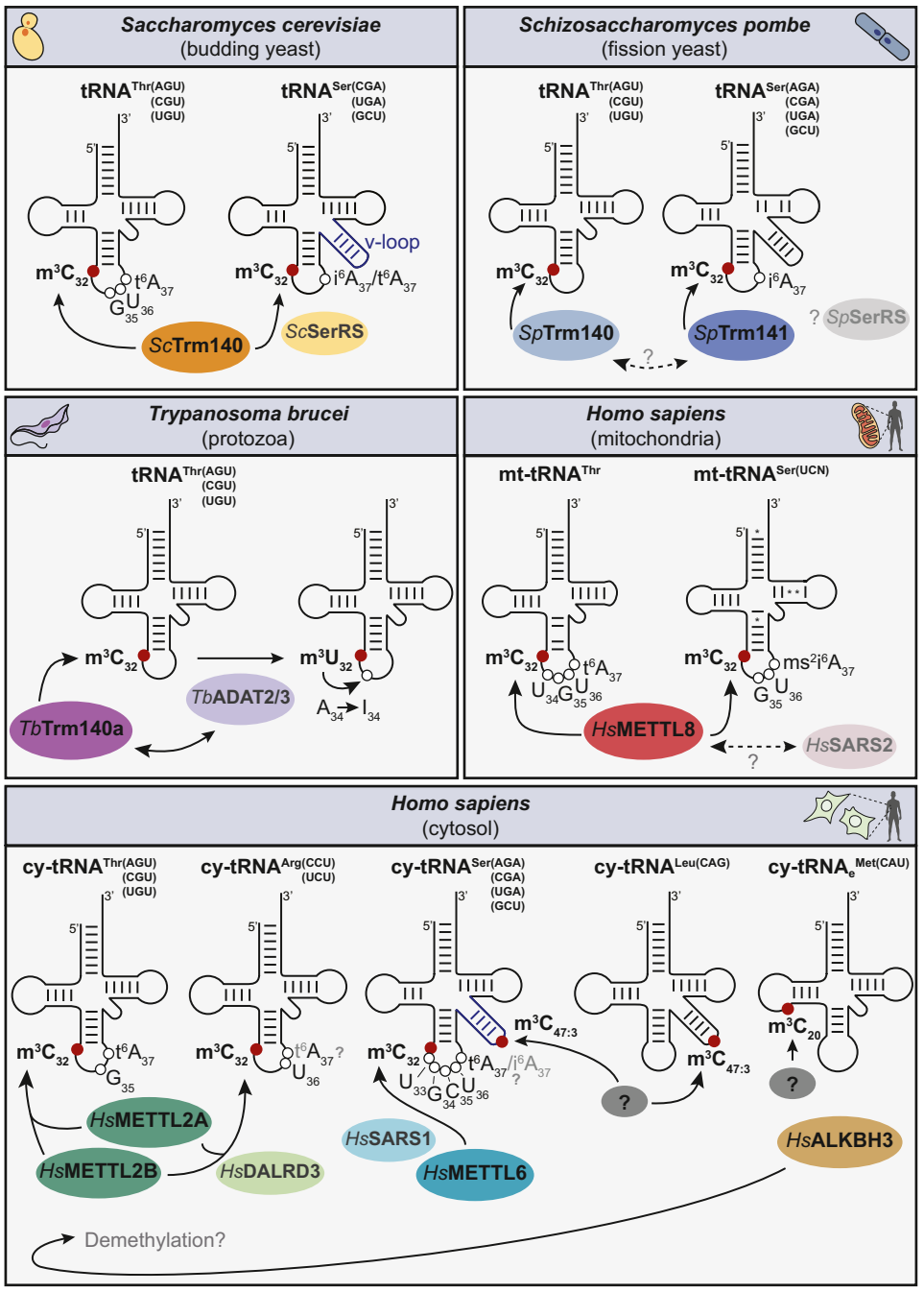
Species	Protein	m^3C position	Localization	tRNA	Isoacceptor	m^3C level[a]	Known identity elements
S. cerevisiae	Trm140	32	Cytoplasm	Thr	AGU	~94% [17]	$G_{35}U_{36}t^6A_{37}$
					CGU	N/A	
					UGU	N/A	
				Ser	CGA	~70% [17]	i^6A_{37} + SerRS
					UGA	~49% [17]	i^6A_{37} + SerRS
					GCU	N/A	t^6A_{37} + v-loop
S. pombe	Trm140	32		Thr	AGU	N/A	?[b]
					CGU	N/A	
					UGU	N/A	
	Trm141			Ser	AGA	N/A	i^6A_{37}
					CGA	N/A	i^6A_{37}
					UGA	N/A	i^6A_{37}
					GCU	N/A	?
Homo sapiens	METTL2A/METTL2B	32		Thr	AGU	From ~75% [16] to 99% [17]	G_{35} and t^6A_{37}
					CGU	~98% [16]	
					UGU	From ~60% [16] to 98% [17]	
				Arg	CCU	From ~30% [16] to 87% [17]	U_{36} and A_{37}
					UCU	From ~55% [16] to 69% [17]	
	METTL6	32		Ser	AGA	From ~45% [16] to 87% [17]	?
					CGA	~38–85% [16]	
					UGA	~79% [16]	
					GCU	~60–85% [16]	U_{33} G_{34} $C_{35}U_{36}$ t^6A_{37} + SARS1 + v-loop
	METTL8	32	Mitochondria	Thr	UGU	From ~85% [16] to 96% [18]	U_{34} G_{35} U_{36} $t^6A_{37}A_{38}$
				Ser (UCN)	UGA	~35% [16]	G_{35} U_{36} $ms^2i^6A_{37}$
	Unknown	47:3	Cytoplasm	Ser	AGA	~74% [16]	?
					CGA	~90% [16]	
					UGA	~74–91% [16]	
					GCU	~65% [16]	
				Leu	CAG	~78% [16]	?
		20	Cytoplasm	eMet	CAU	~68% [16]	?

