Adenine methylation in eukaryotes: Apprehending the complex evolutionary history and functional potential of an epigenetic modification

Lakshminarayan M. Iyer, Dapeng Zhang and L. Aravind

While N6-methyladenosine (m6A) is a well-known epigenetic modification in bacterial DNA, it remained largely unstudied in eukaryotes. Recent studies have brought to fore its potential epigenetic role across diverse eukaryotes with biological consequences, which are distinct and possibly even opposite to the well-studied 5-methylcytosine mark. Adenine methyltransferases appear to have been independently acquired by eukaryotes on at least 13 occasions from prokaryotic restriction-modification and counter-restriction systems. On at least four to five instances, these methyltransferases were recruited as RNA methylases. Thus, m6A marks in eukaryotic DNA and RNA might be more widespread and diversified than previously believed. Several m6A-binding protein domains from prokaryotes were also acquired by eukaryotes, facilitating prediction of potential readers for these marks. Further, multiple lineages of the AlkB family of dioxygenases have been recruited as m6A demethylases. Although members of the TET/JBP family of dioxygenases have also been suggested to be m6A demethylases, this proposal needs more careful evaluation.

Keywords:
- adenine methylation; chromatin; dioxygenases; methyltransferases; modified DNA; restriction modification; transcription regulation

Introduction

In the early 1950s Luria, Anderson, Ralston and co-workers uncovered cellular processes regulating the host range of bacteriophages [1–3]. Subsequent investigations of this phenomenon by Arber, Meselson and co-workers led to the discovery of restriction-modification (R-M) systems, a landmark event in the history of molecular biology [4, 5]. While much subsequent work focused on characterizing restriction enzymes as tools for recombinant DNA technology, the biology and biochemistry of the R-M systems proved to be interesting in their own right [6–8]. These systems are the most widespread prokaryotic biological conflict systems facilitating both discrimination of cellular “self” DNA from invasive “non-self” DNA and destruction of the latter [6, 9–11]. In their most basic form, R-M systems are linked genes (like other prokaryotic operons), which code for a modification enzyme that covalently modifies DNA and a restriction endonuclease that cuts DNA upon recognizing specific sequence signatures [7, 10]. However, R-M systems often exhibit great diversity, and include other linked genes whose products might perform various accessory functions, such as target site recognition, DNA unwinding, long-distance DNA-looping and translocation, and regulation or augmentation of the restriction activity [6, 7, 10–14].

While the most common modification catalyzed by R-M systems is methylation of specific bases in DNA, recent studies suggest that there might be others, including incorporation of different modified bases and modification of the DNA-backbone by replacement of the non-bridging oxygen atom of the phosphate by a sulfur [15–17]. Modification methylases (MTases) methylate either cytosine or...
Cytosine is methylated either on the carbon at the 5 position of the pyrimidine ring (C5) or at the exo-cyclic NH₂ group at the 4 position (N⁴), whereas adenine is methylated on the exocyclic NH₂ group at the 6 position of the purine ring (N⁶) (Fig. 1A). In classic R-M systems, modification of DNA serves as the discriminatory tag, which prevents the restriction of self DNA, while allowing the non-self DNA, which is not modified, to be targeted [8, 10]. Several bacteriophages have evolved counter-strategies against R-M systems in the form of DNA modifications generated by enzymes encoded in their genomes, which inhibit restriction enzymes [10, 22, 23]. These modifications include, N⁶-methyladenine (m⁶A), adenine modified at N⁶ by glycine (momylation), deazaguanines, 5-hydroxymethyluracil, hypermodified thymines, 5-methylcytosine (5mC), and 5-hydroxymethylcytosine (5hmC) [15, 24, 25]. As part of the ongoing arms-race, prokaryotes have in turn evolved several specialized restriction systems targeting invasive DNA with such modifications [15, 26].

Following the discovery of R-M systems, studies pointed to more extensive functions for methyl modifications in prokaryotes and their viruses. m³A was found to mark replication origins of genomic and plasmid replicons, and regulate replication and chromosome segregation [27–29, 30]. Similarly, m⁴A marks also help distinguishing the DNA strands during mismatch repair [31]. Furthermore, in several bacteria, transcription was found to be regulated by specific m⁴A patterns associated with a given gene [31]. m⁴A and other modified bases were found to regulate transcription and facilitate packaging of a genome length of DNA into the phage head following replication [32]. These findings led to the concept that m⁴A could encode information over and beyond that encoded by the bases of DNA (genetic information) – a form of information termed "epigenetic" [33–35].

By the 1980s, it had become clear that DNA modifications were not the unique preserve of prokaryotes – several were discovered in eukaryotes, including m³A, 5mC, and the hypermodified thymine (base J) [24, 36–38]. Of these, 5mC was found to be widely distributed across eukaryotes, including humans and other mammals, thereby making it the subject of intense investigation [39, 40]. While 5mC in eukaryotes was found to be generated by enzymes having evolutionary links to prokaryotic R-M and counter R-M MTases [41], it was found to be an important epigenetic mark with diverse functional consequences in different eukaryotes [42–45]. Recent work has shown that 5mC is not a terminal modification: it is further oxidized by action of the TET/JBP family of 2-oxoglutarate and Fe (II)-dependent dioxygenases (2OGFeDOS) to give rise to 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) [46–50]. While functions of these oxidized 5mC derivatives remain to be fully understood, it is becoming apparent that they might be both epigenetic marks of their own right, as well as intermediates in 5mC demethylation [51].

In contrast to 5mC, m³A (the dominant epigenetic mark in prokaryotes) remained largely neglected in eukaryotes [24, 36]. This has recently changed: multiple groups have reported conclusive, genome-wide evidence for m³A modifications from diverse eukaryotes and potential epigenetic roles for this modification [52–54]. Given that these discoveries are likely to elicit much interest and raise several new questions, in this article we attempt to provide an overview of the natural history of the N⁶A methylation, demethylation, and “reading” apparatus.

**Eukaryotic N⁶A-MTases belong to three broad groups**

Comprehensive genomic analysis revealed that eukaryotes have acquired N⁶A-MTase domains (Box 1) from prokaryotic precursors on at least 13 independent occasions in their evolutionary history (Fig. 1B and C), each defining a distinct clade. These clades in turn belong to three major higher-order groups (groups 1–3), whose primary radiation occurred in bacteria and their phages in R-M and counter R-M systems, and epigenetic systems associated with DNA replication and repair (i.e. the classic Dam MTases). We describe below the eukaryotic clades and their provenance.

**Group-1 contains MTases structurally related to prokaryotic M.MboIIA/M.Mult (circularly permuted) and DnpA (unpermuted)**

Members of this group were acquired by eukaryotes on at least six distinct occasions (Fig. 2). The most widespread of these, the Ime4-like (also called MT-A70) clade [59, 66], with circularly permuted MTase domains, in turn radiated into six distinct eukaryotic sub-clades [53] (Fig. 2). Of these, the subclades typified by human METTL3 (yeast Ime4) and human METTL14 (yeast Kar4) are most conserved, and are typically in a single copy per genome [59, 66]. METTL14 representatives often show disruptions of their active site motifs suggesting that they are inactive versions (Supporting Information). METTL3 and METTL14 cognates are typically subunits of a dimeric enzyme, catalyzing N⁶A methylation of specific positions in mRNAs [67, 68]. Consistent with this, in METTL3 the MTase is fused to N-terminal ssRNA-binding CCCH domains (Fig. 2). Of the other four eukaryotic sub-clades of the Ime4-like/MT-A70 clade that prototyped by METTL3 is widely, albeit patchily, distributed (Fig. 3). Recent work in the nematode *Caenorhabditis elegans* suggests that it is likely to be a DNA MTase [53]. The remaining eukaryotic subclades of the Ime4-like/MT-A70 clade show even more sporadic phylogenetic patterns, distantly related microbial eukaryotes being united close together in the phylogenetic tree (Fig. 2), hence indicating extensive lateral transfer of these genes between them. One of these subclades is typified by fusion of the MTase domain to multiple C-terminal ZZ-domains [20] (Fig. 2), a treble-clef fold Zn-binding domain mediating protein–protein interactions in chromatin [69, 70]. The eukaryotic Ime4-like/MT-A70 clade is nested within a prokaryotic radiation that includes MTases of the BglII R-M system [53] (Fig. 2).

Two further clades of circularly permuted MTases, distinct from the above, representing independent transfers from bacteria, show more restricted distributions. They are characterized by unusual variants of the diagnostic strand-4 associated motif (Fig. 1B). Of these, the sporadically
Figure 1. m^6^A methylation and demethylation reactions, topology, and conserved features of eukaryotic N^6^-MTases. **A:** Groups modifying the nucleotide are colored red and blue. **B:** Topology and anatomy of MTase domains. Cartoon representations of principle groups of eukaryotic MTases grouped according to their higher order relationships (shown to the left). Conserved strands are colored green and helices orange. Additionally, lineage-specific structural elements are shown in gray. Ancestrally conserved residues are shown in gray circles at their structural position, whereas clade specific residues are shown in their respective colors. **C:** Representative domain architectures and gene neighborhoods for different clades within the three groups are illustrated. Genes in operons are shown with the arrow head pointing to the 3’ direction of the coding strand. Proteins are denoted by their gene name if present, species name, and Genbank identifier (GI) separated by underscores. Proteins from species not available in Genbank are given a temporary id, separated by the species name. The full sequence can be accessed in the Supporting Information. Standard abbreviations are used for domain names. Additional non-standard names include: X, domains of uncharacterized function; cpN8-MTase, circularly permuted Group I-like MTase; RAGNYA, RAGNYA fold domain found in the methylase-specificity subunit; Helix, α-helical element that forms coiled coils; ZFCW, PHIDX/ZFCW domain; DUF3872-Ig, an all-b Ig fold domain.
**Box 1**

**Anatomy of N⁶A-MTases-MTase domains**

The majority of nucleic acid MTases belong to a superfamily of enzymes displaying a classical Rossmann fold catalytic domain, and use AdoMet as the methyl group donor [20, 21, 55–57]. This superfamily additionally includes diverse enzymes catalyzing methylation of a wide array of small molecules and proteins. Structurally, it is characterized by a distinctive connector between the first conserved strand and helix of the Rossmann domain, assuming a “double-headed loop” conformation, and binding the ribose moiety of AdoMet (Fig. 1B). Additional contacts with ribose and adenine of AdoMet are mediated by residues from the two downstream conserved strand-helix elements of the domain [19, 57].

