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Article

N^6-Methyladenosine mRNA Modification: From Modification Site Selectivity to Neurological Functions

Published as part of the Accounts of Chemical Research special issue "RNA Modifications".

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Cite This: *Acc. Chem. Res.* 2023, 56, 2992–2999
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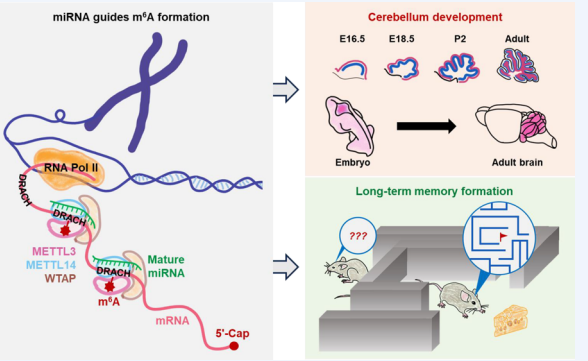
CONSPECTUS: The development of various chemical methods has enabled scientists to decipher the distribution features and biological functions of RNA modifications in the past decade. In addition to modifying noncoding RNAs such as tRNAs and rRNAs, N^6-methyladenosine (m^6A) has been proven to be the most abundant internal chemical modification on mRNAs in eukaryotic cells and is also the most widely studied mRNA modification to date. Extensive studies have repeatedly demonstrated the important functions of m^6A in various biological conditions, ranging from embryonic organ development to adult organ function and pathogenesis. Unlike DNA methylation which is relatively stable, the reversible m^6A modification on mRNA is highly dynamic and easily influenced by various internal or external factors, such as cell type, developmental stage, nutrient supply, circadian rhythm, and environmental stresses.



In this Account, we review our previous findings on the site selectivity mechanisms regulating m^6A formation, as well as the physiological roles of m^6A modification in cerebellum development and long-term memory consolidation. In our initial efforts to profile m^6A in various types of mouse and human cells, we surprisingly found that the sequence motifs surrounding m^6A sites were often complementary with the seed sequences of miRNAs. By manipulating the abundance of the miRNA biogenesis enzyme Dicer or individual miRNAs or mutating miRNA sequences, we were able to reveal a new role of nucleus localized miRNAs, which is to guide the m^6A methyltransferase METTL3 to bind to mRNAs and to promote m^6A formation. As a result, we partially answered the question of why only a small proportion of m^6A motifs within an mRNA could have m^6A modification at a certain time point. We further explored the functions of m^6A modification in regulating brain development and brain functions. We found that cerebellum had the most severe defects when *Mettl3* was knocked out in developing mouse embryonic brain and revealed that the underlying mechanisms could be attributed to aberrant mRNA splicing and enhanced cell apoptosis under m^6A deficit conditions. On the other hand, knocking out *Mettl3* in postnatal hippocampus did not cause morphological defects in the mouse brain but impaired the efficacy of long-term memory consolidation. Under learning stimuli, formation of m^6A modifications could be detected on transcripts encoding proteins related to dendrite growth, synapse formation, and other memory related functions. Loss of m^6A modifications on these transcripts would result in translation deficiency and reduced protein production, particularly in the translation of early response genes, and therefore would compromise the efficacy of long-term memory consolidation. Interestingly, excessive training sessions or increased training intensity could overcome such m^6A deficiency related memory defects, which is likely due to the longer turnover cycle and the cumulative abundance of proteins throughout the training process. In addition to revealing the roles of m^6A modification in regulating long-term memory formation, our work also demonstrated an effective method for studying memory formation efficacy. As the lack of an appropriate model for studying memory formation efficacy has been a long-lasting problem in the field of neural science, our hippocampus-specific postnatal m^6A knockout model could also be utilized to study other questions related to memory formation efficacy.

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Received: July 30, 2023
Published: October 17, 2023

https://doi.org/10.1021/acs.accounts.3c00440
Acc. Chem. Res. 2023, 56, 2992–2999

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INTRODUCTION

The development and survival of humans and other organisms are the results of coordinated functions of billions of molecules, including large biomolecules and small chemical molecules. With the advancements in chemical biology and high-throughput sequencing technologies, multiple layers of new regulatory types have been identified in cells over the past few decades. This has complicated our understanding of the regulatory mechanisms and networks that govern cellular behaviors.[4,5] For example, in addition to the well-recognized regulations controlled by DNA sequences, RNA transcription, RNA splicing, and protein abundance, many new types of regulations have been discovered in the past few decades, including DNA higher structures, DNA methylation, histone codes, noncoding RNAs, and RNA modifications, and have been proven to play essential roles in all types of biological events.[5−12]

