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Means, mechanisms and consequences of adenine methylation in DNA

Konstantinos Boulias[1,2,3], **Eric Lieberman Greer**[1,2,3,✉]

[1]Division of Newborn Medicine, Boston Children's Hospital, Boston, MA, USA.

[2]Department of Pediatrics, Harvard Medical School, Boston, MA, USA.

[3]Harvard Medical School Initiative for RNA Medicine, Boston, MA, USA.

Abstract

N^6-methyl-2′-deoxyadenosine (6mA or m^6dA) has been reported in the DNA of prokaryotes and eukaryotes ranging from unicellular protozoa and algae to multicellular plants and mammals. It has been proposed to modulate DNA structure and transcription, transmit information across generations and have a role in disease, among other functions. However, its existence in more recently evolved eukaryotes remains a topic of debate. Recent technological advancements have facilitated the identification and quantification of 6mA even when the modification is exceptionally rare, but each approach has limitations. Critical assessment of existing data, rigorous design of future studies and further development of methods will be required to confirm the presence and biological functions of 6mA in multicellular eukaryotes.

Directed DNA epigenetic modifications include N^4-methylcytosine (4mC), which has been identified mainly in the genomes of thermophilic bacteria and archaea[1–5]; C^5-methylcytosine (5mC), the most extensively studied and predominant DNA modification in the genomes of more recently evolved organisms[6–9]; and N^6-methyladenosine (6mA or m^6dA; not to be confused with RNA N^6-methyladenosine (m^6A), a prevalent modification of the same nitrogen position that is estimated to occur in approximately 25% of mammalian mRNAs with an average frequency of one to three modifications per transcript[10,11]) (FIG. 1a). Genomic DNA (gDNA) modifications can increase the versatility of nucleic acids by altering the chemical properties of the nucleosides, including their affinity for binding proteins and the stability of the modified substrate. For example, 5mC increases DNA helix stability and chiefly functions to repress gene expression, whereas methylation of adenines destabilizes the helix and causes DNA unwinding[12–14], with 6mA shown to increase base pair stability and destabilize base stacking[15–17] (FIG. 1a).

In prokaryotes and protists, 6mA is actively added to DNA by specific DNA adenine methyltransferases, which primarily use *S*-adenosylmethionine (SAM or AdoMet) as

✉ eric.greer@childrens.harvard.edu .

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the methyl donor[18,19], although the existence of alternative methyl donors such as 5,10-methylene tetrahydrofolate cannot be excluded[20–22]. DNA adenine methylation can be removed via the Fe(ii)- and α-ketoglutarate-dependent dioxygenases of the AlkB family[23] or by conversion into hypoxanthine by a 6mA deaminase[24] followed by base excision repair by hypoxanthine DNA glycosylases of the AlkA family[25] (FIG. 1b). 6mA has been implicated in prokaryotic immunity, the DNA damage response, transcriptional tuning, nucleosome positioning and cell cycle control[7].

Until recently, 6mA was thought to be restricted to the genomes of prokaryotes and protists, but several studies have now reported the presence of 6mA in gDNA from multicellular eukaryotes such as algae, plants, invertebrates and vertebrates, including mammals[7,26–38]. These discoveries have largely been driven by a revolution in the development of new and more sensitive 6mA detection techniques over the past decade. As with all technologies, these methods have limitations that need to be taken into account when interpreting results, leading some in the field to question whether 6mA in multicellular eukaryotic genomes is a bona fide DNA modification or a technical artefact. Others have questioned whether, if present, 6mA is a directed modification that occurs through a mechanism similar to that in bacteria[39] or is instead the consequence of other cellular processes; gDNA methylation could also arise as the result of a Dimroth rearrangement of a non-enzymatic DNA-damaging lesion such as 1mA[40] or through incorporation by DNA polymerases of pre-methylated RNA or DNA nucleosides, such as those generated via the nucleotide salvage pathway[41–45] (FIG. 2).

Here, we review evidence for and against the presence of 6mA in multicellular eukaryotes and whether it is a regulated epigenetic modification, starting with a discussion of the methods used for its detection and the enzymes that have been identified to add, remove and recognize 6mA in various species. We also discuss whether the functions of 6mA in prokaryotes and protists could be conserved in metazoans and assess the evidence for the involvement of 6mA in other biological processes in multicellular eukaryotes, including the stress response, disease and transmission of non-genetic information across generations.

Methods for detecting 6mA in genomes

Detection techniques for quantifying and mapping 6mA have evolved rapidly in the past decade. Owing to the limitations of each of these methods it is necessary to use multiple orthogonal approaches to confirm or exclude the presence of 6mA. Here, we critically evaluate the methods that are used to identify, quantify and localize 6mA and discuss some of the reasons for discrepancies in results between different studies.

Methods for detecting and quantifying 6mA.

Historically, detection and quantification of 6mA DNA methylation relied on relatively insensitive methods including salt crystallization[46], paper chromatography[47–49], ultraviolet absorption spectra[50], dot blots[51] and electrophoretic mobility[49] (TABLE 1). Sensitivity was increased by methods using methylation-sensitive restriction enzymes[52,53], which facilitated the detection of a single methylated base; however, only methylated bases within the restriction enzyme recognition motif are detected using this approach, meaning other

methylation events are missed. More recently, capillary electrophoresis and laser-induced fluorescence (CE–LIF) has been developed, which detects 6mA at a lower limit of 0.01% 6mA per nucleotide[54]. Liquid chromatography[55] has also become increasingly sensitive, with ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) capable of detecting 6mA at levels as low as 0.00001% of adenines[32] (TABLE 1). Owing to its specificity and sensitivity, UHPLC–MS/MS has become the predominant method for verifying the presence of and quantifying 6mA in any given organism. However, a main caveat of UHPLC–MS/MS (and most other 6mA quantification techniques) is that it cannot discriminate the source of 6mA in a given gDNA sample, which may become particularly problematic when 6mA is of very low abundance in the organism of interest compared with potential contaminants, such as DNA from microbiota or other species or from the enzymes used to digest DNA for UHPLC–MS/MS (which are purified from bacteria)[43,56,57]. Thus, when 6mA is sparse it is essential to use multiple orthogonal approaches that do not depend on the same reagent (TABLE 1) to confirm its presence, and also to identify environmental or genetic conditions that change the levels and/or distribution of 6mA significantly to confirm that it is an actively regulated modification in the organism being investigated.

Methods for mapping 6mA.

In addition to quantifying the levels of 6mA in the genome, several methods have been developed to determine its genomic location. Identification of the location of 6mA allows for correlations to be drawn between 6mA and gene expression, DNA damage repair, cell cycle regulation and nucleosome positioning and chromatin regulation. Methylated DNA immunoprecipitation (MeDIP), which uses a methyl-specific antibody to pull down methylated gDNA, has been coupled with quantitative PCR (MeDIP–qPCR[58]) to determine the methylation levels at a particular residue (TABLE 1). Alternative approaches for mapping 6mA at particular genomic sites have used probes that bind to either methylated or unmethylated adenines in specific sequence contexts[59] or rely on radio-active methylation of the DNA followed by restriction digestion, electrophoresis and sequencing[60] (TABLE 1).

MeDIP has also been used with microarray analysis[61] or DNA sequencing (MeDIP–seq[62]) to examine methylation sites genome wide (TABLE 1). However, these techniques are dependent on the 6mA-directed antibody being highly specific. The most commonly used 6mA antibody has a >1,000-fold greater affinity for methylated adenines over unmethylated adenines[26], but if only one or two adenines per million are methylated, as is likely the case in most multicellular eukaryote genomes, this antibody would still result in a relatively high amount of nonspecific binding and pull-down. Furthermore, in addition to their inherent error rates, sequencing-based methods are sensitive to the tendency of all antibodies to bind non-specifically to unmodified repetitive DNA sequences[57,63]; however, over-representation of repetitive sequences can be minimized by avoiding over-amplification during library preparation[64]. MeDIP–seq can give near single-nucleotide resolution when coupled with photo-crosslinking, exonuclease digestion and restriction enzyme digestion[65,66], but this approach detects methylated adenines only within the specific recognition motif.

Finally, long-read sequencing techniques, such as single-molecule real-time sequencing (SMRT-seq) and Oxford nanopore sequencing, identify methylated bases directly in DNA[67]. SMRT-seq provides an antibody-independent means of detecting, at nucleoside resolution, every different DNA modification that produces a unique kinetic signature on the basis of changes in the incorporation rate of complementary bases during sequencing, but it requires high sequencing depth and loses accuracy when 6mA is present at less than 10 parts per million bases (ppm)[35,43,57,68,69] (TABLE 1). Given that some of the earliest SMRT-seq analyses of multicellular eukaryotic genomes were performed using mapping algorithms designed for bacterial species in which 6mA occurs at high abundance in specific motifs[70], it will be important to repeat these analyses with higher sequencing depth to ensure detected methylated bases do not represent false positives[43,69]. The difficulties in mapping 6mA are highlighted by the lack of reproducibility between data generated by SMRT-seq and MeDIP–seq for the same gDNA sample[26].

Discrepancies in reported 6mA locations and levels in metazoans.

Technologies such as SMRT-seq[69] and UHPLC–MS/MS[43] were developed and optimized for prokaryotic genomes containing abundant 6mA. As such, these technologies are less accurate and require further optimization when 6mA is orders of magnitude less abundant, as is likely the case for multicellular eukaryotic genomes. Furthermore, UHPLC–MS/MS reports on all 6mA in the sample, regardless of source, and is therefore sensitive to contaminants such as prokaryotic DNA, which contains substantial levels of 6mA[43]. These inherent methodological inaccuracies and limitations likely contribute to the wide range of 6mA concentrations that have been reported for metazoan genomes[7,43,71] (TABLE 2). For example, in mammals, 6mA has been reported either to not exist at all[41,57] or to occur at levels as low as 0.1–1 ppm[32] and as high as ~1,000 ppm in human glioblastoma-derived stem cells[72]. Similar differing reports exist for most multicellular eukaryotic genomes. A recent study tried to address these discrepancies by developing a quantitative 6mA detection method based on SMRT-seq (termed 6mASCOPE) that deconvolutes 6mA in samples of interest from contamination sources[71]. Application of 6mASCOPE to *Drosophila melanogaster*, *Arabidopsis thaliana* and *Homo sapiens* samples detected low amounts of 6mA in the organisms of interest (~2–3 ppm), while high 6mA was found in DNA from contaminating bacterial sources from the fly gut microbiome or plant soil, suggesting that 6mA is much less abundant in these organisms than previously thought[71]. The recent discovery of the presence of RNA m^6A in RNA–DNA hybrids[73] should also be accounted for as a potential caveat when 6mA antibodies pull down particular regions of DNA or if enzymes consistently localize to specific regions of the genome. Nevertheless, it remains possible that the range of detected 6mA levels reflects true biological variation, such as between cell types, developmental stages or environmental contexts. For example, basal levels of 6mA could be low to undetectable while higher levels could reflect context-dependent changes, such that a failure to detect 6mA may be the result of looking at cells in the wrong biological context. Thus, it is important to consider biological differences such as cell type and culture conditions when making direct comparisons between studies. It is best, therefore, to complement these detection techniques with genetic manipulation of the enzymes required for adding, removing or recognizing 6mA and/or environmental

manipulations that alter the levels of 6mA to ensure the biological relevance of the detected adenine methylation.

Inconsistencies in 6mA localization have also been reported. Between-species differences may reflect real biological differences, for example, 6mA was reported to be enriched on the X chromosome in mice[29], but depleted on the X chromosome in human SMRT-seq samples[74] and in *Caenorhabditis elegans*[43]. By contrast, enrichment of 6mA at young long interspersed nuclear element 1 (LINE-1) retrotransposon elements in both mammals and *D. melanogaster*[29,34,66,69,75] is not always reproducibly detected[71,76,77]. Similarly, inconsistent 6mA localization is reported when different sequencing techniques are applied or when different cell lines have been mapped. Although these inconsistencies could reflect that 6mA is targeted to particular genomic regions instead of to specific sites[43,74], they could also reflect deeper issues of mapping techniques giving false positives owing to secondary structure or RNA contamination[57,63]. Alternative mapping techniques are required to shore up confidence in the mapping of 6mA and the conclusions drawn from correlations between 6mA localization and other phenotypes.

Biological relevance of eukaryotic 6mA

The existence of 6mA in more recently evolved eukaryotes is heavily debated owing to its relative spar sity[26–31,41,43,44,57,78], which often places it at the very limit of current detection methods and makes them prone to reporting false positives[41,43,57,78]. The confirmation of active DNA N^6-methyltransferases, demethylases and 6mA-interacting partners in eukaryotic genomes would support the biological relevance of 6mA as an active epigenetic modification in these species despite its relative scarcity. However, the presence of eukaryotic proteins with sequence homology to prokaryotic enzymatic regulators of 6mA is not definitive proof that 6mA is a bona fide regulated modification in eukaryotes; these proteins could have adopted new functions or new substrates in multicellular eukaryotes and, even if their ability to modify 6mA is retained, this function could be irrelevant under physiological conditions. In this section, we discuss putative multicellular eukaryote 6mA-regulating enzymes and 6mA-interacting proteins and whether they provide sufficient supporting evidence for 6mA as a directed DNA modification. We also discuss evidence that indicates that 6mA is a passive consequence of misincorporation of pre-methylated RNA or DNA nucleosides.

Putative N^6-adenosine methyltransferases in multicellular eukaryotes.

The search for N^6-adenosine methyltransferases in metazoans has focused on proteins that contain the MT-A70 domain, which evolved from the M.MunI-like bacterial 6mA DNA methyltransferases[8]. This domain homology approach identified the DAMT-1 protein in *C. elegans* as a putative N^6-adenosine methyltransferase[26] (TABLE 2). METTL4, the mammalian homologue of DAMT-1, has also been proposed to be an N^6-adenosine methyltransferase that modifies gDNA[79] and has been shown to be active in vitro against mammalian mitochondrial DNA (mtDNA)[80], raising the possibility that 6mA could indeed be a directed active epigenetic modification. However, it has also been suggested, on the basis of both in vitro and ex vivo experiments, that METTL4 catalyses m^6Am on U2 small

nuclear RNAs (snRNAs)[81–83] and so, as for all putative DNA methyltransferases, it will be important to perform kinetic experiments to confirm that its specificity or preference for DNA over alternative substrates is physiologically relevant (TABLE 2). Indeed, in vitro and ex vivo experiments on human N6AMT1, another enzyme that was initially proposed to be a 6mA methylase on the basis of homology (in this case to the 6mA methyltransferase M.TaqI)[76,77], suggest that it is more likely to be a glutamine methyltransferase for eRF1 (REF.[84]). Crystal structures revealed that the active site and substrate binding sites of N6AMT1 are negatively charged and therefore presumably cannot bind to DNA[85]. Additionally, side-by-side comparisons of the in vitro activity of N6AMT1 and bacterial DNA methyltransferases on DNA and eRF1 revealed that N6AMT1 is active only on eRF1 under physiological conditions[86]. As an aside, it is interesting to note that the human RNA m^6A methyltransferase complex METTL3–METTL14 has been shown to have in vitro activity against single-stranded DNA[87]. However, whether this RNA methyltransferase complex has physiologically relevant activity against DNA is unknown. These examples of the promiscuity of putative methyltransferases highlight the importance of confirming that putative N^6-adenosine methyltransferase activity is physiologically relevant.

Putative N^6-adenosine demethylases in multicellular eukaryotes.

The AlkB family of DNA and/or RNA dealkylating enzymes is conserved from bacteria to humans[88,89]. AlkB demethylases use oxygen to oxidize the methyl group of 6mA, which creates the unstable intermediate 6-hydroxymethyladenine (6hmA), which will then spontaneously release formaldehyde and regenerate an unmodified adenine base[88] (FIG. 1b). There are nine AlkB proteins (ALKBH1–8 and FTO) in humans, of which ALKBH1 and ALKBH4 have been proposed to demethylate 6mA[29,72,77] (TABLE 2). ALKBH4 demethylates 6mA in demethylation assays in vitro[79], and its homologue in *C. elegans*, NMAD-1, has also been proposed to be a DNA 6mA demethylase[26], although alternative substrates could potentially be more physiologically relevant[90] (TABLE 2). ALKBH1 has the capacity to demethylate single-stranded DNA in vitro, and gene knockout of *Alkbh1* in mouse embryonic stem cells causes an increase in global 6mA levels, which is rescued by wild-type alleles but not by alleles that disrupt the ALKBH1 catalytic domain[29,66]. Structural studies of mouse and human ALKBH1 suggest that it prefers partially opened or bubbled DNA as a substrate over double-stranded or single-stranded methylated DNA[91,92]. Moreover, a recent report detected 6hmA in rat tissues and human cell lines and demonstrated that ALKBH1 could produce 6hmA both in vitro and ex vivo[93], suggesting that the mechanism of 6mA demethylation is conserved. However, in vitro assays with ALKBH1 and various potential substrates reveal that it preferentially demethylates m1A on tRNAs[94], while another in vitro study showed that it demethylates m^5C on tRNAs[95].

Surprisingly, the ten-eleven translocation (TET) proteins, which demethylate 5mC in metazoans[96–98], have been proposed to demethylate 6mA in *D. melanogaster*, an organism that seems to lack 5mC (REFS[27,99]). Nuclear extracts from flies that lack *Dmad* activity (the *D. melanogaster* gene that encodes the TET homologue) displayed reduced in vitro demethylation activity compared with extracts from wild-type flies, and activity was restored by supplementation with purified DMAD protein[27] (TABLE 2). Although TET family

members do not seem to demethylate purines in bacteria, and crystal structures of several species indicate that TET catalytic domains should not be able to accommodate flipped out purines[100], it remains possible that they could adopt a non-canonical function under specific circumstances, such as the absence of its canonical substrate, as seems to be the case in *D. melanogaster*.

Presence of 6mA binding proteins supports a functional role for 6mA.

One mechanism by which 6mA could alter biological function is through recognizing and recruiting or antagonizing effector molecules (TABLE 2). For example, in bacteria, the MutH and SeqA proteins bind to 6mA in hemimethylated DNA to maintain DNA replication fidelity[101–104] and regulate replication timing[105–108], respectively. The most prevalent and best-defined 6mA binding proteins in prokaryotes are restriction enzymes. Conserved restriction enzyme adenine methylase-associated (RAMA) domains from 6mA-specific restriction enzymes have been computationally identified in eukaryotic proteins[109], as have SeqA domains, HARE-HTH domains and ASCH domains, which, on the basis of their domain architectures, have also been predicted to bind to N^6-adenine methylated DNA[109–112]. The existence of these domains in eukaryotic proteins lends credence to the notion that 6mA could be present and functional in multicellular eukaryotes.

In addition to the proteins with predicted 6mA binding domains, several eukaryotic proteins have recently been identified in which DNA binding is modulated directly or indirectly by 6mA in vitro. Pull-down experiments from human cells followed by quantitative mass spectrometry identified the mitochondrial protein SSBP1 as a 6mA binding protein with an ~2.5-fold increased binding affinity for methylated DNA over unmethylated DNA, as assessed by fluorescence anisotropy[66] (TABLE 2). The functional role of 6mA in mammalian mtDNA is further bolstered by the discovery that the mitochondrial transcription factor TFAM has a slight binding preference for unmethylated DNA and that the addition of 6mA suppressed in vitro transcription of mtDNA[80]. In vitro binding assays suggest that 6mA antagonizes binding of the mammalian transcription factor and chromatin regulator SATB1 to its potential binding sites, and this interaction could explain how 6mA prevents euchromatin spreading into heterochromatic regions during trophoblast stem cell differentiation[64] (TABLE 2). However, when 6mA directly precedes a thymine, as it does in the DNA sequences used for the in vitro SATB1 binding assays, it causes dramatic bending of the DNA helix[113], which could prevent SATB1 binding independently of any effects of 6mA on binding site recognition. Therefore, whether 6mA actively repels SATB1 binding or whether the in vitro synthesized oligo did not contain sufficient space for any transcription factor to bind remains to be determined.