Within this superfamily, nucleic acid MTases belong to two distinct clades, one including all nucleic acid C⁵ MTases [58] and the other uniting enzymes catalyzing methylation of both N⁴C and N⁶A, those modifying the N2 position of guanines in RNA, as well as certain protein MTases methylating the amide group of glutamine in proteins such as the ribosomal protein L3 and peptide release factors (HemK family) [59–61]. Members of the latter clade are characterized by a [DNSH][PPP][YFW] motif at the C-terminus of conserved strand-4 of the Rossmann domain [18, 20, 21, 62] (Fig. 1B). These MTases follow a conserved catalytic mechanism: the target base is held in place by π–π stacking interactions with the aromatic residue [YFW] in the last position of the above motif [62, 63]. The target NH₂ group is the donor for hydrogen bonds with the polar group of the first residue [DNSH] of their conserved motif, and with the backbone carbonyl of the peptide bond between the next two prolines. Consequently, the NH₂ group is primed for a SN₂ reaction with the CH₃ group from AdoMet, and resultant conformational inversion of the newly formed CH₃NH group [63].

In the clade of N⁶A-MTases, those acting on DNA had a single origin, probably being derived from the more ancient and nearly universally conserved protein and rRNA MTases. Among N⁶A-MTases, one clade is characterized by a circular permutation, bringing strand-3 of the conserved core of the Rossmann domain to the N-terminus [59]. This group includes several MTases of R-M systems (e.g. M.MboII and M.MunI; Fig. 1B) [21, 59, 64]. N⁶C-MTases appear to have been derived on multiple occasions within the wider radiation of N⁶A-MTases, and are characterized by a strand-4 associated motif with serine in the first position [64].

**Table 1**

| Clade | Description |
|-------|-------------|
| 1     | RNA-binding PPR domains |
| 2     | Protein MTases |

**Clade 1** is found in distantly related unicellular photosynthetic eukaryotes (Fig. 3). Some of these are fused to the RNA-binding PPR domains [76, 77], suggesting that they might also modify cytosine at the N⁶ position in RNA like the aforementioned clade (Figs. 1 and 3). Several of their prokaryotic counterparts are the MTases of the EcoRII-like R-M systems. Clade 6 is restricted but lineage-specifically expanded in the haptophyte algae, like *Emiliania* (Fig. 3), and are fused to an N-terminal FHA-fold domain [78]. They are derived from prokaryotic versions encoded by the ParB-Terminase large subunit (TIs) locus found in several phages and prophages (Fig. 1C), which are predicted to modify phage DNA as part of the DNA-packaging process [15].

**Group-2 MTases are prototyped by prokaryotic M.EcoKI/M.TaqI**

These MTases are characterized by complete or partial degeneration into coils of the helices before and after...
strand-3 [18, 79]. They also display a helix N-terminal to the core MTase domain with a conserved residue that helps position the asparagine in the strand-4-associated motif in the active site (Fig. 1B). Six clades from this group, representing independent transfers from bacteria, are present in eukaryotes (Fig. 3). The first and most widespread clade in this group is defined by the PCIF1 protein, which is traceable to the last eukaryotic common ancestor (Fig. 3). PCIF1 is usually fused to an N-terminal WW domain (Fig. 1C), which recruits it to the carboxy-terminal tail (CTD) of the RNA polymerase II (RNAPII) largest subunit [80]. The strong conservation of this enzyme, which is typical of RNA-modification enzymes, and interaction with the CTD, which plays an important role as a scaffold for RNA-processing [81], raises the possibility that it might methylate mRNA or a CTD-associated ribonucleoprotein. Also in this clade are MTase domains that are embedded in the polyprotein of DIRS1-type retrotransposons (Figs. 1C and 3) [82], and which were probably derived from the cellular PCIF1. These elements are highly mobile across species, and are seen in diverse eukaryotes (Fig. 3) [20, 82]. However, all copies of the DIRS1 MTase domain are likely inactive because of substitutions affecting catalytic and substrate-binding residues [20]. Hence, they might merely interact with template transcripts of the DIRS1 transposon, or with the CTD, which plays an important role as a scaffold for RNA-processing [81], raises the possibility that it might methylate mRNA or a CTD-associated ribonucleoprotein. Also in this clade are MTase domains that are embedded in the polyprotein of DIRS1-type retrotransposons (Figs. 1C and 3) [82], and which were probably derived from the cellular PCIF1. These elements are highly mobile across species, and are seen in diverse eukaryotes (Fig. 3) [20, 82]. However, all copies of the DIRS1 MTase domain are likely inactive because of substitutions affecting catalytic and substrate-binding residues [20]. Hence, they might merely interact with template transcripts of the DIRS1 transposon, or
mimic endogenous PCIF1 to regulate transposon polyprotein localization by interacting with RNAPII.

The second clade in this group, the “chlorophyte-type Dam” clade, contains two families predominantly found in chlorophyte algae [20]. The first family usually occurs as a single copy in chlorophytes, and exists as fusions to one or more BMB/PWWP and a ZfCW/PHD-X domain (Fig. 1C). These domains indicate that they might interact with modified or...
unmodified histones [73, 83, 84]. The second family, present only in certain chlorophytes and chytrid fungi, is characterized by an N-terminal fusion to a ParB-type helix-turn-helix (HTH) (Supporting Information; Figs. 1C and 3). Prokaryotic members of this clade are found both in phage ParB-Tls loci and DpnII-type R-M systems, where they are the primary modification MTase DpnM (Fig. 1C) [85]. Furthermore, both the chlorophyte-type Dam and the linked ParB-HTH found in the second family are fused in cyanobacteria to ASCH domains, predicted to bind modified nucleic acids (see below).

The third clade in this group, typified by the Chlamydomonas protein CHLREDRAFT_205675 (gi: 159485216), is broadly distributed in microbial eukaryotes (Fig. 3). They often occur as two paralogs, suggesting that they might form a dimer like METTL3-METTL14 [68]. Further, like METTL3, they are often fused to RNA-binding domains, namely CCCH and KH (Fig. 1) [59]. This suggests that at least a subset of this clade is involved in RNA methylation. Their bacterial cognates are encoded by mobile conjugative elements, which might protect from restriction during DNA transfer, and less frequently by R-M systems. In both cases, they might be found alongside a gene for a DNA C5-MTase, and in some cases a second N6A-MTase (Fig. 1C). The fourth clade from this group is represented by paralogous copies seen thus far only in the haptophyte alga Emiliania, and appears to have been derived from a bacteriophage version (Fig. 1C).

MTases of Clades 5 and 6 in this group are restricted to rhizarians and/or basal fungi (Fig. 3). They are fused to the DNA-binding MTase-S domain, which contains a RAGNYA fold, seen in diverse nucleic-acid-binding contexts where it recognizes specific nucleotide sequences [79, 86, 87]. Clade 5 MTases in the rhizarian Reticulomyxa are found in up to five copies, and at least one is fused to an N-terminal restriction endonuclease domain, thereby retaining the ancestral Type I R-M system architecture (Figs. 1C and 3). These are also found in bacterial endosymbionts/parasites, pointing to possible lateral acquisition from such organisms.

**Group-3 MTases are prototyped by Dam MTases of Escherichia coli and bacteriophage T4**

These are characterized by an additional N-terminal helix and a winged HTH domain inserted after the second conserved strand-helix unit, which help in recognition and flipping of the target adenine [62] (Fig. 1). This clade is only seen in the basal eukaryote Trichomonas (up to 10 nearly identical copies), and its members are fused to a bacteriophage tail-fiber domain [20] (Figs. 1C and 3). They are in the vicinity of transposons coding for an A32-like packaging ATPase, suggesting that they might have been dispersed by these transposons [20] (Fig. 1C).

These observations indicate that there were multiple origins for N6A-MTases in eukaryotes involving several independent transitions to RNA-modification upon acquisition from prokaryotic DNA-modification systems (Box 2).

**How do eukaryotic methylomes correlate with the presence of N6-A-MTases?**

Since the 1970s, studies have detected and estimated m6A in DNA from diverse eukaryotes [24, 36, 38]. Recently, some of these have been reproduced using more sensitive and reliable methods, such as ultra-high-performance liquid chromatography coupled with tandem mass spectrometry. For at least a few organisms, methylomes have been directly inferred using technologies, such as single-molecule, real-time (SMRT) sequencing and methylated DNA-immunoprecipitation sequencing (MeDIP-Seq) [34, 35, 52–54, 91, 92]. This allows us to interrogate the correlation between the detection of m6A in an organism and potential N6-A-MTases coded by a genome.

Notable cases include ciliates, which were reported as having 0.8–2.5% of adenosines as methylated [24, 36, 93]. All ciliates code for members from 2–3 distinct sub-clades of the Ime4-like (MT-A70) clade (Fig. 2), suggesting that one or more of these enzymes probably generate the observed m6A. The chlorophyte Chlamydomonas was reported as having 0.5% of its adenosines methylated [24, 36, 52]. A recent study has provided exquisite detail on its methylome [52]: the bulk of the m6A is associated with specific motifs centered on an AT dinucleotide (one third of them mapping to motifs CATG and GATC), with a bimodal distribution around the transcription start site. These m6A-enriched regions show a periodicity of around one per 130–140 bp, being typically localized to inter-nucleosomal linker regions. Additionally, there are lower abundance m6A methylation sites, lacking periodicity, distributed throughout the gene body; these may be only partially methylated. Chlamydomonas has two MTases from two subclades of the Ime4-like (MT-A70) clade (Fig. 2), and multiple chlorophyte-type Dams (Fig. 3), which could collectively account for the observed methylation. Versions with BMB/PWWP and ZfCW/PHD-X domains could interact with histones to set up the observed inter-nucleosomal DNA methylation [52]. These MTases are conserved across chlorophytes but not land plants (Fig. 3), suggesting that such N6A methylation patterns were lost during the emergence of the land plants.

Surprisingly, m6A was also identified in C. elegans in which no type of DNA methylation had previously been observed [53]. In wild-type worms, m6A levels are variable (0.01–0.4% of adenines) but consistently elevated in certain mutant backgrounds (see below). Knockdown of the only candidate DNA m6A MTase gene, damt-1, specifically reduced m6A in genomic DNA and knockouts suppressed mutants with elevated m6A. These results provide strong evidence that in C. elegans damt-1 is the likely DNA methylase [53]. Me-DIP- and SMRT-sequencing suggest that m6A is enriched at certain motifs, namely AGAA and GAGG, the former being only 10–50% methylated and the latter 50–100%. Interestingly, unlike in Chlamydomonas, the C. elegans motifs are asymmetric in that methylation at these motifs will be necessarily limited to a single strand [53]. Recent investigations in Drosophila have revealed that early stage embryos display methylation at ~0.07% of the adenines, which rapidly fell to ~0.001% in late stage embryos and adults [54]. This is unlike C. elegans, where m6A is present ubiquitously, both in...
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Box 2

Evolutionary trends in eukaryotic N6A-MTases

N6A-MTases share several common evolutionary trends with C5-MTases and DNA-modifying 2OGFeDOs of the TET/JBP family (Fig. 3). Both types of MTases have been independently transferred on several occasions from prokaryotes, and their viruses to eukaryotes and their viruses: N6A-MTases and C5-MTases on 13 and 8 occasions, respectively (Fig. 3) [20]. Whereas some transfers occurred in the stem eukaryotes (e.g. PCIF1), others happened only in terminal branches (Fig. 3). Since eukaryotes typically lack R-M systems, the acquired N6A-MTases are reused in different functional capacities [88]. This is often accompanied by fusions to domains, which on the one hand enable specific interactions with methylated histones/other chromatin proteins [83, 84], DNA [73], or both, and on the other hand facilitate interactions with RNA [59]. Convergent fusions to the same type of domain are observed in more than one clade (Fig. 1C), suggesting that there are comparable selective pressures acting on independently acquired N6A-MTases to recruit them in similar functional contexts. A comparable set of multiple, independent fusions to chromatin-related domains are also observed in eukaryotic C5-MTases and TET/JBP proteins, suggesting that such fusions represent a common evolutionary mechanism by which DNA-modifying enzymes of prokaryotic provenance are recruited as generators of epigenetic DNA-modifications in eukaryotic chromatin [20].

While use of N6A-MTases as epigenetic DNA-modifiers can be seen as a functional continuation of their prokaryotic counterparts, a more pronounced functional shift is their repeated recruitment as RNA MTases in eukaryotes. This is known or predicted (based on fusions to RNA-binding domains) to have happened on at least 4–5 occasions. While a similar shift to RNA specificity has been reported among eukaryotic C5-MTases, i.e. DNMT2 [39, 89] and at least in one clade of TET/JBP enzymes [15, 90], it appears to be more common in N6A-MTases. This difference might be related to the distinct C-terminal module in C5-MTases that predisposes them to preferentially bind dsDNA [20]. In contrast, many N6A-MTases (e.g. DpnA from DpnII-type systems) were already targeting ssDNA [74]. When acquired by eukaryotes, they likely encountered abundant pre-mRNA in the nucleus, which potentially mimicked their ancestral ssDNA substrate, thereby enabling a functional shift toward RNA methylation. Interestingly, diverse complements of N6A-MTases are specifically found in phylogenetically distant microbial photosynthetic eukaryotes; in contrast, land plants show few N6A-MTases (Fig. 3). This suggests that methylation of both DNA and (pre)mRNA, including perhaps chloroplast transcripts, is likely to be important for the regulation of physiology in microbial algae [52].

embryos and adults. Again, unlike C. elegans, m6A in Drosophila was found to peak in gene bodies of transposons, but not regions upstream and downstream of them [52]. Drosophila has three members of the Ime4-like (MT-A70) clade (Fig. 3, Supporting Information): two of these likely to constitute the conserved mRNA methylating enzyme. The third (CG14906), an ortholog of C. elegans damt-1, is predicted to be the primary N6A-MTase in Drosophila.

Thus, in principle, other organisms with a METTL4 representative, such as vertebrates (including humans), land plants, and stramenopiles, might possess m6A in DNA. However, in several organisms, such as the fission yeast Schizosaccharomyces pombe and nematodes, a METTL4A representative is the only Ime4-like/MT-A70 clade MTase in the genome (Fig. 3); hence, it might functionally back up the absent METTL3/ METTL4 dyad and operate on ssRNA. This is also consistent with the single-strand biased motifs identified for C. elegans damt-1 [53]. Therefore, at least in some organisms, the METTL4 representative could be predominantly an RNA MTase. While phyletic patterns of N6A-MTases do not suggest the presence of DNA methylation in the last eukaryotic common ancestor, early-branching eukaryotes, such as Trichomonas and Naegleria, have representatives of one or more clades of N6A-MTases, implying that they might possess m6A in their genomic DNA (Fig. 3). Similarly, genomes of basal fungi, which possess one or more N6A-MTase, are predicted to possess a robust m6A signal (Fig. 3).

How are m6A marks reset?

Removal of 5mC marks is part of a key epigenetic resetting mechanism operating at critical developmental junctures in certain eukaryotes [94, 95]. The recent discovery of the TET/JBP family of 2OGFeDOs has cast light on how this might happen via a combination of oxidative modification of 5mC and nucleotide excision repair [51]. With the earlier discovery of a related family of 2OGFeDOs, prototyped by E. coli AlkB, it became apparent that these enzymes could repair DNA with adenine and cytosine methylated, respectively, at the N1 and N3 positions by mutagenic alkylating agents [96–98]. It was also predicted then that certain members of the AlkB family were likely to demethylate m6A in RNA [96]. Subsequently, such demethylation of m6A in RNA was indeed observed to be the mechanism for resetting methyl marks generated by N6A RNA MTases [68, 99]. Oxidation of the methyl group by these enzymes results in formation of N6hmA and N6fa, which restore the original base, releasing formaldehyde and formate [100]. The recent C. elegans study demonstrated via in vitro and in vivo experiments that nmad-1, a member of the AlkB family, demethylated m6A in DNA [53]. This suggested that in addition to repair of DNA damaged by alkylating adducts, members of the AlkB family are likely to be key players in m6A demethylation in DNA. It remains to be seen whether the metastable intermediates, N6hmA and N6fa, have
any independent role in DNA as proposed for modified RNA [100].

While bacteria typically have only a single AlkB representative (Fig. 3), the family underwent explosive radiation in eukaryotes upon being acquired by lateral transfer from bacteria. The basal eukaryotic lineages, the parabasalids and diplomonads, lack members of the AlkB family, suggesting that it was perhaps absent in the ancestral eukaryote (Fig. 3). By the time that other early-braching eukaryotes – such as euglenozoans and heteroloboseans – split off from the remaining eukaryotes the AlkB family had already radiated into at least five distinct clades (prototyped by human AlkBH1, AlkBH4, AlkBH6, AlkBH7, and AlkBH8; Supporting Information). Subsequently, five further widespread clades emerged along with smaller clades restricted to particular eukaryotic lineages. Of these, the most widespread, AlkBH1, retained the ancestral DNA repair role, while the AlkBH4 and FTO clades specialized in RNA modification [101–103]. C. elegans nmad-1 belongs to the AlkBH4 clade defined by a N-terminal Zn-binding domain and might be primarily involved in DNA m^6A demethylation [53]. However, an nmad-1 ortholog is absent in key lineages with confirmed m^6A in DNA (Supporting Information). We observed that these organisms instead possess members of the AlkBH5 clade with fusions to domains such as the PHD finger and the AT-hook DNA-binding motif. Similarly, AlkBH3 also shows fusions to the PHD finger, and different DNA-binding domains (SAD/SRA and AT-hook motif; Fig. 4). Hence, members of the AlkBH5 and AlkBH3 clades conceivably demethylate m^6A in DNA (Fig. 4).

Recently, it was proposed that the *Drosophila* TET enzyme, DmTET, functions as a m^6A demethylase [54]. However, this proposal is at odds with other observations. DmTET is closely related to vertebrate TETs, and retains all features of the active site of the latter enzymes which accommodate a pyrimidine rather than a purine [15, 49]. A diversity of characterized eukaryotic TET/JBP enzymes have been observed only to modify 5-methylpyrimidines rather than purines [49–51, 104, 105]. This is further confirmed by contextual analysis of bacterial and phage TET/JBP family

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**Figure 4.** Domain architectures and gene neighborhoods of N^6A demethylases and predicted modified DNA-binding domains. Domains and gene neighborhoods are grouped based on the principal domain of that group. Domains architectures and operons are labeled as in Fig. 1. X refers to uncharacterized domains.

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members, which are predicted to primarily modify pyrimidines [15, 90]. Consistent with these, purified DmTET demonstrated 5mC oxidation capacity in vitro on DNA substrates [54]. However, all experiments demonstrating its purported m6A demethylation activity were done with additional Drosophila cell extracts [54]. The study did not rule out the presence of the Drosophila mnad-1 ortholog in these extracts, which could have catalyzed the actual demethylation like its C. elegans ortholog [53]. However, a secondary role for DmTET via a pathway involving oxidation of thymines opposite to m6A cannot be ruled out. Hence, until further independent confirmation, the role of TET/JBP enzymes as m6A demethylating enzymes should be treated with caution.

**Multiple domains potentially discriminate m6A marks in DNA**

In several bacteria, proteins containing a SeqA domain specifically bind DNA with hemimethylated m6A sites, and distinguish parent from newly synthesized duplexes [106]. Given the prevalence of m6A modification in eukaryotes, in principle, multiple DNA-binding proteins might recognize it. We had earlier developed a method to predict modified DNA-binding domains by using contextual information from domain architectures and conserved gene-neighborhoods of R-M and counter-restriction systems [15]. By identifying homologs of domains thus recovered in eukaryotes, we had successfully predicted proteins involved in discrimination of DNA with 5mC and its oxidized derivatives. Applying a similar approach, we were able to predict multiple domains that might be involved in recognition of m6A in eukaryotes [15].

Most prevalent of these is a previously unrecognized domain that was initially observed fused to both N6A-MTases and nuclease domains belonging to different structural folds: REase, HNH, URI, ParB, serine-resolvasel, and T5orf172, most of which are restriction endonucleases of N6A-modifying R-M systems (Fig. 4; Supporting Information) [6, 107]. It is also fused to McrB-like AAA+ domains, involved in DNA translocation/looping in certain R-M systems [10, 12], and to DNA-binding domains such as the hemimethylated m6A-binding SeqA domain (Fig. 4). Accordingly, we named it the RAMA (restriction enzyme adenine methylase associated) domain. We also observe that it has been transferred to eukaryotes, and is found in lineages predicted or known to possess m6A, such as animals, chlorophytes, algae, stramenopiles, rhodophytes, and certain eukaryotic organisms (Fig. 3). In eukaryotes, RAMA domains are frequently fused to JAB deubiquitinating peptidase (DUB) domains in paralogs of the MYSM1 enzymes that deubiquitinate the monoubiquitinated histone H2A (H2A-K119u) [108]. Less common domain architectures across eukaryotes combine the RAMA domain in a single polypeptide with (Fig. 4): the chlorophyty-type DAM in the alga Bathycoccus; ankyrin repeats; the histone MTase SET domain; domains recognizing modified histones (PHD finger, Chromo, Tudor, and Bromo domains); a domain recognizing phosphopeptides in chromatin (BRCT); DNA-binding domains (ARID/BRIGHT, TAM/MBD, PARP-zinc finger, and AT-Hook); the ubiquitin E3-ligase (RING) domain [72, 73, 83, 84].

The HARE-HTH (for instance found in human ASXL1) is another domain showing similarity to the RAMA domain in its architectural and functional linkages [109]. In prokaryotes, it is fused to an array of endonuclease domains, which serve as restriction enzymes in N6A-modifying R-M systems, and N6A-MTase domains [109]. In eukaryotes, it is linked to a comparable set of chromatin-related domains (Fig. 4). Thus, both these domains display architectures that in prokaryotes are suggestive of recognition of modified bases in R-M systems (Fig. 4), while in eukaryotes they are consistent with a role in recognition of similar epigenetic marks in chromatin. Strikingly, the HARE-HTH protein AXSL1 is a subunit of the H2A-K119u MYSM1 DUB in vertebrates (MYSM1 itself is fused to a DNA-binding MYB domain) [110]. Hence, both paralogous H2A-K119 DUBs are likely to bind DNA, and possibly recognize modified bases in DNA in conjunction with H2A deubiquitination.

PUA-like domains display a β-barrel fold, and are the common structural denominator of several families of proteins recognizing modified nucleic acids. These include the SAD/SRA, which recognizes 5mC in DNA; EVE, which binds DNA with 5hmC; and the PUA, which binds modified RNA (including the YTH family which binds m6A containing RNA) [20, 68, 111–115]. Another family displaying this fold, the ASCH domain, was predicted to bind several modified bases, and is found fused to or operationally associated with the N6A-MTase domain on multiple occasions in bacteria [15, 112] (Fig. 4). This suggests that eukaryotic ASCH domains might also serve as m6A discrimination modules. This proposal is attractive given that C. elegans, with m6A in DNA, lacks both RAMA and HARE-HTH domains, but has a protein with an ASCH domain. This protein, TRIP4/ASCI, combines the C-terminal ASCH domain with an N-terminal RNA-binding PWI domain, and central Zn-binding domain, which interacts with specific transcription factors (Fig. 4) [112, 116] and the RNA demethylase FTO [117]. TRIP4/ASCI is prevalent across eukaryotes, and is part of the basal transcription apparatus, where it serves as a co-activator, suggesting that recognition of m6A marks by TRIP4/ASCI might have a role in transcription regulation [116]. A divergent version of the SAD(SRA) domain is also fused to an AlkB 20GFeDO domain in several fungi [90] (Fig. 4). While the SAD(SRA) domain typically recognizes single-strand 5mC sites [118–120], this version of the domain has distinct features suggesting that it could potentially recognize m6A in RNA or DNA as opposed to 5mC. Hence, it is conceivable that these AlkB-like proteins also function as m6A demethylases.

**What are the roles for m6A in eukaryotic DNA?**

While research on m6A function in eukaryotes is still in its infancy, certain consistent features are already seen emerging. Genetic evidence in C. elegans [53], biochemical evidence of m6A in intermolecular and interchromosomal linkers [52], and domain architectures of the N6A-MTases in fungi, chlorophytes, and ciliates...
(Fig. 3) suggest that modification of DNA is likely coordinated with recognition of specific histone methylation marks. In *Chlamydomonas*, genes showing m^6^A are significantly associated with active transcription as opposed to those with no m^6^A methylation [52]. In *C. elegans*, loss of function of the LSD1-like of histone H3 methyl/di-methyl lysine 4 (H3K4me1/me2) demethylase, spr-5, causes multi-generational increase in m^6^A levels. Conversely, deletion of the potential N^6^-MTase, damt-1, reduces elevated H3K4me2 levels of spr-5 mutant worms. Knockdown of the H3K9me binding protein eap-1 [121], which reduces H3K4me2 levels in spr-5 mutant worms, also reduced levels of m^6^A in spr-5 mutant worms. This indicates a tight link between H3K4me2 and m^6^A marks in *C. elegans* [53]. Given that across eukaryotes H3K4me2 is acquired during active transcription [125], here too m^6^A might show a link to active transcription. In *Drosophila*, elevated levels of m^6^A in gene bodies of transposons were associated with increased levels of transcription of these elements [54]. Thus, in three model systems m^6^A is potentially associated with active transcription. This might in part relate to early observations that m^6^A in DNA results in lower stability of duplexes, thus favoring strand separation during transcription [122].

In contrast, 5mC shows a conserved association with condensed and transcriptionally silent chromatin across eukaryotes [42, 94, 123]. However, while apparently contrary in their functional consequences, 5mC and m^6^A do not seem to be correlated in eukaryotes. Some eukaryotes, such as nematodes and ciliates, possess only an m^6^A modification system. Others, such as *Naegleria*, chlorophytes and several fungal lineages, show co-occurring systems capable of generating m^6^A, 5mC, oxidized 5mC, and perhaps oxidized thymines [49, 104, 105]. Kinetoeplastids lack m^6^A-generating systems but possess 5mC and oxidized thymine systems [15, 37, 124]. Thus, unlike histone-methylation, which is universal across eukaryotes, DNA modifications are patchily utilized. Therefore, though they might play critical roles in certain lineages, they have been evolutionarily subordinate to histone modifications, which are an essential feature of eukaryotic life [72].

The m^6^A methylomes are very different in the three models in which they have been determined [52–54]. Hence, despite common features of m^6^A modifications in distantly related eukaryotes, there are likely to be functional differences that are currently poorly understood. In *Chlamydomonas*, extensive modification (84% of genes), a clear TSS-associated pattern, and symmetric methylation sites, suggest that the modification might play a role in the organization of transcriptionally active chromatin [52]. In contrast, in *C. elegans* there are no strong local patterns of m^6^A, the levels of m^6^A are “noisy” even in wild-type animals, and methylation motifs are single-strand biased. These observations, along with the remarkable trans-generational increase coupled to H3K4me2 marks in the spr-5 mutant, suggest that m^6^A might reinforce the function of the H3K4me2 mark – the two marks together serving as epigenetic memory for genes that have been actively transcribed [125]. Because a large fraction of genes are actively transcribed during germ-line development, these marks might need to be reset to restore the ground state during meiosis and zygote development. Consistent with this, buildup of m^6^A and H3K4me2 levels is correlated with infertility that emerges after several generations [53]. The asymmetric DNA motifs of m^6^A in *C. elegans* are reminiscent of mRNA motifs of methylation catalyzed by enzymes of the related METTL3/METTL14 clades [126]. Hence, it is possible that damt-1 and other METTL4-like enzymes act on ssDNA generated during transcription.

Conclusions and prospects

Evolutionary analysis of m^6^A MTases adds further evidence to the recent hypothesis that key players in eukaryotic chromatin first emerged in prokaryotic conflict systems [15, 20]. In the latter systems they play a role in self-nonself discrimination or diversification of secondary metabolites to effectively target rivals with antibiotics and evade stealing of products such as siderophores [88, 107]. The origin of the eukaryotic nucleus probably opened niches that selected for prokaryotic modification enzymes and modified DNA readers in new capacities [88]. Interestingly, these were often also deployed in RNA-related roles. This might again relate to the eukaryotic separation of cytoplasmic translation from nuclear transcription allowing for similar enzymes to be utilized in both RNA- and DNA-based regulation [15, 68].

Renewed focus on m^6^A in eukaryotic DNA has added it to the growing list of epigenetic marks in DNA [15, 37, 49, 51, 105]. However, there are still several biochemical questions needing careful consideration in the future: (i) which members of the AlkB family are involved in demethylation of m^6^A? (ii) Is there a genuine, direct role for TET/JBP enzymes in removal of m^6^A marks? (iii) Are partners used by DNA N^6^-MTases and demethylases (such as WTAP in the RNA methylation system)? (iv) How does the methylation- and demethylation-apparatus interact with histone modifications and the transcription machinery? (v) What are the readers of m^6^A marks? (vi) Is binding of transcription factors and chromatin proteins affected by m^6^A? (vii) Do oxidized metastable derivatives of m^6^A have any role in dynamics of m^6^A-based regulation?

In terms of biology, an important question is how prevalent m^6^A marks are in eukaryotes. It remains to be established if METTL4 orthologs outside of nematodes are involved in DNA m^6^A methylation. Further, investigating fungal and basal eukaryotic systems [104, 105] might help understand elements of their functions that are not obvious from mammalian systems.

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