The *je ne sais quoi* of 5-methylcytosine in messenger RNA

MARCO GUARNACCI[1] and THOMAS PREISS[1,2]

[1]Shine-Dalgarno Centre for RNA Innovation, Division of Genome Science and Cancer, John Curtin School of Medical Research, Australian National University, Canberra 2601, Australian Capital Territory, Australia

[2]Victor Chang Cardiac Research Institute, Sydney, New South Wales 2010, Australia

ABSTRACT

The potential presence of 5-methylcytosine as a sparse internal modification of mRNA was first raised in 1975, and a first map of the modification was also part of the epitranscriptomics "big bang" in 2012. Since then, the evidence for its presence in mRNA has firmed up, and initial insights have been gained into the molecular function and broader biological relevance of 5-methylcytosine when present in mRNA. Here, we summarize the status quo of the field, outline some of its current challenges, and suggest how to address them in future work.

Keywords: RNA modification; protein synthesis; RNA processing; RNA stability; methyltransferase; RNA-binding protein

INTRODUCTION

The nucleobase 5-methylcytosine (m^5C) is well studied among RNA methylations and abundant within transfer (t)RNAs but also ribosomal (r)RNA (Motorin and Helm 2022). Early evidence indicated the sparse presence of internal m^5C also in mammalian (Dubin and Taylor 1975) and several viral mRNAs (Dubin and Stollar 1975; Sommer et al. 1976; Dubin et al. 1977). Nevertheless, research into the latter was abandoned for decades until bisulfite treatment of cellular RNA combined with high-throughput sequencing (bsRNA-seq) provided a first noisy map of m^5C positions in human mRNA (Squires et al. 2012). With better sequencing and bioinformatic tools, we now have confidence in the presence of typically several hundred m^5C sites in the mRNAs of mammalian somatic cells or cell lines (e.g., Huang et al. 2019; Schumann et al. 2020; Hussain 2021; Dai et al. 2024).

Cells do not express a dedicated mRNA:m^5C methyltransferase (MTase). Instead, mRNAs have been shown to be modified by members of the NOL1/NOP2/SUN domain family (NSUN1–7) of MTases (Reid et al. 1999; Bohnsack et al. 2019; Chen et al. 2021), chiefly but not exclusively, the tRNA:m^5C MTases NSUN2 and NSUN6 (Squires et al. 2012; Yang et al. 2017; Huang et al. 2019; Schumann et al. 2020; Liu et al. 2021a).

The presence of m^5C can influence RNA structure; for example, it affects tRNA folding or codon–anticodon interactions (Motorin et al. 2010; Bohnsack et al. 2019). In contrast, its roles in mRNA are thought to mostly arise through preferential interaction with RNA-binding proteins (RBPs), which then direct mRNA fate, be it splicing (Ma et al. 2023), export from the nucleus (Yang et al. 2017), or stability (Yang et al. 2019b; Chen et al. 2021). An interdependence between m^5C and mRNA translation has also repeatedly been seen (Huang et al. 2019; Schumann et al. 2020; Liu et al. 2021a), although no mediating RBP has yet been described.

In this Perspective, we summarize important developments in key areas of m^5C in mRNA research. For more in-depth information, the reader is directed to other recent review articles (e.g., García-Vílchez et al. 2019; Trixl and Lusser 2019; Chen et al. 2021; Gao and Fang 2021; Guo et al. 2021; Wiener and Schwartz 2021; Gilbert and Nachtergaele 2023; Sun et al. 2023).

TRANSCRIPTOME-WIDE m^5C MAPPING TECHNOLOGIES

The m^5C-Atlas database catalogs m^5C sites from diverse species by reanalyzing published mapping data (Ma et al. 2022) and it also separately lists sites in mRNA for a range of model organisms. Below, we briefly outline the main mapping approaches for m^5C; see Garcia-Vilchez et al. (2019), Trixl and Lusser (2019), Xue et al. (2020), Gao and Fang (2021), and Guo et al. (2021) for a more complete description.

Corresponding author: thomas.preiss@anu.edu.au

Article is online at http://www.rnajournal.org/cgi/doi/10.1261/rna.079982.124. Freely available online through the *RNA* Open Access option.

The bsRNA-seq approach was used to generate most of the currently available data. It can map m^5C candidate sites and their stoichiometry with single-nucleotide precision (Fig. 1A, top). Bisulfite treatment induces quantitative conversion of cytosine to uracil, while m^5C is resistant. It was first combined with primer extension to detect specific tRNA sites (Gu et al. 2005). 454 next-generation sequencing of PCR amplicons to map tRNA and rRNA sites was the next step (Schaefer et al. 2009), before our group combined the treatment with transcriptome-wide RNA-seq based on the now obsolete SOLiD technology (Squires et al. 2012). BsRNA-seq has since been updated to use Illumina technology and more stringent bioinformatic data analysis and applied in a range of organisms and contexts, including plants (Burgess et al. 2015) as well as archaea, bacteria, and yeast (Edelheit et al. 2013). Along the way, the pendulum swung from quite liberal (e.g., Squires et al. 2012; Amort et al. 2017; Yang et al. 2017) to highly stringent site calls that even raised doubt as to whether m^5C sites were present in mRNA at all (Legrand et al. 2017). Rui Zhang and colleagues then established a set of criteria for conversion efficiency, sequence quality, and coverage, as well as cut-off criteria for site stoichiometry (Huang et al. 2019) that found broader acceptance (Chen et al. 2019; Janin et al. 2019; Schumann et al. 2020; Liu et al. 2021a, 2022; Ortiz-Barahona et al. 2023).

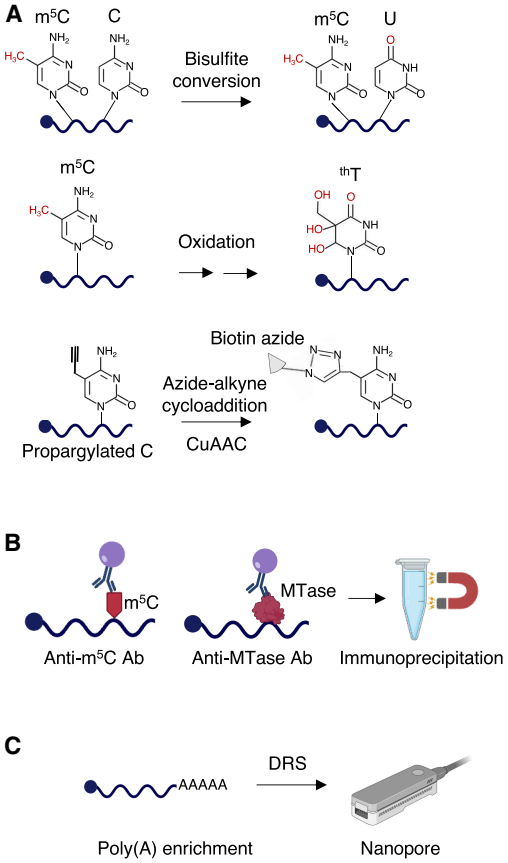

FIGURE 1. m^5C mapping approaches. Schematics of the key principles of current methods are shown. (*A*) Three approaches are based on chemical transformation: bisulfite conversion (*top*) triggers the deamination of nonmethylated cytidines to uridine, while 5-methylcytidine is resistant. TAWO-seq (*middle*) involves two rounds of oxidation of m^5C sites, ultimately leading to a C-to-^{th}T (trihydrooxylated thymidine) conversion; MePMe (*bottom*) relies on generation of a propargylic SAM analog within cells which is then used by MTase enzymes to propargylate their target sites. After cell lysis, propargylate RNA fragments are reacted with biotinazide by click chemistry and enriched on streptavidin-coated magnetic beads. (*B*) Antibody-based methods require immunoprecipitation using an antibody to specifically target either m^5C (meRIP—*left*) or a MTase (miCLIP and Aza-IP—*right*). (*C*) Depiction of a minION sequencer for detection of m^5C by direct poly(A)$^+$ RNA-seq. Some figure panels were created with Biorender.com.