In *D. melanogaster*, the forkhead transcription factor Jumu has been shown to have a slight preference for binding to 6mA-methylated DNA over unmethylated DNA in vitro, and comparison of in vivo Jumu binding sites (determined by ChIP–seq) and 6mA localization (determined by MeDIP–seq) revealed a significant overlap (TABLE 2). 6mA in *D. melanogaster* is enriched at essential genes required for zygotic genome activation, which is consistent with the proposed role of Jumu in binding to and activating transcription of methylated target genes involved in the maternal-to-zygotic transition[75]. By contrast, the

A. thaliana transcription factor WER, which is essential for development, displays reduced binding to motifs that are methylated either at the C^5 position of cytosines or at the N^6 position of adenines[114] (TABLE 2).

Taken together, these examples highlight the potential mechanistic role of 6mA in promoting or preventing binding of effector proteins that translate the putative epigenetic information into biological consequences.

Evidence for indirect incorporation of 6mA in multicellular eukaryotes.

Although the evidence discussed above indicates that 6mA may be a directed methylation event in metazoans, other studies suggest that it is the consequence of misincorporation of salvaged methylated nucleotides by DNA polymerases (FIG. 2). Several groups have demonstrated that administration of exogenous pre-methylated DNA N^6-methyladenine and RNA N^6-methyladenosine to mammalian cells leads to their incorporation into mammalian DNA[41–45]. However, misincorporation of methylated nucleotides does not preclude directed methylation, and these studies did not examine mtDNA methylation or methylation under specific environmental conditions that might reveal directed 6mA. Furthermore, if pre-methylated adenines are incorporated into the gDNA of multicellular eukaryotes in vivo, it does not imply that these ‘misincorporated’ bases are not functionally important. It will be necessary to examine whether, under different conditions, levels of 6mA change in a directed fashion. If 6mA increases in specific locations, even if it is through an indirect mechanism, it could still have important biological functions.

Biological roles of 6mA

6mA is best studied in prokaryotes and protists (such as *Chlamydomonas reinhardtii*, *Tetrahymena thermophila* and *Oxytricha trifallax* (now *Sterkiella histriomuscorum*)) in which it has well-established biological roles. Here, we discuss these roles and consider whether new evidence from multicellular eukaryotes supports conservation of 6mA function throughout evolution.

Restriction–modification systems.

Whereas eukaryotes have evolved complex immune systems, prokaryotes use DNA methylation as a marking system to distinguish self DNA from foreign DNA. Bacteria have developed unique endonucleases that selectively cleave foreign bacteriophage DNA based on the methylation status of their own genome. If the bacteria methylate their own DNA, then their restriction enzymes will selectively cleave unmethylated DNA and, conversely, if their own DNA is unmethylated, their endonucleases will selectively cleave methylated DNA and leave the host genome undigested[8,115] (FIG. 3a). Interestingly, 6mA in LINE-1 retrotransposons in *D. melanogaster* and mammals correlates with transposon repression[29,34,66,75] (FIG. 3a). Thus, 6mA might have a conserved role in recognizing and inhibiting foreign DNA, even if it has integrated into the host genome. However, further proof of this potential role is required, as enriched 6mA in LINE-1 elements is not always reproducibly detected[76].

Effects on transcription.

In addition to the immune functions of restriction–modification systems in bacteria, 6mA also directly regulates the expression of host genes involved in bacterial virulence and in defence against phage infection[115–118]. 6mA correlates with actively transcribed genes in the single-celled eukaryote *C. reinhardtii*[58] and occurs in clusters around the transcriptional start sites of expressed genes in several diverse fungal species[35]. 6mA also correlates with increased transcription of modified genes in *A. thaliana*, rice, barley, tobacco, wheat, zebrafish and of methylated constructs electroporated into mice[30,31,70,119–121]. However, 6mA is correlated with reduced expression of modified genes in the parasite *Trichomonas vaginalis* and with both increased and reduced expression in *D. melanogaster*, depending on the tissue studied[99,122]. These species- and tissue-dependent effects could be due to two potentially opposing consequences of DNA methylation: physical relaxing of the DNA structure, which is conducive to increased gene expression; and the recruitment of 6mA-specific binding proteins, which could either activate or repress gene transcription. 6mA has been shown to directly affect the binding affinity of specific transcription factors[123–125] (FIG. 3b), suggesting that its effects on transcription of specific genes could be dependent on gene regulatory sequences and associated transcription factors. However, in vitro transcriptional elongation experiments using *Saccharomyces cerevisiae* RNA polymerase II revealed that 6mA reduces the incorporation efficiency of uridines and causes the polymerase to stall[126] (FIG. 3b), suggesting that any increases in transcriptional activity will be dependent on overcoming this physical pausing. To move beyond correlations with gene expression, it will be important to perform more experiments with directed methylation to examine whether the 6mA modification is causal for changes in transcription.

Nucleosome positioning.

In *Tetrahymena thermophila*, *C. reinhardtii* and *O. trifallax* 6mA is preferentially localized in linker regions between nucleosomes[58,127–129] (FIG. 3c), raising the possibility that this modification could help to direct nucleosome positioning. In *C. reinhardtii* this increased 6mA is correlated with increased gene expression[42,70] presumably owing to a combination of an increase in euchromatic DNA, a reduction in helix stability and the recruitment of transcription machinery. 6mA is important for nucleosome occupancy of *O. trifallax* DNA in vitro[129]. However, in vivo experiments showed that genomes from *O. trifallax* that lack the gene encoding the putative N^6-adenine methyltransferase, MTA1, did not have altered nucleosome occupancy nor was transcription of genes methylated near their transcriptional start site dramatically changed, suggesting that additional factors can restore appropriate nucleosome positioning in the absence of 6mA[129]. Deficient in DNA methylation 1 (DDM1) has been reported to control C^5-cytosine methylation in vivo in *A. thaliana*[130] and *O. sativa*[131] and has also been shown to shift nucleosomes in vitro in *A. thaliana* independently of the 5mC status of the DNA[132]. Interestingly, deletion of DDM1 causes a 2.5-fold reduction in 6mA in rice genomes, and 6mA is also enriched in linker regions in rice[70], raising the possibility that DDM1 could be important for both 6mA methylation and nucleosome remodelling (FIG. 3c). Whether there is a mechanistic or functional link between 6mA deposition and nucleosome remodelling remains to be determined.

DNA damage control.

In both *Escherichia coli* and the fungus *Penicillium chrysogenum*, mutants that lack DNA adenine methyltransferase function have higher mutation rates and are more sensitive to DNA-damaging agents, suggesting that 6mA could protect against DNA damage or affect the DNA repair process[133,134]. Indeed, in *E. coli* and other Gram-negative bacteria, 6mA helps to maintain fidelity after DNA replication by marking the parental DNA strand and, in the event of mismatches, recruiting 6mA binding proteins to specifically cleave the newly synthesized strand[135] (FIG. 3d). This method of DNA mismatch repair has been demonstrated predominantly in bacteria, with different mechanisms existing in eukaryotes[136]. However, we recently reported that a mutation in the *C. elegans* gene *nmad-1*, which encodes a putative 6mA DNA demethylase, leads to defective expression of DNA repair genes and elevated levels of DNA damage[90], raising the possibility that some aspects of the prokaryotic DNA repair function of 6mA could be conserved in eukaryotes (FIG. 3d).

Cell cycle regulation.

E. coli mutants that lack functional 6mA methyltransferase Dam cannot properly regulate the timing of DNA replication[137,138]. In wild-type *E. coli*, the DNA methyl binding protein SeqA binds to 6mA at the origin of replication, *OriC*, and delays DNA replication until the cell has divided[115,139] (FIG. 3e). Conversely, in the absence of SeqA binding, 6mA lowers the thermal melting temperature and facilitates DNA unwinding at the origin, thereby promoting replication[140]. DNA replication and cell cycle progression are regulated in a similar manner in *Caulobacter crescentus*, another Gram-negative bacterium[141–143]. The addition of 6mA to the genome of PaP1, a phage that infects *Pseudomonas aeruginosa*, significantly decreased the efficiency of the DNA polymerase Gp90 exo$^-$ (REF.[144]), suggesting that DNA polymerase enzymes could be directly affected by the presence of a methylated adenine. Some preliminary evidence hints at a possible role for 6mA in regulating the cell cycle in eukaryotes. For example, mutation of the putative 6mA DNA demethylase-encoding gene, *nmad-1*, in *C. elegans* leads to reduced fertility owing to defects in chromosome segregation accompanied by delayed DNA replication and impaired DNA repair[90]. Moreover, the recent identification of human SSBP1, a mtDNA replication factor, as a 6mA binding protein[66], raises the possibility that 6mA could play a part in regulating mtDNA replication as well. Finally, 6mA decreases the replication efficiency of human DNA poly merase-η in kinetic experiments in vitro[145], suggesting that if 6mA is present in humans it could inhibit DNA replication (FIG. 3e). However, this altered activity could simply reflect changes to the ancestral polymerase that, if 6mA is absent from or present at negligible levels in human genomes, were not selected against. To determine a definitive role for 6mA in cell cycle regulation in multicellular eukaryotes it will be necessary to confirm through multiple independent techniques that 6mA is differentially enriched at origins of replication and to perform directed N^6-adenine methylation or demethylation at origins of replication and measure the consequences on cell cycle progression. It is also possible that 6mA could regulate the cell cycle of metazoans through direct regulation of cell cycle gene expression[90] instead of by marking the origin of replication as it does in bacteria.

Emerging roles for 6mA

Despite controversy surrounding the presence of 6mA in multicellular eukaryotes some potential eukaryote-specific biological roles have emerged in recent years. Here, we critically evaluate the evidence for the role of 6mA in biological processes in multicellular eukaryotes and discuss both the strengths and limitations of these studies.

Stress responses.

Several studies suggest that 6mA could have a regulatory and responsive role to stress in recently evolved eukaryotes. Rice exposed to heat shock displayed a marked increase in 6mA at crucial heat stress genes, as assessed by HPLC–MS/MS and 6mA immunoprecipitation followed by qPCR[70]. Treatment of *C. elegans* with the electron transport chain inhibitor, antimycin, caused an overall increase in 6mA as detected by dot blots[38]. Induction of hypoxia in human cell lines caused an increase in 6mA in mtDNA as assessed by UHPLC–MS/MS and MeDIP–seq[80] (FIG. 4a). In mice, two recent studies measured an increase in overall 6mA levels in the brain in response to either chronic stress[18] or fear extinction learning[80], as assessed by dot blots and LC–MS/MS[34,76]. However, when RNA sequencing was used to examine the expression of genes determined by MeDIP–seq to have increased levels of 6mA, the two studies revealed opposite correlations[34,76], suggesting that whether 6mA functions as an activating or repressive modification in the mouse brain is context dependent. However, it is also possible that stress could lead to an increase in RNA m6A levels and a decrease in RNA stability, which in turn could lead to increased incorporation of pre-methylated RNA nucleosides into gDNA through the nucleotide salvage pathway. This increased misincorporation of modified nucleosides would represent a less directed, but equally important, effect on DNA methylation that could also help to explain opposing effects on gene expression in response to stress.

Chromatin regulation.

6mA has been proposed to have a regulatory role in metazoan gene expression because of its direct effect on DNA folding and transcription factor recruitment, but it also has the potential to recruit other chromatin-regulating enzymes, possibly through communicating with other epigenetic modifications. For example, 5mC has been shown to be important for directing histone methylation, and vice versa, because the modifying enzymes responsible for each of these epigenetic marks can also physically interact with the other epigenetic modifications[146–151]. 6mA correlates with chromatin boundaries[64], the histone variant H2A. X[29] and various histone modifications (including histone H3 lysine 4 dimethylation (H3K4me2)[26], H3K9me3 and H3K27me3 (REFS[72,99])), leading to the supposition that these modifications could communicate with 6mA to reinforce an epigenetic signature. In *C. elegans*, genetic epistasis experiments have revealed coordinated regulation of phenotypes in response to manipulation of the putative 6mA-regulating enzymes and the histone H3K4 methyltransferases[26,38]. In *D. melanogaster*, the proposed DNA demethylase Dmad was shown to bind to Wds, a component of the complex that trimethylates H3K4, and the levels of the activating histone mark H3K4me3 were reduced in loss-of-function *Dmad* mutants[99] (FIG. 4b). In this same study, the authors showed a significant overlap between sites of 6mA modification (assessed by MeDIP–seq) and binding sites for polycomb proteins (assessed by

ChIP–chip), which catalyse histone H3 lysine 27 di- and trimethylation (H3K27me2/me3) associated with transcriptional repression[99]. As 6mA in *D. melanogaster* is associated with reduced transcription, interactions between these chromatin modifications would reinforce a repressed chromatin state. Similarly, coordinated interactions between 6mA binding proteins and polycomb recruitment were demonstrated in mice through manipulation of the putative 6mA methyltransferase gene *Mettl4* and the putative 6mA demethylase gene *Alkbh4* (REF.[79]). It remains to be determined whether 6mA potentially communicates with activating histone-modifying enzymes in organisms in which 6mA correlates with increased transcription.

Cancer.

In humans, dot blot analysis showed that total levels of 6mA were lower in triple negative breast cancer tissue samples than in normal breast tissue samples[152]. Conversely, 6mA was detected at higher levels in glioblastoma stem cell lines and primary samples than in astrocytes by dot blots, UHPLC–MS/MS and immunofluorescence, and, in glioma cell lines, 6mA correlated with an increase in epigenetic modifications associated with heterochromatin[72]. Knock-down of the putative 6mA demethylase gene *ALKBH1* in glioma cell lines led to increased levels of 6mA and gene expression, inhibition of tumour cell proliferation and increased survival of immunocompromised mice implanted with glioma *ALKBH1* knock-down tumour cells[72] (FIG. 4c). Taken together, these data raise the possibility that 6mA might help regulate growth of specific subtypes of cancer in humans.

Transgenerational inheritance.

Because of the semi-conservative nature of DNA replication, 6mA can readily be transmitted through cell divisions and potentially across generations. Labelling experiments have revealed that newly synthesized DNA in *E. coli* is quickly N^6-adenine methylated[153], raising the possibility that this mark could be marking specific residues and this information could be transmitted and rapidly reinforced in newly produced cells. In *C. elegans*, 6mA levels have been shown to increase transgenerationally in mutant animals that lack functional H3K4 demethylase[26] and in response to antimycin, an electron transport chain (ETC) complex III inhibitor[38] (FIG. 4d). Excitingly, elimination of the putative 6mA methyltransferase gene, *damt-1*, eliminated the transgenerational epigenetic phenotypes in both these scenarios, suggesting that the DAMT-1 enzyme is important for transmitting non-genetic information from ancestors to their descendants. Although 6mA could be inherited in a semi-conservative manner and reapplied to newly synthesized strands in a manner similar to inheritance of 5mC, unlike 5mC 6mA does not seem to mark complementary strands of DNA in equivalent locations. Thus, if it is involved in epigenetic inheritance, it seems more likely that it would be reacquired in new cells and new generations through coordinated communication with other chromatin modifications or non-coding RNAs. However, it remains unclear whether 6mA is indeed present in the *C. elegans* genome, and directed epigenetic modification, using a nuclease-null Cas9 fused to a 6mA methyltransferase, is required to demonstrate that directed methylation is responsible for the inheritance of epigenetic information.

Future perspectives

The current evidence that 6mA is present and playing a regulatory part in the genomes of recently evolved eukaryotes is promising but not definitive (TABLE 3). Further experiments using multiple independent orthogonal approaches are required to confirm (or rule out) its existence as a directed epigenetic modification and to determine in which biological processes, if any, it is involved.

Confirmation that 6mA is a directed modification in multicellular eukaryotes.

If 6mA is a directed modification, it would be expected to occur at consistent genomic locations within a given cell or tissue type under the same experimental conditions across studies. By contrast, incorporation of pre-methylated bases from RNA[44] or foreign DNA via nucleotide salvage pathways would be expected to occur with a more random distribution. Initial SMRT sequencing of human gDNA revealed a consistent localization of 6mA[74], but these findings need to be validated by independent 6mA detection techniques. Moreover, alternative explanations need to be ruled out. For example, sequencing errors could give a false impression of localized, regulated modification[63], as could non-directed incorporation of pre-methylated 6mA by DNA polymerase at fragile sites under replication stress[44,45].

To further increase confidence in 6mA mapping techniques, the field would benefit from novel antibody-independent sequencing methods that take advantage of the unique chemistry of 6mA, similar to bisulfite sequencing for 5mC[154]. One potential approach would be to use adenine deaminases that have distinct preferences for unmethylated adenines or N^6-methylated adenines[24,155], to convert either all unmethylated adenines or all 6mA into inosines. As inosines base pair with cytosines, methyl-specific deamination followed by PCR amplification would convert all methylated adenines into guanines, and this change could be read out by sequencing analysis. This method would circumvent current problems of false positives due to the low abundance of 6mA in metazoan DNA and would ensure that the methylated bases were contained within gDNA from the species of interest. However, like all new methodologies, testing would have to be done to ensure that the detected bases occurred at a frequency significantly greater than that expected for spontaneous deamination. A promising new 6mA sequencing technique, nitrite sequencing, uses sodium nitrite under acidic conditions to selectively deaminate unmethylated adenines. N^6-methylated adenines are unaffected by nitrite treatment whereas unmethylated adenines are selectively deaminated to hypoxanthines, which, after polymerase chain reactions and sequencing, are read as guanines[156]. This technique still needs to be thoroughly tested to determine the lower limit of detection to ensure it can accurately detect 6mA at the low concentrations present in multicellular eukaryotes. However, using this technique to take advantage of the unique chemical signature of 6mA could provide a powerful tool for accurate mapping of 6mA.

Consistent 6mA concentration changes in response to environmental factors or biological processes would also support a regulated role for 6mA. Several reports have suggested that 6mA changes its concentration during development. Nanopore sequencing of genomes from whole adult *D. melanogaster* showed that 6mA was more prevalent in developmental genes and positively correlated with their expression[157]. However, in two other studies,

MeDIP–seq analysis of *D. melanogaster* brain and ovaries showed 6mA correlated with gene repression[27,99]. Assuming the techniques used are specifically detecting 6mA, these results suggest that 6mA has a tissue-specific role in regulating gene expression in *D. melanogaster*. UHPLC–MS/MS and immunofluorescence analyses have shown that 6mA accumulates during early cell divisions in gametes and early embryos of zebrafish, mouse and pig[30,158], and dot blotting and SMRT-seq show it to dynamically increase in whole plant extracts during *A. thaliana* development[31]. However, it will be important to confirm that these changes in methylation in multicellular eukaryotes are not due to changes in the relative contribution of microbiota species or foreign bacterial species with prevalent 6mA in their genomes[43,71]. Use of independent methodologies to localize changes in 6mA coupled with genetic experiments to uncover the effects of putative 6mA-regulating enzymes on 6mA levels will help to determine whether it is important for regulating development.

A thorough characterization of the enzymes responsible for adding and removing 6mA is also required to solidify 6mA as a directed DNA modification. In vitro and in vivo characterization of 6mA methylases and demethy lases should include kinetic experiments to ensure physiologically relevant enzymatic activity, particularly in light of the fact that the DNA and RNA methyltransferases and demethylases are part of the same families of enzymes[109]. One of the most promising subcellular locations for bona fide 6mA-regulating enzymes to function is the mitochondria. The ancient origins of the mitochondrion make it plausible that an important regulatory role for 6mA has been retained in this organelle. Two recent reports using a combination of multiple orthogonal approaches found that 6mA was ~100- to 1,000-fold higher in mtDNA than in human gDNA in human cell lines[66,80] and that levels increase further in response to hypoxia[80]. One of the studies demonstrated that METTL4 is present in mitochondria and is necessary for 6mA of mtDNA[80]. Knock-down of METTL4 caused an increase in expression of mtDNA genes and an increase in mtDNA copy number, suggesting that 6mA on the mitochondria repress transcription and mtDNA replication[80] (FIG. 4a). This finding also raises the possibility that changes in 6mA concentration that are detected under specific conditions could be due to changes in the number of mitochondria and/or the amount of 6mA-containing mtDNA in the cell. This caveat needs to be considered when assessing 6mA levels as it could also potentially explain quantification differences between different groups, as could differential efficiencies of mtDNA extraction methods.

Confirmation of biological function.

The confirmation that 6mA is a bona fide directed modification as described above by independent sequencing techniques, consistent changes in 6mA in response to environmental changes or biological conditions, and validation of putative 6mA-regulating enzymes, will facilitate the study of 6mA function. For instance, validation of N^6-adenine methyltransferases and demethylases will enable the biological consequences of their genetic manipulation to be assessed. Similarly, accurate mapping of 6mA in the genome will facilitate targeted epigenomic editing experiments. Fusing a nuclease-null Cas9 with DNA methyltransferases or demethylases and directing the epigenomic editor to specific locations to change the 6mA status at precise genes will allow the specific effects of N^6-adenine methylation on gene expression and resulting functional consequences to be determined, and

will help to demonstrate a causal rather than a correlative role for 6mA. However, it will also be important to fuse a catalytically inactive enzyme to nuclease-null Cas9 to ensure that changes in gene expression are the result of changes in 6mA status rather than binding of the fusion protein.

Conclusions

Despite a resurgence of research on 6mA in eukaryotes[26–31] and the advent of more sensitive and precise mapping techniques, several studies have reported difficulty in detecting this modified base, particularly in the genomes of multicellular eukaryotes, and caution must therefore be exercised when interpreting findings[41,43,44,78]. There is a tendency within the field to either champion or dismiss the existence of 6mA in multicellular eukaryotes. It is our opinion that the preponderance of evidence currently points towards 6mA being much rarer in multicellular eukaryotes than some initial reports suggested. Moreover, if present, 6mA probably only reaches consistently detectable levels under specialized circumstances, such as in response to hypoxia[80] or ETC stress[38], which might help explain discordant results in otherwise similar samples. Because of its likely exceptionally low abundance, a higher standard of proof is required to support a functional role for 6mA in multicellular eukaryotes, and we are encouraged by the increasing rigour that is being applied to this field. If multiple independent techniques can validate the existence and dynamics of 6mA in the DNA of multicellular eukaryotes, identify and confirm the in vivo activity and specificity of 6mA methyltransferases and demethylases, and demonstrate direct functional consequences of specific methylation events, confidence will begin to grow in its biological importance in multicellular eukaryotes.

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Epigenetic

In the context of this article, this term refers to changes to the DNA that do not alter its nucleotide sequence.

Dimroth rearrangement

A passive process by which a methyl group is transferred from the N^1 position to the N^6 position of the same adenine base.

Nucleotide salvage pathway

The process by which intermediates of nucleotide degradation are recovered to be converted back into nucleotides, bypassing de novo synthesis.

Long-read sequencing

Third-generation sequencing approaches that can generate reads >10,000 bases in length, which improves mapping accuracy compared with short-read approaches; however, long-read approaches have higher error rates at individual bases than short-read methods.

RNA–DNA hybrids

Occur when nascent RNA transcripts hybridize with one strand of the DNA template creating a three-stranded structure called an R loop.

MT-A70 domain

This domain binds to *S*-adenosylmethionine and is present in a clade of RNA and DNA methyltransferases.

Hemimethylated DNA

Describes a DNA molecule in which only one of the two complementary DNA strands is methylated.

Triple negative breast cancer

Cancers that are negative for oestrogen receptors, progesterone receptors and excess HER2 protein and therefore do not respond to hormonal therapies or targeting of the HER2 receptor.

Epigenomic editing

Engineered changes to the epigenome that do not alter the DNA sequence that are accomplished using a modified Cas9 nuclease.

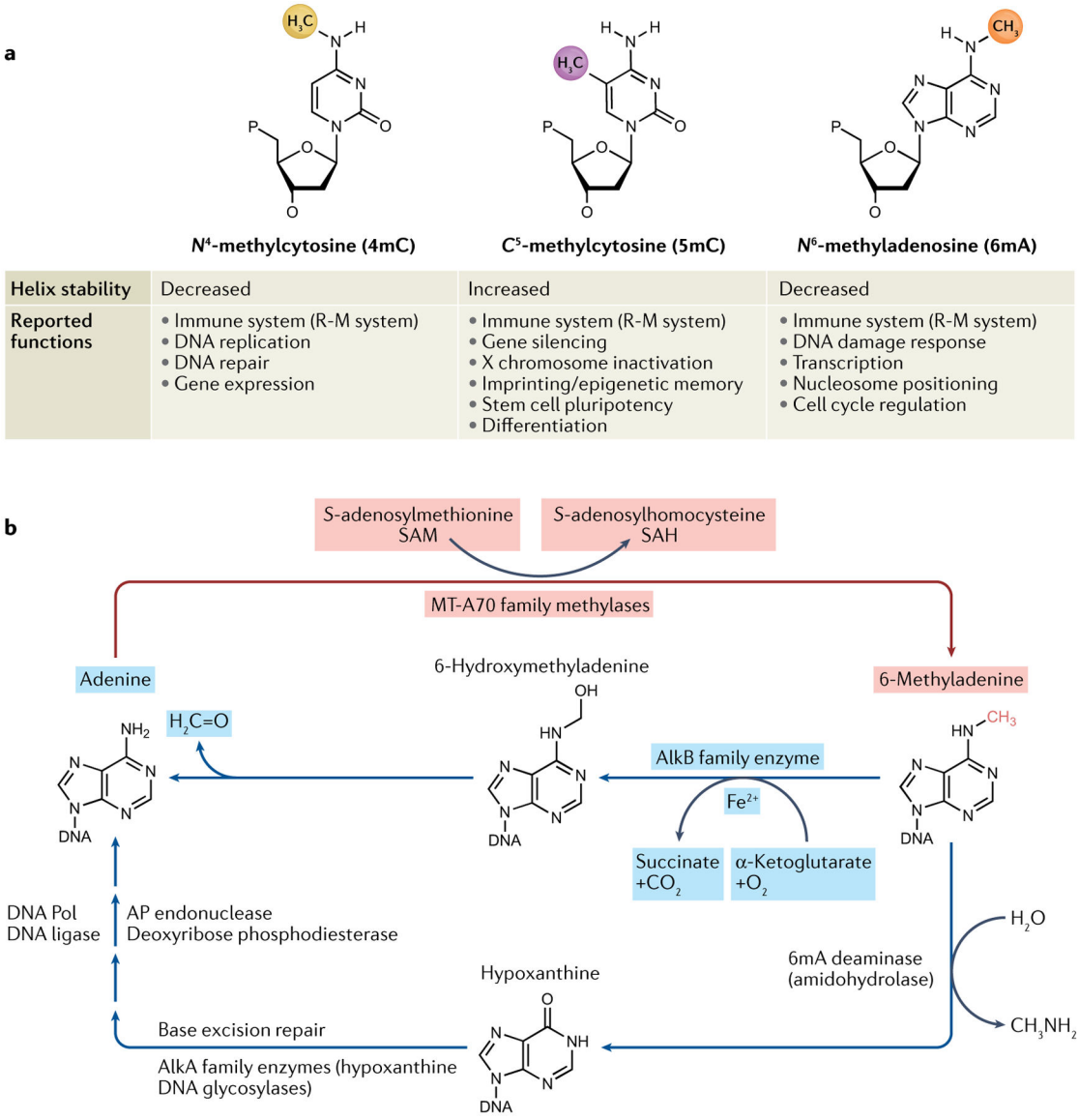

Fig. 1 |. Directed epigenetic DnA methylations.

a | The chemical structures, effects on helix stability and reported biological functions are shown for three directed epigenetic modifications of DNA — N^6-methyladenosine (6mA), C^5-methylcytosine (5mC) and N^4-methylcytosine (4mC)[166–168]. **b** | Depicted are the enzymatic pathways by which 6mA is added to (in red) and removed from (in blue) the genome. MT-A70 family methylases use *S*-adenosylmethionine (SAM) to catalyse the methylation of adenines at the sixth position of the purine ring, generating 6mA. 6mA can be removed by AlkB family demethylase enzymes. These enzymes require α-ketoglutarate and Fe^{2+} and use oxygen to oxidize the methyl group to generate 6-hydroxymethyladenine (6hmA), which then releases a formaldehyde group to generate adenine. Alternatively, in prokaryotes, 6mA can be deaminated and excised from the DNA by the base excision repair pathway, whereby a deaminase hydrolyses the methylamine to generate hypoxanthine, which is then recognized by AlkA enzymes as a damaged base. They cleave the glycosyl

bond to remove the base, after which apurinic (AP) endonuclease cleaves the phosphodiester backbone at the abasic site, thereby exposing the residual 5′ deoxyribose phosphate group, which is removed by deoxyribose phosphodiesterase. DNA polymerase I (DNA Pol) will repair the DNA by incorporating an unmodified adenine while DNA ligase catalyses the formation of the phosphodiester bond. It remains to be determined whether a similar mechanism exists in eukaryotes. R-M, restriction–modification. Part **b** reprinted from REF.[7], Springer Nature Limited.

