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The epigenetic roles of DNA N^6-Methyladenine (6mA) modification in eukaryotes

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ABSTRACT

The DNA N^6-methyladenine (6mA) modification is a prevalent epigenetic mark in prokaryotes, but the low abundance of 6mA in eukaryotes has recently received attention. The possible role of 6mA as an epigenetic mark in eukaryotes is starting to be recognized. This review article addresses the epigenetic roles of 6mA in eukaryotes. The existence of 6mA in metazoans and plants, the correlation of 6mA with gene expression, the enzymes catalyzing the deposition and removal of the 6mA modification, the relationship of 6mA to nucleosome positioning, the 6mA interaction with chromatin, its role in tumorigenesis and other physiological conditions/diseases and technical issues in 6mA detection/profiling and bioinformatics analysis are described. New directions and unresolved issues (e.g., the base-pair-resolution 6mA-sequencing method and gene activation vs. repression) in 6mA research are discussed.

1. The prevalence of 6mA in prokaryotes and its functions

Prevalent DNA N^6-methyladenine (6mA or m^6dA) modifications in prokaryotes (as much as 1.5% of total adenine in *E. coli* K-12) have been demonstrated [1]. 6mA belongs to the classical EcoK restriction/modification system that mainly plays a role in host defense [1–3]. 6mA also plays crucial roles in regulating bacterial DNA repair, replication, transposition, and gene expression [1–3]. One notable example shows that 6mA inhibits different types of prokaryotic DNA polymerase, such as *Sulfolobus solfataricus* Y-family DNA polymerase Dpo4 and *Pseudomonas aeruginosa* phage DNA polymerase PaP1 [4,5]. The functions of 6mA in prokaryotes have been extensively reviewed [1–3] and are not discussed in this review.

2. The presence of 6mA in lower eukaryotes, *Drosophila,* and *C. elegans*

The 6mA modification is very rare in eukaryotes (ranging from 6-7 ppm–6000 ppm) [6–8]. Unlike DNA 5-methylcytosine (5mC), which is a well-characterized epigenetic mark in mammalian genomes that has multiple functions [9], the role of 6mA modification in eukaryotes is just starting to be elucidated and remains to be extensively characterized [6–8]. The revival of the functional significance of 6mA in eukaryotes has recently been initiated. Recent research results show that detectable levels of 6mA exist in the unicellular eukaryote *Chlamydomonas reinhardtii* (green algae), *Drosophila,* and *C. elegans* [10–12]. In *Chlamydomonas reinhardtii*, 6mA modifications are present in 84% of genes [10]. 6mA mainly occurs at ApT dinucleotides around the transcription start sites (TSS) and is correlated with active gene expression [10]. Further studies of nucleosome positions show that 6mA shows a negative correlation with nucleosome position [10]. These results indicate that 6mA may play a regulatory role in eukaryotic gene expression [10]. In *Drosophila*, 6mA is located mostly in the gene bodies of transposons and is correlated with transposon activation [11]. The removal of 6mA during the embryonic stage results in transposon suppression [11]. In *C. elegans*, 6mA is enriched in two different sequence motifs (GAGG and AGAA) [12]. The function of 6mA in *C. elegans* is linked to transgenerational inheritance [12]. Furthermore, there is a crosstalk between

Abbreviations: 6mA, DNA N^6-methyladenine; DMAD, DNA 6mA demethylase; NMAD-1, N6-methyl adenine demethylase 1; Alkbh1, Alkb homolog 1; MT-A70, DNA methyltransferase family A70; Mettl4, methyltransferase like 4; AMT1, adenine MTase 1; N6AMT1, N6 adenine methyltransferase 1; DAMT-1, DNA N6-adenine methyltransferase 1; MTA1, methyltransferase family A1; MedIP, methylated DNA-immunoprecipitation; SMRT-seq, single molecule-real-time-sequencing; ChIP, chromatin immunoprecipitation; qChIP, quantitative chromatin immunoprecipitation.

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histone H3K4 methylation and 6mA, since a mutant lacking the putative *C. elegans* H3K4me2 demethylase *spr-5* causes 6mA accumulation transgenerationally [12]. According to the results described above, 6mA has been demonstrated to play significant roles in different aspects of biology in *Chlamydomonas/Drosophila/C. elegans*, and its role in eukaryotic biology has started to be recognized [10–14].

3. The existence of 6mA in different species of metazoans/fungi/plants and its functional significance

The initial discovery of 6mA marks in *Chlamydomonas, C. elegans*, and *Drosophila* ignited the search for the presence of 6mA in vertebrates, fungi, and plants. 6mA has been detected in vertebrates including *Xenopus laevis*, mice, and humans [15]. 6mA is also present in different cell types but is depleted from gene exons [15]. Other results showed that 6mA is present up to 0.1–0.2% of total adenines during the early embryogenesis of zebrafish and pigs, but decreases to background levels with the progression of embryo development [16]. During this process, 6mA marks are usually located in repetitive regions of the genome [16]. These results suggest that 6mA marks may be correlated with 5mC reprogramming in embryonic development [8,16]. Further results showed that abundant 6mA is associated with transcriptionally active genes in early-diverging fungal lineages [17], among which up to 2.8% of all adenines are methylated [17]. 6mA occurs symmetrically at ApT dinucleotides and is concentrated in densely methylated adenine clusters surrounding the TSS of expressed genes, similar to the situation in *Chlamydomonas* [10,17]. The distribution of 6mA is inversely correlated with 5mC [17]. Another study showed that 6mA is found at approximately 0.051% of all adenine within the human genome [18]. [G/C]AGG [C/T] is the motif associated with 6mA modification [18]. 6mA sites are enriched in the coding region and mark actively transcribed genes in human cells [18]. 6mA has also been detected in plants [19]. Using a DpnI cleavage system coupled with size-exclusion ultrafiltration method, bacterial DNA containing 6mA can be excluded, and the 6mA marks in plants and mammals can be measured by high-resolution mass spectrometry analysis [19]. This result demonstrated the universal presence of 6mA marks in mammals and plants [19]. A subsequent study showed that 6mA sites are widely distributed within the *Arabidopsis thaliana* genome and are enriched in pericentromeric heterochromatic regions [20]. 6mA marks are more frequently found in gene bodies and are positively correlated with gene expression in *Arabidopsis* [20]. The presence of 6mA specifies the transition from vegetative to reproductive growth in *Arabidopsis*, suggesting a physiological role [20]. Finally, 6mA has been identified in the rice genome and woodland strawberry [21,22]. A total of 0.2% of all adenines are 6mA methylated in the rice genome [21]. 6mA sites mark the promoters of silent genes but are correlated with gene activity in gene bodies [21]. In woodland strawberry, the presence of 6mA is correlated with active transcription [22]. All the results described above demonstrate the existence of 6mA in metazoans/fungi/plants [15–22]. Overall, the distribution of 6mA in eukaryotes can be species- and developmental stage-specific [10–12,15–22].

The presence of 6mA in various locations within genomes is correlated with different outcomes of gene expression [8]. Although the locations of 6mA in different species may vary, 6mA is linked to transcriptionally active genes in *Chlamydomonas*, early-diverging fungi, *Drosophila*, *C. elegans*, *Xenopus*, zebrafish, pigs, humans, *Arabidopsis*, rice, and woodland strawberry [10–12,15–22]. However, 6mA may also be correlated with gene silencing. In mouse embryonic stem cells, 6mA is correlated with transcriptional silencing of young LINE-1 transposon elements (<1.5 million years old) together with their neighboring enhancers and genes but not old LINE-1 transposon elements (>6 million years old) [23]. In addition, since L-1 elements are enriched on the X chromosome, 6mA also silences genes located on the X chromosome [23]. This epigenetic silencing mediated by 6mA can resist gene activation signals during embryonic stem cell differentiation [23]. This role in epigenetic silencing in mammalian evolution is distinct from its role in gene activation in other organisms [10–12,15–22]. Similarly, Phytophthora species (*Oomyces*, a plant pathogen) exhibit measurable levels of 6mA (0.04–0.05%) [24]. The 6mA marks detected in Phytophthora are usually located in transposable elements and are correlated with gene repression [24]. Other contrasting examples of 6mA dictating gene repression will be discussed in later sections. The linkage of 6mA with either gene activation or repression strongly suggests that 6mA is an epigenetic mark that regulates eukaryotic gene expression.

4. Enzymes catalyzing the deposition and removal of 6mA

For 6mA to be considered a putative epigenetic mark, enzymes catalyzing the deposition and removal of 6mA need to be identified, and their activities must be demonstrated. Regarding the possible enzymes removing 6mA, DMAD (DNA 6mA demethylase) has been identified to be a 6mA demethylase in *Drosophila* based on its sequence containing a conserved Fe2+ and 2-oxoglutarate-dependent dioxygenase domain and a DSBH (double-stranded β-helix) domain showing homology to the bacterial AlkB protein, which mediates 6mA demethylation [11]. Further assays demonstrated that removal of 6mA from the transposon region by DMAD is correlated with transposon suppression [11]. However, in another study in *Drosophila*, DMAD was shown to interact with the trithorax-related complex protein Wds to maintain active transcription by demethylating intragenic 6mA in a group of genes involved in neurodevelopment and neuronal functions [25]. These contrasting results may indicate that 6mA located in different regions and different gene groups may lead to different transcriptional outcomes [11,25]. In *C. elegans*, the enzyme removing 6mA has been shown to be NMAD-1 (N6-methyl adenine demethylase 1) on the basis of its sequence homology to ALKB family members, its ability to modulate fertility defects of *spr-5* mutant worms, and its biochemical activity demethylating 6mA [12]. Alkbh1 (Alkb homolog 1) was identified as a 6mA demethylase in mouse embryonic stem cells due to its sequence homology to bacterial demethylase Alkb and an increase in 6mA levels in Alkbh1 knockout mouse embryonic stem cells [23]. Alkbh1-deficient mouse embryonic cells show transcriptional silencing of young LINE-1 transposon elements, which is consistent with the role of *Drosophila* DMAD in the expression of genes involved in neurodevelopment and neuronal functions [23,25]. ALKBH1 has also been characterized as a 6mA demethylase in human mitochondria on the basis of its sequence homology to bacterial AlkB and its location in mitochondria [26]. Asymmetric 6mA clusters in human mitochondria regulate mitochondria functions, and the loss of ALKBH1 decreases mitochondrial oxidative phosphorylation [26]. In another study in humans, ALKBH1 was demonstrated to be a 6mA demethylase due to its homology with mouse Alkbh1 and its biochemical ability to demethylate 6mA [18]. The function of ALKBH1 as a human 6mA demethylase was also confirmed in human glioblastoma cells by the same group [27]. In rice, OsALKBH1 has been identified as a 6mA demethylase on the basis of its sequence homology to mouse Alkbh1 and the finding that the disruption of OsALKBH1 increases 6mA levels [20]. Structural analysis of OsALKBH1 revealed a DSHB domain, and the mutation of its enzymatic site abolishes its demethylase activity [21]. Furthermore, a study in mice shows that Alkbh4 is a 6mA demethylase according to its sequence homology to the *Drosophila/C. elegans* 6mA demethylases and its ability to demethylate oligonucleotides and genomic DNAs containing 6mA [28].

Regarding the enzymes mediating 6mA deposition, homologs of MT-A70 family members (DNA methyltransferase family A70) can be identified on the basis of evolutionary relationships [13,14]. DAMT-1 (DNA N6-adenine methyltransferase 1) was shown to be a 6mA methyltransferase in *C. elegans* according to its homology with MT-A70 family members and its knockdown in *C. elegans,* leading to decreased 6mA levels in extracted gDNAs [12–14]. In *Tetrahymena*, AMT1 (adenine MTase 1) was shown to be a 6mA methyltransferase on the basis of its homology with MT70-domain of methyltransferases, and deletion of

AMT1 in *Tetrahymena* significantly decreases 6mA levels [29]. N6AMT1 (N6 adenine methyltransferase 1) was identified as a 6mA methyltransferase in humans since it contains an adenine methyltransferase-characterized amino acid motif (DPPW), indicating a potential AdoMet-dependent methyltransferase activity, and silencing of N6AMT1 decreases 6mA levels in human cells [18]. However, Mettl4 (methyltransferase like 4) has been shown to be a mouse 6mA methyltransferase that increases 6mA levels via *in vitro* overexpression experiments, and Mettl4 null mouse embryonic stem cells show depleted 6mA levels [28]. In *Oxytricha* ciliates, MTA1 (methyltransferase family A1), is a subunit of the *Oxytricha* MTA1c complex, containing MTA1, MTA9, p1, and p2 [30]. MTA1 and MTA9 belong to the MT-A70 family, whereas p1 and p2 are homeobox-like proteins exhibiting DNA-binding activity [30]. Among these four components, only MTA1 shows the 6mA catalytic activity, and the disruption of MTA1 gene significantly decreases overall 6mA levels in *Oxytricha* [30]. Therefore, MTA1 is a putative 6mA methyltransferase in *Oxytricha* [30]. Finally, recent reports using isotopic labeling coupled to ultrasensitive mass spectrometry showed the presence of a low level of 6mA modification in mammalian DNA and suggested that these 6mA marks may be derived from recycled m6A marks on RNA through the nucleotide-salvage pathway and misincorporated into the mammalian genome by DNA polymerases [31,32]. In these reports, neither Alkbh1, Alkbh4, nor Mettl4 was shown to be either a demethylase or methyltransferase of 6mA marks [31,32]. Considering all the above results, the elucidation of the true identity of both the 6mA methyltransferase and demethylase will require further characterization and confirmation. A summary of putative 6mA methyltransferases and demethylases is presented (Fig. 1).

5. 6mA, nucleosome positioning, and DNA replication

The ability of 6mA to regulate nucleosome positioning and affect chromatin organization is an important issue. Earlier results obtained in *Chlamydomonas* by comparing high-resolution 6mA-sequencing and nucleosome footprinting results, followed by high-throughput sequencing, showed that 6mA presents a negative correlation with nucleosome position and that 6mA marks DNA linkers between adjacent nucleosomes around the TSS of actively transcribed genes [10]. Further result in *Tetrahymena* showed a negative correlation between 6mA and nucleosomes [33]. *In vitro* nucleosome assembly using 6mA-containing DNAs showed that nucleosome-protected DNA contained less 6mA marks, indicating that a single 6mA site is able to alter the preference of DNA wrapping around the nucleosome [33]. Additional biophysical experiments showed that 6mA changes the curvatures and causes the rigidification of dsDNA structures, disfavoring nucleosome wrapping [33]. Another study in *Tetrahymena* using MNase-seq to map nucleosome distributions showed that linker regions with 6mA marks are flanked by well-positioned nucleosomes or H2A.Z-containing nucleosomes in Pol II-transcribed genes [34]. Finally, as described above in the ciliate *Oxytricha*, the 6mA methyltransferase complex MTA1c deposits 6mA at ApT dinucleotides [30]. *In vitro* chromosome assembly using native gDNAs (with 6mA) vs. PCR-amplified DNAs (without 6mA) with *Oxytricha* histone octamers followed by MNase-seq showed low nucleosome occupancy with increasing 6mA levels, indicating that 6mA directly disfavors nucleosome occupancy *in vitro* [30]. This pattern occurs in a local and quantitative manner independent of the DNA sequence, and the chromatin remodeler ACF (ATP-utilizing chromatin assembly and remodeling factor) can restore nucleosome occupancy across 6mA sites [30]. All these results delineate the role of 6mA in regulating nucleosome positioning in different species. However, the role of 6mA in regulating nucleosome positioning in mammalian cells has not been demonstrated.

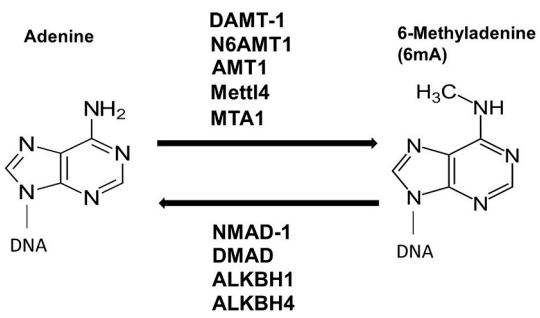

Fig. 1. A summary of different 6mA methyltransferases and demethylases from different species described in the text. Abbreviations for different species are: ce, *C. elegans*; te, *Tetrahymena*; ox, *Oxytricha*; d, *Drosophila*; m, mouse; h, human.

The presence of 6mA has been shown to inhibit human DNA polymerases iota and η by reducing dTMP incorporation, next-base extension, burst-rate incorporation, and the binding affinity of DNA for either polymerase with varying degrees of inhibition [35,36]. These results indicate that 6mA plays a role in inhibiting mammalian DNA replication, rather than regulation of gene expression, similar to the observation of the inhibition of bacterial DNA polymerases by 6mA as described above [4,5].

6. 6mA, stress response, and physiological conditions

The current literature shows that overall 6mA levels are significantly elevated under stress [37–39]. Specifically, 6mA levels are significantly increased under stress in the prefrontal cortex of mouse brain, although gain-of-6mA and loss-of-6mA regions can both be identified [37]. 6mA is correlated with the downregulation of LINE transposon expression in the mouse prefrontal cortex under stress [37]. Genes bearing stress-induced 6mA changes (i.e., loss of intragenic 6mA) significantly overlap with a set of upregulated neuronal genes related to neuropsychiatric disorders in the mouse brain [37]. This result indicates a strong link of dynamic changes in 6mA with neuropsychiatric disorders [37]. In *C. elegans*, global 6mA levels are significantly elevated following mitochondria perturbation, and 6mA modifications are required for the transmission of mitochondrial stress adaptions to progeny [38,39]. 6mA marks mitochondria stress response genes and promotes their transcription to alleviate mitochondria stress in progeny, making 6mA a transgenerational epigenetic mark [38,39]. This result is consistent with an earlier report that 6mA plays a role in transgenerational inheritance in *C. elegans* [12]. Another report showed that 6mA marks are the results of deposition along promoters and coding sequences in activated prefrontal cortical neurons in adult mice trained in fear extinction [40]. Genome-wide occupancy of N6amt1 was correlated with 6mA modification and extinction-induced gene expression [40]. 6mA modification of the P4 promoter in *Bdnf* (brain-derived neurotrophic factor) drives *Bdnf* exon IV mRNA expression in the infralimbic prefrontal cortex (ILPFC) of mice and mediates the formation of fear extinction memory, providing another example of the role of 6mA in the neuronal functions of mice [40]. All the results from the mouse brain and *C. elegans* support the role of 6mA as an epigenetic mark that modulates the stress response [37–40]. For other physiological conditions, increased 6mA levels in peripheral mononuclear cells are associated with systemic lupus erythematosus [41]. Overall, the significance of changes in 6mA levels is starting to be recognized as being related to different physiological conditions, and 6mA marks likely play regulatory roles in these conditions.

7. Roles of 6mA in tumorigenesis

The recent literature shows that 6mA plays different roles in human tumorigenesis. In one report, a high 6mA density appears in exons and is positively correlated with gene transcription [18]. Notably, G-protein coupled receptor (GPCR) genes, which are well-known receptors involved in cancer, have a high 6mA modification ratio [18]. Further

analyses of a TCGA dataset showed that increased ALKBH1 levels and deceased N6AMT1 levels are associated with tumorigenesis [18]. 6mA levels have been shown to be lower in gastric and liver cancers, and the downregulation of 6mA levels promotes tumorigenesis [18]. Specifically, a decrease in 6mA levels caused by the depletion of N6AMT1 promotes cell growth, colony formation, migration, and invasion of different tumor cell lines [18]. In contrast, silencing of ALKBH1 represses tumor cell growth, colony formation, migration and invasion [18]. The results from mouse xenograft experiments and *in vivo* metastatic assays are consistent with these *in vitro* tumor cell line results when either N6AMT1 or ALKBH1 is manipulated to control the 6mA levels that are correlated with the stages of tumor progression [18].

However, another report showed that 6mA levels are elevated in glioblastoma, especially in glioblastoma stem cells [27]. In glioblastoma, 6mA is enriched in heterochromatin regions and colocalizes with heterochromatin H3K9me3 and H3K27me3 marks [27]. Tumor suppressor genes, including CDKN3, AKAP6, and RASSF3, are shown to be repressed by 6mA and H3K9me3 [27]. Interestingly, hypoxia-related genes are downregulated following ALKBH1 knockdown under hypoxic conditions, whereas DNA damage and p53 pathway genes are upregulated [27]. Therefore, 6mA provides a good therapeutic target for glioblastoma [27]. By comparing these two discoveries [18,27], the difference in tumor types and the different groups of tumor suppressors or oncogenes regulated by 6mA may explain the different correlation patterns of 6mA levels and tumorigenesis. It is obvious that 6mA levels in various tumor types may be elevated or repressed depending on the specific gene groups regulated by 6mA.

A recent report showed that the levels of *N6*-hydroxymethyl-2′-deoxyadenosine (h6mA or hm^6dA), a hydroxylation derivative of 6mA, are significantly increased in lung carcinoma tissues [42]. ALKBH1 is shown to convert 6mA to h6mA *in vitro* and *in vivo*, and an increase in h6mA levels in lung carcinoma tissues is consistent with increased *ALKBH1* mRNA levels in these tissues [42]. The functional role of h6mA in lung carcinoma remains to be further investigated.

8. The 6mA interaction with chromatin and its contribution to tumorigenesis

The role of 6mA in interacting with different chromatin-modifying complexes has been demonstrated in various model systems. In *Drosophila*, DMAD interacts with Trithorax-related complex protein Wdrs to maintain active transcription [25]. 6mA accumulation caused by depleting DMAD functions in coordination with polycomb proteins and contributes to transcriptional silencing of these genes, which are involved in neurodevelopment and neural functions [25]. In *C. elegans*, there is crosstalk between histone H3K4 methylation and 6mA since a mutant lacking the putative *C. elegans* H3K4me2 demethylase *spr-5* causes 6mA accumulation transgenerationally [12]. In mice, 6mA deposition triggers the proteolytic destruction of components of the polycomb repressive deubiquitinase complex, MPND deubiquitinase and ASXL1, to preserve histone H2A-K119Ub levels and polycomb silencing [28]. In glioblastoma, 6mA levels are significantly increased, and 6mA colocalizes with H3K9me3/H3K27me3 heterochromatin regions, indicating gene silencing [27]. The repression of tumor suppressor genes by 6mA and H3K9me3 promotes glioblastoma stem cell survival [27]. The depletion of ALKBH1 in glioblastoma causes the transcriptional silencing of oncogenic pathways, providing a therapeutic target for glioblastoma [27]. The above results demonstrate the interaction between 6mA and different chromatin-modifying complexes, leading to transcriptional silencing in various species [25,27,28].

9. 6mA sites and 6mA-binding proteins

As described above, 6mA has been linked to transcriptionally active genes in various species [10–12,15–22,29,33]. 6mA enrichment at ApT dinucleotides and its concentration in densely methylated adenine clusters surrounding the TSS of expressed genes are correlated with gene expression in early-diverging fungi, *Chlamydomonas*, and *Oxytricha* [10,17,30]. In *Drosophila*, *Xenopus*, zebrafish, pigs, and mice, 6mA sites located in either transposons or repetitive elements are correlated with gene expression [11,15,16]. In liver and gastric cancers, 6mA sites are enriched in coding regions, and a higher 6mA density in exons caused by ALKBH1 depletion is associated with gene transcription [18]. 6mA sites located in gene bodies are correlated with gene expression in *Arabidopsis*, rice, and woodland strawberry [20–22]. In rice, 6mA is positively correlated with salt and heat stress and is associated with actively transcribed genes involved in heat stress [43]. In contrast, 6mA located in young transposable elements or intergenic regions is correlated with gene repression in mouse embryonic stem cells or glioblastoma, respectively [23,27]. It is possible that the locations of 6mA and the proteins binding to 6mA may dictate the differential transcriptional outcomes of different gene groups. The positions of 6mA sites and their transcriptional outcomes in different species are summarized (Table 1).

Regarding the identification of 6mA-binding proteins, a 6mA DNA affinity purification approach has been used to identify the Fox-family protein Jumu in *Drosophia* [44]. Jumu binds to 6mA and acts as a maternal factor to control the maternal-to-zygotic transition (MZT) [44]. 6mA marks coding genes in early embryo [44]. Jumu controls proper zygotic genome activation (ZGA) in early *Drosophila* embryos by regulating the expression of *zelda* marked by 6mA [44]. These findings demonstrate that 6mA epigenetic mark can be read by a specific transcription factor [44]. Using a similar affinity purification method, single strand DNA-binding protein 1 (SSBP1) has also been identified as a 6mA-binding protein [26]. In this study, 6mA was shown to label the heavy strand of mitochondria chromosomes, and SSBP1 favors its binding to the heavy strand of mitochondria chromosomes [26]. Whether SSBP1 binds to 6mA sites in genomic DNAs remains to be determined. More 6mA-binding proteins from different species and their functions will be identified and further characterized in the future.

Table 1
The positions of 6mA marks and their transcriptional outcomes.

Species/Organs	Locations of 6mA	Transcriptional outcomes	References
Chlamydomonas	TSS[a]	Activation	10
C. elegans	Wide distribution; Mitochondria stress response genes	Activation	12, 38
Arabidopsis thaliana	Pericentromeric chromatin regions	Activation	20
Early diverging fungi	TSS	Activation	17
Mouse embryonic stem cells	Transposable elements	Repression	23
Xenopus, mice, humans	Wide distribution (depleted in exons)	Activation	15
Woodland strawberry	Wide distribution	Activation	22
Zebrafish, pigs	Repetitive elements	Activation	16
Humans/ glioblastoma	Intergenic regions	Repression	27
Humans/liver, lung cancer	Coding regions	Activation	18
Drosophila	Transposon	Activation	25
Oxytricha	+1∼+3 nucleosomes downstream of TSS	Activation	30
Tetrahymena	TSS; Linker DNA between nucleosomes	Activation	33, 34
Mice/brain	Neuronal genes, transposable elements/ Extinction learning genes	Repression/ Activation	37, 40
Rice	Wide distribution	Activation	21, 43
Phytophthora	Transposable elements	Repression	24

[a] TSS: transcription start site.

10. Sequencing methods and bioinformatics

It is conceivable that the profiling of 6mA-modified genomic locations and the characterization of 6mA regulatory biology relies heavily on how the numbers and positions of the 6mA marks are quantified and profiled. The quantification of 6mA levels requires ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) [6–8], which is a standard technology for measuring the absolute quantities of 6mA marks since the levels of 6mA modification are extremely low, usually around the detection limit [6–8]. To profile the locations of 6mA sites, different sequencing techniques described in the published literature have been used, including 6mA-IP--seq, MedIP-seq, 6mA-CLIP-exo-seq, SMRT-seq, and Oxford Nanopore-seq (for a summary, see Table 2). Although SMRT-seq seems to be the most common technology used to date, a recent report showed that this method usually overestimates the amount of 6mA marks measured in eukaryotic genomes [45]. Contaminations by the enzymes used to digest the DNA and bacterial contamination in the prepared samples comprise two of the sources for 6mA measurement artifacts [45]. In addition, a portion of the 6mA modifications in mammalian cells may be derived from the incorporation of exogenous methylated nucleosides into the mammalian genome from bacteria in food or microbiota in *C. elegans*. The absolute concentration of 6mA measured by SMRT-seq was significantly higher than that was measured by UHPLC-MS/MS, as determined by comparing the sequencing results from many different species [45]. All these aforementioned factors should be considered before concluding that an accurate measurement of the 6mA concentration in mammalian cells has been obtained [45]. The 6mA-CLIP-exo-seq or DA-6mA-seq (DpnI-assisted 6mA sequencing) method may exhibit a more accurate resolution in identifying 6mA positions [46,47]. The recently developed Oxford Nanopore sequencing technology has been reported to be able to profile 6mA locations within the genome, a claim that requires confirmation by different laboratories [48]. Therefore, the improvement of the precise and accurate whole-genome profiling of 6mA sites within the genome remains a pressing issue.

Another approach to profiling genomic 6mA marks is the use of a bioinformatics strategy. Various computer algorithms and platforms have been developed [49–56]. For example, MetSMRT was the first platform resource created for browsing 6mA and 4mC methylomes in different species according to public PacBio SMRT-seq data [49]. In addition, i6mA-Pred, i6mA-DNCP, MM-6mAPred, and 6mA-RicePred are four computational methods developed to detect the 6mA sites within the rice genome [50–53]. Among the methods used, the support vector machine is used in the i6mA-Pred and i6mA-DNCP methods, whereas the Markov model is used for the MM-6mAPred method [50–52]. The combination of the Markov model for feature extraction and the support vector machine for model training is used in the 6mA-RicePred methods [53]. These methods have shown varying degrees of successful prediction rates, as determined by jack-knife test (i6mA-Pred: 83.13%; i6mA-DNCP: 86.65%) or by 10-fold cross validation (MM-6mAPred: 89.72%; 6mA-RicePred: 85.6%) [50–53]. iDNA6mA-PseKNC is the first tool utilized to identify 6mA sites in DNA sequences from cross-species analysis, and it uses a support vector machine, which is also associated with a web server and is easy to use [54]. The 6mA-Finder tool combines all the analysis methods and is claimed to better predict 6mA sites [55]. SNNRice6mA is a tool applying artificial intelligence and neural network deep learning to predict 6mA sites and seems to be the best for identifying 6mA sites in rice [56]. It is obvious that more algorithms and platforms will be developed to improve the prediction of 6mA sites from the sequencing data of different species.

Table 2
Different sequencing methods used to profile genome-wide 6mA marks.

Species/Organs	Sequencing methods	References
Chlamydomonas	6mA-IP-seq, 6mA-CLIP-exo-eq	10
C. elegans	MeDIP-seq, SMRT-seq, MeDIP-seq	12, 38
Arabidopsis thaliana	SMRT-seq	20
Early diverging fungi	SMRT-seq	17
Mice/embryonic stem cells	SMRT-ChIP-seq	23
Xenopus, mice, humans	MeDIP-seq	15
Woodland strawberry	SMRT-seq	22
Zebrafish, pigs	6mA-IP-seq	16
Humans	6mA-IP-seq	18, 27
Drosophila	6mA-IP-seq	25
Oxytricha	SMRT-seq	30
Tetrahymena	6mA-IP-seq,	33, 34
Mice/brain	6mA-IP-seq, DA-6mA-seq	37, 40
Rice	SMRT-seq, SMRT-PacBio-seq	21, 43
Phytophthora	MeDIP-seq	24

6mA-IP-seq: 6mA immunoprecipitation-sequencing; 6mA-CLIP-exo-seq: 6mA immunoprecipitation-photocrosslinking-exonuclease digestion-sequencing; MedIP-seq: 6mA methylated DNA-immunoprecipitation-sequencing; SMRT-seq: single molecule-real-time-sequencing; SMRT-ChIP-seq: single molecule-chromatin immunoprecipitation-sequencing; DA-6mA-seq: DpnI-assisted 6mA sequencing; Oxford Nanopore-sequencing: sequencing by Oxford Nanopore technology; SMRT-PacBio-seq: single molecule-PacBio sequencing technology.

11. Conclusions

In this review, the current research on 6mA related to its prevalence in eukaryotes, the correlation of 6mA with gene expression, the enzymes catalyzing and removing this mark, the relationship of 6mA with nucleosome positioning, the 6mA interaction with chromatin, its role in tumorigenesis and other physiological conditions/diseases and technical issues of 6mA detection/profiling and bioinformatics analysis are presented. The locations of 6mA marks and their correlations with gene activation/repression are summarized (Table 1). The 6mA levels in different species are also summarized (Table 3). Taken together, these results begin to elucidate the function of 6mA as a putative epigenetic mark. Various biological processes other than gene regulation are summarized (Fig. 2). However, further research is clearly required to fully understand the biological functions of 6mA in eukaryotes, especially its role in tumorigenesis.

Although different 6mA methyltransferases and demethylases have been identified and characterized, it is possible that these methyltransferases or demethylases are only components of large protein complexes, such as the MTA1c complex in *Oxytricha* [30]. The identification and characterization of cofactors in 6mA methyltransferase or demethylase protein complex and their possible modulatory roles may further expand the knowledge of the mechanisms of 6mA deposition and removal.

Table 3
Comparison of 6mA levels in prokaryotes and eukaryotes.

Species	Abundance of 6mA levels (ppm)[a]	References
E. coli K-12	~15000	1
Chlamydomonas	4000–6000	8, 10
C. elegans	~100–4000	8, 12, 38
Drosophila	10–700	8, 25
Early diverging fungi	Up to 28000	17
Xenopus	~90	8, 15
Zebrafish	30–2000	8, 16
Mice	6–7	8, 23
Humans	~6–1000[b]	8, 18, 27
Arabidopsis thaliana	60–1380	20
Rice	~2000	21, 43
Woodland strawberry	~1390	22
Phytophthora	400–500	24
Ciliates	1800–25000	30
Tetrahymena	~8000	8, 33, 34

a: The abundance of 6mA levels is reported as ppm (6mA sites per million dAs).b: The 6mA levels in human cells are similar to the levels in mice; whereas a hundred fold 6mA levels are observed in glioblastoma stem cells.

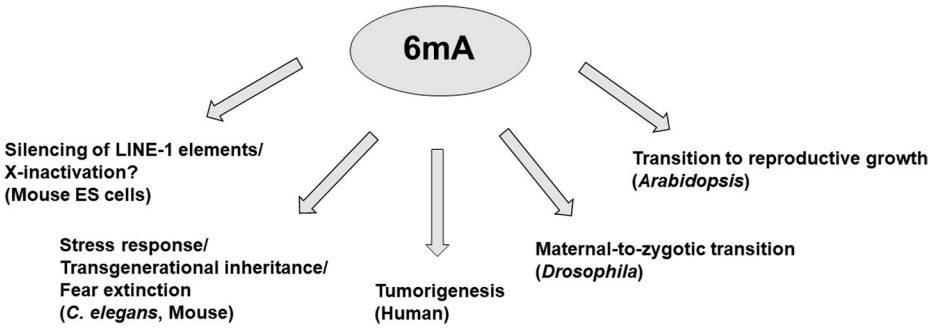

Fig. 2. A summary of various biological processes in different species regulated by 6mA. Regulation of gene expression by 6mA is not included.

From an evolutionary point of view, higher abundance of 6mA marks, leading to gene activation, tends to be observed in lower eukaryotes whereas mammalian species exhibit far fewer 6mA marks. In addition, gene repression mechanisms are easily observed in mammalian species, suggesting that sophisticated epigenetic mechanisms have evolved in more complex organisms to accommodate the 6mA epigenetic mark to modulate gene expression. The difference in the abundance of 5mC and 6mA in lower eukaryotes vs. mammalian species is another evolutionary aspect worth investigating [8]. In addition, the ability of 6mA to inhibit human DNA polymerases [35,36] indicates that the lower abundance of 6mA marks in humans allows higher fidelity of human DNA replication. In contrast, higher 6mA levels in glioblastomas may facilitate genomic instability to promote tumor progression [27]. It is obvious that other evolutionary roles played by 6mA will start to be illuminated once more functional significance of 6mA marks can be delineated.

Although 6mA is starting to be recognized as an epigenetic mark in eukaryotes (especially in mammalian cells), many unresolved issues must be addressed. First, the sequencing methods used to profile 6mA sites in the whole genome need to be improved. The present techniques using anti-6mA-specific antibodies to pull down genomic 6mA marks, followed by sequencing, cannot achieve a base-pair resolution. The single molecule real-time sequencing (SMRT-seq) method also tends to overestimate the amount of 6mA sites [45]. A non-antibody method (e.g., using specific chemical modification of 6mA sites followed by sequencing) may be an alternative strategy for accurately profiling genomic 6mA sites. The identification and characterization of the 6mA sites, their consensus sequences, and their locations will reveal more information about how 6mA is correlated with gene activation or repression depending on the gene context and its interaction with chromatin. The knowledge obtained will be considered in concert with the current knowledge of chromatin modifications regarding gene expression. Although a *Drosophila* 6mA reader (Jumu) has been identified [44], the next high-priority issues are the identification of mammalian 6mA-binding proteins and the further characterization of their functions in the current context of transcriptional regulation. The interplay between 6mA readers and the eukaryotic transcription factor/transcription initiation complex will provide new insight into gene transcription. For the 6mA marks located in LINE-1 transposable elements causing gene silencing on the X chromosome [23], understanding the molecular mechanism of how 6mA contributes to X chromosome gene silencing will be another interesting topic. Since 6mA has been shown to be linked to stress conditions [37–40], it will be interesting to test what other stress conditions increase 6mA levels and to further understand the role of 6mA under such stress conditions. Furthermore, whether 6mA plays a crucial role in many different biological processes remains to be demonstrated. Regarding the role of 6mA in tumorigenesis, as 6mA levels can be either increased or decreased depending on the tumor type [18,27], it will be important to determine 6mA levels in different types of cancer and perform the whole-genome profiling of 6mA sites in these different types of cancer to delineate the functional significance of the 6mA sites during tumorigenesis. Other roles played by 6mA that contribute to other human diseases (e.g., neuropsychiatric disorders) will require similar approaches to better understand the functional significance of 6mA in these diseases [37,40]. With respect to the above discussion, the field of 6mA research must further expand both technically and conceptually to answer all these unresolved issues.

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Declaration of competing interest

The author declared that no competing interests existed.

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