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Shaping eukaryotic epigenetic systems by horizontal gene transfer

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Abstract

DNA methylation constitutes one of the pillars of epigenetics, relying on covalent bonds for addition and/or removal of chemically distinct marks within the major groove of the double helix. DNA methyltransferases, enzymes which introduce methyl marks, initially evolved in prokaryotes as components of restriction-modification systems protecting host genomes from bacteriophages and other invading foreign DNA. In early eukaryotic evolution, DNA methyltransferases were horizontally transferred from bacteria into eukaryotes several times and independently co-opted into epigenetic regulatory systems, primarily via establishing connections with the chromatin environment. While C5-methylcytosine is the cornerstone of plant and animal epigenetics and has been investigated in much detail, the epigenetic role of other methylated bases is less clear. Recent addition of N4-methylcytosine of bacterial origin as a metazoan DNA modification highlights the prerequisites for foreign gene co-option into the host regulatory networks, and challenges the existing paradigms concerning the origin and evolution of eukaryotic regulatory systems.

Graphical Abstract

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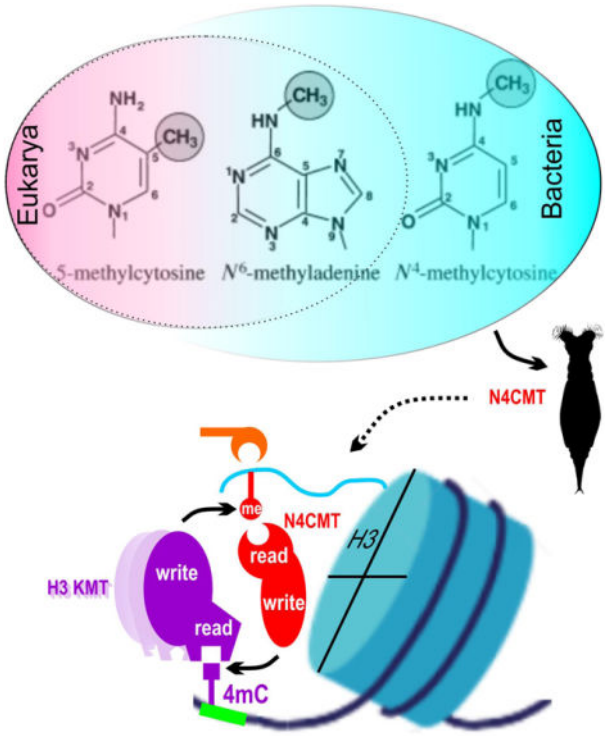
Conflict of interest

The authors declare no conflict of interest.

Supporting information: Supplementary Figure S1; Supplementary Table S1.



Three major types of DNA methylation from bacteria to eukaryotes, with an example of recruitment of a horizontally transferred bacterial N4C-methyltransferase into a eukaryotic epigenetic silencing system involving histone modifications. Cross-talk between DNA and histone epigenetic layers is mediated by catalytic ("write") and recognition ("read") domains of DNA and histone methyltransferases.

Keywords

DNA methylation; N4-methylcytosine; amino-methyltransferase; epigenetic silencing; transposable elements; regulatory evolution; lateral gene transfer

INTRODUCTION

One of the most quintessential components of epigenetics is DNA methylation, i.e., enzymatic addition of a methyl group to a nucleobase, which introduces subtle but chemically distinct changes into cytosines and adenines without changing the underlying genetic code. These changes are central to numerous biological processes that take advantage of the presence of a heritable and potentially removable mark on DNA, forming the basis for many epigenetic phenomena. Methylation is the most common form of DNA modification, and three main types of methylated bases have been discovered, with varying distribution patterns across prokaryotes and eukaryotes: N6-methyladenine, 5-methylcytosine and N4-methylcytosine [1–5] (Box 1; Fig. 1A). Although methylated bases in RNA (N^6-methyladenosine, N^1-methyladenosine, N^6,2'-O-dimethyladenosine, 5-methylcytidine, 5-hydroxylmethylcytidine) gained much prominence in recent years, we do

not consider epitranscriptomics here due to substantially different enzymology, substrate requirements, and biological effects.[6]