RNA modification refers to the addition or changes of chemical compounds to RNA molecules. For example, over 38 types of modifications on adenosine have been recorded (Figure 1).[13] For only the *N*^{6} position of adenosine, there could be at least 13 types of modifications, including *N*^{6}-methyladenosine (m^{6}A), *N*^{6}-hydroxymethyladenosine (hm^{6}A), *N*^{6}-acetyladenosine (ac^{6}A), *N*^{6}-formyladenosine (f^{6}A), etc. In the recently updated version of the MODOMICS database, the number of modified RNA residue entries increased from less than 170 to 334, including 180 modified nucleotide residues, 152 modified nucleoside residues, and 3 modified bases.[13,14] Among these, m^{6}A is the most abundant internal modification found on mRNAs in eukaryotic cells.[15]

The discovery of m^{6}A modification on RNAs can be traced back to the late 1960s;[16,17] however, due to technical limitations, the distribution and functions of m^{6}A modification were not elucidated until 2010s.[18−20] With the development of antibodies against m^{6}A modification and new biochemical methods to detect m^{6}A modification,[5] scientists nowadays are able to profile m^{6}A modification sites genome-wide at single nucleotide resolution[21] or at single cell level[22,23] and also discovered the diverse functions of m^{6}A modification in regulating organism development as well as various physiological or pathological responses. Although scientists were excited about these new discoveries, an important question remains to be addressed: how are the selectivity and dynamics of m^{6}A modification regulated? Our previous studies have revealed some clues to these questions. In this Account, we give a review of the mechanisms underlying the site selectivity of m^{6}A modification and the functions of m^{6}A modification in regulating cerebellum development and long-term memory formation.

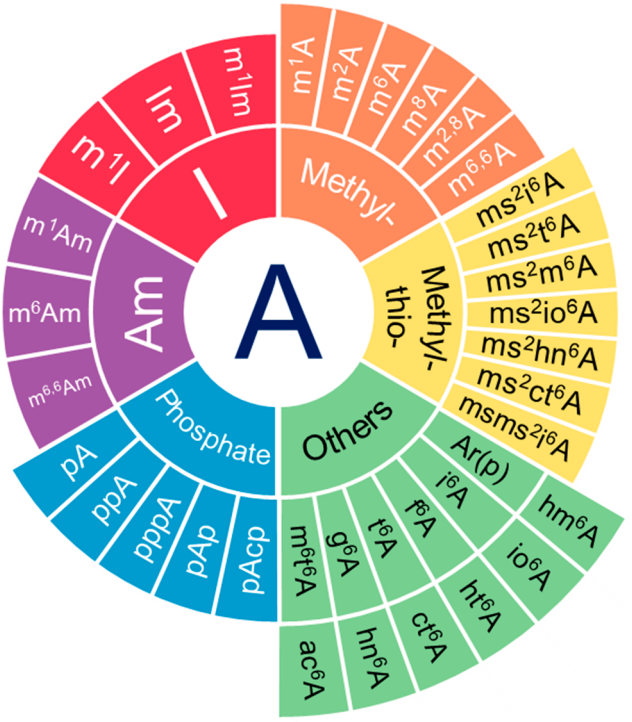

Figure 1. Pie chart summary of chemical modifications on adenosine documented in the MODOMICS database. Adenosine modifications are classified into 6 groups: 1. Deamination (I), 2. Methylation on adenosine (Methyl-), 3. Methylthiolation on adenosine (Methyl-thio-), 4. Methylation on 2′-*O*-methyladenosine (Am), 5. Phosphorylation (Phosphate), 6. Other types of chemical moieties added on adenosine (Others).

SITE SELECTIVITY OF m^{6}A MODIFICATIONS

Unlike DNA methylation, modifications on RNAs are more dynamic and emerge or diminish in a more rapid manner.[24,25] The formation of m^{6}A modification is mainly catalyzed by a methyltransferase complex (known as the m^{6}A writer complex) with METTL3, METTL14, and WTAP as the core components, of which METTL3 serves as the primary catalytic methyltransferase.[26] Reversely, the demethylation of m^{6}A is mediated by FTO or ALKBH5 (termed as m^{6}A erasers).[25] Multiple proteins within cells can recognize and bind to m^{6}A modifications (termed as m^{6}A readers), including the well-known group of proteins with a YTH (YT521-B homology) domain (namely YTHDF1, YTHDF2, YTHDF3, YTHDC1,

