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The cap epitranscriptome: Early directions to a complex life as mRNA

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Abstract

Animal, protist and viral messenger RNAs (mRNAs) are most prominently modified at the beginning by methylation of cap-adjacent nucleotides at the 2′-*O*-position of the ribose (cOMe) by dedicated cap methyltransferases (CMTrs). If the first nucleotide of an mRNA is an adenosine, PCIF1 can methylate at the N^6-position (m^6A), while internally the Mettl3/14 writer complex can methylate. These modifications are introduced co-transcriptionally to affect many aspects of gene expression including localisation to synapses and local translation. Of particular interest, transcription start sites of many genes are heterogeneous leading to sequence diversity at the beginning of mRNAs, which together with cOMe and m^6Am could constitute an extensive novel layer of gene expression control. Given the role of cOMe and m^6A in local gene expression at synapses and higher brain functions including learning and memory, such code could be implemented at the transcriptional level for lasting memories through local gene expression at synapses.

KEYWORDS
capping, CMTr, FMRP, m6A, Mettl3, mRNA modifications, 2′-*O*-ribose methylation, YTHDF

INTRODUCTION

In recent years several nucleotide modifications in messenger RNA (mRNA) have emerged as important regulators of gene expression at multiple levels in the nucleus and cytoplasm.[1,2] These modifications can be broadly classified as cap modifications and internal modifications based on whether they occur at the beginning or the body of the mRNA (5′UTR, 3′UTR and coding region).[3] Among the most prominent modifications are methylation at the 2′-*O*-position of the ribose, mostly of cap adjacent nucleotides (cOMe), methylation of adenosine at the N^6position (m^6A), methylation of cytosine (m^5C), editing of adenosine by deamination into inosine and conversion of uridine to pseudouridine. Other more rare modifications include internal 7-methylguanosine (m^7G), m^1A (N^1-methyladenosine), N^6,N^6-dimethyladenosine ($m^6{}_2A$), ac4 (N^4-acetylcytidine), m^3C (3-methylcytidine), hm^5C (5-hydroxymethylcytidine), 8-oxoG (7,8-dihydro-8-oxoguanosine) and C-U editing.[3,4]

A striking feature of the main mRNA methylation pathways (cOMe and m^6A) is their co-transcriptional installation in the first steps of mRNA processing. As shown for m^6A, this can affect splicing,[5–9] but also regulates expression far away from the nucleus at dendrites and synapses.[5,10,11] Despite its early installation in gene expression, roles for cOMe prominently include regulation of translation and localisation of untranslated mRNAs to synapses.[12–14] Hence, the main importance of cOMe may lay in connection with the heterogeneity of transcription start sites impacting on later steps in gene expression including local translation at synapses. Here, we review our current understanding of the role of cOMe in gene expression with a particular

Abbreviations: CMTr, cap methyltransferase; IFIT, interferon-induced protein with tetratricopeptide repeats; TOP, terminal oligopyrimidine tract.

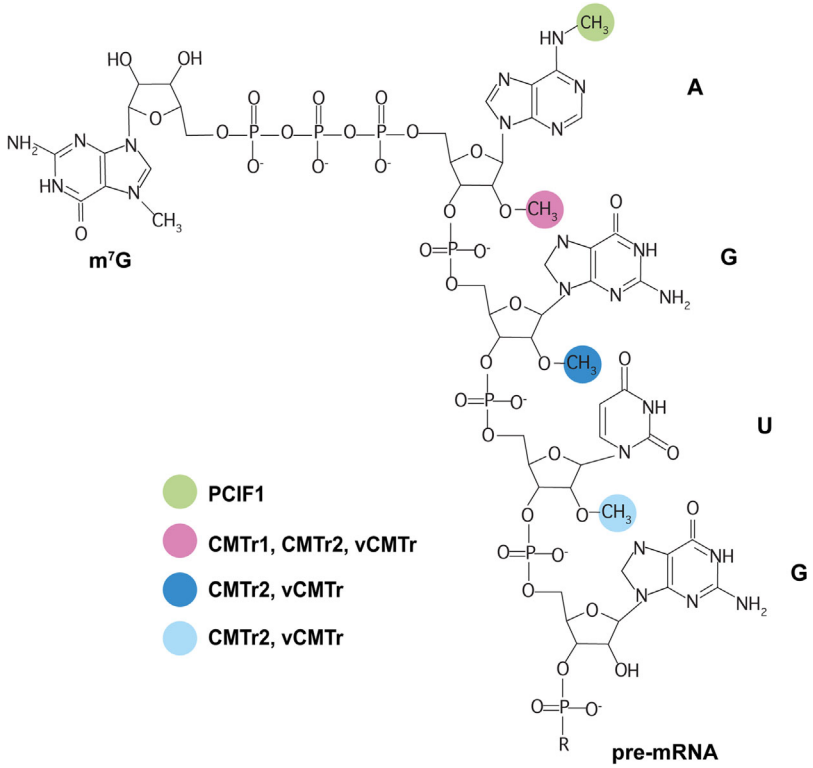

FIGURE 1 The mRNA cap structure consisting of a 5′-5′ linked m^7G. If the first nucleotide of an mRNA is an adenosine, it can be *N6*-methylated by PCIF1 (green). The ribose can be methylated at the 2′-*O*-position by CMTr1, CMTr2 and vCMTr at the first position (purple) and at the second position by CMTr2 and vCMTr (dark blue). For a transcript starting with the sequence AGUG, human CMTr2 and vCMTr (light blue) can add 2′-*O*-methylation in vitro.[14,19] m^7G, 7-methylguanosine; CMTr, cap methyltransferase; mRNA, messenger RNA; vCMTr, vaccinia CMTr.

emphasis on local gene expression at synapses and its convergence with m^6A regulation by its YTH reader and fragile X mental retardation protein (FMRP) in regulating local translation.

CO-TRANSCRIPTIONAL INSTALLATION OF CAP-ADJACENT 2′-*O*-RIBOSE METHYLATION

After transcription initiation by RNA Pol II, the first step in processing of an mRNA includes the addition of a cap to the first nucleotide consisting of inversely added guanosine in a characteristic 5′-5 linkage (Figure 1). Subsequently, cap-adjacent nucleotides predominantly at first and second positions can then be methylated at the ribose (2-*O*-ribose methylation, cOMe, Figure 1).[12,13,15,16] Generally, the first nucleotide is methylated to a high level in insect and vertebrate model organisms and human cell lines. In contrast, only about half of the second nucleotides are methylated to various degrees between tissues and transcripts in fish and mice, and human cell lines, while in *Drosophila* cOMe is mainly present on the first nucleotide.[17–20]

cOMe is introduced by dedicated cap methyltransferases (CMTrs) that bind to the cap structure (Figure 1).[21–23] Animals generally have two *CMTr* genes (*CMTr1* and *CMTr2*) which introduce cOMe on the first two nucleotides, while trypanosomes have three CMTrs, which introduce cOMe on the first four nucleotides.[24] Moreover, many viruses including corona viruses have their own *CMTr* gene.[25,26] For instance, the CMTr from smallpox virus (vaccinia) preferentially adds cOMe to the first nucleotide but can also methylate additional nucleotides (Figure 1).[14,19] Structural comparisons between methyltransferase domains of animal, trypanosomes and viruses revealed strong similarities between both animal CMTrs and CMTr1 from trypanosomes, while the methyltransferase domains of viral CMTrs are more divergent and align best with CMTr2 and 3 from trypanosomes.[19] Accordingly, both *Drosophila* and human CMTrs can methylated the first cap-adjacent nucleotide, while CMTr2 seems kinetically slower to methylate the second cap-adjacent nucleotide.[19]

In animals, CMTr1 interacts with RNA Pol II, is primarily nuclear, and in *Drosophila* it localises to sites of transcription. Even though CMTr1 and 2 act redundantly in *Drosophila*, CMTr2 localises to distinct sites on polytene chromosomes suggesting target preference but based on its primarily cytoplasmic localisation potentially could add cOMe later to regulate gene expression.[14,19]

In vertebrates and some other organisms, additional substantial cap adjacent methylation can be introduced by PCIF1 on adenosine when it is the first nucleotide of the mRNA (Figure 1). However, the mechanism for cap adenosine N^6-methylation is different from internal methylation of adenosine.[18,27–34] Levels of m^6Am vary between different fish and mouse tissues, but intriguingly, even though insects such as *Drosophila* and honeybees have a PCIF1 gene, no m^6Am is detectable in polyA mRNA.[19]

Like cOMe, other mRNA modifications such as m^6A, A-to-I editing and pseudouridine also occur co-transcriptionally and before splicing.[14,33,35] Installation of internal m^6A is directed by Mettl3 as part of a conserved 900 kDa complex including Mettl14, Fl(2)d (WTAP), Virilizer (Virma), Flacc, Nito (RBM15) and Hakai in a consensus DRACH motif (D: A, G or U; R: A and G; H: A, C or U).[33,36,37] A-to-I editing by ADARs requires double-stranded RNA often forming between exons and distal sites in introns,[38] while pseudouridine synthase localisation to the nucleus directs conversion before splicing,[] but regulation

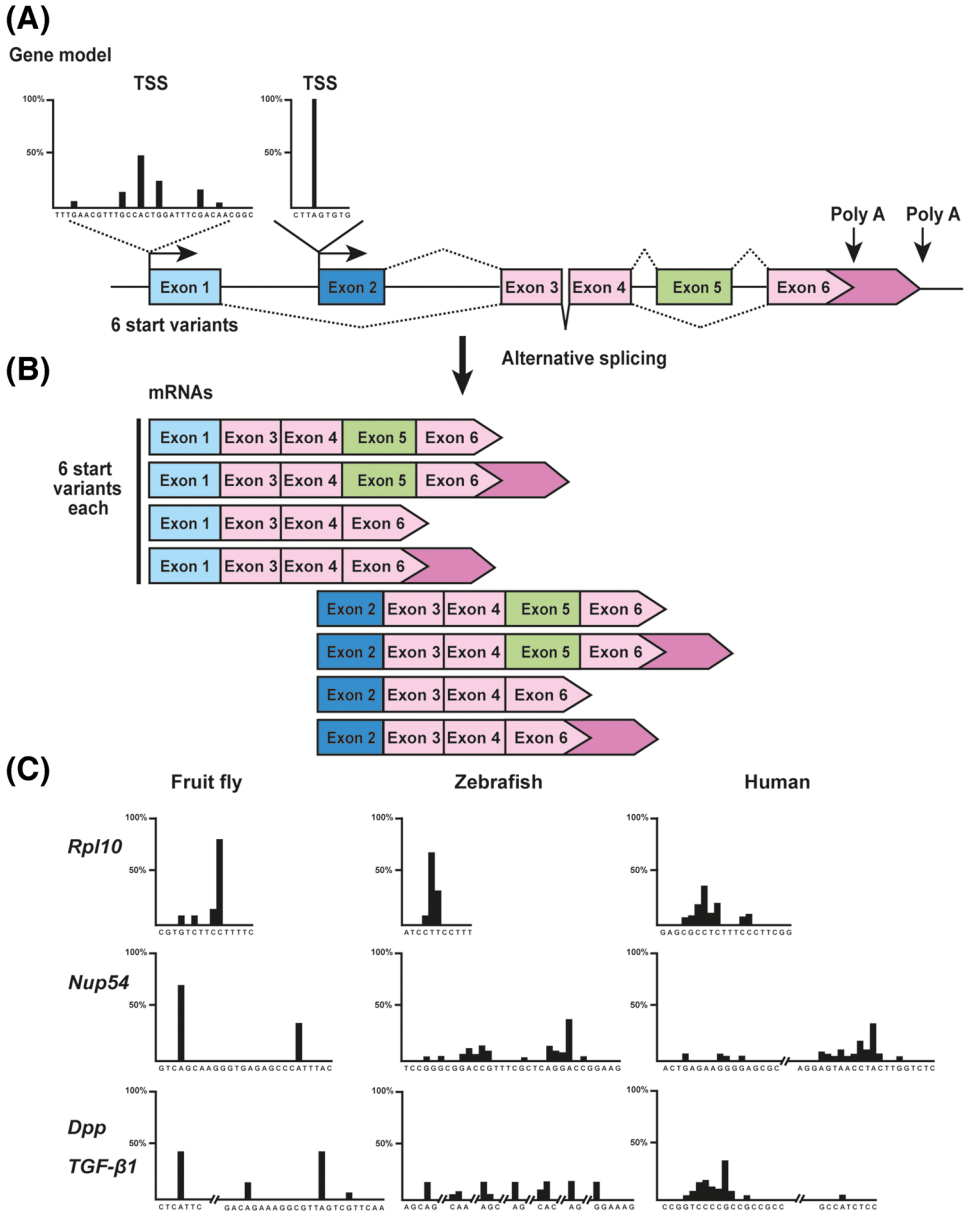

FIGURE 2 Multiple transcription start sites contribute to messenger RNA (mRNA) diversity. Depiction of a generic gene model showing transcribed parts as boxes and introns or intergenic regions as lines (A). Constant exons are shown in pink, alternative start exons 1 and 2 in light and dark blue, the alternatively spliced exon 5 is in green and the 3′UTR extension from alternative polyadenylation is in purple. Constant and variable mRNA processing are indicated in solid and dashed lines, respectively. Transcription start sites (TSS) for alternative start exons 1 and 2 are shown on top with the sequence at the bottom and initiation frequency on the left (in %). All different combinations of mRNAs (28 variants) are shown below the gene model (B). Examples for transcription start heterogeneity (C) is shown for fruit flies (*Drosophila melanogaster*), zebrafish (*Danio rerio*) and humans (*Homo sapiens*) for the ribosomal protein gene *Rpl10* (top, TOP message initiating with pyrimidine, see text), the nuclear pore protein gene *Nup54* (middle) and the developmental regulatory gene *Dpp/TGF-b1* (bottom). Note that *Nup54* is ubiquitously expressed but has regulatory roles,[43,44] while *Dpp/TGF-b1* is a morphogen involved in many aspects of development.[45,46]

of gene expression beyond splicing has not been explored for this modification.

HETEROGENEOUS TRANSCRIPTION START SITES: A NOVEL LAYER OF GENE EXPRESSION REGULATION

Alternative transcription start sites associated with alternative 5′ exons are now a recognised common feature of many genes which is used to diversify expression (Figure 2A). In addition to tissue-specific expression, such diversification can impact on further processing of mRNAs including alternative splicing and polyadenylation, but also on the cytoplasmic fate of an mRNA through regulatory elements in the 5′UTR. However, there is an additional element of diversity in mRNAs, namely an inherent property of most promoters to start transcription at multiple initiation sites with varying first nucleotides identified from CAGEse (Cap Analysis of Gene Expression Sequencing) accurately mapping the first nucleotide in mRNA[39–42] (Figure 2A,B).

Mammalian promoters consist of two types either containing a TATA box directing initiation at well-defined sites or CpG-rich promoters with more spread initiation.[47–49] Recent whole genome efforts to determine transcription start sites by CAGEseq confirmed previous identification of an mRNA start consensus in *Drosophila* of AGU in most mRNAs, but mRNA start sequences in humans, mice and zebrafish are much more diverse.[39–42] The meaning of such diversity at the start of most mRNAs remains to be explored, but one functional example are transcripts of most ribosomal protein genes, which contain a 5′ terminal oligopyrimidine tract (5′ TOP) motif immediately after the 5′cap.[50] Some ribosomal proteins contain a snoRNA in an intron accompanied by dual transcription initiation from either purine or pyrimidine generating preference for ribosomal protein or snoRNA expression during development.[51] Translation of TOP RNAs is tightly regulated, and their miss-regulation including transcriptional regulation is a prominent feature of many cancers also involving MYC.[52–54] Key to the expression of TOP messages is the RNA binding protein LARP, which binds the cap and adjacent CU-rich sequences to inhibit translation under starvation conditions.[55,56] Upon nutrient-induced phosphorylation by mTOR, LARP binding to the cap is inhibited and instead LARP associates with PABP at the mRNA polyA tail to induce translation.[57] The exact role of cOMe in the expression of TOP message genes has not been determined, but in the absence of CMTr1, ribosomal protein and histone genes are downregulated.[58]

Recently, variation of the first nucleotide after the cap was found to affect translation efficiency, particularly under stress conditions, but whether this is due to cOMe has not been determined.[59] A systematic analysis of the first seven nucleotides adjacent to the cap revealed up to 200-fold difference in mRNA expression and identified the TOP motive as key regulatory element affecting translation efficiency.[60] Although this analysis has some bias from interference with transcription for some sequences, in vitro transcribed mRNAs transfected into cells were differentially processed and translated in two different cell types depending on sequence and methylation status.[61]

FUNCTIONS OF THE CAP AND METHYLATED CAP-ADJACENT NUCLEOTIDES IN GENE EXPRESSION

The main function of the cap is to protect mRNAs from degradation and to recruit translation initiation factors, but also to promote splicing and 3′end processing.[62,63] The cap is initially bound by the nuclear cap binding complex (CBC), consisting of CBP20 and CBP80, and upon export from the nucleus, is replaced by the rate limiting translation initiation factor eIF4E, which is predominantly cytoplasmic.[64,65] *N7* methylation of the cap guanosine is critical for both CBC and eIF4E binding, however, cOMe affects the binding of only CBC and not of eIF4E.[14,66]

Early studies showed that cOMe can enhance translation in trypanosomes and of *c-mos* mRNA in *Xenopus* oocytes.[67,68] Later it was shown that cOMe can also stimulate protein synthesis in in vitro translations and from mRNA transfected into human cells.[18,61] In *Drosophila*, cOMe was found to increase CBC binding and a knock-out of both CMTrs reduces protein synthesis at synapses and the ability to learn upon reward conditioning.[14] Intriguingly, the CBC prominently localises to synapses together with another nuclear complex, the exon junction complex (EJC), which has roles in synaptic protein expression.[69] The EJC is deposited upon splicing and removed in a pioneer round of translation,[64,65,70] and thus another marker for untranslated mRNA. In the absence of cOMe in *Drosophila*, both the CBC and EJC are reduced at synapses indicating that cOMe provides a mark for certain mRNAs to be transported in an untranslated state to synapses for local translation.[14,71]

Intriguingly, CMTr2 targets in *Drosophila* are enriched in transcripts also targeted by FMR1, the homologue of human FMRP which has been shown to regulate protein synthesis.[14,72] In fact, the translational repressive role of FMRP in arresting ribosomes on mRNAs is consistent with a role of cOMe in directing untranslated mRNAs to synapses.[73–75]

Export and stability of m^6A containing mRNAs requires FMRP to direct neuronal differentiation.[76,77] Furthermore, a significant proportion of synaptically localised mRNAs carry m^6A.[78,79] Also, m^6A regulates mRNA stability and local translation at synapses and is required for learning and memory.[80–82] FMR1 together with the m^6A reader YTHDF has been shown to inhibit translation of key transcripts directing synaptic growth in *Drosophila*[83] and FMR1's preferential binding to m^6A-modified mRNAs contributes to clearance of maternal mRNA early in *Drosophila* embryonic development.[77] Additionally, different m^6A readers are enriched pre- and post-synaptically in neurites and have been shown to compete for binding of m^6A modified transcripts.[72,79,84,85]

In *Drosophila*, cOMe is mostly found on the first cap-adjacent nucleotide and required for reward learning, while internal Mettl3/14 complex deposited m^6A is required for memory consolidation in classic aversive conditioning.[14,86] Given the presence of m^6Am at the first and cOMe on the second cap-adjacent nucleotide in vertebrates, mRNA methylation has diversified consistent with more elaborate higher brain functions. Hence, cap modifications together with internal modifications might constitute an epitranscriptomic code[87] that has key regulatory roles in subcellular localisation and translation of neuronal mRNAs (Figure 3).

The role of cOMe and other modifications in mRNA fate is likely multifaceted and involves gene-specific responses. In addition to regulating translation, cOMe could regulate the stability of certain mRNAs as suggested by downregulation of ribosomal protein and histone genes upon knock-out of *CMTr1* in mammalian cells.[58] In *Drosophila*, however, cOMe seems not to generally protect mRNAs from degradation as determined by incubation of capped mRNA in cellular extracts and evaluated from significantly more upregulated genes in *CMTr* double knockouts. However, since there are several decapping enzymes present in metazoans, this could include more transcript specific effects on stability as has been found for transcripts with m^6Am at the first nucleotide.[28,88]

To elucidate the functions of cOMe in vivo, knockouts made in trypanosomes and viruses revealed substantial impact on propagation,

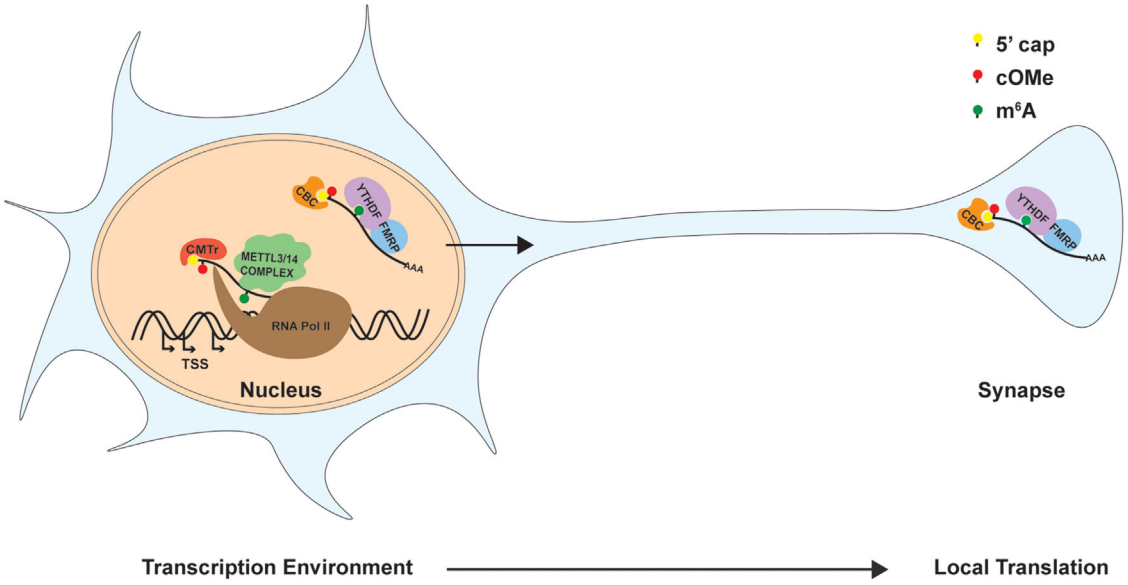

FIGURE 3 Transcription environment linked to local translation at synapses of neurons. Genes transcribed by RNA Pol II (brown) from different TSS can be differentially packaged into ribonucleoprotein particles dependent on mRNA modifications (cOMe, red and m^6A, green, added by CMTr, red and the Mettl3/14 writer complex, green, respectively) and RNA binding proteins (FMRP, light blue, m^6A reader YTHDF, purple and CBC, orange), and transported to synapses for local translation.

but the exact molecular mechanisms are still not well understood.[12,26] CMTrs in mice are essential and their knock-out is lethal displaying severe neurological defects in a *CMTr1* brain knockout.[89] Surprisingly, *Drosophila* with both *CMTrs* knocked out are viable, but display neurological phenotypes including reward learning disabilities, likely because of perturbed local mRNA translation.[14,19]

Lastly, cap functions in higher eukaryotes have expanded by the presence of an additional CBC protein NCBP3, which can substitute CBC20.[90,91] *NCBP3* knock-out mice are viable, but express less interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and have a reduced antiviral defence.[92] Human IFIT1 binds to capped RNA lacking cOMe and inhibits translation. Moreover, NCBP3 recruited to select HIV mRNAs containing a tri-methylated cap, typical to snRNAs involved in splicing, can induce eIF4e independent translation.[90,93,94]

IMMUNE SYSTEM INDUCED mRNA TURN-OVER

Viral infection can trigger an immune response through interferon signalling by detection of viral RNA by various nucleic acid sensors. These sensors exploit differences in viral RNA from host mRNA, mostly dsRNA and triphosphate 5′ ends.[25,26] By generally being processed in the same way as host mRNAs, including a cap and cOMe, viral RNAs have adapted to evade host detection. Accordingly, many viruses have their own capping and cap-modifying enzymes that associate with viral RNA polymerases to prevent detection.[25,26] Alternatively, Alphaviruses, which lack cOMe, can evade an immune response through a cap-adjacent secondary structure.[95,96] Intriguingly, immune gene expression in *CMTr1* knock-out mice, or in *CMTr1/2* double knock-out *Drosophila* remain basal. Hence, the role of cOMe in gene expression regulation is clearly more divers than just defending against non-self RNA.[14,89] However, during viral infection host translation is shut-down, for example, by Nsp1 in SARS-CoV-2, and concomitant degradation of host mRNAs might constitute the main trigger for the interferon response.[97,98]

To ensure success of viruses to propagate, massive multiplication of viral RNAs is required. This change in expression levels is likely also a trigger to a more diversified immune response towards abundant RNAs. Moreover, high levels of viral RNA expression likely also increase generation of erroneous transcripts harbouring features detected by nucleic acid sensors of the immune system. Intriguingly, *CMTr1* was first identified as an interferon induced gene, but itself can also regulate expression of other interferon stimulated genes.[99] A prominent class of interferon-induced genes in vertebrates are the diverse family of interferon-induced proteins with tetratricopeptide repeats (IFITs).[92] A key emerging feature of IFITs is that protein–protein interactions direct optimal action of IFITs, for example, to suppress translation by competing with cap recognition. Hence, strong expression of viral RNAs, that like host mRNAs are capped and carry cOMe, might result in shortage of cap-binding proteins or associated factors and this way may expose viral mRNA to become apparent to the immune system.

RNA modifications have also been attributed roles in tuning down an immune response to prevent fatal cytokine overload. In particular, ADAR mediated A to I editing is key to reduce immunogenicity of dsRNA and its expression is enhanced by internal m^6A.[100] Whether cOMe is part of an intricate network of mRNA modifications essential to tune an immune response to appropriate levels, however, is currently not known.

Multiple transcription initiation sites with concomitant alternative first nucleotides in mRNAs further offers advantages in diversifying an immune response to viruses that rely on a unique mRNA start sequence. Such sequence restriction likely is exploited by the immune system. Likewise, viral cOMe carrying transcripts remain sensitive to IFIT1 inhibition of translation in some viruses suggesting also a more diverse role of IFITs.[101] Potentially, IFITs could regulate a specific set of mRNAs in response to changing cellular conditions depending on the sequence of cap-adjacent nucleotides and whether they carry cOMe or not. Since not all host mRNAs carry cOMe, IFIT1 could be used to shut down translation of a specific set of transcripts, which direct viral propagation. Of note, invertebrates do not have an interferon response, but many of their viruses contain cOMe. Likewise, *Drosophila* does not have homologues of human IFITs. Although they have number of tetratricopeptide genes, they have not appeared differentially regulated in the immune response.[102]

CONCLUSIONS

For a long time, translation of an mRNA into protein has been viewed as a regulatory step independent from transcription in the nucleus. However, number of recent studies have revealed links between the regulation of transcription in the nucleus and translation in the cytoplasm. Slower transcription has been found associated with lower translation efficiency.[97,103] Hereby, co-transcriptional addition of m^6A contributes to RNA Pol II pausing and reduced transcription and translation.[104,105] Moreover, the 5′UTR sequence immediately after the transcription start site can result in up to 200-fold differences in mRNA expression and identified the TOP motive as key regulatory element affecting translation efficiency,[60] but the impact of cOMe remains to be evaluated in detail. However, in vitro transcribed mRNAs transfected into cells were differentially processed and translated in two different cell types depending on sequence and methylation status.[61] Hence, 5′UTR heterogeneity resulting from RNA Pol II promoters with multiple initiation sites can impact on translation efficiency (Figure 2) for differential regulation during development or in response to changing conditions. Here, addition of cOMe to some transcripts but not others could further increase the potential for differentially regulating gene expression, particularly in the context of local translation in neurons.[14,106,107]

Clearly, progress in the field has been slow by the difficulty in detecting cOMe and identifying the modifying enzymes.[3,19] Although analysis of the first nucleotide from small amounts of mRNA is well established using molecular labelling techniques and separation of individual nucleotides on 2D TLCs,[18] analysing individual transcripts is only exceptionally possible. The analysis of the cOMe status on nucleotides after the first has recently become possible for small amounts of mRNA using molecular labelling techniques and will facilitate characterisation of modifying enzymes and the impact on gene expression of cOMe.[19] To advance the field of cap epitranscriptomics, however, new methodologies need to be developed to examine how sequence variation at the beginning of mRNAs together with variable methylation of all cap-adjacent nucleotides in individual transcripts impact on gene expression in a biological context.

During transcription mRNAs are packaged into ribonucleoprotein complexes (RNPs) through *cis*-regulatory elements in mRNAs (known as zip-codes) recognised by RNA binding proteins (RBPs) that determine the fate of the mRNA.[38,108,109] Such large RNPs are then transported to specific sites in the cell including axons and dendrites in neurons to excerpt diverse functions. In fact, over 70% of mRNAs are specifically localised in *Drosophila* embryos.[110] Localisation of mRNAs to specific sites has been shown to be mediated by several RBPs including Staufen and FMRP, but mRNA modifications and their readers have also been assigned key roles in mRNA localisation, serving essential roles in neurons such as in learning and memory.

Intriguingly, both transcriptional regulation and heterochromatin states have been implicated in the consolidation and maintenance of long-term memory in the brain.[111–114] Whether epigenetic states stored in heterochromatic DNA exert cell-specific local translation programs in synapses to form the long-sought basis for how the brain encodes long-lasting memories remains to be explored in more detail. Certainly, epigenetic heterochromatin states can diversify transcription start sites and transcription dynamics, packing into different RNPs, deposition of mRNA modifications and alternative splicing and polyadenylation.[114] These extensive resources to diversify mRNPs would have the capacity for extensive coding of different mRNA fates localised to synapses in individual cells for higher brain functions including long-lasting memory.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available.[39–42]

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