Other m^5C mapping approaches rely on antibodies. m^5C-RNA immunoprecipitation (m^5C-RIP) entails RNA fragmentation followed by immunoprecipitation with anti-m^5C antibody and sequencing of enriched RNA (Fig. 1B, left; Edelheit et al. 2013). m^5C-RIP is universally deployable and can report approximate site positions. It was used in a range of organisms, including archaea (Edelheit et al. 2013), Arabidopsis (Cui et al. 2017; Yang et al. 2019a), mouse (Luo et al. 2023), and human (Luo et al. 2022; Hu et al. 2023; Wang et al. 2023b; Yang et al. 2023).

5-Azacytidine-mediated RNA immunoprecipitation (Aza-IP) (Fig. 1B; Khoddami and Cairns 2013) and methylation-individual nucleotide resolution cross-linking immunoprecipitation (miCLIP) (Fig. 1B, right; Hussain et al. 2013; George et al. 2017) both rely on trapping the RNA:m^5C MTase under investigation as a covalently attached catalytic intermediate before RNA fragmentation, immunoprecipitation, and sequencing. Both methods can directly identify m^5C site coordinates due to frequent C-to-G transversions where the enzyme was covalently bound. Aza-IP relies on prior incorporation of 5-azacytidine into cellular RNA. This cytidine analog then reacts with MTases but inhibits their release from RNA. Aza-IP was used to characterize targets of human NSUN2 and tRNA aspartic acid methyltransferase 1 (TRDMT1) but could be used to identify RNA targets of endogenous RNA:m^5C MTases in any context if 5-azacytidine incorporation can be ensured and suitable anti-MTase antibodies are available. In miCLIP, a tagged and mutated RNA:m^5C MTase is transiently expressed in cells. Mutation of a highly conserved cysteine residue in the catalytic domain to alanine again renders the enzyme unable to release from its RNA target. miCLIP was used to study human NSUN2 (Hussain et al. 2013) and NSUN6 (Van Haute et al. 2016) but could be deployed more broadly as long as mutation and overexpression of the RNA:m^5C MTase is feasible.

Apart from the already mentioned features and advantages, each of the above methods also has its own conceptual and technical limitations. All three antibody-based methods lack the ability to report on the extent of m^5C modification at a given site. While m^5C-RIP is

straightforward and simple, it does not pinpoint m^5C positions and will likely remain hampered by the limited affinity and specificity of available anti-m^5C antibodies. miCLIP and Aza-IP overcome the latter limitations, but they introduce new challenges as they reduce MTases from multiple catalytic cycles to a single encounter with the substrate. For MTases at or near endogenous expression level, this means that only their most abundant substrates might realistically be discoverable. miCLIP compensates for that by overexpression of the enzyme, which might run the risk of collateral methylation of RNAs that would not normally be targeted.

bsRNA-seq in turn is not completely selective for m^5C. While bisulfite treatment was reported to also quantitatively convert 3-methylcytosine, N4-acetylcytosine and 2′-*O*-methylcytidine, 5-hydroxymethylcytosine (hm^5C), and N4,2′-*O*-dimethylcytidine were found to be resistant (Gu et al. 2005; Schaefer et al. 2009; Amort and Lusser 2017). The most severe problem with bsRNA-seq is, however, the threat of false positives due to incomplete conversion of cytosines in structured regions of RNA. Prolonged exposure to harsh thermal and pH conditions is meant to guard against this but in turn causes severe RNA fragmentation. Application of semi-arbitrary cut-offs during data analysis, for example, ≥10% cytosine nonconversion level and exclusion of clustered sites, combined with the sparseness and often substoichiometric level of m^5C in mRNA, can suppress false positives but at the expense of increasing the rate of false negatives.

What is needed are better ways to map m^5C transcriptome-wide, and recent developments indicate that they are now within reach. Ultrafast bisulfite sequencing (UBS-seq) is an optimization of bsRNA-seq (Fig. 1A, top) that features optimized incubation temperature and time to increase m^5C site calling confidence (e.g., ≥5% retained cytosine cut-off) (Dai et al. 2024). Two new methods do not rely on the complete conversion of unmodified cytosines but instead detect an active change at modified sites, thus largely overcoming false positive issues. Tet-assisted peroxotungstate oxidation sequencing (TAWO-seq) converts m^5C via hm^5C to trihydrooxylated thymine, which is detected as a C-to-T conversion (Fig. 1A, middle; Yuan et al. 2019). TAWO-seq has not yet been applied transcriptome-wide. Metabolic propargylation for methylation sequencing (MePMe-seq) maps m^5C as well as m^6A through metabolic labeling with a clickable precursor of *S*-adenosyl-L-methionine, ultimately to cause reverse-transcription stops in short-read sequencing data (Fig. 1A, bottom; Hartstock et al. 2023). It remains to be seen if these new methods can be further developed to also measure the m^5C/C ratio at detected sites.

Many in the epitranscriptomics field are looking to nanopore direct RNA sequencing (DRS) as the panacea for modification mapping (Fig. 1C; Begik et al. 2022). In principle, DRS should be able to distinguish most naturally occurring modifications from "regular" nucleosides without chemical treatment or other manipulation of the input RNA. Given DRS is a long-read technology, it can further identify mRNA isoform-specific modifications and co-occurrence/dependence of modifications within the same mRNA molecule. Nevertheless, the potentially subtle and variable signal changes at the pore necessitate advanced computational tools for accurate and sensitive data interpretation. Thus, the application of DRS and advanced algorithms to m^5C mapping in mRNA has been limited thus far but progress is being made. For example, CHEUI (CH3 [methylation] estimation using ionic current) is a new approach that processes signals with convolutional neural networks to achieve high single-molecule accuracy in detecting m^6A and m^5C sites and quantifying their stoichiometry (Mateos et al. 2024).

ENZYMOLOGY OF m^5C IN mRNA

Humans have eight RNA:m^5C MTases, NSUN1–7 and TRDMT1. While most of these have recognized "canonical" tRNA or rRNA targets (Bohnsack et al. 2019), several have been shown to also methylate mRNA. NSUN2 (Motorin and Grosjean 1999; Brzezicha et al. 2006; Auxilien et al. 2012; Van Haute et al. 2016) was first to be implicated and emerged as the main mRNA:m^5C MTase, with NSUN6 (Haag et al. 2015; Long et al. 2016; Liu et al. 2017) a close second (Squires et al. 2012; Yang et al. 2017; Huang et al. 2019; Schumann et al. 2020; Liu et al. 2021a; Selmi et al. 2021). Very recently, improved bsRNA-seq data analysis uncovered appreciable mRNA methylation by NSUN1 and NSUN5 as well (Liu et al. 2023). Beyond that, there is some evidence for the modification of at least specific mRNAs by Nsun4 (Yang et al. 2022), NSUN7 (Ortiz-Barahona et al. 2023), and TRDMT1 (Xue et al. 2019).