https://doi.org/10.1021/acs.accounts.3c00440
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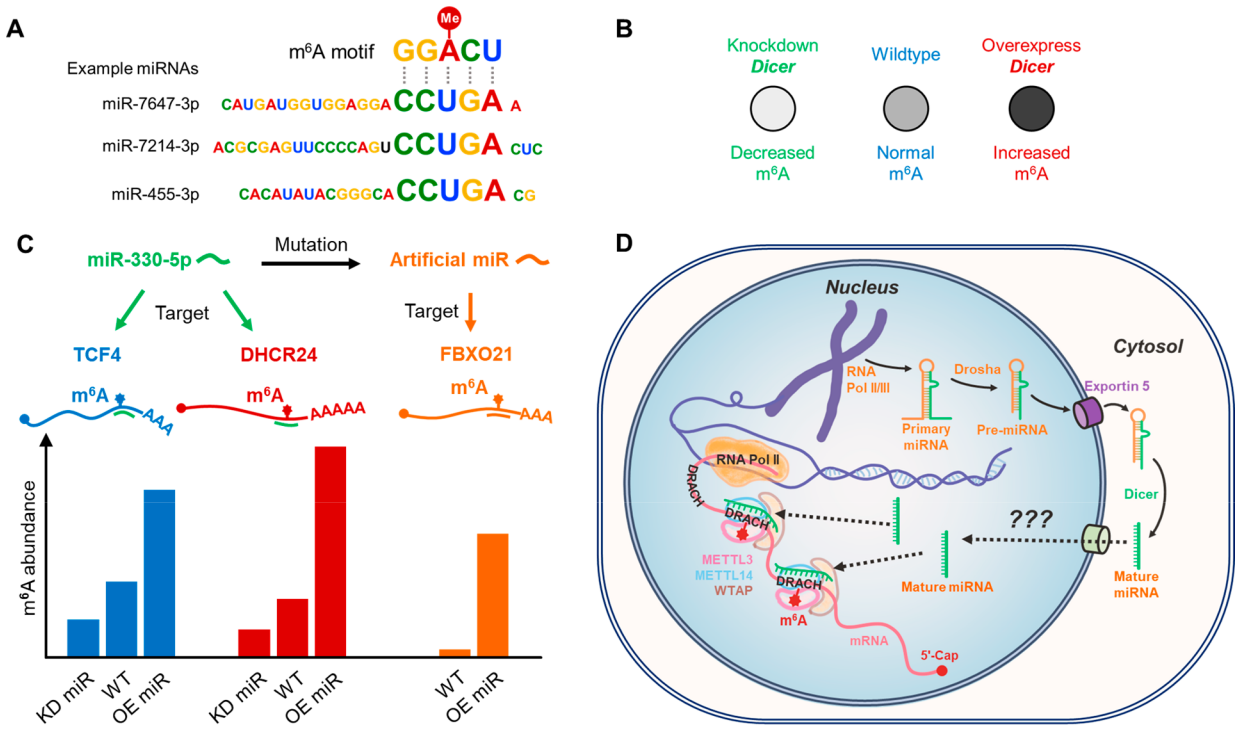

Figure 2. Illustration of miRNA-guided regulation on site-specific m^{6}A formation. (A) Pairing relationships between m^{6}A motif and example miRNAs. (B) Knockdown or overexpression of *Dicer* leads to decreased or increased m^{6}A abundance in cells, respectively. (C) Alteration of m^{6}A abundance on specific genes by miRNA. Knockdown or overexpression of miR-330-5p can specifically decrease or increase m^{6}A abundance on its native targets TCF4 and DHCR24; mutating miR-330-5p to make it artificially target FBXO21 can create a new m^{6}A modification site on FBXO21. (D) Proposed model for miRNA-guided regulation on site-specific m^{6}A formation. m^{6}A is installed co-transcriptionally inside the cell nucleus by the m^{6}A writer complex (METTL3−METTL14−WTAP). Primary miRNAs are mostly transcribed by RNA polymerase II or III and are first processed into pre-miRNAs (stem−loop structure) in the nucleus, then transported into the cytosol and further processed into mature miRNAs. Mediated by unknown mechanisms, some mature miRNAs could be shuttled back into cell nucleus, where they bind to complementary sequences around DRACH motifs on nascent mRNAs. Such miRNA pairing can enhance the binding of m^{6}A writer complex to mRNAs and facilitate m^{6}A formation.

and YTHDC2) and some newly identified m^{6}A readers without a YTH domain, such as HNRNPC, HNRNPG, and IGF2BPs.[27]

m^{6}A modification tends to occur on adenosine within the DRACH motif in human and mouse cells, where D represents G/A/U, R represents G/A, and H represents A/U/C. However, although the DRACH motif could theoretically occur in every 57 randomly ordered nucleotides ($3/4 \times 1/2 \times 1/4 \times 1/4 \times 3/4$), most RNA transcripts contain fewer than 3 m^{6}A modification sites.[28] Moreover, which DRACH motif is selected for adding m^{6}A modification is dynamically regulated, even for transcripts from the same gene. In different cell types or the same cell type under different physiological conditions, the profiles of m^{6}A modification could be quite different. When we started to work on m^{6}A modification about ten years ago, how cells know which DRACH motif on mRNAs to modify with m^{6}A was an important yet unaddressed question.

Based on our years of research experience on microRNAs (miRNAs), we tried to align the enriched m^{6}A motifs with miRNA sequences when analyzing the m^{6}A modification profiles in mouse embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), neural stem cells (NSCs) and testicular sertoli cells (SCs).[1] We surprisingly found that most 8-nt long sequence motifs surrounding m^{6}A modification sites exhibited reverse complementary pairing relationships with the seed sequences of miRNAs (Figure 2A). In contrast, such preference could not be found among randomly generated control sequences with similar nucleotide composition to miRNAs. Thus, we hypothesized that the selectivity of m^{6}A modification sites might in part be mediated by miRNA binding. Inspired by this hypothesis, we first tested the effects of Dicer (the major miRNA processing enzyme[29]) on the overall m^{6}A abundance in NSCs and HeLa cells. In line with our hypothesis, down-regulating *Dicer* expression resulted in a decrease in m^{6}A abundance, while upregulating *Dicer* led to an increase in m^{6}A level (Figure 2B).[1] We further used individual miRNAs to demonstrate that manipulating miRNA levels could indeed impact the abundance of m^{6}A on adenosines within the miRNA binding sites (Figure 2C).[1] Moreover, by introducing mutations into the miRNA sequences to target sites that were previously unmodified by m^{6}A, we successfully generated new m^{6}A modifications on these artificial miRNA target sites (Figure 2C).[1] These findings provide evidence that miRNAs could serve as guide sequences to direct site-specific m^{6}A formation (Figure 2D).

Although the above lines of evidence are quite strong, still something appears to be contradictory to the traditional understanding of miRNAs. This contradiction lies in the inconsistency between the locations of miRNAs and m^{6}A formation. The m^{6}A methyltransferase METTL3 is localized in the nucleus,[1] whereas previous studies have shown that in mammalian cells, miRNAs are processed from their precursors to the ~22 nt mature functional forms in the cytosol.[30] Thus, it seems that the cytosolic miRNAs are unable to influence

https://doi.org/10.1021/acs.accounts.3c00440
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nucleic m^{6}A formation. However, nuclear localization of some miRNAs in mouse and human cells has been detected by many research groups[31] and ourselves (Figure 3), demonstrating that

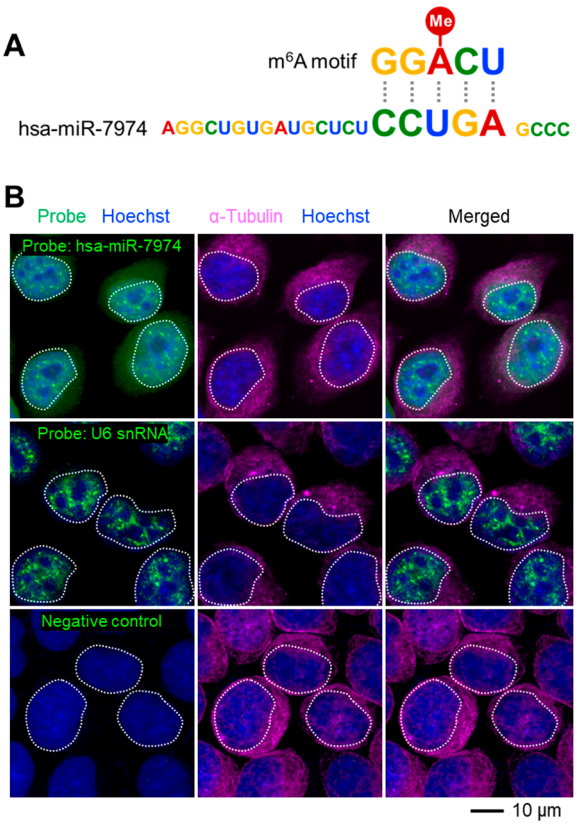

Figure 3. Subcellular localization of miRNA hsa-miR-7974. (A) Complementary relationship between m^{6}A motif and hsa-miR-7974 mature sequence. (B) Fluorescent *in situ* hybridizations show the nuclear localization of hsa-miR-7974 in HeLa cells. Nuclei are highlighted in white dashed circles. Probes targeting nuclear localized U6 snRNA are used as positive controls; probes with no predictable targets are used as negative controls.

some miRNAs could be transported back to the nucleus after cytosolic processing, thereby having the opportunity to physically interact with the writer complex and their target DRACH sites. Indeed, we have also found that miRNAs could affect the ability of METTL3 to bind to their target mRNAs,[1] further supporting the function of miRNAs in mediating m^{6}A formation.

Later on, Prof. Jianjun Chen, Prof. Jianhua Yang, Prof. Chuan He and their colleagues reported that H3K36me3 could guide m^{6}A formation co-transcriptionally by interacting with METTL14,[32] which explains another possible mechanism that regulates the site selectivity of m^{6}A formation. However, as H3K36me3 is a transcription elongation-associated histone modification which can be found throughout entire mRNAs,[33] the H3K36me3 guidance theory still cannot explain why most mRNAs have only 1−3 m^{6}A modification sites, although there are many other positions that could be methylated. It is possible that the sequence guidance function of miRNAs is still required in the theory of H3K36me3-directed m^{6}A formation, further experiments are needed to explore the relationships among miRNAs, H3K36me3, and the site selectivity of m^{6}A modification.

REQUIREMENT FOR m^{6}A MODIFICATION IN CEREBELLUM DEVELOPMENT

The primary function of m^{6}A modification is to regulate embryogenesis and organ formation. Mouse epiblasts or embryonic stem cells without the m^{6}A methyltransferase METTL3 experienced early embryonic lethality;[34] accordingly, our previous collaborative research also showed that knocking down *Mettl3* reduced the expression of pluripotent genes (*Oct4*, *Sox2*, and *Nanog*) and impaired cell reprogramming efficacy.[1] A large number of publications have demonstrated that m^{6}A modification is necessary for the development of nearly every organ in mouse.[24] Therefore, we directed our attention to studying the role of m^{6}A modification in brain development.

By utilizing *Nestin-Cre* transgenic animals,[35] we were able to specifically knockout *Mettl3* in the neural system (*Mettl3-Nestin*-cKO) of mouse embryos. Although the *Mettl3-Nestin*-cKO embryos were able to produce live pups, the newborns were significantly smaller in body size, had impaired movement ability, and died within 3 weeks after birth.[2] Similar to previously published *Mettl14-Nestin-Cre* cKO results,[36] the *Mettl3-Nestin*-cKO mice also had enlarged brain ventricles but reduced overall brain sizes, with the most significant shrinkage observed in the cerebellum compared to other brain regions.[2] Such more severe developmental defects in cerebellum may be attributed to the fact that cerebellum has higher intrinsic m^{6}A abundance than other brain regions,[37] which makes it more vulnerable to *Mettl3* knockout. Through various histological and molecular experimental approaches, we demonstrated that such *Mettl3-Nestin*-cKO related defects in the cerebellum were caused by abnormal expression and aberrant splicing of m^{6}A modified genes involved in neural development (e.g., *Atoh1*, *Sox2*, *Yap1*, and *Dapk1*) and apoptotic signaling pathways (e.g., *Grin1*, *Atp2b3*, *Grm1*, and *Lrp8*) (Figure 4).[2]

Around the same time as our work, a paper published in *Genome Biology* also investigated the role of m^{6}A modification in regulating the postnatal development of mouse cerebellum.[38] They observed significant changes of m^{6}A modifications in mouse cerebellum from postnatal day 7 to day 60. Specifically, they found an increase of m^{6}A peaks around the start codon regions of mRNAs and a decrease of m^{6}A peaks around the stop codon regions. As expected, genes with dynamic m^{6}A modifications during cerebellum development were enriched in functions related to cell cycle, DNA damage response, neural development, and synaptic plasticity. In line with our findings, they also observed abnormal cerebellar development when knocking down *Mettl3* or knocking out the m^{6}A eraser enzyme *Alkbh5*.[38]

m^{6}A MEDIATES THE EFFICACY OF LONG-TERM MEMORY FORMATION

After discovering the developmental regulatory function of m^{6}A modification in the cerebellum, we proceeded to explore its roles in adult brains. As a dynamic RNA modification type, m^{6}A modification has been proven to function in multiple physiological and pathological processes, such as circadian rhythm, immune responses, metabolism, and various cancers.[24] We believe that the necessity of RNA modifications for cells lies in their responses to various internal or external stimuli. For adults, one of the major stimuli to the neural system is learning and memory. Thus, we hypothesized that m^{6}A modification could play a role in memory regulation.

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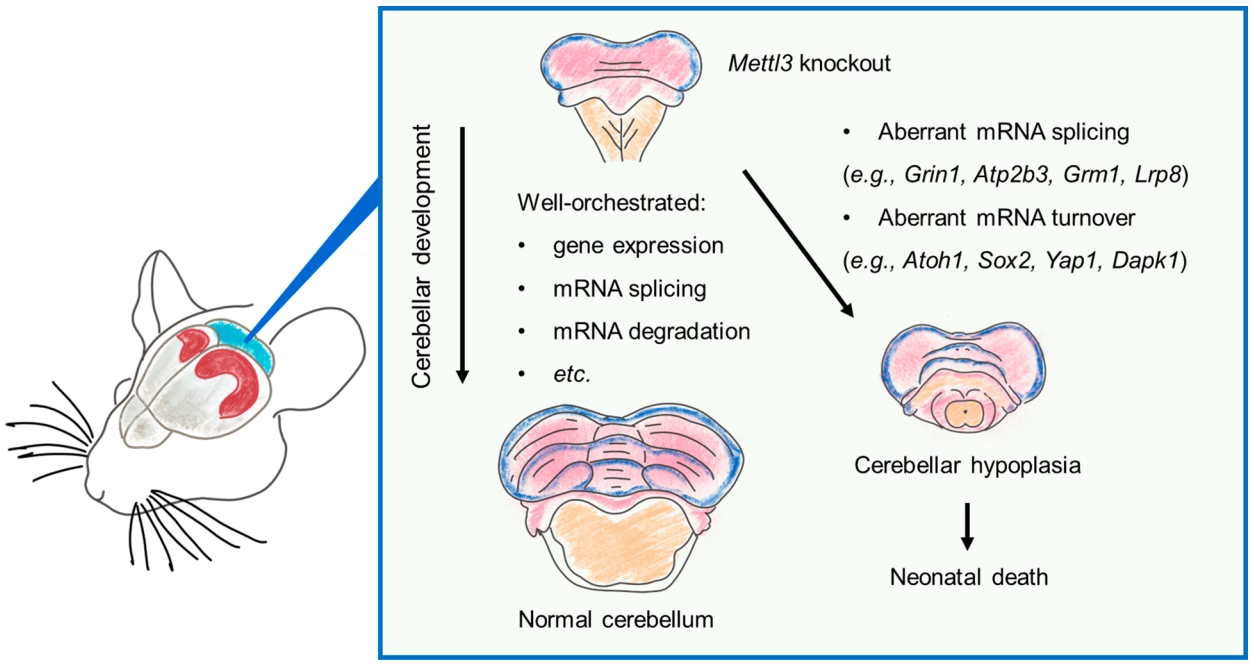

Figure 4. Cartoon summary for the functions of m^{6}A in cerebellar development. Embryonic brain development is a complex process that must be tightly controlled by well-orchestrated gene expression, mRNA splicing, and mRNA degradation. Depletion of m^{6}A writer *Mettl3* in the embryonic mouse brain leads to aberrant mRNA splicing, turnover of essential genes, and cerebellar hypoplasia and neonatal death.