[a] m^3C stoichiometries derive from [16–18], as indicated.
[b] ?, unknown.

catalyzed by the deaminases ADAT2/3 [27] (Figure 1, middle left). In mice, three homologs of Trm140/Trm141 were identified, namely the methyltransferase-like (METTL) proteins *Mm*METTL2, *Mm*METTL6, and *Mm*METTL8 [13]. Similarly, four human homologues of yeast Trm140/Trm141 exist: the METTL2 paralogs *Hs*METTL2A and *Hs*METTL2B, *Hs*METTL6, and *Hs*METTL8 [13]

Key figure

m^3C modifications in tRNAs



(See figure legend at the bottom of the next page.)

(Figure 1, middle right and bottom). Consistent with their homology to Trm140 and Trm141, respectively, *Hs*METTL2A/B are responsible for m^3C_{32} methylation of cytoplasmic *Hs*tRNA$^{Thr(AGU/CGU/UGU)}$ (*Hs*tRNA^{Thr}), whereas *Hs*METTL6 mediates methylation of cytoplasmic *Hs*tRNA^{Ser} isoacceptors (*Hs*tRNA$^{Ser(AGA/CGA/UGA/GCU)}$) [13,28,29]. Notably, m^3C_{32} is also detected in a subset of cytoplasmic *Hs*tRNA^{Arg} isoacceptors (*Hs*tRNA$^{Arg(CCU/UCU)}$) in higher eukaryotes and *Hs*METTL2A/B have been revealed as the enzymes responsible for introducing these methylations [20,30] (Figure 1, bottom). In contrast to *Hs*METTL2A/B/METTL6, *Hs*METTL8 localizes to the mitochondrial matrix where it installs m^3C_{32} in mitochondrial (mt-)tRNA$^{Thr(UGU)}$ (*Hs*mt-tRNA^{Thr}) and *Hs*mt-tRNA$^{Ser(UCN)}$ [31–34,75] (Figure 1, middle right). Consistent with the dual functionality of *Hs*METTL8 in methylating both *Hs*mt-tRNA^{Thr} and *Hs*mt-tRNA$^{Ser(UCN)}$, it is most closely related to *S. cerevisiae* Trm140, which is similarly capable of methylating both *Sc*tRNA^{Thr} and *Sc*tRNA^{Ser} [20]. The enzyme(s) responsible for methylating $C_{47:3}$ of *Hs*tRNA$^{Ser/Leu(CAG)}$ and C_{20} of *Hs*tRNA$^{Met(CAU)}_e$ remain elusive and it is unclear if the target spectra of *Hs*METTL2A/B/METTL6/METTL8 extend to these sites or whether hitherto unidentified m^3C methyltransferases exist in human cells.

It is striking that in both *T. brucei* and humans, but not mice, specific m^3C methyltransferase paralogs have been identified. *Tb*TRM140a is an active methyltransferase, whereas lack of *Tb*TRM140b was not observed to impact m^3C levels [27]. Similarly, although both *Hs*METTL2A and *Hs*METTL2B are expressed to comparable levels, *Hs*METTL2B displays significantly less catalytic activity *in vitro* [29], implying that *Hs*METTL2A may be functionally predominant. In yeast and humans, the genes encoding the known m^3C methyltransferases are not essential [13,23,28,31,32]; however, their functional importance is emphasized by observations that their perturbation impairs development and/or contributes to various pathogenic conditions (Box 3 and references therein).

Mechanisms of substrate recognition and requirements for methylation activity

Identification of methylation targets of each of the m^3C_{32} methyltransferases suggests that *Sp*Trm141/*Hs*METTL6 are dedicated exclusively to *Sp*/*Hs*tRNA^{Ser}, whereas *Sc*Trm140/*Hs*METTL2A/B/*Hs*METTL8 have broader substrate spectra that encompass more than one type of tRNA isoacceptor [13,20,23,28,30–32,35]. The question therefore arises of how these enzymes achieve substrate specificity, especially as some m^3C_{32} methyltransferases coexist in the same cellular compartments (Table 1). Across eukaryotes, the m^3C_{32}-containing (mt-)tRNAs share some common features but differences are also apparent and most of the shared elements are also found in non-m^3C_{32}-containing (mt-)tRNAs. Identification of features and factors necessary for installation of m^3C_{32} modifications by the different m^3C_{32} methyltransferases reveals a complex interplay of different elements.

Sequence motifs

Recognition of particular sequence motifs is a mechanism commonly employed by RNA modification enzymes to specifically identify appropriate target RNAs within the RNA-dense cellular environment. For example, the m^6A methyltransferase complex of *Hs*METTL3/*Hs*METTL14 preferentially binds **DRACH motifs** and target nucleotides of the pseudouridine synthetase *Hs*PUS7 lie within a UGUAR context [36,37]. Likewise, nucleotides present at specific positions

Figure 1. Schematic tRNAs are shown with m^3C positions highlighted in red. The methyltransferases responsible for introducing the modifications in each species are indicated. Currently unidentified enzymes are indicated by '?'. Modified and unmodified nucleotides within these tRNAs that are required for specific binding and/or methylation by the methyltransferases are marked. Cofactor proteins required for specific target binding and/or methylation are shown in grey. Abbreviations: m^3C, 3-methylcytosine in RNA; mt, mitochondrial; cy, cytoplasmic; tRNA, transfer RNA.

Box 3. m^3C in human disease

Several genetic and functional studies connect m^3C_{32}-installing proteins to human diseases, including inflammatory, neoplastic, metabolic, and developmental pathologies. Changes in m^3C_{32} methyltransferase expression levels are observed in various cancers and, in several cases, the altered methyltransferase levels correlate with differences in prognosis and/or survival. For example, elevated *Hs*METTL8 expression in pancreatic adenocarcinoma is linked to stimulated respiration rates, increased cell proliferation, and high mortality [31]. However, in low-grade gliomas, higher *Hs*METTL8 expression is associated with survival [65]. *Hs*METTL2A/B upregulation also significantly correlates with survival in several neoplasias, including kidney, prostate, breast, and pancreatic cancers [65], whereas in hepatocarcinoma, their overexpression is linked to poor prognosis [66]. Increased *Hs*METTL6 expression in breast and hepatic cancers is associated with poor prognosis [66]. Depletion of *Hs*METTL6 in a hepatic cancer cell line revealed that METTL6 is important for tumor cell growth, which correlates with the observation that patients with low *Hs*METTL6 levels have increased cancer survival rates [28]. Interestingly, depletion of HsMETTL6 in lung cancer cells reduces susceptibility to the chemotherapeutic alkylating agent cisplatin [67]. Beyond changes in expression levels, a number of mutations are observed in genes encoding m^3C_{32} methyltransferases in different tissue-derived neoplasias [68]. For example, *Hs*METTL8 displays a frameshift mutation linked to the development of colon cancer [69].

Several lines of evidence highlight links between m^3C methyltransferases and inflammatory conditions. For example, *Hs*METTL2B and *Hs*METTL6 are downregulated in eosinophils derived from asthmatic patients after a methacholine challenge, potentially connecting m^3C with hypersensitivity responses. Also genome-wide association screens revealed *Hs*METTL2B expression levels are associated with monocyte counts and IL1B levels [70,71].

Alterations in metabolism are also observed upon impairment of m^3C_{32} methyltransferases; *Hs*METTL6 KO mice showed reduced metabolic activity, altered glucose hemostasis, and changes in liver size [28], while *Hs*METTL6 variants are linked to diverticular disease, a condition highly associated with metabolic syndrome [72,73]. Moreover, lack of *Hs*METTL8 perturbs mitochondrial function, thus impacting the metabolic status of cells [31].

A role of m^3C during development is suggested by the findings that loss of *Hs*METTL6 induces differentiation in mouse embryonic stem cells, reducing stemness and inducing early loss of pluripotency [28]. Moreover, the gene encoding *Hs*DALRD3, necessary for m^3C_{32} formation in $tRNA^{Arg(CCU/UCU)}$, is mutated in patients with developmental delay and early onset epileptic encephalopathy [30].

Intriguingly, recent evidence also implicates infection with RNA virus with alterations in cellular m^3C levels [74], but functional consequences of these changes remain to be explored.

within the anticodon loop have emerged as important recognition elements for m^3C_{32} methyltransferases. G_{35} and U_{36} are present in all $tRNA^{Thr}$ isoacceptors containing m^3C_{32} and these nucleotides are both necessary and sufficient for *Sc*Trm140-mediated methylation of C_{32} [35] (Figure 1, upper left). In contrast, the *Sc*$tRNA^{Ser}$ isoacceptors containing m^3C_{32} lack this motif, demonstrating that while nucleotide recognition by *Sc*Trm140 is an important mechanism for $tRNA^{Thr}$ identification, it is not the sole way in which this modification enzyme distinguishes its target RNAs. G_{35} and U_{36} have also been highlighted as important for the methylation activity of *Hs*METTL2A/B on *Hs*$tRNA^{Thr}$ and *Hs*$tRNA^{Arg(CCU/UCU)}$, respectively [22,23] (Figure 1, bottom). In this case, the critical role of G_{35} and U_{36} in discrimination of substrate and nonsubstrate tRNAs by *Hs*METTL2A/B is emphasized by the finding that substitution of C_{35} of $tRNA^{Ser(GCU)}$ with G enables *Hs*METTL2A/B-mediated m^3C_{32} methylation of this nonendogenous substrate and that mutation of U_{36} to G in $tRNA^{Arg(CCU)}$ abrogates *Hs*METTL2A/B-mediated methylation [29,30]. Nucleotide substitutions of U_{34}, G_{35}, and U_{36} of *Hs*mt-$tRNA^{Thr}$, and U_{34} and G_{35} of *Hs*mt-$tRNA^{Ser(UCN)}$, were likewise found to impair or abolish methylation by METTL8 *in vitro* [32]. Intriguingly, substitution of A_{38} for C in both *Hs*$tRNA^{Ser}$ and *Hs*mt-$tRNA^{Ser(UCN)}$ leads to enhanced methylation by *Hs*METTL6 and *Hs*METTL8, respectively, indicating that this position is also recognized by these methyltransferases [29,32]. Interestingly, in *S. cerevisiae*, substitution of A_{38} for U or C leads to mildly reduced binding of *Sc*$tRNA^{Thr}_{(CGU)}$ by *Sc*Trm140 [35] and abolished *Hs*METTL8-mediated methylation of *Hs*mt-$tRNA^{Thr}$ [32], further supporting that this position is recognized by m^3C_{32} methyltransferases, but implying that the precise influence may depend on other elements within the tRNA or the specific enzyme.

Other RNA modifications

Beyond recognizing specific nucleotides within the anticodon loop, it has emerged that the m^3C methyltransferases also sense the presence of modified nucleotides at position 37 of substrate tRNAs. The presence of **N^6-isopentyladenosine (i^6A)** at position 37 of *Sc*tRNA^{Ser} (Figure 1, upper left) and its hypermodified version **2-methylthiol-N^6-isopentyladenosine (ms^2i^6A)** in *Hs*mt-tRNA$^{Ser(UCN)}$ (Figure 1, middle right) strongly enhances m^3C_{32} installation [32,35]. The yeast and human cytoplasmic tRNA$^{Ser(GCU)}$ isoacceptor carries instead **N^6-threonylcarbamoyladenosine (t^6A)** at position 37, as is typical for tRNAs decoding ANN codons, and the presence of this modified nucleotide also enhances C_{32} methylation by METTL6 [29] (Figure 1, bottom). t^6A_{37} is also present in yeast and human cytoplasmic and mitochondrial tRNA^{Thr} isoacceptors, where it similarly enables/increases *Sc*Trm140/*Hs*METTL2A/*Hs*METTL8-mediated C_{32} methylation [29,32,35] (Figure 1, upper left, bottom, and middle right). While all m^3C_{32}-containing tRNAs carry $(ms^2)i^6A_{37}/t^6A_{37}$, these modifications are also present in other (mt-)tRNAs with C_{32}, meaning that although these modifications are important elements for C_{32} methylation, they are not strict determinants.

Dependencies of modification enzymes targeting different nucleotides on prior RNA modifications in their substrates is a recurring theme, with the obligate stepwise 2′-*O*-methylation of C_{32} and G_{34}, and conversion of m^1G_{37} into wybutosine in tRNA^{Phe} being a prominent example in yeast and humans [38,39]. Notably, the requirement of $(ms^2)i^6A_{37}$ for C_{32} methylation typically is more pronounced than the need for t^6A_{37}. Although *Hs*METTL2A-mediated methylation of *Hs*tRNA^{Thr} is negligible in the absence of t^6A_{37}, human cells devoid of the mitochondrial threonylcarbamoyltransferase OSGEPL1 display approximately 65% m^3C_{32} in *Hs*mt-tRNA^{Thr} and m^3C_{32} in *Sc*tRNA^{Thr} is substantially reduced, but not absent, in yeast lacking Sua5 and consequently t^6A_{37} [29,35,40]. Although t^6A_{37} cannot therefore be classified as an essential prerequisite for m^3C_{32}, it remains unclear what the kinetics of these two modification steps are in cells and whether substantial populations of non-t^6A_{37}-containing m^3C_{32} modified tRNA naturally occur. If this is the case, it would be interesting to determine if the presence of m^3C_{32} reciprocally stimulates installation of t^6A_{37}. Interestingly, in *T. brucei*, position 37 of *Tb*tRNA^{Thr} is almost fully modified with t^6A [41] and the presence of m^3C_{32} is indeed a prerequisite for downstream RNA modification events; not only is m^3C_{32} of tRNA^{Thr} a precursor of m^3U_{32}, but also this modification drives A-I editing at position 34 of the tRNA [27] (Figure 1, middle left). In the case of m^3C_{32}, it appears that the influence of other modified nucleotides extends beyond those within the anticodon loop. *S. cerevisiae* cells lacking *Sc*Trm1, which is responsible for installing $m_2^2G_{26}$, display twofold lower levels of m^3C_{32} in *Sc*tRNA$^{Ser(UGA)}$ than those expressing *Sc*Trm1 [17]. This implies that the broader tRNA modification landscape also contributes to efficient installation of m^3C_{32} in cellular tRNAs.

Interplay between substrate binding and methylation

The identification of modified and unmodified anticodon loop nucleotides as important determinants of m^3C_{32} installation raises the question of whether these elements influence modification enzyme binding and/or catalysis. In the case of *Sc*Trm140, only the *Sc*tRNA$^{Thr/Ser}$ substrates are efficiently recovered in binding assays, while other nonsubstrate tRNAs are not, and substitution of the essential G_{35} and U_{36} nucleotides of *Sc*tRNA$^{Thr(CGU)}$ abolished interaction, indicating that, in this case, substrate recognition drives C_{32} methylation [35]. In contrast, *Tb*TRM140a can form interactions with target and nontarget tRNAs and synergistic binding with *Tb*ADAT2/3 leads to C_{32} methylation [42]. Furthermore, *Hs*METTL8 has recently been shown to possess robust, non-substrate-specific RNA binding *in vitro* and no strong preference for i^6A_{37}-containing RNA [32], implying that for this enzyme, the identified specificity elements are more important for methylation activity that RNA binding.

Interactions with other proteins

During the characterization of *Sp*Trm140 and *Sp*Trm141, it was observed that, despite the clear substrate specificity of these two enzymes, depletion of one protein leads to reduced m^3C_{32} levels in the nonsubstrate tRNA, raising the possibility that these enzymes may function co-operatively [26]. However, the first evidence that other proteins can contribute to m^3C methyltransferases accomplishing specificity came with the discovery that the action of *Sc*Trm140 on *Sc*tRNA^{Ser} is enhanced by the presence of the seryl-**tRNA synthetase** (*Sc*SerRS) [35] (Figure 1, upper left). The *in vitro* methylation activity of an *Sc*Trm140 fragment on *Sc*tRNA$^{Ser(CGA/UGA)}$ was increased by the presence of purified *Sc*SerRS and the m^3C_{32} levels of *Sc*tRNA$^{Ser(CGA/UGA)}$ were increased upon overexpression of *Sc*SES1 [35]. *Sc*SerRS binds to the unique long variable loop of *Sc*tRNA^{Ser} and, consistently, this region of the tRNA is also important for *Sc*Trm140-mediated C_{32} methylation. It remains unclear whether *Sc*SerRS directly influences the catalytic activity of *Sc*Trm140 or whether it plays a role in optimizing tRNA^{Ser} structure to enhance *Sc*Trm140-mediated methylation. The human ortholog of *Sc*Trm140, *Hs*METTL6, also robustly coprecipitates the human cytoplasmic seryl-tRNA synthetase *Hs*SARS1 [13,29,43], and it was recently shown that the presence of *Hs*SARS1 increases METTL6-mediated C_{32} methylation of human tRNA$^{Ser(GCU)}$ [29] (Figure 1, bottom). The interdependence of *Sc*Trm140 and *Sc*SerRS, and *Hs*METTL6 and *Hs*SARS1, raises the possibility that installation of m^3C_{32} is coordinated with tRNA^{Ser} aminoacylation, which may be a mechanism of ensuring that only correctly modified tRNAs enter the translation pool. Unlike its cytoplasmic and fungal counterparts, human mt-tRNA$^{Ser(UCN)}$ does not possess an extended variable loop and the mitochondrial seryl-tRNA synthetase *Hs*SARS2 instead interacts via the **D and TΨC loops** [44]. Interestingly, *Hs*SARS2 does not appear to be essential for methylation of C_{32} by METTL8 and lack of m^3C_{32} does not affect aminoacylation of mt-tRNA$^{Ser(UCN)}$ [31,32,75] (Figure 1, middle right).

Beyond its evolutionarily conserved role in modifying *Hs*tRNA^{Thr}, *Hs*METTL2A/B also methylates C_{32} of *Hs*tRNA$^{Arg(CCU/UCU)}$, where they require interaction with *Hs*DALRD3 [30] (Figure 1, bottom). Strikingly, *Hs*DALRD3 has a C terminal domain homologous to the anticodon-binding domain of the arginyl-tRNA synthetase (*Hs*RARS), but in contrast to *Hs*RARS, *Hs*DALRD3 specifically binds *Hs*tRNA$^{Arg(CCU/UCU)}$, displaying preferential interaction with non-m^3C_{32}-containing versions for subsequent methylation. The *Hs*tRNA$^{Arg(CCU/UCU)}$ isoacceptors are unique among the five *Hs*tRNA^{Arg} versions as they possess U_{36} and t^6A_{37}, both also present in *Hs*tRNA^{Thr} isoacceptors, implying that a combination of sequence specificity and protein cofactor-mediated recruitment drive substrate specificity.

In *T. brucei*, m^3C_{32} in *Tb*tRNA^{Thr} is further modified to m^3U_{32} by *Tb*ADAT2/3 and, surprisingly, the enzymes involved in catalyzing these two distinct modification reactions are tightly interlinked [27]. Although methylation of C_{32} is a strict prerequisite for deamination, *Tb*ADAT2/3 is essential for *Tb*TRM140a activity (Figure 1, middle left). The interdependence of these enzymes further extends to the fact that *Tb*TRM140a is required for m^3U_{32} formation in *Tb*tRNA^{Thr} already containing m^3C_{32}.

Functional implications of m^3C tRNA modifications

Despite m^3C being detected in tRNAs approximately 50 years ago, its molecular function(s) have remained challenging to uncover.

Regulation of translation

Studies in *Escherichia coli* highlighted interplay between positions 32 and 38 of tRNA anticodon loops as being important for ribosome binding and translation fidelity [45,46], leading to the notion that m^3C_{32} is likely relevant for translation regulation. In line with this, an observable phenotype of yeast lacking *Sc*Trm140 is a mild sensitivity to the translation inhibitor cycloheximide when the $m^2_2G_{26}$

methyltransferase *Sc*Trm1 is also absent [24]. Similarly, depletion of *Tb*TRM140a leads to impaired cell proliferation and cell death in the presence of cycloheximide [27]. However, it is important to note that yeast lacking *Sc*Trm140 grow as wild type [24], and human m^3C_{32} methyltransferases knockout cell lines are viable [13,28,31,32], implying that effects of m^3C_{32} on translation are mild. Translation of a reporter construct carrying consecutive codons recognized by either m^3C_{32}-containing or m^3C_{32}-lacking *Hs*tRNA^{Arg} isoacceptors coupled to luciferase expression was not significantly affected by lack of the *Hs*METTL2A/B cofactor *Hs*DALRD3 [30]. Similarly, while ribosome profiling in cells lacking *Mm*METTL6 revealed substantial changes in ribosome occupancy compared with wild type cells, these changes were accompanied by extensive alterations in gene expression that accounted for most of the observed differences in ribosome footprints [28]. However, in the mitochondrial system, although loss of METTL8 does not affect *Hs*mt-tRNA$^{Thr/Ser(UCN)}$ aminoacylation or ribosome-association, it does lead to detectable changes in the synthesis of mitochondrial-encoded proteins [31,32]. Mitochondrial ribosome profiling revealed that mitoribosomes in METTL8 knockout cells display longer dwell time on the serine UCA and UCG codons in P-site and UCA in A-site [31]. Although loss of METTL8 does not cause mitoribosome stalling at threonine codons, overexpression of METTL8 leads to enrichment of threonine codons, especially ACC, in the mitoribosomal A-site. These effects manifest in reduced incorporation of the mitochondrial-encoded proteins ND6 and ND1 into the respiratory chain complex I, which correlates with the reduced respiratory chain function observed in cells lacking the catalytic activity of METTL8 [31].

RNA structure

The molecular basis of how m^3C_{32} affects translation remains unclear. However, based on the interplay between positions 32 and 38 with the anticodon loop it has been suggested that m^3C_{32} contributes to maintaining the anticodon loop in an optimal conformation for tRNA function [47,48]. The first experimental evidence supporting this notion came from the recent observation that mt-tRNA$^{Thr/Ser(UCN)}$ from METTL8 knockout cells migrate differently on native polyacrylamide gels than those from wild type cells [32]. Indeed, thermal melting curves, imino proton nuclear magnetic resonance spectroscopy, and circular dichroism measurements of unmodified, m^3C_{32}-containing and m^3C_{32}- and $(ms^2)i^6A_{37}$-containing anticodon stem loops support differences in anticodon stem loop structure, depending on the presence of m^3C_{32} [32]. Although the structure of mt-tRNA$^{Thr/Ser(UCN)}$ is affected by lack of m^3C_{32}, the stability of these mt-tRNAs is not substantially altered. Consistent with the mild effects of lack of these enzymes on translation, this implies that the structural influence of m^3C_{32} is not in preventing gross misfolding but rather that this modification fine-tunes the architecture of the tRNA to optimize its functionality.

Regulation of m^3C modification levels

Several studies report high stoichiometry of m^3C sites in tRNAs, particularly in (mt-)tRNA^{Thr}, suggesting many of these tRNAs are almost fully modified [16,17,31,40]. However, independent transcriptome-wide m^3C mapping techniques also suggest much lower m^3C levels in some tRNAs (Table 1). As in some cases diverging m^3C stoichiometries are reported, it remains to be determined whether these differences represent technical limitations of different detection methods or whether there is extensive biological variation. Interestingly, there is also evidence that the levels of m^3C change in response to certain stress conditions, such as in the presence of alkylating agents [49].