In prokaryotes, the presence of a DNA modification is usually a hallmark of a host restriction-modification (R-M) system, which protects methylated bacterial DNA from phage infections by destroying unmethylated non-self DNA with a cognate restriction endonuclease (REase).[16,17] R-M systems can travel horizontally across prokaryotic species as selfish genetic elements, with the same target sequence recognized by the REase and the MTase to safeguard genome integrity.[18] Additionally, stand-alone or "orphan" MTases, such as *Dam*-like and *CcrM*-like, which are not associated with any REase, can be involved in DNA replication, mismatch repair, control of cell cycle, gene expression, and transposition.[19–21] In eukaryotes, C5-methylation is overwhelmingly prevailing, while amino-methylation is far from typical. Equally atypical for eukaryotes are the corresponding N4/N6 modifying enzymes, homologies to which were sporadically reported in ciliates, fungi, and even a few mammals, although their functionality in the latter is questionable, as they have not been linked with N4/N6 base modifications in DNA.[11,22,23]

While 5mC constitutes a well-recognized cornerstone of mammalian and plant epigenetics, it is more difficult to assign straightforward epigenetic roles to eukaryotic 6mA methylation marks, and the function, abundance and even presence of 6mA in DNA of multicellular eukaryotes have been debated on multiple occasions (see [24,25] for review). Recently, the repertoire of methylated bases in eukaryotes was expanded to include the third modification, 4mC, added by N4-MTase which was horizontally transferred from a bacterial donor to the common ancestor of bdelloid rotifers about 60 million years ago.[26] Its relatively recent recruitment into the host epigenetic system provides an opportunity to infer how the newly acquired DNA modifications can turn into epigenetic marks, and to understand the requirements for a successful evolutionary transition from decorating the DNA double helix with a previously unseen chemical modification to its integration into the complex regulatory mechanics of the host cell. Since 4mC was previously known only in bacterial DNA, below we seek to explore how a eukaryotic genome can acquire a bacterial modification mark, how it can be reliably detected and experimentally validated, and how it can become incorporated into the complex regulatory networks of a eukaryotic host to yield evolutionary innovations that did not exist in bacteria.

HORIZONTAL GENE TRANSFER FROM BACTERIA AS THE SOURCE OF EUKARYOTIC DNA METHYLTRANSFERASES

All known eukaryotic DNA MTases are of bacterial origin, having been acquired independently from different bacterial lineages and preserved by evolution following successful adaptation to their new eukaryotic host. In other words, they originated by horizontal gene transfer (HGT), which is also called lateral gene transfer (LGT); these terms are fully interchangeable and denote transmission of genetic information by means other than parent-to-offspring (vertical). The evolutionary history of eukaryotic DNA MTases has been described in several reviews, which covered the origin of C5-MTases such as Dnmt1 and Dnmt3 from bacterial Dcm enzymes,[27] as well as polyphyletic origins of N^6A-MTases,

which were divided into three large groups (Group-I, M.MboIIA/M.MunI-like; Group-2, M.EcoKI/M.TaqI-like; Group-3, Dam-like).[28]

In eukaryotes, HGT is a rare phenomenon in contrast to bacteria, and has long been thought to be a relatively minor force in adaptive evolution in comparison with gene duplication, regulatory evolution, etc. However, certain metazoan taxa were discovered to be capable of capturing massive amounts of foreign genes, with bdelloid rotifers representing a prime example where 8–10% of genes have originated from non-metazoan sources.[29,30] Gradually, evidence began to accumulate that non-vertical processes such as HGT can add substantially to the adaptive potential of recipient taxa by bringing in previously absent functions.[31] Nevertheless, most of these functions are confined to the so-called operational genes responsible for a specific biochemical reaction, not involving informational or other complex interconnected regulatory systems ("the complexity hypothesis").[32,33]