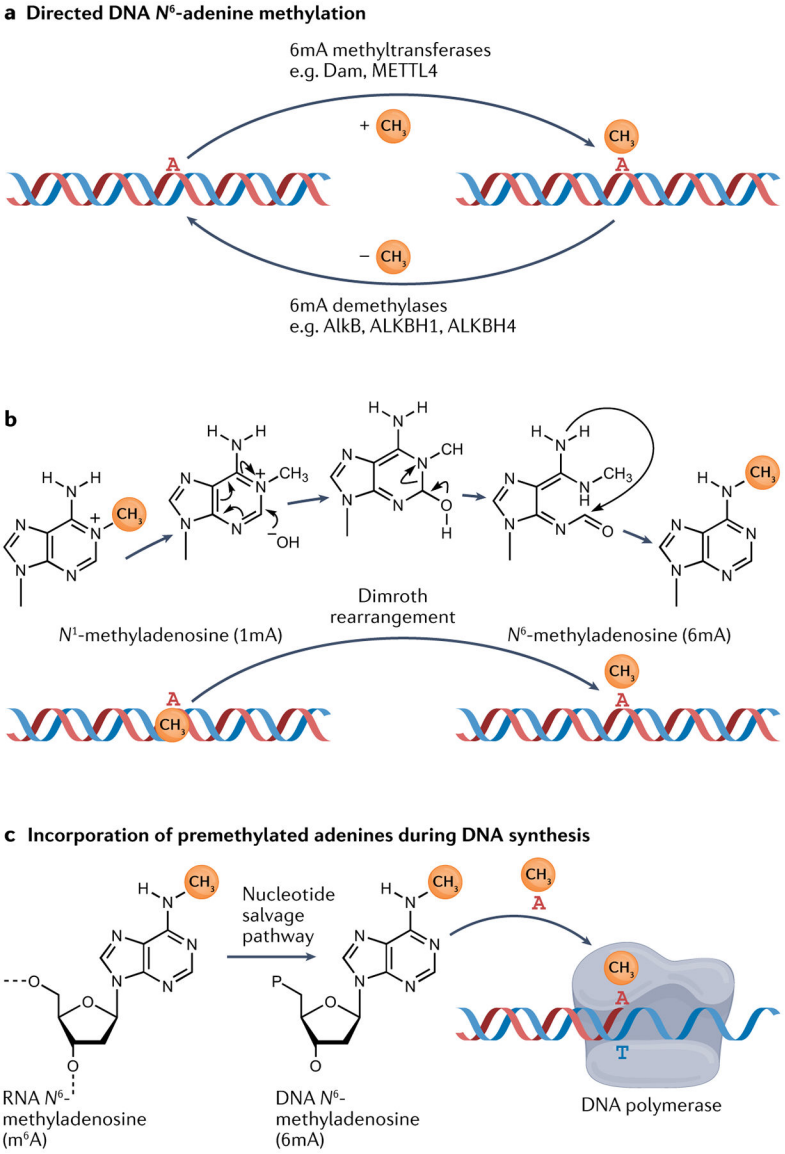

Fig. 2 |. Potential modes of 6mA enrichment.

a | 6mA could be added to and removed from genomes by 6mA-specific methyltransferases (such as Dam in prokaryotes and METTL4, a putative metazoan methyltransferase) and demethylases (such as AlkB in prokaryotes or putative metazoan demethylases ALKBH1 and ALKBH4). **b** | Adenines in the genome that are methylated at the N1 position (1mA) could undergo a Dimroth rearrangement whereby the methyl group is transferred to the N^6 position. **c** | Pre-methylated nucleotides could be incorporated into the genome by DNA polymerase. Pre-methylated nucleotides could be generated by the nucleotide salvage pathway via recycling of RNA N^6-methyladenosine (m^6A) or DNA N^6-methyladenosine (6mA) repurposed from foreign organisms.

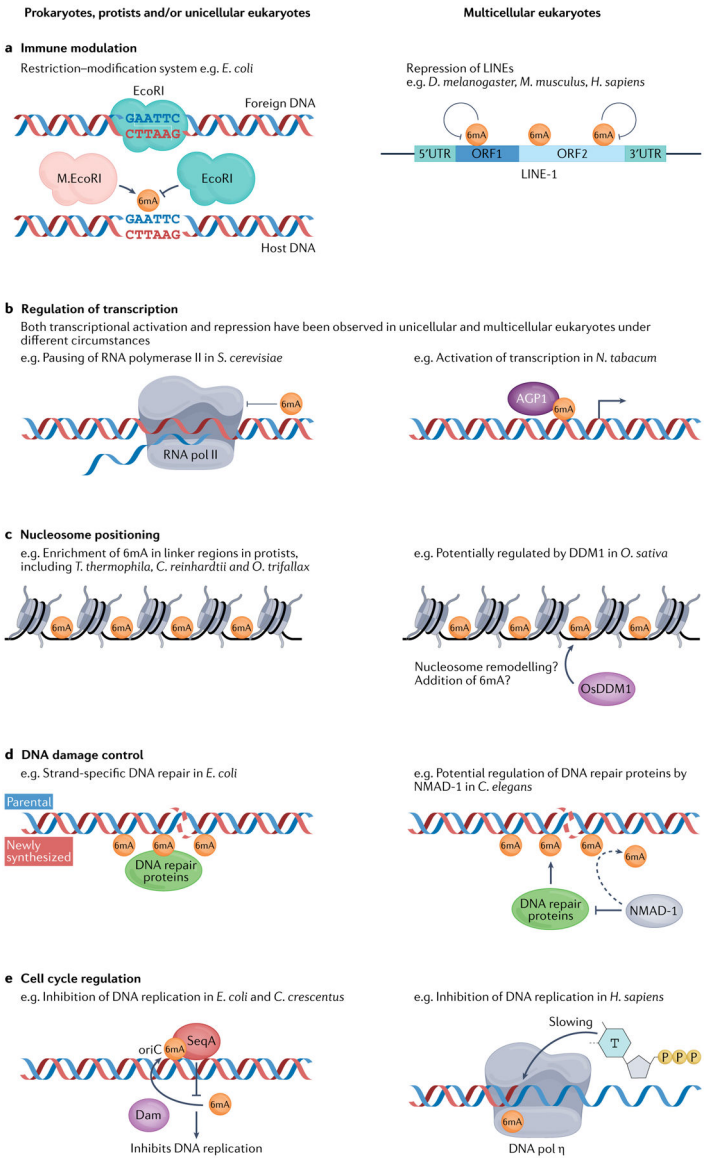

Fig. 3 |. Biological roles of DnA adenine methylation potentially conserved in unicellular and multicellular organisms.

a | In prokaryotes (left), DNA adenine methylation provides a basic immune system in the form of modification enzymes that methylate the host DNA and restriction enzymes that recognize and digest foreign unmethylated DNA. Reciprocal examples also exist. Here, the *Escherichia coli* system is depicted, which uses EcoRI as the restriction enzyme and M.EcoRI as the modification enzyme. In multicellular eukaryotes (right), N^6-methyladenosine (6mA) has been detected in long interspersed nuclear element (LINE) retrotransposons and is associated with their transcriptional silencing. Thus, 6mA might have a conserved role in recognizing and inhibiting foreign DNA. **b** | 6mA is postulated to repress transcription in some unicellular and multicellular eukaryotes through repelling some transcription factors and in *Saccharomyces cerevisiae*, 6mA has been shown to cause RNA polymerase II pausing[126] (left), which would decrease transcription rates. 6mA has also been shown to activate transcription in other unicellular and multicellular

eukaryotes by reducing DNA duplex stability to facilitate transcription, and 6mA can also enhance the binding of alternative transcription factors, such as AGP1, to increase transcription (right). **c** | 6mA is enriched in the linker region between nucleosomes in several protists, raising the possibility that it could actively regulate nucleosome positioning (left). Increasing the separation of nucleosomes increases chromatin accessibility and gene expression. In *Oryza sativa*, DDM1 could regulate nucleosome positioning through nucleosome remodelling activity and/or through N^6-adenine methylating linker regions where 6mA is enriched (right). **d** | As elucidated in *E. coli*, DNA adenine methylation allows DNA repair proteins to identify the parental strand and replace the sequence of the newly synthesized mutated strand (left). In *Caenorhabditis elegans*, deletion of the putative 6mA demethylase NMAD-1 causes misregulation of DNA damage repair genes (right), but it remains to be determined whether 6mA has a conserved role in directly regulating DNA damage in eukaryotes. **e** | In prokaryotes such as *E. coli* and *Caulobacter crescentus*, the 6mA binding protein SeqA will bind to hemimethylated DNA at the origin of replication and will prevent the methyltransferase Dam from methylating the newly synthesized strand (left). DNA replication is thereby inhibited until SeqA is released and both strands of DNA are methylated. In vitro studies with *Homo sapiens* DNA polymerase-η show that 6mA slows the incorporation of thymines during DNA replication (right), raising the possibility that 6mA could have a conserved role in inhibiting DNA replication. *C. reinhardtii*, *Chlamydomonas reinhardtii*; *D. melanogaster*, *Drosophila melanogaster*; *N. tabacum*, *Nicotiana tabacum*; *O. trifallax*, *Oxytricha trifallax*; *T. thermophila*, *Tetrahymena thermophila*.