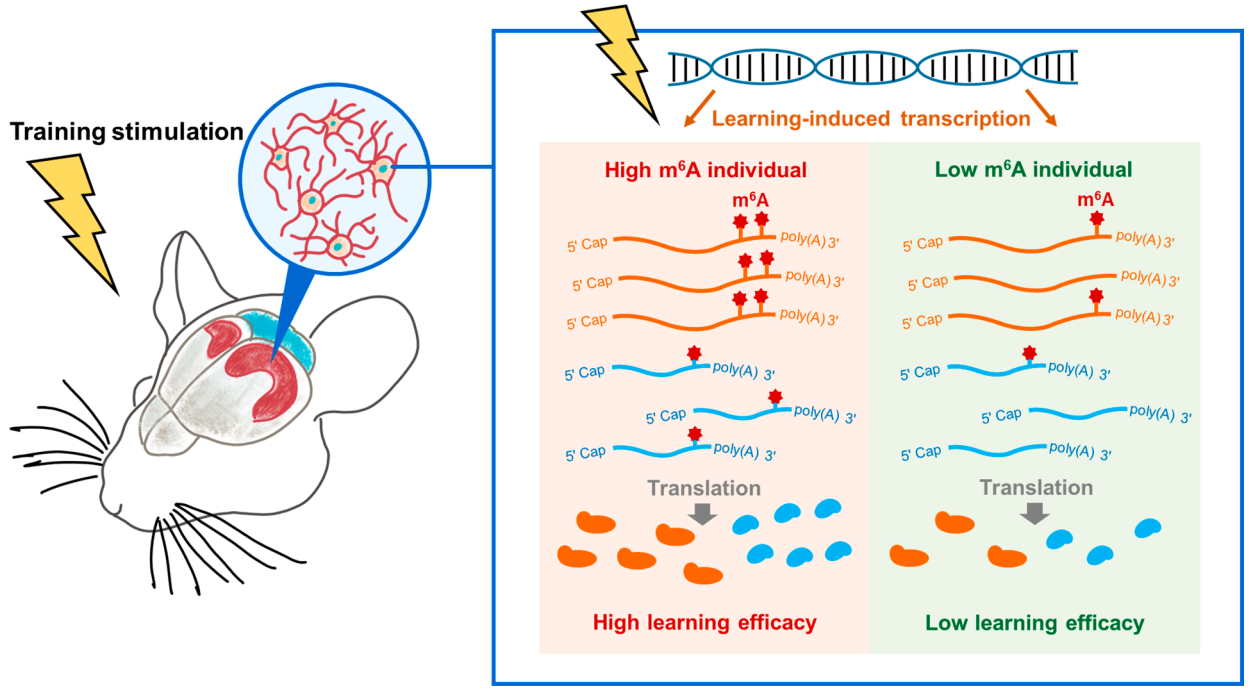

Figure 5. Cartoon illustrating the positive regulatory functions of m^{6}A on the efficacy of long-term memory formation. Behavior training will induce gene expression in mouse hippocampus. Such learning-based gene activation is essential for the brain to reshape its neural network to encode new information as memory. After learning stimulation, m^{6}A modifications would be added to transcripts of learning-induced genes and further enhance mRNA translation, neural network long-term potentiation, and the efficacy of mouse long-term memory formation.

To test this hypothesis, we generated *Mettl3* conditional knockout mice using *CaMKIIα-Cre* (*Mettl3-CaMKIIα*-cKO), which depleted *Mettl3* in the excitatory neurons in hippocampus and cerebral cortex from postnatal day 1.[39] The *Mettl3-CaMKIIα*-cKO mice exhibited normal brain morphology and motor ability, without any detectable developmental or psychological defects.[3] The *Mettl3-CaMKIIα*-cKO mice behaved normally in short-term memory tests but showed reduced long-term memory formation efficacy in both the Morris water maize test and fear conditioning test.[3] We further proved that such *Mettl3* depletion associated long-term memory defects were indeed caused by the lack of m^{6}A modification using mutagenesis of the key enzymatic site in the METTL3 methyltransferase domain.[3] Interestingly, after

https://doi.org/10.1021/acs.accounts.3c00440
Acc. Chem. Res. 2023, 56, 2992−2999

repeated training sessions or increased training intensity (10 training times for Morris water maize test or 3 consecutive electronic shocks for fear conditioning test), the *Mettl3-CaMKIIα*-cKO mice performed similarly to the wild-type control mice,[3] indicating that extended training sessions or increased training intensity can compensate for the memory defects caused by m^{6}A modification deficiency.

Molecularly, the m^{6}A modification related memory consolidation is achieved by modulating the translation efficacy of genes that regulate dendrite development, synapse organization, cellular protein localization, and other processes related to memory formation, especially the translation of immediate early genes (e.g., *c-Fos*, *Egr1*, *Arc*, *Npas4*, and *Nr4a1*) that are essential for neuronal quick responses to learning training (Figure 5).[3] By manipulating the abundance of m^{6}A reader protein YTHDF1, another collaborative team also demonstrated that m^{6}A facilitates hippocampus-dependent learning and memory by promoting protein translation, which aligns with our findings.[40] Yet they did not observe the compensatory effects of extensive training for the lack of m^{6}A modification, probably due to differences in m^{6}A perturbation approaches (knockout of m^{6}A methyltransferase *Mettl3* vs knockout of m^{6}A reader *Ythdf1*) or training procedures.

We also demonstrated that overexpressing *Mettl3* in hippocampus could significantly improve memory ability to create "super smart" mice; however, such superiority only exists in the early training stages; after repeated training, wild-type mice could also reach the same memory level as the "super smart" mice.[3] Such phenomenon is in accordance with one Chinese proverb which is "diligence can make up dullness", and may be explained by the accumulative effects of memory-related proteins and their regulations on the neural circuits.[41] Due to the longer turnover rate of proteins, the abundance of memory regulatory proteins would accumulate after each round of training. If there is a functional saturation level for proteins in regulating neural connections and long-term memory formation, under excessive training conditions, the depletion or overexpression of *Mettl3* would only affect the time needed to reach the saturated protein level of m^{6}A modified transcripts, but not the final learning outcomes. In a comment on our work written by Prof. Pico Caroni, he pointed out that the underlying mechanisms of memory strength were poorly understood; our work identified "an endogenous learning-related molecular process with a role in modulating memory strength" and thus "makes an important contribution to molecular studies of learning and memory".[42]

CONCLUSIONS AND OUTLOOK

In summary, the past years of effort from our team and our collaborators have identified a class of guiders used by cells to select specific adenosine sites for adding m^{6}A modifications when necessary and also revealed the functions of m^{6}A in regulating cerebellum development and long-term memory formation. Based on these, there are a few questions worth exploring in the future.

First, how do miRNAs and m^{6}A-writer/reader/eraser proteins function together to respond to different biological signals? As both m^{6}A modification and miRNA expression play essential roles in daily physiological activities such as metabolism, circadian rhythm, learning, and immune responses,[24,43] they could be highly dynamic in cells. One potential working model for the cause of such dynamic m^{6}A changes could be that certain internal or external signals trigger the conditional generation and nuclear shuttling of miRNAs to guide m^{6}A modifications on mRNAs. However, the mechanisms by which these signals are converted into miRNA expression and nucleus transportation remain to be investigated. Similar questions also arise regarding pathological related m^{6}A changes. In addition, as miRNAs would affect the binding of METTL3 to mRNAs, how METTL3 can efficiently interact with specific miRNAs under different conditions also awaits attention.

Second, regarding the enhancement role of m^{6}A in long-term memory consolidation, most of the reported works trained mice using context-dependent behavior protocols, which primarily examine the spatial memory (e.g., spatial cues in the Morris water maze test and the conditioned box in the fear conditioning test). But for human beings, learning is a complicated process that involves the combination of multiple types of memories, such as emotional memory, social memory, and implicit memory.[44] Therefore, it is important to examine the extent to which m^{6}A modification participates in other types of memory, and whether other types of RNA modifications also play roles in learning and memory formation. Knocking-out *Mettl3* in mouse brain regions other than the hippocampus and designing new behavioral test protocols are also desired. In addition, due to the lack of m^{6}A profiling information in human samples, it is unclear whether aging associated memory decline is related to reduced m^{6}A abundance or METTL3 activity. On the other hand, designing or screening for chemical compounds that could enhance m^{6}A modifications would be beneficial for improving long-term memory formation efficacy. However, due to the diverse functions of m^{6}A, such a strategy should be applied with caution regarding the target specificity of m^{6}A modifications.

Finally, most reported m^{6}A studies either focus on the global profiling of m^{6}A modifications or the dynamic changes of m^{6}A on particular genes;[5] little attention has been paid to the coordinated changes of m^{6}A modifications on different genes as well as the coordinated functions of m^{6}A and other types of modifications on the same transcripts. The latter is to a large extent constrained by technical limitations. Hopefully, with the development of more sensitive RNA modification detection methods, we will be able to uncover the relationship between m^{6}A and other RNA modifications in the near future.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Zeyu Zhang** conceptualization, formal analysis, investigation, methodology, project admin-

https://doi.org/10.1021/acs.accounts.3c00440
Acc. Chem. Res. 2023, 56, 2992−2999

istration, resources, validation, visualization, writing-review & editing; **Xiujie Wang** conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing-original draft, writing-review & editing.

Funding

We acknowledge funding from the National Key Research and Development Program of China (2019YFA0802203), Beijing Natural Science Foundation (Z200020), and Chinese Academy of Sciences for Young Scientists in Basic Research Project (YSBR-073) to X.-J.W.

Notes

The authors declare the following competing financial interest(s): The authors have filed a patent pertaining to mechanisms described in this Account.

Biographies

Zeyu Zhang received his Ph.D. in Bioinformatics from Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, in 2022 under the guidance of Prof. Xiu-Jie Wang. Then he continued his research work as a postdoctoral fellow in Prof. Wang's lab. His major research interests include stem cell biology, neuroscience, and RNA modifications.

Xiu-Jie Wang received her Ph.D. in Bioinformatics from Rockefeller University in 2004 and joined the Institute of Genetics and Developmental Biology of Chinese Academy of Sciences as a principal investigator in the same year. She is now also a professor at the University of Chinese Academy of Sciences. A major research focus of her lab is to utilize bioinformatics and experimental approaches to decipher the functions and underlying mechanisms of noncoding RNAs and RNA modifications in mouse and human.

■ ACKNOWLEDGMENTS

The authors would like to thank former and present members in Xiu-Jie Wang's lab and all collaborators who participated in the research described in this Account, especially Prof. Yun-Gui Yang for his long-term collaboration.

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