Recently, two eukaryotic N^4C-MTases were shown to be similarly acquired by HGT, as evident from complete lack of detectable eukaryotic homologs. In the phylum Rotifera, the enzyme N4CMT, fused to a eukaryotic chromodomain, helps to epigenetically suppress transposable elements (TEs) in rotifers of the class Bdelloidea;[26] and an independent N4-MTase acquisition from bacteria occurred in the liverwort Marchantia (Viridiplantae; Streptophyta), where MpDN4MT1 is essential for spermiogenesis.[34] In the latter case, however, no apparent cross-talk with histone-based epigenetics has been established, as the liverwort enzyme contains no extra domains and deposits 4mC marks rather indiscriminately, except when hampered by pre-existing 5mC marks.

To find out if other metazoans may have captured intact N4/N6-MTases from bacteria, we turned to the sensitive hidden Markov model (HMM) profile searches, which could reveal additional HGT instances. In the Pfam database,[35] use of the PF01555 profile as a query returned 86 sequences from 33 eukaryotic species (Fig. 2B, compared with nearly one-half of C5-MTases coming from eukaryotes). However, ORFs in 14 of these species lack catalytic residues, and 9 more species were filtered out as bacterial contaminants (Supplementary Table 1). The remaining 10 species were placed in the following taxonomic groups: 1 in Viridiplantae (green plants), 3 in Rhodophyta (red algae), 1 in Parabasalia (flagellated protists), 3 in Haptophyta (photosynthetic microalgae), 1 in Oomycetes (stramenopiles), and 1 in Glomeromycete fungi (Table 1). Upon clustering, however, only MTases from *Marchantiopsida* (Viridiplantae) showed similarity to rotifers, grouping with bacterial enzymes which methylate CpG targets and contain the SPPY catalytic motif characteristic of N4-MTases (Fig. 2A,C). Other HGT candidates are clustered with N6-MTases retrieved from REBASE,[36] and harbor the DPPY catalytic motif likely to methylate adenine (Fig. 2C). The search is by no means exhaustive, as only annotated proteins in the Pfam database were inspected. Recent horizontal transfers can escape annotation due to poor recognition of alien ORFs by annotation pipelines trained on eukaryotic proteomes, evading detection. Nevertheless, the rarity of N4-MTase acquisition/retention in comparison with N6-MTases is notable, and may reflect the known bias in amino-MTase distribution in the donor prokaryotic taxa and/or lack of successful functional recruitment.

Of note, 4mC represents a prominent modification in the genomes of giant DNA viruses, such as mollivirus and pandoraviruses, which infect amoebas of the genus *Acanthamoeba*.[37] The corresponding N4-MTases (the same permuted Type II subtype β[38]) are scattered among bacterial type II N4-MTase clusters denoted by specific target sequences (Fig. 2C), but are not paired with cognate REases, and are thought to be used for protection against R-M systems of co-infecting bacteria or viruses.

Out of the previously described groups of eukaryotic amino-MTases not presented here, most enzymes correspond to adenine N6-MTases.[27,28] The only exception are the non-permuted MTases forming Clade 5 of Iyer et al.,[28] found in Chlorophyta (green algae), SAR and Haptista. Most of these Clade 5 ORFs feature SPPY as the catalytic motif, making them good candidates for the role of cytosine N4-MTases (except for SCPY motif in unicellular green algae *Ostreococcus spp*. and GPPY in the dinoflagellate *Symbiodinium minutum*). Again, the non-permuted N4-MTases from giant viruses infecting amoebae (pandoraviruses) display homology to bacterial MTases with the same recognition sequence, rather than to eukaryotic hosts.[37]

Note that it is not possible to reliably discriminate between the ability of an MTase to modify N4-cytosine or N6-adenine based on sequence homology alone, as they could be quite similar to each other in amino acid sequence and even functionally interchangeable.[39] Indeed, 6mA and 4mC bases are chemically similar, while being quite distinct from 5mC (Fig. 1A). Nonetheless, the homology-based approach can be used for preliminary assessment of N4 *vs* N6 enzyme specificity until further experimental validation (see below). So far, none of the SPPY-carrying MTases were shown to methylate N6-adenine, confirming that this signature motif can serve as a good indicator of specificity for N4-cytosine (the reverse does not apply, as there are many examples to the contrary). Recognition of any adjacent bases in the DNA target, however, can be inferred only tentatively, on the assumption that base specificity depends on amino acid residues in the target recognition domain (TRD) interacting with target bases, so that similarities in TRD should at least partially reflect similarities in the target sequence (e.g., sharing the CpG target, Fig. 2C), but would have no correlation with bacterial phylogeny.

