PERSPECTIVE

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DNA N^6-methyladenine in metazoans: functional epigenetic mark or bystander?

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The DNA-adenine modification N^6-methyladenine (6mA), initially thought to be mainly restricted to prokaryotes and certain unicellular eukaryotes, has recently been found in metazoans. Proposed functions vary from gene activation to transposon suppression. However, since most metazoan genomes possess 5-methylcytosine (5mC) as a dominant epigenetic mark, it raises the question of why 6mA is required. This Perspective summarizes the latest discoveries and suggests potential functional roles for 6mA in metazoan genomes.

An essential component for the storage of genetic information, DNA is composed of only four building blocks, the nucleobases adenine, guanine, cytosine, and thymine. These bases can be chemically altered through exposure to a variety of environmental agents[1]. There are, however, also naturally occurring base modifications, such as 5mC, which is produced by cellular enzymes in most metazoans to regulate gene expression[2]. 6mA, another naturally occurring modified base, was initially discovered as a widespread DNA modification in restriction-modification systems in prokaryotes[3]. Certain unicellular eukaryotes have also been known to possess relatively abundant genomic 6mA[4–6]. In addition, studies implied the presence of genomic 6mA in insects and plants[7–9], but the modification was not thought to be widespread in metazoans.

One barrier preventing the extensive study and characterization of genomic 6mA had been its low frequency in complex eukaryotes[6]. The recent development of highly sensitive MS approaches and high-throughput sequencing technologies has made such investigations possible[10,11]. In 2015, three independent papers reported the genome-wide distribution of 6mA in three different eukaryotes, *Chlamydomonas reinhardtii*, *Caenorhabditis elegans*, and *Drosophila melanogaster*[12–14]. These studies proposed regulatory functions for 6mA other than as a marker to distinguish invading foreign DNA from resident host DNA, which is its role in prokaryotic restriction-modification systems. Adenine methylation has since been reported in the genomes of vertebrates and mammals, raising widespread interest in its biological role as a potential epigenetic mark[15–18]. Genomic 6mA was observed in species of frog[16], mouse[16,17], and fish[16,18,] and genome-wide distribution patterns have been investigated using high-throughput sequencing. 6mA methyltransferases and demethylases were also proposed[13,14,17], although further investigations are required to validate the suggested functions.

Interestingly, the genomic distribution of 6mA appears to vary among species. Four distinct patterns of 6mA distribution in eukaryotic genomes have been observed: enrichment around transcription start sites (TSS) in green alga[12], broad distribution in *C. elegans*[13], depletion around TSS in frog and mouse[16], and enrichment at repeat elements in *Drosophila*, zebrafish, and mouse[14,17,18]. Accordingly, the proposed functions range from transcriptional activation to transcription silencing, as well as trans-generational chromatin regulation[11,15,19]. In unicellular eukaryotes, such as ciliated protozoa and green algae, the levels of 6mA in genomic DNA are relatively high. However, in metazoans, and mammals in particular, the overall abundance of 6mA is very low (6–7 6mA per million adenine residues, or lower)[16,17,20], which raises questions regarding its biological relevance. In addition, most metazoan genomes possess 5mC as a dominant epigenetic mark, so why should 6Ma be needed?

Here, we summarize recent discoveries on genomic distribution, biogenesis, turnover, and potential functions of 6mA in eukaryotes. We attempt to rationalize these results and speculate about the roles of 6mA in metazoans, with particular focus on its potential role in complementing 5mC. Despite the lower frequency of 6mA compared to 5mC in most circumstances, we propose 6mA as a functional, precisely regulated DNA mark, rather than a bystander in metazoans.

Distribution and dynamics of 6mA

5mC exhibits a unique genomic distribution pattern. In mammals, the majority of methylated sites are in a C-G context, but the mark is depleted in CpG islands[21]. Gene promoters enriched with C-G dinucleotides are usually hypomethylated unless permanently silenced in specific cell lineages[22]. In the ciliated protozoan *Tetrahymena thermophila*[23,24] and in the unicellular green alga *C. reinhardtii*[12,25], 6mA is mainly located in nucleosome-linker DNA with an A-T sequence motif. The genomic distribution pattern of 6mA diverges in metazoans: 6mA is evenly distributed in *C. elegans*[13] but depleted at TSS in frog and mouse[16]. In mouse, 6mA is enriched at regions with high levels of the histone variant H2A.X and at LINE-1 elements[17]. Although 6mA does not appear to associate with genes in *Drosophila* and zebrafish, it is enriched at repetitive elements[14,18]. This nonrandom distribution indicates regulated deposition, which suggests potential unique functions of 6mA in these species. Studies in green algae and in *C. elegans* mutants lacking *spr-5*, a histone H3K4me2 demethylase, indicate a heritable nature of 6mA in these species[12,13].

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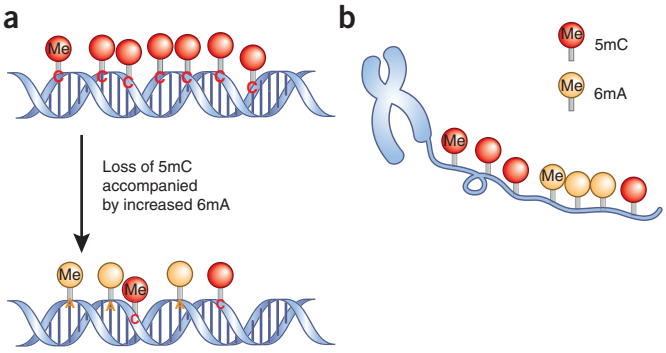

Figure 1 6mA might be regulated in spatiotemporal manners. (**a**) During early embryo development, most of 5mC is temporarily erased, and elevated levels of 6mA may serve as a complementary epigenetic mark to control transcription. (**b**) Certain regions of the mammalian genome may not or cannot be 5mC modified for various reasons. 6mA methylation could provide an alternative DNA mark in these regions.

Rigorously regulated dynamics is a characteristic feature of 5mC as an epigenetic mark. In mammals, the entire epigenome of the zygote undergoes reprogramming right after fertilization, and a large proportion of 5mC is erased[26]. This process allows the establishment of tissue- and cell-type-specific methylation patterns during embryo development[27]. DNA methyltransferases, DNA demethylases, and a variety of auxiliary proteins are involved in this highly orchestrated process[28]. Intriguingly, an accumulation of 6mA has been found in vertebrates during the same time period. In zebrafish, as well as in pig, neither sperm nor oocyte contains substantial 6mA; however, after fertilization, 6mA levels are significantly elevated before the sphere stage (4 h after fertilization) and then rapidly decline[18]. This timing is precisely that of 5mC reprogramming. Although the exact functions of 6mA during this period of early embryo development have yet to be elucidated, it is tempting to speculate that the noticeable increase of genomic 6mA is not a coincidence. Rather, it might complement the temporary loss of 5mC in the genome and facilitate embryonic development (**Fig. 1a**).

Establishment and turnover of 6mA

6mA in green alga occurs at palindromic sequences (ApT), resembling 5mC CpG methylation in mammals[12]. Palindromic motif sequences with symmetric methylation ensure that information is faithfully inherited during DNA replication. However, no similar palindromic motif has been reported for 6mA in other metazoans. In frog and mouse, 6mA is instead proposed to occur in multiple motifs without palindromic sequences[16,17], suggesting *de novo* methylation. This raises the question of how information is maintained between cell generations.

In mammals, DNMT1 methylates hemimethylated DNA to produce 5mC on the nascent daughter strand, whereas DNMT3A and DNMT3B mediate methylation of unmethylated DNA as *de novo* methyltransferases[22]. A putative DNA-adenine methyltransferase, DAMT-1, has been reported in *C. elegans*[13]. Deletion of the *damt-1* gene decreased the overall 6mA level, while overexpression of the gene increased the 6mA level. A methylation target sequence has yet to be identified. DAMT-1 belongs to the MTA70 protein family, a large class of proteins widely conserved in eukaryotes. Two human homologs of this family of proteins, METTL3 and METTL14, form a heterodimer that mediates N^6-methyladenosine (m^6A) RNA methylation[29–32]. The broad MTA70 family may contain other, as yet unidentified, DNA 6mA methyltransferases[33].

Both DNA and RNA methylation are removed by demethylase enzymes. As members of the AlkB family of dioxygenases, FTO and ALKBH5 are known RNA demethylases that erase the m^6A methylation from RNA[34,35]. The first DNA 6mA demethylase candidate was proposed in *Drosophila*[14]. It belongs to the TET protein family, which is known to facilitate 5mC oxidation and demethylation[28]. An AlkB family 6mA demethylase was also reported in *C. elegans*[13]. Most recently, the human and mouse AlkB homolog ALKBH1 was shown to mediate DNA 6mA demethylation in mouse embryonic stem cells (mESCs)[17]. ALKBH1 also catalyzes tRNA demethylation[36]. While the precise contribution of ALKBH1 to the DNA 6mA landscape requires further study, the presence of potential 6mA methyltransferases and demethylases suggest that 6mA could be actively regulated. Therefore, it is unlikely to be randomly generated DNA damage or a mere bystander, suggesting biological regulatory functions of 6mA (**Table 1**).

Readout of 6mA

A variety of 5mC-specific binding proteins have been identified in mammals. For example, MECP2 specifically binds methylated gene promoters to repress transcription[37]. More generally, methylation-binding proteins recruit partners to remodel the chromatin structure, mainly to silence transcription[2,38]. Reader proteins that specifically recognize 6mA on DNA have yet to be identified. In *C. elegans*, 6mA accumulates transgenerationally in mutants lacking *spr-5*, a histone H3K4me2 demethylase. Conversely, deletion of the putative 6mA methyltransferase mitigates elevated H3K4me2 levels in *spr-5* mutants, suggesting crosstalk between histone H3K4 and 6mA[13]. In the green alga *Chlamydomonas*, 6mA sites and nucleosomes are mutually exclusive, indicating a reciprocal regulation, which could be a more general mechanism that impacts nucleosome positioning. Furthermore, in this organism, 6mA accumulation is associated with gene activation, suggesting the existence of an unidentified pathway that reads the mark and communicates with the transcription machinery[12]. Whether these observations apply to other organisms still requires further investigation.

Table 1 A summary of recent studies of 6mA in eukaryotes

Species	Abundance (p.p.m.)	Distribution	Methyltransferase	Demethylase	Refs.
T. thermophila	~8,000	Enriched in linker DNA	–	–	4,23,24
C. reinhardtii	4,000–6,000	Enriched around TSS	–	–	12,25,45
C. elegans	~100–4,000	Evenly across the genome	DAMT-1	NMAD-1	13
D. melanogaster	10–700	Enriched on repeat elements	–	DMAD	14
Danio rerio	30–2,000	Enriched on repeat elements	–	–	16,18
Xenopus laevis	~90	Depleted at TSS	–	–	16
Mus musculus	6–7	Enriched on LINE-1	–	ALKBH1	16,17
Homo sapiens	Similar to mouse	–	–	–	16

The overall abundance is represented as p.p.m. (6mA sites per million dAs). Genomic distributions, corresponding methyltransferases, and demethylases are listed for each model organism. *T. thermophila*, ciliated protozoan; *C. reinhardtii*, green alga; *C. elegans*, nematode (roundworm); *D. melanogaster*, fruit fly; *D. rerio*, zebrafish; *X. laevis*, African clawed frog; *M. musculus*, house mouse; *H. sapiens*, human.

Uncertainties in metazoans

Although DNA methylation is acknowledged to be broadly involved in gene regulation, how pivotal a role it plays has been the center of discussion for a long time. Disruption of *de novo* methylation (DNMT3A and DNMT3B) in mESCs does not prevent their proliferation[39]. Organisms such as *D. melanogaster* and *C. elegans* do not seem to possess appreciable levels of 5mC[40,41]. The discovery of 6mA in fly and worm imply critical roles of DNA modifications, albeit in a chemically distinct form, in most complex life forms[42]. More intriguing is the identification of 6mA in vertebrates, in which 5mC is known to be a key epigenetic mark[19]. While 5mC comprises between 2% and 8% of all cytosine in mammalian nuclear DNA, the overall abundance of 6mA in the genomes of vertebrates is exceedingly low under most circumstances: it is present at 6–7 p.p.m. in the genome of mESCs and at even lower levels in frogs[16,17]. This is almost equivalent to the levels of common DNA base lesions[43]. Most recently, ultrasensitive UHPLC-MS further highlighted the extremely low levels of 6mA and N^4-methyldeoxycytosine (4mC) in the genomes of mESCs and tissues[20]. To what extent these limited 6mA levels actually play a biological role needs to be further investigated, with a focus on loci that show temporally or locally enriched 6mA (**Table 1**).

In contrast to the relatively defined roles of 5mC in transcriptional repression in vertebrates, the distribution and functions of 6mA are more elusive. In *Chlamydomonas*, 6mA is present near TSS and is associated with gene activation[12]. In *Drosophila*, 6mA is proposed to activate transcription of transposons, because removal of 6mA during embryonic development correlates with transposon suppression[14]. Analysis of the genome-wide distribution of 6mA in multiple frog tissues and mouse kidney using immunoprecipitation coupled with high-throughput sequencing demonstrated a unique pattern in which 6mA is depleted right after the TSS[16]. Conversely, by using SMRT sequencing, a third-generation sequencing method that reads base modification at single-base resolution, Wu *et al.* focused on chromatin regions enriched with the histone variant H2A.X in mESCs and observed an enrichment of 6mA in transposons. Furthermore, they showed, contrary to what was seen in *Drosophila*, that 6mA appears to silence transposon expression and nearby genes[17]. These observations may reflect highly divergent features of 6mA; however, it is also possible that some percentage of 6mA modification is due to rare, nonspecific, or aberrant methylation events mediated by cellular DNA or RNA methyltransferases. This would add to the difficulty in elucidating a role of 6mA.

Notably, the quantification of 6mA requires stringently sterile sample preparation because of the potential for contamination by prokaryotic DNA, in which 6mA is prevalent and abundant[3]. In cultured cell lines, mycoplasma contamination is a potential confounder that should never be neglected. In one large-scale study analyzing published RNA-seq data, mycoplasma contamination was suspected in at least 10% of mammalian cell lines[44]. In addition to exogenous contamination, endogenous nonnuclear DNA or free nucleotides might also confound the measurement of 6mA.

Outlook

With 5mC playing a dominant role as a DNA epigenetic mark in vertebrate genomes, how might 6mA influence gene expression given that it is present at 10^2- to 10^3-fold lower levels? Here, we suggest possible scenarios in which 6mA could mediate a biological function because of its temporal or spatial enrichment (**Fig. 1**).

A complementary mark at specific development time windows

As a chemically distinct mark, 6mA could play critical roles during specific developmental time windows during which 5mC levels are depleted. The observed spike of 6mA levels right after fertilization in zebrafish and pig suggest that it might serve as a replacement during the programmed loss of 5mC[18] (**Fig. 1a**). In this case, the activation of 5mC demethylation proteins, such as the TET enzymes, might signal the upregulation of DNA-adenine methylation, perhaps through a well-integrated signaling network, to induce 6mA as a way to offset the loss of 5mC. The TET enzymes themselves might even be directly involved in 6mA methylation and demethylation[33]. It is tempting to speculate that accumulation of 6mA could repress transposons in the absence of 5mC or control transcription critical to early embryonic development. Future studies analyzing the level and distribution of 6mA during early embryogenesis and its interplay with 5mC demethylation should reveal further mechanistic details. It should be noted that *Chlamydomonas* is unusual in that its nuclear DNA contains both 6mA and 5mC, and they are present at roughly similar levels[12,45,46]. This situation makes this organism particularly suited for investigating the roles of the two methylations.

A unique mark at specific genomic loci

Although 6mA might act as an 'alternative' DNA mark at times when 5mC is depleted during reprogramming or other differentiation processes, 6mA could also play unique roles at specific genomic loci devoid of 5mC. A large portion of the metazoan genome is composed of repetitive elements that are subject to 5mC repression; however, other regions are low or devoid of CpG. The discovery of 6mA in some of these regions suggests an alternative way to control these elements independent of 5mC-based regulation (**Fig. 1b**). The effects could be due to direct binding by 6mA-specific 'reader' proteins or transcriptional factors through the positioning of nucleosomes or through other chromatin factors. Therefore, despite the overall low abundance of 6mA in the genome, certain loci may possess spatially enriched 6mA for regulation.

Lastly, enzymes such as METTL3–METTL14 and ALKBH1 have been known to mediate mRNA methylation and tRNA demethylation, respectively[36,47]. These enzymes are known to exist in the nucleus, and some of them could interact with DNA[17]. More extensive studies to identify and characterize proteins involved in 6mA writing, reading, and erasing will be essential to understand the underpinnings of its presence and functional relevance in the future.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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