One mechanism via which m^3C levels may be regulated is by the expression levels of the writer methyltransferases. As upregulation of m^3C methyltransferase levels are observed in different types of cancer (Box 3 and references therein), it is possible that normally substoichiometric m^3C modifications are increased upon tumorigenesis. Indeed, this is reported to be the case

for m^3C_{32} in mt-$tRNA^{Ser(UCN)}$ in the pancreatic cancer cell line PNAC-1, where METTL8 levels are elevated compared with noncancerous pancreatic cells [31]. Importantly, m^3C regulation at the level of differential methyltransferase expression only affects nascent tRNAs and thus has intrinsically slow kinetics due to its dependency on tRNA turnover. It still remains unknown how the expression levels of m^3C methyltransferases are regulated in cells and whether activity of these enzymes can also be post-translationally modulated.

A potentially more efficient way of regulating m^3C levels is active demethylation by m^3C erasers. Proteins from the human AlkB family of dioxygenases (*Hs*ALKBH1 and *Hs*ALKBH3) have been show to demethylate m^3C nucleosides and m^3C-containing RNAs *in vitro* [50,51] (Figure 1, bottom). *Hs*ALKBH3 localizes to the cytoplasm but while it shows a robust demethylation activity towards m^1A and m^3C *in vitro*, the effect of loss of *Hs*ALKBH3 on m^3C sites in cellular tRNAs is very modest. It is possible that under normal conditions the activity of *Hs*ALKBH3 towards its cytoplasmic substrates is limited to avoid excessive m^3C removal but it may be that, in particular circumstances, *Hs*ALKBH3 activity is upregulated to more strongly reduce m^3C levels. Interestingly, *Hs*ALKBH3 contributes to cell proliferation and cancer progression [51,52]. Although this could reflect a relevance of altered cellular m^3C levels, it is important to note that *Hs*ALKBH3 also localizes to the nucleus where it removes alkylated 3meC lesions in DNA, which could also affect gene expression and, thus, cell growth [53].

Concluding remarks

Knowledge on the sites of m^3C modifications in cellular RNAs, the enzymes that install them, and their functional relevance has increased significantly in recent years. The identification of the m^3C_{32} methyltransferases has opened the door to mechanistic studies dissecting elements necessary for modification and also highlighted potential links to disease. However, numerous aspects of the m^3C methylome still remain incompletely understood (see Outstanding questions) and future work will likely aim towards answering these open questions. Building on the extensive body of biochemical work on m^3C methyltransferases with structural analyses of these enzymes and their target RNAs will likely reveal how the identified recognition/methylation elements endow substrate specificity and help define what precisely the effects of this modification on tRNA architecture are. A key challenge for the future will be to decipher how dynamic m^3C levels are in different contexts, to elucidate the mechanistic basis of such regulation, and to demonstrate direct functional links between changes in m^3C levels and alterations in gene expression.

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Declaration of interests

No interests are declared.

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Outstanding questions

Are m^3C modifications present in mRNAs? If so, in which transcripts and how do the contexts of such modifications compare with the mapped m^3C sites in tRNAs?

Recent evidence indicates that m^3C_{32} affects the structure of mt-$tRNA^{Thr/Ser(UCN)}$. What precisely are these structural alterations and why are they particularly required in these tRNAs? Is this also the case for other m^3C_{32}-containing tRNAs? Mechanistically, how do these changes in structure affect tRNA function?

What structural features of m^3C methyltransferases endow their specificity for their tRNA substrates? How do the structures of the enzymes that target individual substrates compare with those that are able to methylate several different tRNA types? Are there structural differences between m^3C methyltransferases for which tRNA binding drives methylation and those capable of binding multiple tRNAs?

How do cofactor proteins influence m^3C methyltransferase recruitment and/or activity? What is the mechanistic basis of how *Sc*SerRS and *Hs*SARS1 influence methylation of $tRNA^{Ser}$, and why is HsSARS2 not necessary for HsMETTL8-mediated mt-$tRNA^{Ser(UCN)}$ methylation?

To what extent do m^3C levels in tRNAs vary in different cell types and growth conditions? How accurate are the m^3C stoichiometries estimated using different m^3C mapping techniques? Do alterations in m^3C levels contribute to cell type identity and cellular adaptation by modulating gene expression in different conditions?

Mapping approaches reveal m^3C modifications outside tRNA anticodons, but do the currently known m^3C methyltransferases install these methyl groups or are hitherto unidentified enzymes responsible? What are the functions of these modifications and why are $m^3C_{47:3}$ in type II tRNAs and m^3C_{20} in $tRNA_e^{Met(CAU)}$ only present in mammals but not other eukaryotes?

Are any m^3C modifications in cellular RNAs specifically recognized by

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