It stands to reason that these enzymes will not abandon their established substrate preferences when encountering mRNA. Indeed, several studies have shown that NSUN2 (Huang et al. 2019; Schumann et al. 2020) as well as NSUN6 (Liu et al. 2021a; Selmi et al. 2021) recognize mRNA regions that resemble their respective tRNA substrates. Rui Zhang and colleagues introduced a scheme to classify m^5C sites in the transcriptome and attribute each to one of four NSUN family members (Fig. 2; Liu et al. 2021a, 2023). NSUN2 is the major m^5C:MTase for tRNAs, modifying several positions in multiple tRNAs, but most commonly at positions C:48–50, methylating up to three consecutive cytosines at the junction between the variable loop and the TψC stem–loop (Bohnsack et al. 2019). NSUN2/Type I sites in mRNA best resemble tRNA C:49 (Fig. 2A). Flanking regions exhibit strong predicted base-pairing to mimic the TψC stem–loop and approximate at least parts of the anticodon stem, with the modified C followed by a 3′ G-rich motif

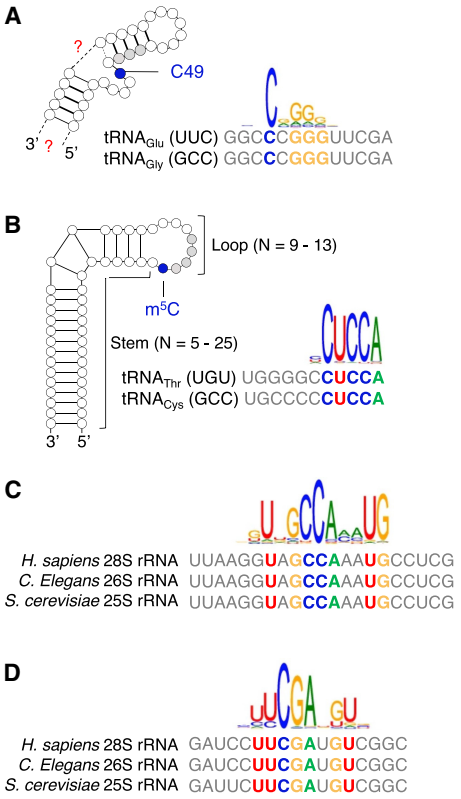

FIGURE 2. mRNA site preferences of m^5C MTases. (*A*) Schematic of secondary structure (*left*; predicted based on neighboring base-pairing propensity; "?" indicates uncertain/broken linkage within mRNA strand) and enriched sequence context (logo on *right*) found for Type I sites modified by human NSUN2 in mRNA. Type I sites mimic the characteristics of C49 situated within the variable loop between two stem sections of NSUN2 tRNA substrates (sequence context of two such tRNA are shown *below* the Type I sequence logo, *right*). (*B*) As in *A* but for Type II sites modified by NSUN6 in mRNA. (*C*) Sequence logo of Type III sites modified by NSUN5 in mRNA (*top*), aligned with the conserved context of its canonical sites in 28S rRNA of *Homo sapiens*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*. (*D*) As in *C* but for Type IV sites modified by NSUN1 in mRNA. Sequence logos in each panel were taken from Liu et al. (2023).

matching the canonical tRNA context (Fig. 2A). NSUN6 modifies position C:72 at the 3′ end of the acceptor stem in specific tRNAs (Bohnsack et al. 2019). NSUN6/Type II sites form a predicted hairpin (Fig. 2B). Based on mutation analyses and modeling of Type II sites into the human NSUN6-tRNA co-crystal structure (Liu et al. 2017), it was shown that the based-paired section mimics the acceptor stem and other key portions of the 3D tRNA structure while the loop correctly presents the CUCCA motif to the enzyme (Liu et al. 2021a). NSUN5/Type III and NSUN1/Type IV sites consist of extended sequence motifs that match the highly conserved contexts of their targets, C:3782 and C:4113, respectively, in relatively unstructured regions of 28s rRNA (Fig. 2C,D; Liu et al. 2023).

Multiple areas of m^5C enzymology deserve further exploration. Not enough is currently known about the remaining NSUN family enzymes and TRDMT1, regarding either their canonical and/or their mRNA targets, to ascertain consensus substrate requirements. It is not clear why NSUN2 near exclusively modifies a single cytosine position in mRNA while it often modifies three consecutive positions within tRNAs. NSUN2-modified sites are enriched in the 5′ region of mRNAs (Liu et al. 2023), while NSUN6 sites are concentrated in the 3′ region (Selmi et al. 2021). What are the additional features/processes guiding each MTase to different mRNA regions? Part of the answer is that 5′ end enrichment of Type I sites in mammals correlates with the evolutionary emergence of more structured 5′ mRNA regions (Liu et al. 2022). Region-specific placement of m^6A follows an exon exclusion mechanism based on steric hindrance by the exon-junction complex (Uzonyi et al. 2023). Could something similar be at play for m^5C?

Further, at what stage in the mRNA life does it become modified? A role of m^5C in mRNA export (Yang et al. 2017) and the occurrence of at least a proportion of m^5C sites in intronic regions (e.g., Schumann et al. 2020) suggest modification during initial pre-mRNA processing, but is this the case for all m^5C sites and all MTases involved? The latter will critically depend on the intracellular localization of the enzyme. NSUN2, NSUN7, and TRDMT1 are at least partly localized in the nucleus, while NSUN6 is a cytoplasmic protein. NSUN1 and NSUN5 are predominantly found in the nucleolus, while NSUN3 and NSUN4 are mitochondrial enzymes (Bohnsack et al. 2019). bsRNA-seq of nuclear and cytoplasmic fractions suggests that NSUN2/Type I sites are made early on during nuclear mRNA processing, while NSUN6/Type II are formed on mature mRNA in the cytoplasm (Liu et al. 2021a); this should be expanded upon in follow-up work. How can the nucleolar MTases have effective encounters with mRNA? The answer might lie in the observation that, contrary to Type I and II sites, NSUN5/Type III and NSUN1/Type IV m^5C sites are rare in common experimental conditions. Instead, they were identified in data from nocodazole-treated HeLa cells (Type III/IV) and early stage vertebrate development (Type III) (Liu et al. 2023). These conditions feature nuclear envelope breakdown allowing persistent contact between nuclear/nucleolar MTases with mRNA in the cytoplasm (Liu et al. 2022).

The expression level of relevant MTases will also affect the pattern and extent of m^5C deposition on mRNAs. For example, during mouse embryogenesis, members of the NSUN family are broadly expressed during gastrulation but take on distinct tissue-selective patterns as development proceeds (Chi and Delgado-Olguín 2013). Several NSUN genes and TRDMT1 are dysregulated in cancer and some other pathologies (García-Vílchez et al. 2019). There is thus much scope in searching for (patho-) physiological conditions where the action of a given MTase on its mRNA is both, more prominent and functionally relevant.

There is also evidence that m^5C can be "erased" in mRNA, with oxidation to hm^5C by the ten to eleven translocation 2 (TET2) dioxygenase as the first step (Shen et al. 2018). If and how this contributes to dynamic transcriptomic changes in m^5C level is not as clear; mRNAs continually turn over and thus changes in de novo methylation can achieve as much.

MOLECULAR FUNCTIONS OF m^5C IN mRNA

Much of the current information on the molecular function of m^5C in mRNA comes from transcriptome-wide bsRNA-seq analyses with cell lines, studying the correlation of m^5C sites with other mRNA features. A relative enrichment of sites near mRNA 5′ ends, that is, around start codons, was seen in mouse and human cells (Amort et al. 2017; Yang et al. 2017; Chen et al. 2019; Schumann et al. 2020; Hartstock et al. 2023), suggesting a link to translation (Fig. 3A) consistent also with a negative correlation between m^5C presence and translation state measured by polysome/ribosome profiling (Huang et al. 2019; Schumann et al. 2020; Liu et al. 2021a). This correlation was strongest for 5′ region sites and weak/absent for 3′ sites (Schumann et al. 2020; Liu et al. 2021a), suggesting context-dependence of m^5C function. The strongest association of 5′ sites with ribosome density, diminished upon NSUN2 inactivation, was again reported very recently (Dai et al. 2024) although, confusingly, this time a positive correlation between m^5C and mRNA translation was seen. Although the above parallels NSUN2's preference for 5′ (Dai et al. 2024) and NSUN6's for 3′ sites (Selmi et al. 2021), the functional link to site location seems stronger than with the MTase responsible, at least in model cell lines such as HeLa and HEK293. Finally, a transcriptome-wide correlation between m^5C site presence and increased mRNA steady-state level, suggesting a role in promoting mRNA stability, was also repeatedly noted (Chen et al. 2019; Yang et al. 2019b; Schumann et al. 2020).

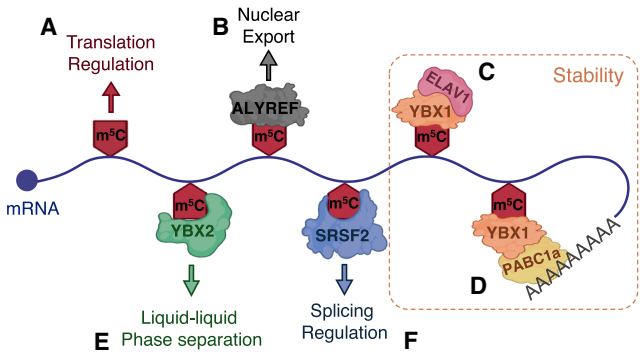

FIGURE 3. Molecular functions of m^5C in mRNA. Each of the proposed functions of m^5C is illustrated along an mRNA schematic. (*A*) Translation efficiency is affected through an unknown intermediary. Several RBPs have been shown to preferentially recognize m^5C in mRNA to affect different outcomes: (*B*) ALYREF mediates nuclear export; YBX1 enhances mRNA stability by interacting with ELAV1 (*C*) or PABC1a (*D*); (*E*) YBX2 mediates liquid–liquid phase separation; (*F*) SRSF2 regulates splicing. Some figure panels were created with Biorender.com.

m^5C function was also tested for individual mRNAs or reporters in specific contexts (Gao and Fang, 2021). For example, NSUN2-mediated methylation in the cyclin-dependent kinase inhibitor p27 mRNA 5′ UTR reduced translation (Tang et al. 2015), while modification of interleukin-17A (IL17a) mRNA in the coding region (Wang et al. 2017) and cyclin-dependent kinase 1 (CDK1) mRNA in the 3′ UTR (Xing et al. 2015) both enhanced translation. NSUN2 enhanced the stability of fatty acid-binding protein 5 (FABP5) mRNA via m^5C methylation during osteosarcoma progression (Yang et al. 2023). NSUN2-deposited methylation at several sites in interferon regulatory factor 3 (IRF3) mRNA decreased its stability, reducing inflammation during viral infection (Wang et al. 2023a). It was reported that NSUN2 and METTL3 cooperatively modify the 3′ UTR of cyclin-dependent kinase inhibitor p21 mRNA to enhance its translation during cellular senescence (Li et al. 2017). In bone marrow-derived mesenchymal stem cells, 3′ UTR located m^5C deposition by NSUN4 cooperates with METTL3-mediated m^6A to enhance SRY-related high-mobility group box 9 (Sox9) mRNA translation (Yang et al. 2022). NSUN1 modification of xeroderma pigmentosum gene D (XPD) mRNA was linked to its elevated level in hepatocarcinoma cells (Sun and Ding 2023). Similarly, NSUN5-dependent methylation of the mRNA for zinc finger BED domain-containing protein 3 (ZBED3) was shown to correlate with enhanced mRNA level in hepatocellular carcinoma (Gu et al. 2024). Several NSUN6-modified sites were transferred to reporter constructs yielding, compared to C-to-T point-mutated controls, slightly decreased expression level (Liu et al. 2021a). NSUN7-mediated methylation in the coding sequence of coiled-coil domain-containing 9B (CCDC9B) mRNA enhanced stability in liver cancer cell lines (Ortiz-Barahona et al. 2023).

The main thrust to identify m^5C molecular function has been through the identification and further study of RBPs that preferentially bind methylated mRNA motifs. Aly/REF export factor (ALYREF) selectively promotes target mRNA export from the nucleus (Fig. 3B; Yang et al. 2017), with recognized roles in controlling cell migration (Xu et al. 2020) and adipogenesis (Liu et al. 2021b). Y-box-binding protein 1 (YBX1) preferentially binds m^5C-decorated mRNAs through its cold shock domain, and this is structurally well understood (Chen et al. 2019; Yang et al. 2019b; Zou et al. 2020a). In human urothelial carcinoma of the bladder (UCB), YBX1 recruits the ELAV-like 1 (ELAVL1) RBP to increase the stability of the mRNA for the oncogene heparin-binding growth factor (HDGF) (Fig. 3C; Chen et al. 2019). In early zebrafish embryos, YBX1 promotes the stability of m^5C-containing maternal

mRNAs through recruitment of the poly(A)-binding protein cytoplasmic 1a (PABC1a) (Fig. 3D; Yang et al. 2019b). Another YBX family member, testis-enriched YBX2, binds m^5C-containing mRNA in a similar mode to YBX1. YBX2 features an intrinsically disordered domain and has propensity to undergo liquid–liquid phase separation. m^5C-containing RNA was shown to promote the formation of YBX2 droplets in vitro. Extending observations to live HeLa cells, YBX2 footprints were enriched near 5′ region m^5C sites deposited by NSUN2, and the formation of YBX2 droplets was diminished after NSUN knockout (Fig. 3E; Wang et al. 2022). Serine/arginine-rich splicing factor 2 (SRSF2) was shown to regulate the splicing of mRNA targets in chronic myeloid leukemia cells (Fig. 3F; Ma et al. 2023).

Despite an abundance of information, there are many open questions regarding the true extent and diversity of molecular functions of m^5C in mRNA. The mRNA context of a given m^5C site (sequence, structure, location relative to other *cis*-acting elements or bound, *trans*-acting RBPs) is likely key. To address this, more reliable and more extensive data on site enrichment along mRNA in different cellular conditions will be needed. Many mappable sites in mRNA are modified only at a low level; why is this? Are only mRNA species with near stoichiometric methylation level likely to show an appreciable effect of the modification? Low-level methylation of a site could just be due to collateral action of MTases present in the cells under investigation. Such sites might have no function in this context but might "echo" physiological scenarios, yet to be studied for m^5C, where the modification level will be much higher. Alternatively, the modification might be present at a high level, and thus consequential, only on minor mRNA (splice-)isoforms or on mRNA molecules in specific functional or subcellular contexts.

It is quite possible that RBPs other than the already recognized examples are affected by the presence of m^5C at, or near, their binding sites. To make a start in this direction, well-curated, larger m^5C site sets could be used to identify coenrichment with footprints of known RBPs, available, for instance, through enhanced UV cross-linking and immunoprecipitation sequencing data sets from ENCODE (Van Nostrand et al. 2020). Several NSUN family members have recurrently been identified by mRNA interactome studies (Yang et al. 2017; Huang et al. 2019; Schumann et al. 2020; Liu et al. 2021a, 2023; Ortiz-Barahona et al. 2023) suggesting that they might "linger" at modification sites and thus themselves affect mRNA function. This is perhaps not such a left-of-field suggestion, given that NSUN enzymes can form covalent complexes with their methylated substrates in a manner regulated by the *S*-adenosylmethionine-to-*S*-adenosylhomocysteine ratio (Moon and Redman 2014) and the precedent of the mRNA:m^6A MTase METTL3 in a similar role (Schumann et al. 2016).

Although mRNA processing, export, stability, and translation have all been implicated in m^5C function, there is still a relative lack of dedicated mechanistic studies with exemplary (model) mRNAs. Prototypical cellular conditions and m^5C site contexts that feature specific molecular functions still need to be identified for such in-depth study, and as seen recently in the m^6A field, may throw up surprises (Guca et al. 2024).

Rather than just being "read out" by an RBP, m^5C might also act more directly through influencing local mRNA structure. Although the methyl group is not on the Watson–Crick hydrogen-bonding face of the nucleobase, it does promote base stacking and can alter RNA structure. Further, depending on context, it can either strengthen canonical hydrogen bonding with guanine or, as in the case with methylated C48 in tRNA, promote an unusual Levitt pairing with G15 to assist in tRNA tertiary folding (Väre et al. 2017; Bohnsack et al. 2019).

Finally, the potential for oxidized forms of m^5C functioning in mRNA is currently under-explored. For example, hm^5C was found in mRNA and suggested to either directly enhance translation (Delatte et al. 2016) or else appear as an intermediate in the removal of m^5C to relieve inhibition of editing by ADAR1 (Shen et al. 2018).

PHYSIOLOGICAL AND PATHOLOGICAL FUNCTIONS OF m^5C IN mRNA

The NSUN MTases are well recognized for their regulation in development, link to genetic disease, and aberrant expression in various cancers, as summarized in Nombela et al. (2021), Bohnsack et al. (2019), and García-Vílchez et al. (2019). For example, NSUN2 mutation causes autosomal-recessive intellectual disability (Khan et al. 2012). Human NSUN2 was first described as a target of the Myc oncogene that is up-regulated in skin carcinomas and a range of tumors (Frye and Watt 2006). The functional studies of m^5C deposition in specific mRNAs described above thus often started by observing the differential expression of one of the NSUN genes. This was then expanded to show the molecular and sometimes also phenotypic effects of preventing m^5C in the selected mRNA. In this way, links to infection, cell cycle and cancer were recurrently made. It remains difficult, however, to exclude the possibility that NSUN alteration drove the observed changes at least in part through dysregulated modification of its canonical tRNA or rRNA substrates.

Studies that reported convergent results with both, manipulating the relevant NSUN MTase as well as the m^5C-sensing RBP, are thus on firmer ground even though these RBPs are also known not to act exclusively through binding m^5C-containing mRNAs. Such is the case with a study investigating UCB (Chen et al. 2019). The authors found that compared to adjacent normal tissue, UCB featured frequent up-regulation of both NSUN2 and YBX1, as well

as mRNA hypermethylation correlating with mRNA overexpression. mRNA hypermethylation was enriched in oncogenic pathways. Focusing on HDGF mRNA they showed that its up-regulation by NSUN2 and YBX1 was through an m^5C site in its 3′ UTR (Fig. 3C). Using a mouse xenograft model they further showed that tumor growth was reduced by either NSUN2 or YBX1 depletion. Importantly, HDGF knockdown similarly inhibited UCB growth, and this could be restored by overexpressing WT HDGF mRNA but not with a point-mutated variant that lacked m^5C in the 3′ UTR (Chen et al. 2019).

Notwithstanding the UCB case above, notable physiological effects are perhaps more likely to be driven by MTase action on multiple mRNA species in a cellular transcriptome. That way, potentially subtle molecular effects on individual transcripts can converge on a strong outcome. Remarkably, bsRNA-seq of developmental stages in six vertebrate and invertebrate species (including flies, fish, frogs, mice, and humans) showed a strong build-up of m^5C sites mainly in maternal mRNAs during oocyte maturation and into fertilized embryos, that declined again rapidly after the maternal-to-zygotic transition. The extent of this m^5C site upswell increased during evolution; higher animals gained many NSUN2-mediated at mRNA 5′ ends, whereas humans were unique in acquiring thousands of NSUN6-mediated sites across the CDS and UTR regions (Liu et al. 2022). NSUN5 sites were also present in data from human oocytes (Liu et al. 2023), and in mice, NSUN5 was shown to be involved in oocyte function through mRNA modification (Ding et al. 2022).

Several lines of evidence indicate a key role of m^5C in maternal mRNA for proper maternal-to-zygotic transition. In *Drosophila*, where NSUN2 is the major MTase for maternal mRNA, knockout of the gene in female flies led to early developmental delay in offspring produced with wild-type males (Liu et al. 2022). This concurs with findings that YBX1 depletion led to early gastrulation arrest during zebrafish embryogenesis (Yang et al. 2019). Further, the *Drosophila* YBX1 homolog Ypsilon schachtel (YPS) is important for germline stem cell production in the ovary (Zou et al. 2020b). Thus, this is another case of convergent phenotypes driven by either NSUN2 or YBX1 depletion.

What we know thus far has confirmed early development, the brain and cancer as scenarios where m^5C in mRNA is highly likely to be of considerable relevance. More broadly, it suggests further searches for physiological scenarios, where changes to mRNA:m^5C MTases, m^5C-sensing RBPs, and m^5C deposition in mRNA of considerable magnitude combine, as promising avenues for an exploration of the biological role of this mRNA modification. An experimental tool of considerable value would be NSUN enzyme mutants that have selectively lost their ability to modify mRNA but not their canonical substrates, either by restricting their intracellular location away from mRNA or through engineering their substrate specificity. An NSUN6 mutant that selectively lost tRNA modification ability has already been described (Liu et al. 2021a).

CONCLUSIONS

As a survey of the literature has demonstrated, there are promising leads for a molecular function of m^5C in mRNA, and how specific RPBs mediate it. These need to be tested further and expanded upon. How the cellular encounters between mRNAs and the relevant MTases are facilitated is a related issue to explore more fully. Whereas mRNA site numbers and their m^5C/C ratio tend to be low in typical cell culture conditions, they can swell to impressive levels in specific biological contexts. We will need better insight into the range of conditions where this occurs, and this will critically depend on promising new developments in accurate transcriptome-wide m^5C mapping technology. Thus, the pursuit of that certain something about m^5C in mRNA should continue and will continue to offer surprises and rewards.

ACKNOWLEDGMENTS

The authors wish to thank Rippei Hayashi and Eduardo Eyras for help with resourcing this work. This research was funded by the Australian Research Council (DP210102385 to T.P., R.H. and E.E.) and by the National Health and Medical Research Council of Australia (APP2018363 and APP1135928 to T.P.).

REFERENCES

Acera Mateos P, Sethi AJ, Ravindran A, Srivastava A, Woodward K, Mahmud S, Kanchi M, Guarnacci M, Xu J, Yuen ZWS, et al. 2024. Prediction of m6A and m5C at single-molecule resolution reveals a co-occurrence of RNA modifications across the transcriptome. *Nat Commun* (in press). doi:10.1101/2022.03.14.484124

Amort T, Lusser A. 2017. Detection of 5-methylcytosine in specific poly(A) RNAs by bisulfite sequencing. *Methods Mol Biol* **1562:** 107–121. doi:10.1007/978-1-4939-6807-7_8

Amort T, Rieder D, Wille A, Khokhlova-Cubberley D, Riml C, Trixl L, Jia XY, Micura R, Lusser A. 2017. Distinct 5-methylcytosine profiles in poly(A) RNA from mouse embryonic stem cells and brain. *Genome Biol* **18:** 1. doi:10.1186/s13059-016-1139-1

Auxilien S, Guérineau V, Szweykowska-Kulińska Z, Golinelli-Pimpaneau B. 2012. The human tRNA m^5C methyltransferase Misu is multisite-specific. *RNA Biol* **9:** 1331–1338. doi:10.4161/rna.22180

Begik O, Mattick JS, Novoa EM. 2022. Exploring the epitranscriptome by native RNA sequencing. *RNA* **28:** 1430–1439. doi:10.1261/rna.079404.122

Bohnsack KE, Höbartner C, Bohnsack MT. 2019. Eukaryotic 5-methylcytosine (m^5C) RNA methyltransferases: mechanisms, cellular functions, and links to disease. *Genes (Basel)* **10:** 102. doi:10.3390/genes10020102

Brzezicha B, Schmidt M, Makałowska I, Jarmołowski A, Pieńkowska J, Szweykowska-Kulińska Z. 2006. Identification of human tRNA: m^5C methyltransferase catalysing intron-dependent m^5C formation in

the first position of the anticodon of the pre-$tRNA^{Leu}_{(CAA)}$. *Nucleic Acids Res* **34:** 6034–6043. doi:10.1093/nar/gkl765

Burgess AL, David R, Searle IR. 2015. Conservation of tRNA and rRNA 5-methylcytosine in the kingdom Plantae. *BMC Plant Biol* **15:** 199. doi:10.1186/s12870-015-0580-8

Chen X, Li A, Sun BF, Yang YYG, Han YN, Yuan X, Chen RX, Wei WS, Liu Y, Gao CC, et al. 2019. 5-methylcytosine promotes pathogenesis of bladder cancer through stabilizing mRNAs. *Nat Cell Biol* **21:** 978–990. doi:10.1038/s41556-019-0361-y

Chen YS, Yang WL, Zhao YL, Yang YG. 2021. Dynamic transcriptomic m^5C and its regulatory role in RNA processing. *Wiley Interdiscip Rev RNA* **12:** e1639. doi:10.1002/wrna.1639

Chi L, Delgado-Olguín P. 2013. Expression of NOL1/NOP2/sun domain (*Nsun*) RNA methyltransferase family genes in early mouse embryogenesis. *Gene Expr Patterns* **13:** 319–327. doi:10.1016/j.gep.2013.06.003

Cui X, Liang Z, Shen L, Zhang Q, Bao S, Geng Y, Zhang B, Leo V, Vardy LA, Lu T, et al. 2017. 5-methylcytosine RNA methylation in *Arabidopsis thaliana*. *Mol Plant* **10:** 1387–1399. doi:10.1016/j.molp.2017.09.013

Dai Q, Ye C, Irkliyenko I, Wang Y, Sun H-L, Gao Y, Liu Y, Beadell A, Perea J, Goel A, et al. 2024. Ultrafast bisulfite sequencing detection of 5-methylcytosine in DNA and RNA. *Nat Biotechnol* **2024:** 1–12. doi:10.1038/s41587-023-02034-w

Delatte B, Wang F, Ngoc LV, Collignon E, Bonvin E, Deplus R, Calonne E, Hassabi B, Putmans P, Awe S, et al. 2016. RNA biochemistry. Transcriptome-wide distribution and function of RNA hydroxymethylcytosine. *Science* **351:** 282–285. doi:10.1126/science.aac5253

Ding C, Lu J, Li J, Hu X, Liu Z, Su H, Li H, Huang B. 2022. RNA-methyltransferase Nsun5 controls the maternal-to-zygotic transition by regulating maternal mRNA stability. *Clin Transl Med* **12:** e1137. doi:10.1002/ctm2.1137

Dubin DT, Stollar V. 1975. Methylation of Sindbis virus "26S" messenger RNA. *Biochem Biophys Res Commun* **66:** 1373–1379. doi:10.1016/0006-291X(75)90511-2

Dubin DT, Taylor RH. 1975. The methylation state of poly A-containing messenger RNA from cultured hamster cells. *Nucleic Acids Res* **2:** 1653–1668. doi:10.1093/nar/2.10.1653

Dubin DT, Stollar V, Hsuchen CC, Timko K, Guild GM. 1977. Sindbis virus messenger RNA: the 5′-termini and methylated residues of 26 and 42 S RNA. *Virology* **77:** 457–470. doi:10.1016/0042-6822(77)90471-8

Edelheit S, Schwartz S, Mumbach MR, Wurtzel O, Sorek R. 2013. Transcriptome-wide mapping of 5-methylcytidine RNA modifications in bacteria, archaea, and yeast reveals m^5C within archaeal mRNAs. *PLoS Genet* **9:** e1003602. doi:10.1371/journal.pgen.1003602

Frye M, Watt FM. 2006. The RNA methyltransferase Misu (NSun2) mediates Myc-induced proliferation and is upregulated in tumors. *Curr Biol* **16:** 971–981. doi:10.1016/j.cub.2006.04.027

Gao Y, Fang J. 2021. RNA 5-methylcytosine modification and its emerging role as an epitranscriptomic mark. *RNA Biol* **18:** 117–127. doi:10.1080/15476286.2021.1950993

García-Vílchez R, Sevilla A, Blanco S. 2019. Post-transcriptional regulation by cytosine-5 methylation of RNA. *Biochim Biophys Acta Gene Regul Mech* **1862:** 240–252. doi:10.1016/j.bbagrm.2018.12.003

George H, Ule J, Hussain S. 2017. Illustrating the epitranscriptome at nucleotide resolution using methylation-iCLIP (miCLIP). *Methods Mol Biol* **1562:** 91–106. doi:10.1007/978-1-4939-6807-7_7

Gilbert WV, Nachtergaele S. 2023. mRNA regulation by RNA modifications. *Annu Rev Biochem* **92:** 175–198. doi:10.1146/annurev-biochem-052521-035949

Gu W, Hurto RL, Hopper AK, Grayhack EJ, Phizicky EM. 2005. Depletion of *Saccharomyces cerevisiae* $tRNA^{His}$ guanylyltransferase Thg1p leads to uncharged $tRNA^{His}$ with additional m^5C. *Mol Cell Biol* **25:** 8191–8201. doi:10.1128/MCB.25.18.8191-8201.2005

Gu X, Li P, Gao X, Ru Y, Xue C, Zhang S, Liu Y, Hu X. 2024. RNA 5-methylcytosine writer NSUN5 promotes hepatocellular carcinoma cell proliferation *via* a ZBED3-dependent mechanism. *Oncogene* **43:** 624–635. doi:10.1038/s41388-023-02931-z

Guca E, Alarcon R, Palo MZ, Santos L, Alonso-Gil S, Davyt M, de Lima LHF, Boissier F, Das S, Zagrovic B, et al. 2024. N^6-methyladenosine in 5′ UTR does not promote translation initiation. *Mol Cell* **84:** 584–595.e6. doi:10.1016/j.molcel.2023.12.028

Guo G, Pan K, Fang S, Ye L, Tong X, Wang Z, Xue X, Zhang H. 2021. Advances in mRNA 5-methylcytosine modifications: detection, effectors, biological functions, and clinical relevance. *Mol Ther Nucleic Acids* **26:** 575–593. doi:10.1016/j.omtn.2021.08.020

Haag S, Warda AS, Kretschmer J, Günnigmann MA, Höbartner C, Bohnsack MT. 2015. NSUN6 is a human RNA methyltransferase that catalyzes formation of m^5C72 in specific tRNAs. *RNA* **21:** 1532–1543. doi:10.1261/rna.051524.115

Hartstock K, Kueck NA, Spacek P, Ovcharenko A, Hüwel S, Cornelissen NV, Bollu A, Dieterich C, Rentmeister A. 2023. MePMe-seq: antibody-free simultaneous m^6A and m^5C mapping in mRNA by metabolic propargyl labeling and sequencing. *Nat Commun* **14:** 7154. doi:10.1038/s41467-023-42832-z

Hu S, Yang M, Xiao K, Yang Z, Cai L, Xie Y, Wang L, Wei R. 2023. Loss of NSUN6 inhibits osteosarcoma progression by downregulating EEF1A2 expression and activation of Akt/mTOR signaling pathway via m^5C methylation. *Exp Ther Med* **26:** 457. doi:10.3892/etm.2023.12156

Huang T, Chen W, Liu J, Gu N, Zhang R. 2019. Genome-wide identification of mRNA 5-methylcytosine in mammals. *Nat Struct Mol Biol* **26:** 380–388. doi:10.1038/s41594-019-0218-x

Hussain S. 2021. The emerging roles of cytosine-5 methylation in mRNAs. *Trends Genet* **37:** 498–500. doi:10.1016/j.tig.2021.02.001

Hussain S, Sajini AA, Blanco S, Dietmann S, Lombard P, Sugimoto Y, Paramor M, Gleeson JG, Odom DT, Ule J, et al. 2013. NSun2-mediated cytosine-5 methylation of vault noncoding RNA determines its processing into regulatory small RNAs. *Cell Rep* **4:** 255–261. doi:10.1016/j.celrep.2013.06.029

Janin M, Ortiz-Barahona V, de Moura MC, Martínez-Cardús A, Llinàs-Arias P, Soler M, Nachmani D, Pelletier J, Schumann U, Calleja-Cervantes ME, et al. 2019. Epigenetic loss of RNA-methyltransferase NSUN5 in glioma targets ribosomes to drive a stress adaptive translational program. *Acta Neuropathol* **138:** 1053–1074. doi:10.1007/s00401-019-02062-4

Khan MA, Rafiq MA, Noor A, Hussain S, Flores J V, Rupp V, Vincent AK, Malli R, Ali G, Khan FS, et al. 2012. Mutation in NSUN2, which encodes an RNA methyltransferase, causes autosomal-recessive intellectual disability. *Am J Hum Genet* **90:** 856–863. doi:10.1016/j.ajhg.2012.03.023

Khoddami V, Cairns BR. 2013. Identification of direct targets and modified bases of RNA cytosine methyltransferases. *Nat Biotechnol* **31:** 458–464. doi:10.1038/nbt.2566

Legrand C, Tuorto F, Hartmann M, Liebers R, Jacob D, Helm M, Lyko F. 2017. Statistically robust methylation calling for whole-transcriptome bisulfite sequencing reveals distinct methylation patterns for mouse RNAs. *Genome Res* **27:** 1589–1596. doi:10.1101/gr.210666.116

Li Q, Li X, Tang H, Jiang B, Dou Y, Gorospe M, Wang W. 2017. NSUN2-mediated m5C methylation and METTL3/METTL14-mediated m6A methylation cooperatively enhance p21 translation. *J Cell Biochem* **118:** 2587–2598. doi:10.1002/jcb.25957

Liu RJ, Long T, Li J, Li H, Wang ED. 2017. Structural basis for substrate binding and catalytic mechanism of a human RNA:m^5C methyltransferase NSun6. *Nucleic Acids Res* **45:** 6684–6697. doi:10.1093/nar/gkx473

Liu J, Huang T, Zhang Y, Zhao T, Zhao X, Chen W, Zhang R. 2021a. Sequence- and structure-selective mRNA m^5C methylation by NSUN6 in animals. *Natl Sci Rev* **8:** nwaa273. doi:10.1093/nsr/nwaa273

Liu Y, Zhao Y, Wu R, Chen Y, Chen W, Liu Y, Luo Y, Huang C, Zeng B, Liao X, et al. 2021b. mRNA m5C controls adipogenesis by promoting CDKN1A mRNA export and translation. *RNA Biol* **18:** 711–721. doi:10.1080/15476286.2021.1980694

Liu J, Huang T, Chen W, Ding C, Zhao T, Zhao X, Cai B, Zhang Y, Li S, Zhang L, et al. 2022. Developmental mRNA m^5C landscape and regulatory innovations of massive m^5C modification of maternal mRNAs in animals. *Nat Commun* **13:** 2484. doi:10.1038/s41467-022-30210-0

Liu J, Huang T, Yao J, Zhao T, Zhang Y, Zhang R. 2023. Epitranscriptomic subtyping, visualization, and denoising by global motif visualization. *Nat Commun* **14:** 1–16. doi:10.1038/s41467-023-41653-4

Long T, Li J, Li H, Zhou M, Zhou XL, Liu RJ, Wang ED. 2016. Sequence-specific and shape-selective RNA recognition by the human RNA 5-methylcytosine methyltransferase NSun. *J Biol Chem* **291:** 24293–24303. doi:10.1074/jbc.M116.742569

Luo G, Xu W, Chen X, Wang S, Wang J, Dong F, Hu DN, Reinach PS, Yan D. 2022. NSUN2-mediated RNA m^5C modification modulates uveal melanoma cell proliferation and migration. *Epigenetics* **17:** 922–933. doi:10.1080/15592294.2022.2088047

Luo G, Xu W, Chen X, Xu W, Yang S, Wang J, Lin Y, Reinach PS, Yan D. 2023. The RNA m^5C methylase NSUN2 modulates corneal epithelial wound healing. *Invest Ophthalmol Vis Sci* **64:** 5. doi:10.1167/iovs.64.3.5

Ma J, Song B, Wei Z, Huang D, Zhang Y, Su J, De Magalhães JP, Rigden DJ, Meng J, Chen K. 2022. m^5C-Atlas: a comprehensive database for decoding and annotating the 5-methylcytosine (m^5C) epitranscriptome. *Nucleic Acids Res* **50:** D196–D203. doi:10.1093/nar/gkab1075

Ma HL, Bizet M, Soares Da Costa C, Murisier F, de Bony EJ, Wang MK, Yoshimi A, Lin KT, Riching KM, Wang X, et al. 2023. SRSF2 plays an unexpected role as reader of m^5C on mRNA, linking epitranscriptomics to cancer. *Mol Cell* **83:** 4239–4254.e10. doi:10.1016/j.molcel.2023.11.003

Moon HJ, Redman KL. 2014. Trm4 and Nsun2 RNA:m^5C methyltransferases form metabolite-dependent, covalent adducts with previously methylated RNA. *Biochemistry* **53:** 7132–7144. doi:10.1021/bi500882b

Motorin Y, Grosjean H. 1999. Multisite-specific tRNA:m^5C-methyltransferase (Trm4) in yeast *Saccharomyces cerevisiae*: identification of the gene and substrate specificity of the enzyme. *RNA* **5:** 1105–1118. doi:10.1017/S1355838299982201

Motorin Y, Helm M. 2022. RNA nucleotide methylation: 2021 update. *Wiley Interdiscip Rev RNA* **13:** e1691. doi:10.1002/wrna.1691

Motorin Y, Lyko F, Helm M. 2010. 5-methylcytosine in RNA: detection, enzymatic formation and biological functions. *Nucleic Acids Res* **38:** 1415–1430. doi:10.1093/nar/gkp1117

Nombela P, Miguel-López B, Blanco S. 2021. The role of m^6A, m^5C and Ψ RNA modifications in cancer: novel therapeutic opportunities. *Mol Cancer* **20:** 1–30. doi:10.1186/s12943-020-01263-w

Ortiz-Barahona V, Soler M, Davalos V, García-Prieto CA, Janin M, Setien F, Fernández-Rebollo I, Bech-Serra JJ, De La Torre C, Guil S, et al. 2023. Epigenetic inactivation of the 5-methylcytosine RNA methyltransferase NSUN7 is associated with clinical outcome and therapeutic vulnerability in liver cancer. *Mol Cancer* **22:** 83. doi:10.1186/s12943-023-01785-z

Reid R, Greene PJ, Santi DV. 1999. Exposition of a family of RNA m^5C methyltransferases from searching genomic and proteomic sequences. *Nucleic Acids Res* **27:** 3138–3145. doi:10.1093/nar/27.15.3138

Schaefer M, Pollex T, Hanna K, Lyko F. 2009. RNA cytosine methylation analysis by bisulfite sequencing. *Nucleic Acids Res* **37:** e12. doi:10.1093/nar/gkn954

Schumann U, Shafik A, Preiss T. 2016. METTL3 gains R/W access to the epitranscriptome. *Mol Cell* **62:** 323–324. doi:10.1016/j.molcel.2016.04.024

Schumann U, Zhang H-NN, Sibbritt T, Pan A, Horvath A, Gross S, Clark SJ, Yang L, Preiss T. 2020. Multiple links between 5-methylcytosine content of mRNA and translation. *BMC Biol* **18:** 1–23. doi:10.1186/s12915-020-00769-5

Selmi T, Hussain S, DIetmann S, Heiß M, Borland K, Flad S, Carter JM, Dennison R, Huang YL, Kellner S, et al. 2021. Sequence- and structure-specific cytosine-5 mRNA methylation by NSUN6. *Nucleic Acids Res* **49:** 1006–1022. doi:10.1093/nar/gkaa1193

Shen Q, Zhang Q, Shi Y, Shi Q, Jiang Y, Gu Y, Li Z, Li X, Zhao K, Wang C, et al. 2018. Tet2 promotes pathogen infection-induced myelopoiesis through mRNA oxidation. *Nature* **554:** 123–127. doi:10.1038/nature25434

Sommer S, Salditt-Georgieff M, Bachenheimer S, Darnell J, Furuichi Y, Morgan M, Shatkin A. 1976. The methylation of adenovirus-specific nuclear and cytoplasmic RNA. *Nucleic Acids Res* **3:** 749–766. doi:10.1093/nar/3.3.749

Squires JE, Patel HR, Nousch M, Sibbritt T, Humphreys DT, Parker BJ, Suter CM, Preiss T. 2012. Widespread occurrence of 5-methylcytosine in human coding and non-coding RNA. *Nucleic Acids Res* **40:** 5023–5033. doi:10.1093/nar/gks144