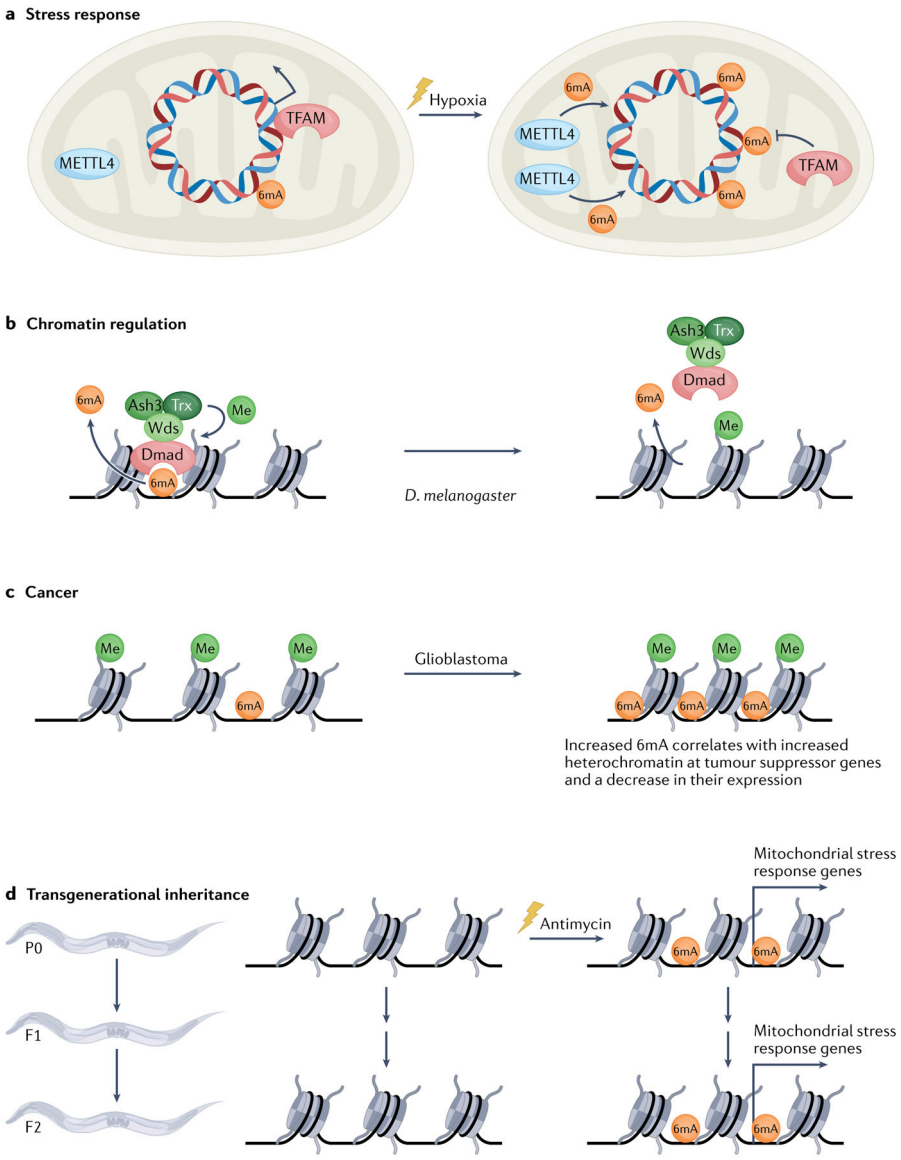

Fig. 4 |. emerging potential roles of 6mA in metazoa.

a | Hypoxia induces an increase in the levels of METTL4 and mitochondrial DNA (mtDNA) N^6-methyladenosine (6mA), which in turn inhibits the binding of the mitochondrial transcription factor TFAM[80]. TFAM facilitates both transcription of mitochondrial genes and mitochondrial replication. Increased 6mA on mtDNA caused by hypoxia stress leads to decreased mtDNA transcription and reduced mtDNA copy number. **b** | In *Drosophila melanogaster*, the proposed DNA demethylase Dmad binds to the H3K4 trimethylation complex protein Wds[99], a component of the protein complex that trimethylates histone H3 at lysine 4 (H3K4Me3, indicated by green circles), thereby suggesting a potential mechanism for chromatin modification crosstalk. **c** | It has been proposed that 6mA becomes upregulated in glioblastomas and that this increase in 6mA correlates with increased heterochromatin at tumour suppressor genes[72]. Therefore, elevated 6mA could facilitate tumorigenesis. **d** | It has been shown in *Caenorhabditis elegans* that levels of 6mA increase

in response to the oxidative phosphorylation inhibitor antimycin. This elevated 6mA and the resulting adaptation to mitochondrial stress is inherited by untreated progeny of antimycin-treated individuals, suggesting that 6mA may have a role in transgenerational inheritance[38].

Table 1 |

techniques for detecting methylation of adenine in genomic DnA

Method	Description	Resolution	specificity	sensitivity	Limitations
6mA-sensitive restriction enzyme digestion[52,53]	Restriction endonuclease cleavage of methylated motifs	Single site	High	High — can detect a single methylated base in the genome	Cannot detect 6mA outside of restriction recognition sites and is therefore mostly used to validate 6mA sites
Dot blot[51]	Antibody-dependent semi-quantitative detection of 6mA	Low	Moderate — can also detect m^6A in RNA or RNA–DNA and 1mA	Low to moderate, depending on the antibody	–
IF[26,30,159]	Antibody-dependent detection of 6mA	Cellular instead of nucleoside-level resolution	Moderate — can also detect m^6A in RNA or RNA–DNA and 1mA	Likely low and antibody dependent	–
UHPLC–MS/MS[26,27,29,32,43,44,55,58]	Chemical separation and detection by tandem mass spectrometry	NA	High	High — 0.0001%	Need to ensure the absence of contaminant (such as *Mycoplasma)* and that digestion enzyme mixtures do not contain methylated bases
CE–LIF[54]	The BODIPY FL EDA probe binds covalently to the phosphate group of deoxyrib-onucleotide after activation by carbodiimide reagent. CE-LIF then distinguishes different bases based on migration time	NA	None	Moderate — 0.01% 6mA/nucleotide limit	Can distinguish 6mA from 5mC but untested for 1mA
SMRT–seq[26,43,67,71]	Long-read sequencing; the kinetics of sequencing are altered when bases are methylated	Single nucleotide	Moderate — 6mA and 1mA are indistinguishable.	High when 6mA is abundant Low when 6mA is rare (<10 ppm)	Expensive and requires high coverage. Third-generation sequencing is prone to errors High false positive rate when 6mA is rare
MeDIP–seq[62,65]	Antibody-dependent method for identifying genomic regions harbouring 6mA	~150 bp region	Moderate — requires that the antibody has a higher preference for 6mA than non-methylated A to eliminate nonspecific binding, and nonspecific binding of IgG to repetitive DNA sequences can occur	Moderate — antibody dependent	Typical problems of antibody pull-downs, including identification of regions instead of specific sites of methylation
MeDIP–qPCR[65]	Antibody-dependent method for validating genomic regions harbouring 6mA	~150 bp region	Moderate — requires that the antibody has a higher preference for 6mA than unmethylated A to eliminate nonspecific binding	Moderate — antibody dependent	Typical problems of antibody pull-downs, including identification of regions instead of specific sites of methylation
6mA-specific probes[59]	DNA probe containing a formyl group on the O^6 position of a G base discriminates between adenine and 6mA via formation of an interstrand crosslink (ICL). 6mA cannot form ICL. ICLs detected by PAGE or HPLC	Single site	High, but untested for other modifications (such as 1mA)	High — can detect a single methylated base in the genome	Can only be used for validation of candidate 6mA sites

6mA, N^6-methyladenosine; CE–LIF, capillary electrophoresis and laser-induced fluorescence; IF, immunofluorescence; MeDIP–qPCR, methylated DNA immunoprecipitation with quantitative PCR; MeDIP–seq, methylated DNA immunoprecipitation with sequencing; m^6A, RNA N^6-methyladenosine; NA, not applicable; SMRT-seq, single-molecule real-time sequencing; UHPLC–MS/MS, ultra-high performance liquid chromatography coupled with tandem mass spectrometry.

Table 2 |

enzymes proposed to regulate or interpret 6mA in eukaryotes

Organism (range of reported 6mA as a percentage of total A)	Protein	Proposed substrate	supporting evidence	comments
6mA methyltransferases				
Caenorhabditis elegans (ND–0.44)	DAMT-1	6mA DNA	In vivo, ex vivo and genetic[26]	Homologue of *Homo sapiens* METTL4
Mus musculus (ND–0.003)	METTL 4	6mA DNA	Ex vivo[79]	Homologue of *H. sapiens* METTL4
H. sapiens (ND–0.1 for gDNA and 0.04 for mtDNA)	METTL4	6mA mtDNA[80] and gDNA[165] m^6Am snRNA[81,82] m^6A snRNA[83]	In vitro, in vivo and genetic In vitro, ex vivo and genetic In vitro, in vivo and genetic rescues in *Drosophila melanogaster*	Homologue of *C. elegans* DAMT-1. Several groups have identified contradictory substrates for METTL4
6mA demethylases				
C. elegans (ND–0.44)	NMAD-1	6mADNA	In vitro, in vivo and genetic[26]	Homologue of *M. musculus* ALKBH4
D. melanogaster (ND–0.07)	Dmad	6mADNA	In vivo, in vitro and genetic[27]	Homologue of *H. sapiens* TET1. Crystal structures of other organisms suggest it should not be able to accommodate flipped out purines[100]
M. musculus (ND–0.003)	ALKBH1	6mA DNA	In vitro, ex vivo and genetic[29]	See comments for *H. sapiens* ALKBH1
M. musculus (ND–0.003)	ALKBH4	6mA DNA	In vitro[79]	Homologue of *C. elegans* NMAD-1
H. sapiens (ND–0.2 for gDNA and 0.04 for mtDNA)	ALKBH1	6mA DNA	In vitro, ex vivo, in vivo and genetic[72,77]	Conflicting reports have suggested that ALKBH1 preferentially demethylates m^1A on tRNAs[94] or m^5C on tRNAs[95]
6mA DNA binding proteins				
Nicotiana tabacum	AGP1	6mA DNA	In vitro[125]	6mA enhances transcription factor binding to activate transcription of target genes
D. melanogaster	Jumu	6mA DNA	In vitro and genetic[75]	–
H. sapiens	SSBP1	6mA DNA	In vitro[66]	Binding of a mitochondrial protein to 6mA bolsters arguments that 6mA could be predominantly found in mitochondria in metazoans
DNA binding proteins antagonized by 6mA				
Arabidopsis thaliana	WER	–	Structural and in vitro[114]	Shows reduced binding to 5mC or 6mA compared with unmethylated C or A.
M. musculus	SATB1	–	Structural and in vitro[64]	The in vitro assays used DNA sequences that undergo dramatic bending when modified by 6mA[113], and the bending may prevent SATB1 binding independently of 6mA
H. sapiens	TFAM	–	In vitro[80]	–

Excluded from this table are the prokaryotic 6mA-specific methyltransferases (such as Dam[133,160,161], EcoRI[162] and M.MunI[163]), demethylases (AlkB[23,164]) and methyl binding proteins (such as SeqA[105,106], MutH[102]). 6mA, N^6-methyladenosine; gDNA, genomic DNA; m^6A, RNA N^6-methyladenosine; mtDNA, mitochondrial DNA; snRNA, small nuclear RNA.

Table 3 |

summary of studies investigating 6mA levels and/or distribution in eukaryotes

Organism	Levels and distribution of 6mA	Proteins that regulate or interpret 6mA	Validated biological functions	Additional evidence and/or validation required
Caenorhabditis elegans	Initially 6mA was detected at ~100–400 ppm by UHPLC–MS/MS and SMRT-seq[26] Revised UHPLC–MS/MS accounting for bacterial contamination of enzymes used to digest gDNA led to lower 6mA values (0.1–1 ppm)[43] 6mA was mapped throughout *C. elegans* genome by MeDIP–seq and SMRT-seq[26]	Potential 6mA methyltransferase: DAMT-1 (REFS[26,38]) Potential 6mA demethylase: NMAD-1 (REF.[26])	6mA dynamically regulated in vivo in two paradigms of transgenerational inheritance by the opposing enzymatic activities of DAMT-1 and NMAD-1 (REFS[26,38])	NMAD-1 may have additional enzymatic activities towards RNA[90] The enzymatic activities and physiological preference of DAMT-1 and NmAD-1 towards 6mA need to be validated in vivo and in vitro by UHPLC–MS/MS using gDNA samples free of contamination
Drosophila melanogaster	6mA detected and levels shown to change during development (10–700 ppm) by UHPLC–MS/MS[27] 6mA DIP–seq showed enrichment of 6mA at transposons and within coding regions[27,75] SMRT-seq-based deconvolution method, 6mASCOPE, reported lower 6mA levels (2 ppm) and suggests that elevated UHPLC–MS/MS values are due to contaminating microbiota with high levels of 6mA[71]	Potential 6mA demethylase: Dmad (REF.[27]) 6mA binding protein: Jumu[75]	6mA is dynamically regulated during early embryonic development and is proposed to regulate transcription and transposon silencing[27,99]	Identification and characterization of 6mA methyltransferase UHPLC–MS/MS measurements may need to be repeated to ensure that samples are free of bacterial contamination during isolation and digestion
Danio rerio	6mA was quantified at 100–1,000 ppm by UHPLCCC–MS/MS and shown to decrease during zebrafish embryogenesis[30,158] Bacteria adhering to zebrafish chorion can artificially elevate 6mA levels[43]. 6mA was mapped throughout the zebrafish genome by MeDIP–seq and was found to be enriched in repetitive elements[30]	–	–	UHPLC–MS/MS measurements may need to be repeated to ensure that samples are free of detectable bacterial contamination during gDNA isolation and digestion for MS Identification of 6mA methyltransferase and 6mA demethylase and characterization of 6mA-regulated biological functions
Arabidopsis thaliana and *Oryza sativa*	6mA was detected at ~60–1,500 ppm by UHPLC–MS/MS and SMRT-seq and shown to increase during *A. thaliana* development[31] and UHPLC–MS/MS, and dot blots show increasing 6mA during *O. sativa* development[70] SMRT-seq found 6mA to be enriched at actively expressed genes in *A. thaliana* and *O. sativa*[31,70] SMRT-seq-based deconvolution method, 6mASCOPE, reported lower 6mA concentrations (3 ppm) and suggested that elevated 6mA values by UHPLC–	Potential 6mA methyltransferase: DDM1 (REF.[70]) Binding of the developmentally important transcription factor WER is antagonized by 6mA[114]	Mutation of DDM1 caused decreased plant growth[70]	UHPLC–MS/MS measurements may need to be repeated to ensure that samples are free of detectable bacterial contamination during gDNA isolation and digestion for MS The in vitro activity of DDM1 needs to be demonstrated to show that it is a direct methyltransferase

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Organism	Levels and distribution of 6mA	Proteins that regulate or interpret 6mA	Validated biological functions	Additional evidence and/or validation required
	MS/MS are due to contaminating soil bacteria with high levels of 6mA[71]			
Mus musculus	6mA detected at ~5–7 ppm by UHPLC–MS/MS in mESCs[29] and in the mouse brain[34] UHPLC–MS/MS analyses from other groups detected 6mA at levels at (0.3 ppm)[80] or below (0.1–1 ppm) the detection limit of the technology in mouse cell lines and adult tissues[41,57] SMRT-ChIP mapped 6mA throughout H2A.X-associated genomic regions in mESCs[29] MeDIP–seq mapped 6mA in mouse brain. Candidate sites were validated using a 6mA-sensitive restriction enzyme digestion method[34]	Potential 6mA methyltransferase: METTL4 (REF.[79]) Potential 6mA demethylases: ALKBH1 (REF.[29]) and ALKBH4 (REF.[79]) 6mA prevents binding of SATB1 to chromatin[64]	Genetic manipulation of 6mA-modifying enzymes is associated with X chromosome inactivation, LINE-1 transposon silencing[29] and Polycomb silencing[79] 6mA levels increase in the mouse brain in response to stress, correlating with gene expression and LINE-1 transposon silencing[34]	UHPLC–MS/MS measurements may need to be repeated to resolve any discrepancies and ensure that samples are free of detectable bacterial contamination during gDNA isolation and digestion for MS METTL4 and ALKBH1 may have additional enzymatic activities towards other substrates[81–83,94,95], which may contribute to gene expression regulation
Homo sapiens	6mA detected at ~1,000 ppm by UHPLC–MS/MS in human glioblastoma samples[72] and at ~500 ppm by UHPLC–MS/MS and SMRT-seq in human cancerous cell lines[77]. However, 6mASCOPE and UHPLC–MS/MS performed independently detected 6mA at 2–3 ppm in glioblastoma samples and 17 ppm in peripheral blood mononuclear cells[71] UHPLC–MS/MS analyses from other groups either failed to detect 6mA above the detection limit (0.1–1 ppm) in mammalian cell lines[57] or detected 6mA at the detection limit (0.3 ppm)[43,80] MeDIP–seq analysis showed enrichment of 6mA in heterochromatic regions in glioblastoma cells[72] whereas SMRT-seq and MeDIP–seq mapped 6mA to exons of transcriptionally active genes in human hepatocellular carcinoma cells[77]	Potential 6mA methyltransferase: N6AMT1 (REF.[77]) Potential 6mA demethylase: ALKBH1 (REFS[72,77])	Genetic manipulations that reduce 6mA levels are associated with increased tumorigenesis in glioblastoma cells and various human cancer cell lines[72,77]	UHPLC–MS/MS measurements may need to be repeated to resolve any discrepancies and ensure that samples are free of detectable bacterial contamination during gDNA isolation and digestion for MS Data from in vitro and ex vivo experiments suggest that N6AMT1 is a glutamine methyltransferase for eRF1 (REF.[84]). If this is true then an alternative gDNA methyltransferase (such as METTL4) would need to be identified and validated
Mammalian mtDNA	6mA detected at ~400–1,500 ppm by UHPLC–MS/MS in mtDNA of mammalian cell lines[80] MeDIP–seq mapped 6mA to 23 sites in the mtDNA of hepatocellular carcinoma HepG2 cell line, which were validated by 6mA IP-qPCR[80]	Potential 6mA methyltransferase: METTL4 (REF.[80]) Potential 6mA demethylase: ALKBH1 (REF.[66]) SSBP1 has been identified as a 6mA binding protein[66] and TFAM has been identified	Depletion of METTL4 correlated with reduced mtDNA transcription and mtDNA copy number[80] Depletion of ALKBH1 correlated with reduced mitochondrial oxidative phosphorylation[66]	METTL4 and ALKBH1 may have additional enzymatic activities towards other substrates[81–83,94,95], which may contribute to regulation of mitochondrial function

Organism	Levels and distribution of 6mA	Proteins that regulate or interpret 6mA	Validated biological functions	Additional evidence and/or validation required
		as protein repelled by 6mA[80]		

6mA, N^6-methyladenosine; 6mA IP–qPCR, 6mA immunoprecipitation followed by quantitative real-time PCR; gDNA, genomic DNA; MeDIP–seq, methylated DNA immunoprecipitation with sequencing; mESC, mouse embryonic stem cell; mtDNA, mitochondrial DNA; SMRT-seq, single-molecule real-time sequencing; UHPLC–MS/MS, ultra-high performance liquid chromatography coupled with tandem mass spectrometry.