METHODOLOGICAL CHALLENGES IN IDENTIFYING NON-CANONICAL BASE MODIFICATIONS

The number of methods developed to detect canonical DNA methylation, either genome-wide or at single-gene level, has steadily increased over the past few decades. Most of these methods were focused on identifying methylation in eukaryotes, where 5mC DNA methylation is prevalent.[40] The main approaches include digestion by REases differing by sensitivity to methylation; liquid chromatography; affinity enrichment of methylated DNA fragments using methyl-binding proteins or antibodies; and chemical conversion (e.g. with bisulfite) (Box 2). Third-generation DNA sequencing technologies such as PacBio and Oxford Nanopore, in combination with improved bioinformatic approaches, have greatly increased the sensitivity and resolution of methylation analysis, and stimulated identification of low-abundance DNA methyl groups.[41–43] These include the still controversial low-

abundance 5mC in Drosophila,[44,45] as well as 6mA (reviewed in [24,25]) and the recently added 4mC marks[26,34] in other eukaryotes, which comprise non-canonical base modifications, as opposed to the 'canonical' abundant 5mC typical of mammalian genomes. Such non-canonical modifications often exhibit non-uniform spatial or temporal distribution (e.g., TE-associated or cell type-/developmental stage-restricted) and constitute a minor fraction of modified *vs* unmodified bases in the genome. While modern techniques offer new advantages for detecting the elusive methylated bases, it is important to highlight potential pitfalls and possible ways to avoid artifacts in the analysis (Box 2).

Independently of the technique chosen for detection of non-canonical modifications, contamination is a major problem when methylated DNA is of very low abundance in the target organism. Misincorporation of salvaged pre-methylated RNA or DNA nucleosides, either from m^6A-rich cellular RNA or from DNA of commensal bacteria, by DNA polymerases has sometimes been invoked as a potential source of 6mA in eukaryotic DNA.[25] By analogy, m^4C could be misincorporated from mitochondrial rRNA, to which it is added by METTL15,[62] or perhaps 4mC could be borrowed from rare bacterial DNA. Such misincorporation, however, is expected to be distributed randomly across the genome, without regard to annotated genomic features.

Improvement of methods for 4mC detection in eukaryotes is only beginning. Its partial sensitivity to bisulfite deamination makes discrimination from 5mC possible but cumbersome.[34,63] While nanopore sequencing and modified bisulfite sequencing (4mC-TAB-seq) were used to detect 4mC in bacteria,[52,64] a promising approach is further development of chemical conversion *via* nitrosylation, followed by PCR amplification.[65] However, application of this method to eukaryotes may be complicated by DNA degradation and by PCR amplification biases. Resistance of 4mC to enzymatic deamination by APOBEC3A (4mC-AMD-seq) was used to discriminate 4mC from C and 5mC in the radioresistant bacterium *Deinococcus radiodurans*.[66] This method avoids excessive DNA degradation and can be applied to low-input samples. Further improvement of 4mC detection methods in eukaryotes will be essential for establishment of validated 4mC reference datasets for training of bioinformatic pipelines, and for eventual elucidation of its biological functions.

CHALLENGES IN ASSIGNMENT AND VALIDATION OF EUKARYOTIC METHYLTRANSFERASE FUNCTION

Since 2017, over 30 publications aimed at characterizing 4mC distribution in eukaryotic genomes were devoted to continuous improvement of 4mC prediction accuracy using machine learning approaches ([67,68] and references therein). However, the value of these methods is uncertain, as they were developed and trained on reference datasets[69,70] which have not been rigorously proven to harbor the 4mC mark. As explained above, the principal techniques employed for detection of 4mC and database creation, such as SMRT-seq or UHPLC-MS/MS, are prone to inaccuracies (high signal-to-noise ratio, bacterial and/or RNA contamination).[50] Thus, in addition to confirming the presence of 4mC modified bases in sequenced genomes by orthogonal methods, it is crucial to identify the corresponding MTase

enzyme before making any claims regarding the presence of 4mC in a genome. If 4mC is identified by SMRT-seq analysis in a genome containing only C5-MTase homologs (which include DNMTs),[71–73] the most natural explanation is the erroneous identification of 5mC marks as 4mC by the software used.

Amino-MTases responsible for incorporation of 4mC and 6mA into DNA are somewhat less conserved than C5-MTases, and are not easily distinguishable from each other *in silico*. Both C5- and amino-MTases contain the catalytic domain demarcated by ten conserved motifs I-X and the variable DNA target recognition domain (TRD) (Fig. 2A). The subdomains can be permuted in several MTase types,[74,75] but it is difficult to predict methylation specificity based solely on the order and sequence of motifs. While DNA C5-MTases reliably group together, the diversity of amino-MTases also includes MTases acting on RNA and/or proteins (e.g. HemK family).[28] Based on multiple sequence alignments and hidden Markov models (HMM) profile searches, most amino-MTases would be assigned to the N6_N4_MTase domain family (PF01555) originating from the Pfam protein database.[35] The general mechanism of catalysis with base flipping, implied by the [DNSH]PP[YFW] amino acid string, is reviewed in [28,76,77].

Although the current algorithms for genome assembly are generally accurate, cases of misassembly still occur, implying that MTase DNA sequences should be verified using additional genomic or transcriptomic data. Homology searches are used to identify novel MTase sequences following prediction of protein-coding genes in a *de novo* genome assembly (Fig. 3). Positive MTase hits must be checked for the presence of eukaryotic genes in the immediate genomic environment to rule out bacterial origin, even for large contigs, as entire endosymbiont genomes can be assembled with sufficient coverage (Supplementary Table S1). Typically, intron accumulation constitutes good evidence for domestication of a formerly bacterial gene. REBASE provides a source of MTases with known recognition sequences and methylation specificity, which can be used to create a multiple sequence alignment.[36] To correctly assign a novel MTase candidate to the appropriate group, it is imperative to identify catalytic amino acids, which are part of motif IV. Most N4C-MTases have the SPPY catalytic string. With sufficient representation of amino- and C5-MTases and simple clustering approaches, any candidate MTase may be tentatively assigned to 4mC-, 6mA- or C5-modifying types.

No matter how reliable a protein alignment is, it cannot serve as proof that a novel MTase possesses the predicted enzymatic activity. Only experimental verification can decisively assess the specificity of a eukaryotic MTase (Fig. 3). To determine whether an MTase shows enzymatic activity, and if so, to characterize it, biochemical approaches should be employed. Purifying the putative MTase protein directly from host cells, or producing a recombinant enzyme in a heterologous expression system, are the easiest solutions. However, it is not always possible to obtain pure protein of sufficient quality and quantity for *in vitro* studies. If so, one can perform indirect studies by expressing putative MTases in bacteria or tissue culture and analyzing DNA methylation status. Substitution of catalytic residues on either side of the prolines in the [DNSH]PP[YFW] domain to alanines should eliminate the corresponding methyl marks from DNA in control assays.[78]

In vivo knockout/knockdown (KO/KD) experiments became the *de facto* gold standard for validation of biological function and establishment of causal links between phenotypes and candidate loci. However, KO/KD of the corresponding gene *in vivo* is possible mostly in model organisms, and may yield inconclusive results due to indirect and off-target effects on other cellular components, and/or co-existence of several methyl marks. Indeed, N6AMT1 was implicated in N^6A methylation based on KD experiments,[79] but is a HemK-like methylase acting on N^5 in glutamine.[80–82] Similarly, the candidate N6AMT2 was shown *in vitro* to act not on N^6A in DNA, but on N^5 in lysine of EF1A.[83] On the other hand, using recombinant enzymes paired with their catalytic mutants (change to APPA in motif IV) can often give a clear answer about MTase specificity and recognition sequence. Use of [^{3}H]-AdoMet or validated anti-4mC/anti-6mA antibodies in combination with DNA substrates to detect methylation *in vitro* are feasible approaches, although optimization of experimental conditions may be required.[26,84] In sum, although assignment of prokaryotic enzyme specificity is now largely achieved *via* high-throughput analysis of genomes and methylomes,[85,86] proper assignment of specificity to novel eukaryotic MTases requires a thorough manual curation of database search outputs, as well as experimental verification of sequence specificity and DNA modification activity.

PREREQUISITES FOR FUNCTIONAL RECRUITMENT BY EUKARYOTIC HOSTS

If 4mC in eukaryotes is so rare that it can be regarded as more of an evolutionary oddity than as a recurring phenomenon, it may be asked what we can learn from it about the general process of horizontal acquisition of non-canonical DNA modifications and their subsequent engagement with pre-existing eukaryotic networks. When an MTase is transferred from bacteria, it would initially be intronless, but can be ***expected to acquire introns*** (Table 1; Fig. 2A). Furthermore, to act on DNA, it should become capable of entering eukaryotic nuclei via ***acquisition of a nuclear localization signal*** (NLS). Indeed, NLS is found in most eukaryotic enzymes shown in Fig. 2A, including giant viruses, in N- or C-terminal extensions to the MTase domain. Although an MTase could retain some target specificity inherited *via* its TRD, there is no *a priori* reason to expect that a bacterial enzyme would exhibit any regional specificity in a eukaryotic host without first acquiring the respective domain. Thus, the most natural adaptation that a bacterial DNA MTase could undergo is the transition from modifying nucleosome-free DNA to acting on DNA packaged into chromatin *via* ***fusion to chromatin-binding domains***, signifying a substantial departure from bacterial circular chromosomes organized into a membrane-less nucleoid consisting mainly of DNA. Finally, if the methyl mark on a stretch of DNA is expected to exert a biological function, it should be recognized by another host component, unless it affects the physical properties of the DNA itself. At this step, gene duplication can play an important role, allowing adaptive evolution of newly emerging paralogs into ***specialized interacting partners***.

The above steps can be traced over evolutionary time at different timescales. In case of eukaryotic C5-MTases, functional recruitment occurred such a long time ago that the ancestry of the corresponding orthologs can be traced back to early eukaryotes, when the present-day domain configurations were established for Dnmt1- and Dnmt3-like MTases

to ensure interaction with chromatin and accessory proteins.[27] It is somewhat easier to observe the initial steps of MTase recruitment if the acquisition is more recent and can be measured in tens rather than hundreds of millions of years ago.

Enzymes acting on DNA in a eukaryotic cell acquire the means for interacting with DNA packaged into chromatin largely *via* domain fusion. Acquisition of the chromodomain (CHD) was the decisive step for the bdelloid amino-MTase, enabling it to "read" silent chromatin marks located over TEs and tandem repeats, such as H3K9me3 and H3K27me3, and to deposit 4mC marks over these regions (Fig. 1B).[26] This is not the only example of a successful MTase-CHD fusion: in plants, CHD was inserted within a C5-MTase to form chromomethylases (CMT), enzymes present throughout the plant kingdom. Here, the degree of convergent evolution extends even further: while bdelloids contain a SETDB1 (SET domain bifurcated) histone H3 lysine (H3K) MTase variant with preference for "reading" 4mC marks (Fig. 1B), the plant CMT3, which methylates C^5 in the CHG context, forms a similar self-reinforcing feedback loop with the histone lysine MTase KRYPTONITE (KYP), which methylates H3K9 *via* its SET domain, allowing further H3K9me recognition and C5-methylation by CMT3.[87]

While this feedback loop design is similar between plants and rotifers, there are also other differences besides C5 *vs* N4C methylation. KYP recognizes 5mC *via* its SAD/SRA DNA-binding domain, which performs 5mC base flipping for discrimination. In contrast, SETDB1 paralogs in rotifers contain the MBD/TAM DNA-binding domain, which is not capable of base flipping, but can interact directly with the major groove of DNA in the nucleosome context.[88] Despite MBD being a generic DNA-binding domain, which does not always require methyl groups for DNA-protein interaction, development by bdelloids of even a modest preference for N^4-methylated base by a paralog may have had a substantial cumulative effect. Similarly, the SRA domain of UHRF2 binds hemihydroxymethylated and fully hydroxymethylated DNA approximately 1.5 and 3.2 times more tightly than hemi-methylated DNA, respectively, with slight alterations in the binding pocket providing enough discriminatory power to classify it as 5hmC-specific reader in comparison with 5mC-specific UHRF1, the Dnmt1 cofactor.[89]

As known from the highly complex domain architecture of the plant and mammalian *Dnmt1/Dnmt3* orthologs, a large variety of chromatin-interacting domains in addition to the chromodomain can be fused to MTases to ensure productive interactions with chromatin.[87,90] In histone KMTases, a similarly high variety of domains can be observed, capable of interacting with repressive or active chromatin marks, and sometimes with both. For example, the Tudor domain can bind both H3K4me3 and H4K20me3 active marks,[91,92] while within SETDB1 the triple Tudor domain binds to doubly modified H3 containing the repressive H3K9me3 and the active H3K14ac marks, forming bivalent chromatin.[93] In bdelloid rotifers, although the interactions of the triple Tudor domain in SETDB1 paralogs have not yet been examined, the silent marks for H3K9me3 and H3K27me3 largely overlap, indicating the lack of a strict subdivision of heterochromatin into constitutive and facultative.[26] While TE transcriptional activity shows a downward trend in H3Kme3-marked areas, it is possible that some of these regions may contain other marks indicative of bivalent nature.

An important factor contributing to epigenetic recruitment may be ***DNA accessibility*** provided by chromatin remodelers. In flowering plants and mammals, nucleosomes typically obstruct action of most types of DNA MTases, which preferentially act on nucleosome-free DNA in all sequence contexts, unless nucleosomes are de-compacted by remodelers such as DDM1/Lsh.[94] However, in deep-branching marine algae C5-methylation by other Dnmt-like MTases is mostly confined to the inter-nucleosomal linker region, likely contributing to nucleosome positioning.[95] Linker preference is also observed for symmetric N^6A-methylation in ciliates, which is performed by a member of the MT-A70 family of amino-MTases.[58] Bdelloids do have a *Ddm1* ortholog and the corresponding capacity to remodel nucleosomes for efficient methylation, and amino-MTases depend on the same base-flipping mechanism for cytosine methylation as C5-MTases. On the other hand, recognition of DNA methyl groups in the major groove by an MBD/TAM domain in rotifer SETDB1-like H3K9-KMTases does not involve base flipping and could potentially be performed in the nucleosome context. In mammals, SETDB1 proteins are best studied in the context of KRAB-KAP1 and HUSH complexes which establish TE repression.[96–98] In contrast, the SAD/SRA domain in H3K9-KMTases, such as KYP and SUVH9, and the Dnmt1 cofactor UHRF1, requires base flipping for recognition of C5-methylated bases in flowering plants and mammals, enhancing the need for chromatin remodeling to ensure H3K9 methylation.[87]

It should be interesting to find out in what ways other domain combinations, which differ from those in the best-studied systems, can ***organize the cross-talk between DNA and histone modifications***. Domain configuration of Dnmt1-like C5-MTases has remained relatively unchanged throughout the evolution of multicellular eukaryotes, while Dnmt3-like MTase domain architectures were configured independently in plants and animals for *de novo* C5 methylation.[27,99,100] At the same time, other metazoan taxa with more taxonomically restricted and/or more evolutionarily recent MTases should offer additional opportunities to explore epigenetically relevant domain configurations. In addition to C5- and N6-MTase types identified in previous studies,[27,28] