N^6-methyladenine functions as a potential epigenetic mark in eukaryotes

Qinmiao Sun[1)], Shoujun Huang[2)3)], Xiaona Wang[2)], Yuanxiang Zhu[2)], Zhenping Chen[2)] and Dahua Chen[2)]**

N^6-methyladenine (6mA) is one of the most abundant types of DNA methylation, and plays an important role in bacteria; however, its roles in higher eukaryotes, such as plants, insects, and mammals, have been considered less important. Recent studies highlight that 6mA does indeed occur, and that it plays an important role in eukaryotes, such as worm, fly, and green algae, and thus the regulation of 6mA has emerged as a novel epigenetic mechanism in higher eukaryotes. Despite this intriguing development, a number of important issues regarding its biological roles are yet to be addressed. In this review, we focus on the 5mC and 6mA modifications in terms of their production, distribution, and the erasure of 6mA in higher eukaryotes including mammals. We perform an analysis of the potential functions of 6mA, hence widening understanding of this new epigenetic mark in higher eukaryotes, and suggesting future studies in this field.

Keywords:
DNA methylation; epigenetic control; eukaryotes; gene regulation
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[1)] State Key Laboratory of Membrane Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China
[2)] State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China
[3)] Center for Developmental Biology, School of Life Sciences, Anhui Agricultural University, Hefei, China

***Corresponding authors:**
Dahua Chen
E-mail: chendh@ioz.ac.cn
Qinmiao Sun
E-mail: qinmiaosun@ioz.ac.cn

Abbreviations:
5caC, 5-carboxylcytosine; **5hmC,** 5-hdryoxymethylcytosine; **5mC,** N5-methylcytosin; **6mA,** N^6-methyladenine; **ATr,** AT dinucleotide repeat; **DNMT,** DNA methyltransferase; **MBD,** methyl-CpG binding domain; **wadmtase,** wheat adenine DNA-methyltransferase.

Introduction

DNA base methylation has been found in genomic DNA from diverse species, and regulates a diverse array of cellular and developmental processes. In general, two types of base methylation have been studied, namely, methylation damage and methylation modification. Methylation damage, such as N3-methylcytosine (3mC), O6-methylguanine (m6G), N1-methyladenine (1mA), and N3-methyladenosine (3mA), is a product generated by exogenous or endogenous methylation agents, and is cytotoxic or mutagenic to cells by blocking or altering Watson–Crick base-pairing [1–4]. Drugs that can induce cytotoxic or mutagenic DNA damage have been widely used to treat some cancers [5, 6]. In contrast, several other methylated bases, including 5-methylcytosine (5mC), N^6-methyladenine (6mA), and N4-methylcytosine (4mC), are found in genomic DNA from prokaryotes, archaea, and eukaryotes, and these methylated bases have been shown to be products of post-replicative DNA modification generated by specific DNA methylases [7–11]. For example, the Dam and CcrM methylases control 6mA modification, and the Dcm methylase is required for 5mC formation in bacteria [12–15], while DNA methyltransferase (DNMT) family members mediate 5mC modification in vertebrates [16]. In mammals, 5mC is thought to be the predominant, though not only, type of methylated base: this DNA modification has been demonstrated to play a critical role in regulating target gene expression and maintaining chromatin architecture during embryonic development and in tissue homeostasis [16]. The 5mC modification is also involved in controlling other important biological processes, such as X-chromosome inactivation and maintenance of genome stability [16, 17]. Dysregulation of 5mC appears to lead to developmental abnormalities, and has also been associated with numerous human diseases, including cancers [18].

Unlike 5mC, 6mA, and 4mC are found at significant levels in the genomic DNA of bacteria, protists, fungi, and eukaryotes [19–29]. Particularly in bacteria, 6mA plays an important role in controlling a number of biological functions, such as DNA replication [30], cell cycle progression [31], DNA mismatch repair [32], host-pathogen interactions [33], and virulence and gene expression [25]. Previous studies have shown that 6mA is present in

higher eukaryotes, such as insects and mammals at very low levels, and its roles, if any in eukaryote cells, are thought to be minor [24, 25, 34]. However, recent studies including our own highlight that the 6mA modification does indeed occur in eukaryotic DNA and plays important conserved roles in regulating gene expression in multiple species, suggesting that 6mA functions as a novel epigenetic mark in eukaryotes [35–37]. Regulation of 6mA has emerged as a new epigenetic mechanism in eukaryotes, but a number of important issues regarding its biological roles remain to be addressed. In this review, we summarize recent research advances regarding the mechanisms that control dynamic regulation of methylated modification of DNA bases and their biological roles in eukaryotes. We focus on the 5mC and 6mA modification in terms of their production and distribution, and the erasure of 6mA in higher eukaryotes including insects and mammals, and perform an analysis toward understanding mechanisms underlying the potential epigenetic roles of 6mA in regulating gene expression and development, and its possible roles in evolution.

Types and patterns of DNA methylation are variable in eukaryotes

In most cases, the DNA sequence from a specialized cell is identical to that of the zygote from which it is descended. However, the cell identity is primarily determined by its specific patterns of gene expression, which are due to various epigenetic controls. DNA methylation is one of the most important epigenetic mechanisms; it does not change DNA sequence, but is thought to regulate target gene expression during development and in tissue homeostasis [38]. Thus, understanding the molecular mechanism of target gene regulation via DNA methylation, and how it is distributed in the genome, is critical for the fields of both epigenetic and developmental biology [39].

5mC is generated by the addition of a methyl group from S-adenosyl-L-methionine (AdoMet/SAM) at the 5-carbon position of cytosine in DNA by certain methyltransferase enzymes in a motif-specific manner. For example, in bacteria, methyltransferase of restriction-modification systems (R-M) can produce 5mC in specific motifs, and the 5mC modification thus ensures the protection of host genomic DNA against endonuclease digestion [40]. Contrary to the limited role of 5mC in bacteria, the spectrum of DNA methylation levels and function in animals is very broad, and its patterns also vary in time and space during development [38, 41]. It has been shown that approximately 1–6% of cytosine bases are methylated as the 5mC form in vertebrate, and that the important feature of DNA 5mC methylation in vertebrates is the presence of CpG islands in their genomes [42]. Particularly in mammals, most of 5mC modification exists in CpG dinucleotide, and 60–80% of CpG dinucleotides carry cytosine methylation on both strands in some tissues [42]. Recent studies have revealed that the methylation state of 5mC in mammals is dynamic and reversible: it is controlled by methyltransferases and/or demethylases (Fig. 1A) [41, 43]. Several DNA methyltransferases including DNMT1, DNMT3A/B, and cofactor DNMT3L, have been identified to be important for 5mC modification in mammals. DNMT1 maintains cytosine methylation during DNA replication, while DNMT3A/B and DNMT3L have de novo roles in methylating DNA during development (Fig. 1A) [44–46]. The dynamic and reversible processes of 5mC modification are controlled by several mechanisms, including passive and active demethylation [41]. Although passive demethylation is attributed to successive cell divisions that cause a progressive loss of 5mC on a genome scale, active demethylation of 5mC is executed through oxidation by ten-eleven translocation (TET) family dioxygenases [47–50]. In this process, 5mC can be oxidized by the TET enzymes in an iterative manner to produce 5-hdryoxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC); and both 5fC and 5caC can be removed by thymine DNA glycosylase and enzymes in the base excision repair pathway [51–53]. Thus, the activities of both methyltransferases and demethylases together determine the DNA methylation levels and patterns in animals. Demethylation of 5mC is also realized through deamination via the conversion of 5mC to thymine (T) by the deaminases AID/APOBEC: if the resultant T remains unpaired in cells, it can be removed by the mismatch repair system [49, 50, 54–56].

Of note, in addition to its role in DNA demethylation, deamination of 5mC has a mutagenic effect in the mammalian genome, because the mismatch of T:G probably gives rise to a C to T transition [57]. For example, the tumor suppressor gene *p53* is frequently mutated in cancer, and more than half of *p53* mutations are C to T transitions [58]. Interestingly, the idea of a mutagenic effect of 5mC has evolutionary significance, because the "C to T transition" has most likely led to a massive depletion of CpG dinucleotides during evolution. Bioinformatics analyses reveal that the observed-to-expected CpG dinucleotide ratio is approximately 0.2–0.25 in mammalian genome, whereas the observed-to-expected CpG ratio in the *Drosophila* genome is very close to 1.0 [59]. This predicts that 5mC modification might not be present in *Drosophila*. In fact, experimental data from a number of studies including ours suggest that 5mC is either undetectable or present at very low levels (as measured by mass spectrometry analysis) in fly [35, 60]. A recent study using bisulfite sequencing assays also suggests that the *Drosophila* genome lacks a defined 5mC pattern [61]. Thus, both theoretical analysis and experimental data reciprocally support the notion that 5mC does not exist in fly, or that its function – if it has one – should be very minor. Recent studies suggested that genomic methylation of 5mC is present in the fly, albeit in small quantities and in unusual patterns [62, 63]. However, on the basis of the observation that the rate of observed-to-expected CpG is close to 1.0 in the fly, it could theoretically be assumed that the activity of 5mC DNA demethylation mediated by the ALKB-like family DNA dioxygenase (e.g. Tet) is dispensable for this organism. Nevertheless, one would argue that low levels of 5mC in *Drosophila* could be attributed to the existence of a high-5mC demethylase activity. Interestingly, the *Drosophila* genome harbors a gene, CG2083, which encodes a Tet-like protein (referred to as DMAD in our recent paper, according to its biochemical function). However, our genetic assays showed that loss of *DMAD* does not affect levels of 5mC or 5hmC, revealing that DMAD has no

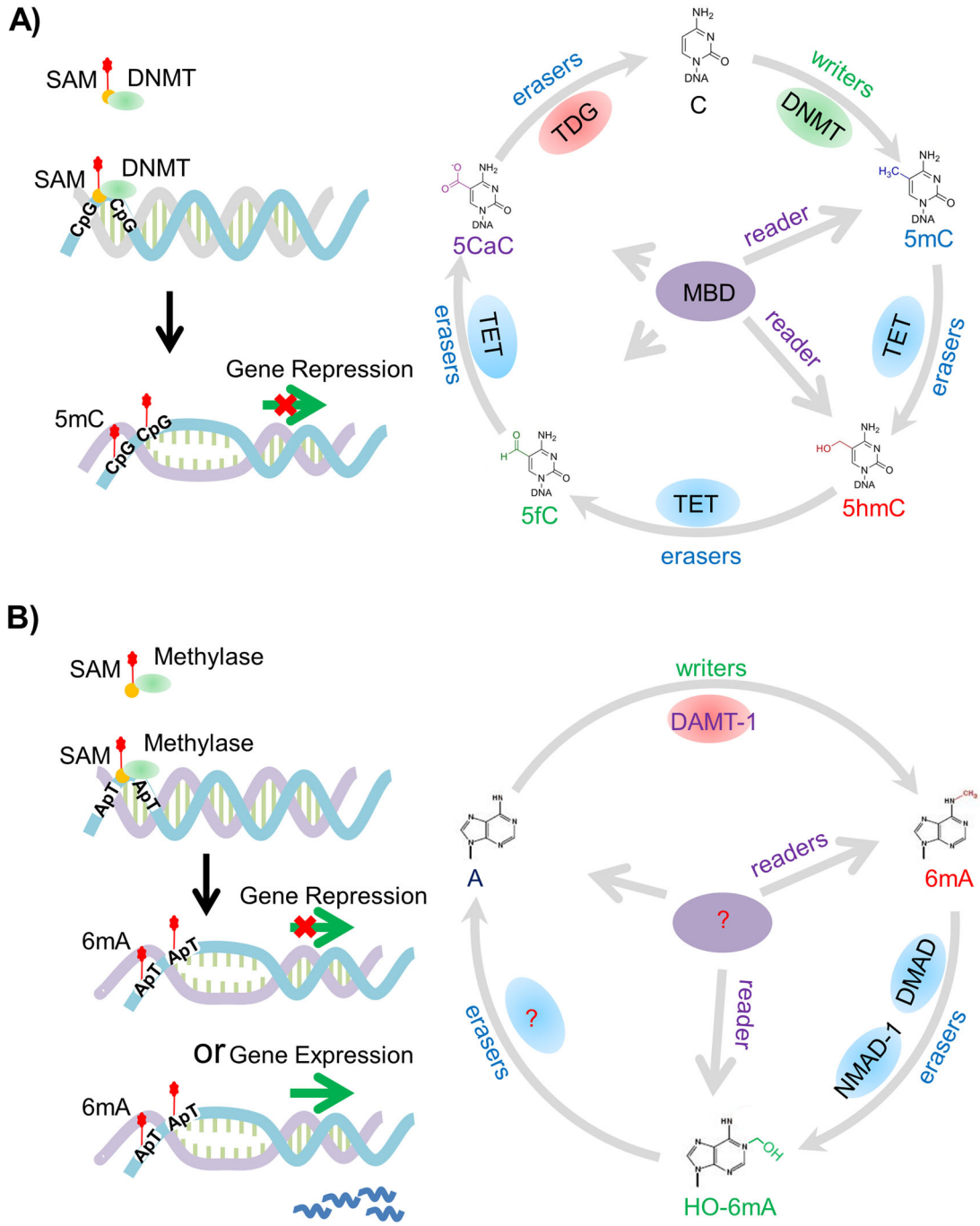

Figure 1. Regulation of 5mC and 6mA modification. **A:** Left panel: In higher eukaryotes, DNA methyltransferases (DNMT) are responsible for 5-methylcytosine (5mC) production at CpG dinucleotides. S-adenosyl-L-methionine (SAM) is the methyl group donor. 5mC in promoter regions represses target gene transcription and regulates multiple processes. Right panel: Active demethylation of 5mC by enzymes of ten-eleven translocation (TET) family. 5mC can be hydroxylated by TET to form 5-hydroxymethylcytosine (5hmC); further oxidation produces 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5caC is readily converted to cytosine (C) by the glycosylase TDG of the base excision repair (BER) pathway. 5mC and 5hmC also serve as stable epigenetic marks and are recognized by proteins of Methyl-CpG binding domain family (MBDs). **B:** Left panel: In unicellular eukaryotes and model animals (e.g. fly and worm), specific methylases are responsible for N^6-methyladenine (6mA) production, mainly in ApT enriched sequences. 6mA methylase also uses SAM as the methyl group donor. 6mA modification in DNA lowers the thermodynamic stability of DNA, and has a positive correlation with gene activation. Right panel: Methylation and demethylation cycle of 6mA in eukaryotes. DAMT-1 is the 6mA methylase that has been identified in *C. elegans*. Hydroxylation of 6mA produces HO-6mA, which is mediated by NMAD-1 and DMAD in *C. elegans* and *Drosophila*, respectively. It is still unknown how HO-6mA is converted to Adenine (A). The 6mA reader has not yet been identified.

apparent role in regulating the demethylation of 5mC [35]. In support of this concept, 5mC is also undetectable in another invertebrate, *Caenorhabditis elegans*, the genome of which lacks the gene(s) encoding a 5mC DNA methyltransferase [36, 64]. Whether DNA methylation plays a role in worm remains unexplored territory.

The prevailing view is that DNA methylation plays conserved epigenetic roles in a wide array of organisms from bacteria to mammals [8]. From an evolutionary perspective, we reason that if their DNA is not methylated at cytosine, animals likely use other types of methylated base(s), such as methylated adenine (6mA), to fulfill the related function of 5mC in mammals. Without a doubt, *Drosophila melanogaster* and *C. elegans*, provide attractive model systems to explore this intriguing possibility. In support of this view, we and others recently provided evidence showing that 6mA modification occurs in DNA from three different eukaryotes, including *D. melanogaster* and *C. elegans*, and that it plays conserved epigenetic roles in regulating gene expression (Fig. 1B) [35–37].

6mA DNA modification has potential roles in eukaryotes

6mA is one of the most abundant types of DNA methylation in bacteria, and its functions have been well studied in bacteria. It has been reported that the 6mA modification occurs in a number of unicellular eukaryotes [20]. For example, the protozoan *Tetrahymena* is a single-celled organism that contains two nuclei: the germline micronucleus is diploid and transcriptional inactive, whereas the somatic macronucleus is polyploid and transcriptionally active in supporting cell growth and differentiation. Notably, abundant 6mA is only detected in the macronucleus. Previous studies have shown that ~0.8% of adenine is modified as the 6mA form in the macronucleus, and that 6mA modification exists in the palindromic ApT dinucleotide [65–68]. Further nucleosome position-mapping analysis showed that 6mA was found preferentially in linker regions between two adjacent nucleosomes [65–68]. Recently, a higher resolution analysis further contributed evidence, showing a similar pattern of 6mA in green algae (see next section) [37]. Interestingly, once it is established during macronucleus development, the methylation pattern of 6mA appears to be relatively stable through changes in the physiological state of *Tetrahymena*. However, the molecular basis for the maintenance of the 6mA pattern – and what its roles are in the macronucleus – remain to be explored in this organism [41, 68]. Besides *Tetrahymena*, 6mA has been found in the genomic DNA of other ciliates [26, 69–71]. The green algae *Chlamydomonas reinhardtii* have both 6mA and 5mC modification in their genomic DNA, and the levels of 6mA in *Chlamydomonas* are approximately 0.5% of all adenine. Similarly to *Tetrahymena*, 6mA modification occurs also in the palindromic ApT dinucleotide in *Chlamydomonas* [20, 37]. In addition, 6mA levels were decreased by ~40% during the DNA synthesis phase, and shortly after DNA replication the 6mA methylation pattern was re-established and was stably maintained during cell proliferation in *Chlamydomonas* [37], suggesting a yet-to-be identified mechanism that controls the dynamics and maintenance of 6mA pattern in this organism.

6mA has also been found in plants. In *Arabidopsis thaliana*, a gene encoding domains rearranged methyltransferase (DRM2) has been shown to be modified by 6mA in some GATC sequences of the gene, implying that the enzymatic activity for 6mA modification exists in the organism [22]. In wheat, levels of 6mA abundance are up to 0.1% (6mA/dA) [29]. Wheat adenine DNA-methyltransferase (Wadmtase) purified from the vacuolar vesicle fraction of wheat coleoptiles has been show to have a 6mA methyltransferase enzymatic activity. In the presence of SAM, Wadmtase can methylate the first adenine residue in the TGATCA sequence. An in vitro enzymatic assay showed that single-stranded DNA but not double-stranded DNA is the preferred substrate of Wadmtase. Notably, although Wadmtase is encoded by nuclear DNA, it seems to be responsible for adenine methylation of mitochondrial DNA, and might be involved in the regulation of replication of mitochondria in plants [23]. It would be interesting to identify other 6mA methyltransferase(s) that are responsible for modifying the genomic DNA in plants. Intriguingly, 6mA exists in viruses, and several 6mA methyltransferases have been identified in *Chlorella* virus, implying that virus methyltransferases might be involved in host-cell DNA modification [72]. As mentioned above, 6mA modification normally exists in the palindromic ApT dinucleotide in unicellular eukaryotes (e.g. *Tetrahymena* and *Chlamydomonas*). Furthermore, the rice genome harbors endogenous rice tun gro bacilliform virus-like sequences (ERTBVs), and many of ERTBVs are present between AT dinucleotide repeats (ATrs). ATrs also incorporate repeat sequences, including transposable elements, suggesting that the insertion DNAs might be trapped by ATrs in the plant genome in a host-dependent manner [73, 74]. One could speculate that ATrs contain the hotspots of 6mA modification, and could be considered as genomic dumping sites that have trapped various invading DNA sequences. It is worth mentioning that deamination of 5mC can produce thymidine, which is a natural base of DNA, and that T:G mismatch repair is relatively ineffective in cells [57], thus the 5mC deamination provides a possibility of mutagenic effects during evolution. By contrast, deamination of 6mA would chemically produce hypoxanthine, which can readily be recognized and efficiently removed by the repair machinery [75]. In this regard, it is unlikely that the existence of 6mA has a mutagenic effect in genomes. Interestingly, it has been shown that although it does not change A:T base-pairing, methylation at the N6 position in adenine theoretically affects hydrogen bond strength, and lowers the thermodynamic stability of DNA [76, 77]. Importantly, previously experiments also showed that 6mA can enhance or induce DNA curvature [78]. This structural property of 6mA implies that modification of 6mA may facilitate DNA unwinding or regulate DNA-protein interactions, thereby potentially affecting genome stability and target gene expression as well. Collectively, we speculate that the ApT dinucleotide sequence is a potential site of 6mA modification that may have exerted a powerful evolutionary force.

In mammals, the important function of 5mC modification has attracted much interest in the field of epigenetic control. However, the issues of whether adenine methylation occurs, and what its

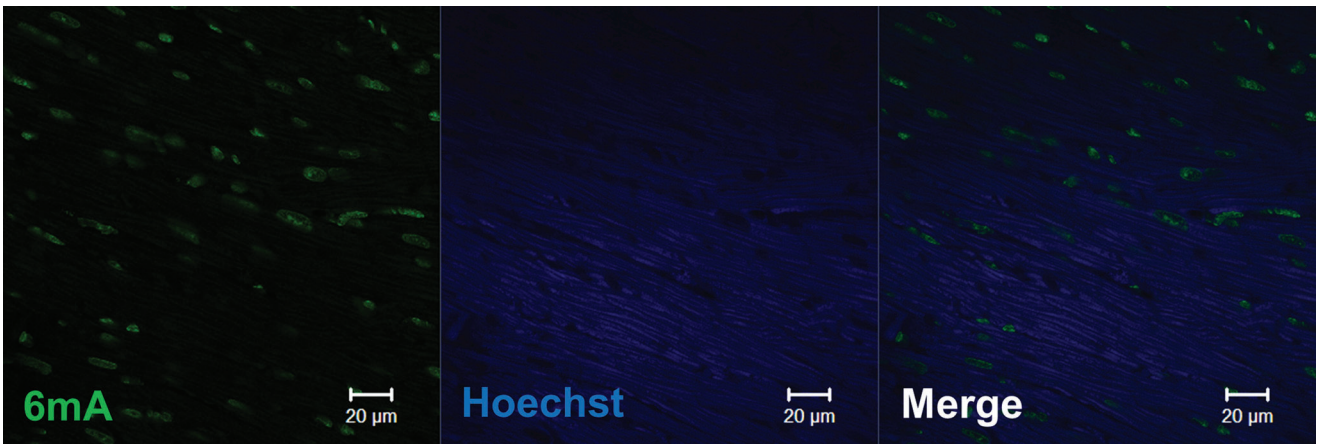

Figure 2. The 6mA immunostaining pattern in mouse heart tissues. Heart tissues from wild-type C57/B6 mouse were fixed in 4% paraformaldehyde, and then embedded in paraffin. After paraffin removing, the sections were stained with anti-6mA antibody and Hoechst.

potential roles are in mammals, have been thought to be less important, and they remain largely unresolved. Despite this, indirect evidence from assays by using 6mA-methylation-sensitive-enzymatic digestion suggested that 6mA was present in the mouse MyoD1 gene and in the rat type2 steroid5α reductase gene, implying that 6mA might contribute to regulating expression of these genes [24, 25]. Previous studies using the HPLC-electrospray ionization tandem mass spectrometry (HPLC-MS/MS) assays suggest that less than one of bases as the 6mA form in 10^6 nucleosides was detected in genomic DNA from some of mouse tissues [34]. Thus, it appears that overall levels of 6mA in mouse tissues are extremely low [34]. However, an important question that remains is whether the overall low levels of 6mA reflects low levels of 6mA in all cells and/or in all tissues. Our recent findings suggest that levels of 6mA display a significantly variable pattern in different cell types in fly ovaries, with relatively high levels of 6mA in the germaria, but very low levels or no detectable 6mA in egg chambers in the late stages [35]. On the basis of these observations, we speculate that 6mA in mammals might have biological significance in a lineage-dependent regulatory fashion if it occurs in specific lineage-dependent genomic loci. In fact, our preliminary results from immuno-staining experiments show that while the majority of cells have no apparent 6mA staining, a small portion of cells exhibit relatively high levels of 6mA in certain types of cells. For example, we performed immunostaining experiments using anti-6mA antibody to examine the patterns of 6mA in mouse heart cells. As shown in Fig. 2, a staining signal of 6mA could be significantly detected in a small potion of cardiac muscle cells, although most of cells from the heart tissue had no apparent 6mA staining, It would be interesting to compare the 6mA distribution pattern in genomic DNA between high- and low-level-6mA cells in the future.

How is 6mA modification regulated and interpreted?

The regulatory factors of 5mC including "writer," "reader," and "eraser" have been known in mammals for several years, and their biochemical and biological functions have been extensively studied (Fig. 1A). In chromatin, 5mC inhibits gene expression mainly in two ways. First, 5mC directly affects DNA structure, and thus modulates binding of transcriptional factors to their target sites. Second, specific 5mC binding proteins are recruited to 5mC sites and regulate histone modification and chromatin architecture [79]. Methyl-CpG binding domain (MBD) proteins, including MeCP2 and MBD1-4, have been identified to function as "readers" of 5mC [80], which can recognize a 5mC region and recruit the histone deacetylase (HDAC) complex. Deacetylation of histones promotes chromatin compaction, and thus inhibits gene transcription [81–83]. *MeCP2* is X-linked, and subject to X inactivation during development; and mutation of *MeCP2* gene has been revealed to be the major cause of Rett syndrome, a neurodevelopmental disorder that causes mental retardation [84–86]. In terms of 6mA, its "writer" in eukaryotes has to date been identified only in wheat and *C. elegans* (Fig. 1B) [43, 87]. We and others identified DMAD and NMAD-1 as "eraser" of 6mA in *D. melanogaster* and *C. elegans*, respectively (Fig. 1B) [35, 36]. The "reader" of 6mA is still unknown in eukaryotes. Identification and characterization of these factors in eukaryotes will bring novel insight into the molecular mechanisms regulated by 6mA modification.

6mA functions as an epigenetic mark and correlates with gene expression in eukaryotes

In bacteria, 6mA has been shown to function as an epigenetic mark that regulates a number of cellular processes. For example, 6mA was shown to regulate DNA replication initiator factors during the cell cycle [87]. 6mA was also reported to be involved in promoting transcriptional initiation by reducing the stability of base pairings through, hence, favoring by lowering the energy to open DNA duplexes [88]. However, similarly to the controversy over 5mC modification in *Drosophila*, the role of 6mA in higher eukaryotes including mammals has long been the

subject of discussion. We and others recently provided evidence showing that 6mA modification occurs in DNA from three different eukaryotic organisms, *C. reinhardtii*, *C. elegans*, and *D. melanogaster*, and that it plays conserved epigenetic roles in regulating gene expression. In our recent study, we found that 6mA exists in the *Drosophila* DNA at a relatively high level at the very earliest embryonic stages, but at low levels at the late embryonic stages, suggesting that 6mA is dynamic during *Drosophila* embryonic development. This dynamic change of the 6mA pattern involved an active demethylation event, which is primarily regulated by the *Drosophila* DMAD protein. Genetic analyses showed that loss of *DMAD* significantly increases the levels of 6mA in multiple adult tissues, supporting the idea that DMAD suppresses DNA 6mA modification in vivo. Importantly, *DMAD* mutant animals display strong developmental defects, suggesting that DMAD-mediated 6mA demethylation is essential for development and tissue homeostasis. 6mA-DNA-immunoprecipitation-sequencing analyses reveal that DMAD determines 6mA distribution in transposon regions in *Drosophila* ovary DNA, and that the regulation of DMAD demethylase activity is correlated with transposon expression [35]. Thus, our study suggests a potential epigenetic role of the DMAD-6mA regulatory axis in controlling development in *Drosophila*. In line with our findings, Shi and co-workers showed that 6mA modification is present in *C. elegans* and is regulated by a DNA demethylase, NMAD-1, and a potential DNA methyltransferase, DAMT-1. Notably, they found that 6mA and the histone modification H3K4me2 coordinately contribute to trans-generationally epigenetic control in worm. These data from the worm provide further evidence suggesting that this methyl mark may have an epigenetic role in eukaryotes [36]. Interestingly, the another study by He and colleagues showed that 6mA is mainly found at ApT dinucleotides surrounding transcription start sites (TSS) with a bimodal distribution, and appears to mark active genes in *Chlamydomonas* (green algae). The genome-wide mapping of 6mA and its genomic distribution in the *Chlamydomonas* suggest a potential epigenetic role of 6mA in regulating gene expression [37]. These findings together suggest that 6mA functions as an epigenetic mark in eukaryotes. Of note, although the DMAD-mediated 6mA modification is essential for development in fly, 6mA modification has a trans-generational role in worm, suggesting distinct roles of 6mA in different species.

Conclusions and outlook

The 6mA modification has been shown to play important roles in bacteria. Although it has been known for some time that 6mA is widely present in unicellular eukaryotes, the distribution and the related functions of 6mA in genomic DNA remain elusive. Most recent studies, including ours, suggest that 6mA is indeed present in multiple model organisms, and its status is dynamically regulated by methyltransferase(s) and demethylase(s) [35–37]. These findings together support a model of 6mA functioning as an epigenetic mark in higher eukaryotes including plants, insects and mammals.

Despite this emerging conceptual advance, the mechanisms that link 6mA modification to epigenetic regulations in higher eukaryotes (e.g. invertebrate) are only just beginning to be identified. A number of important issues regarding the role of the 6mA modifications have yet to be addressed. First, although the roles of 6mA have been determined in several invertebrate model systems, it will be important to explore whether 6mA modification occurs in vertebrates, especially in mammals. Second, enzymes responsible for 6mA methylation and demethylation in different organisms need to be identified. In particular, given that DMAD was shown to control 6mA demethylation in *Drosophila*, it will be interesting to search for double-stranded beta-helix-domain-containing dioxygenases responsible for 6mA demethylation in mammals. In addition, the identification of 6mA methyltransferases is important for understanding how 6mA is regulated in mammals. Third, although levels of 6mA in mouse and human are extremely low, 6mA might have important roles in a developmental stage-dependent and/or cell lineage-dependent manner. Thus, immunofluorescence analysis should be performed to monitor 6mA distribution in tissues, and single cell isolation and sequencing methods would be valuable for 6mA analysis. Fourth, in recent studies, members of the YTH domain-containing family were found to "read" RNA m6A signals and regulate RNA degradation and translation [89, 90]. A similar method could possibly be used to identify a DNA 6mA "reader" in higher eukaryotes. Fifth, the relationship between 6mA and human diseases such as cancer, require further clarification.

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