Sun GF, Ding H. 2023. NOP2-mediated m5C methylation of XPD is associated with hepatocellular carcinoma progression. *Neoplasma* **70:** 340–349. doi:10.4149/neo_2023_230110N17

Sun H, Li K, Liu C, Yi C. 2023. Regulation and functions of non-m^6A mRNA modifications. *Nat Rev Mol Cell Biol* **24:** 714–731. doi:10.1038/s41580-023-00622-x

Tang H, Fan X, Xing J, Liu Z, Jiang B, Dou Y, Gorospe M, Wang W. 2015. NSun2 delays replicative senescence by repressing p27 (KIP1) translation and elevating CDK1 translation. *Aging* **7:** 1143–1155. doi:10.18632/aging.100860

Trixl L, Lusser A. 2019. The dynamic RNA modification 5-methylcytosine and its emerging role as an epitranscriptomic mark. *Wiley Interdiscip Rev RNA* **10:** e1510. doi:10.1002/wrna.1510

Uzonyi A, Dierks D, Nir R, Kwon OS, Toth U, Barbosa I, Burel C, Brandis A, Rossmanith W, Le Hir H, et al. 2023. Exclusion of m6A from splice-site proximal regions by the exon junction complex dictates m6A topologies and mRNA stability. *Mol Cell* **83:** 237–251.e7. doi:10.1016/j.molcel.2022.12.026

Van Haute L, Dietmann S, Kremer L, Hussain S, Pearce SF, Powell CA, Rorbach J, Lantaff R, Blanco S, Sauer S, et al. 2016. Deficient methylation and formylation of mt-$tRNA^{Met}$ wobble cytosine in a patient carrying mutations in NSUN3. *Nat Commun* **7:** 12039–12039. doi:10.1038/ncomms12039

Van Nostrand EL, Freese P, Pratt GA, Wang X, Wei X, Xiao R, Blue SM, Chen J-YY, Cody NAL, Dominguez D, et al. 2020. A large-scale binding and functional map of human RNA-binding proteins. *Nature* **583:** 711–719. doi:10.1038/s41586-020-2077-3

Väre VYP, Eruysal ER, Narendran A, Sarachan KL, Agris PF. 2017. Chemical and conformational diversity of modified nucleosides affects tRNA structure and function. *Biomolecules* **7:** 29. doi:10.3390/BIOM7010029

Wang N, Tang H, Wang X, Wang W, Feng J. 2017. Homocysteine upregulates interleukin-17A expression via NSun2-mediated RNA methylation in T lymphocytes. *Biochem Biophys Res Commun* **493:** 94–99. doi:10.1016/j.bbrc.2017.09.069

Wang X, Wang M, Dai X, Han X, Zhou Y, Lai W, Zhang L, Yang Y, Chen Y, Wang H, et al. 2022. RNA 5-methylcytosine regulates YBX2-dependent liquid-liquid phase separation. *Fundam Res* **2:** 48–55. doi:10.1016/j.fmre.2021.10.008

Wang H, Feng J, Zeng C, Liu J, Fu Z, Wang D, Wang Y, Zhang L, Li J, Jiang A, et al. 2023a. NSUN2-mediated m^5c methylation of IRF3 mRNA negatively regulates type I interferon responses during various viral infections. *Emerg Microbes Infect* **12:** 2178238. doi:10.1080/22221751.2023.2178238

Wang N, Chen RX, Deng MH, Wei WS, Zhou ZH, Ning K, Li YH, Li XD, Ye YL, Wen JH, et al. 2023b. m^5C-dependent cross-regulation between nuclear reader ALYREF and writer NSUN2 promotes urothelial bladder cancer malignancy through facilitating RABL6/TK1 mRNAs splicing and stabilization. *Cell Death Dis* **14:** 139. doi:10.1038/s41419-023-05661-y

Wiener D, Schwartz S. 2021. The epitranscriptome beyond m^6A. *Nat Rev Genet* **22:** 119–131. doi:10.1038/s41576-020-00295-8

Xing J, Yi J, Cai X, Tang H, Liu Z, Zhang X, Martindale JL, Yang X, Jiang B, Gorospe M, et al. 2015. NSun2 promotes cell growth via elevating cyclin-dependent kinase 1 translation. *Mol Cell Biol* **35:** 4043–4052. doi:10.1128/MCB.00742-15

Xu X, Zhang Y, Zhang J, Zhang X. 2020. NSun2 promotes cell migration through methylating autotaxin mRNA. *J Biol Chem* **295:** 18134–18147. doi:10.1074/jbc.RA119.012009

Xue S, Xu H, Sun Z, Shen H, Chen S, Ouyang J, Zhou Q, Hu X, Cui H. 2019. Depletion of TRDMT1 affects 5-methylcytosine modification of mRNA and inhibits HEK293 cell proliferation and migration. *Biochem Biophys Res Commun* **520:** 60–66. doi:10.1016/j.bbrc.2019.09.098

Xue C, Zhao Y, Li L. 2020. Advances in RNA cytosine-5 methylation: detection, regulatory mechanisms, biological functions and links to cancer. *Biomark Res* **8:** 1–13. doi:10.1186/s40364-019-0180-0

Yang X, Yang YY-G, Sun B-F, Chen Y-S, Xu J-W, Lai W-Y, Li A, Wang X, Bhattarai DP, Xiao W, et al. 2017. 5-methylcytosine promotes mRNA export—NSUN2 as the methyltransferase and ALYREF as an m^5C reader. *Cell Res* **27:** 606–625. doi:10.1038/cr.2017.55

Yang L, Perrera V, Saplaoura E, Apelt F, Bahin M, Kramdi A, Olas J, Mueller-Roeber B, Sokolowska E, Zhang W, et al. 2019a. m^5C methylation guides systemic transport of messenger RNA over graft junctions in plants. *Curr Biol* **29:** 2465–2476.e5. doi:10.1016/j.cub.2019.06.042

Yang Y, Wang L, Han X, Yang WL, Zhang M, Ma HL, Sun BF, Li A, Xia J, Chen J, et al. 2019b. RNA 5-methylcytosine facilitates the maternal-to-zygotic transition by preventing maternal mRNA decay. *Mol Cell* **75:** 1188–1202.e11. doi:10.1016/j.molcel.2019.06.033

Yang L, Ren Z, Yan S, Zhao L, Liu J, Zhao L, Li Z, Ye S, Liu A, Li X, et al. 2022. Nsun4 and Mettl3 mediated translational reprogramming of Sox9 promotes BMSC chondrogenic differentiation. *Commun Biol* **5:** 1–13. doi:10.1038/s42003-021-02997-z

Yang M, Wei R, Zhang S, Hu S, Liang X, Yang Z, Zhang C, Zhang Y, Cai L, Xie Y. 2023. NSUN2 promotes osteosarcoma progression by enhancing the stability of FABP5 mRNA via m^5C methylation. *Cell Death Dis* **14:** 125. doi:10.1038/s41419-023-05646-x

Yuan F, Bi Y, Siejka-Zielinska P, Zhou YL, Zhang XX, Song CX. 2019. Bisulfite-free and base-resolution analysis of 5-methylcytidine and 5-hydroxymethylcytidine in RNA with peroxotungstate. *Chem Commun* **55:** 2328–2331. doi:10.1039/C9CC00274J

Zou F, Tu R, Duan B, Yang Z, Ping Z, Song X, Chen S, Price A, Li H, Scott A, et al. 2020a. *Drosophila* YBX1 homolog YPS promotes ovarian germ line stem cell development by preferentially recognizing 5-methylcytosine RNAs. *Proc Natl Acad Sci* **117:** 3603–3609. doi:10.1073/pnas.1910862117

Zou F, Tu R, Duan B, Yang Z, Ping Z, Song X, Chen S, Price A, Li H, Scott A, et al. 2020b. *Drosophila* YBX1 homolog YPS promotes ovarian germ line stem cell development by preferentially recognizing 5-methylcytosine RNAs. *Proc Natl Acad Sci* **117:** 3603–3609. doi:10.1073/pnas.1910862117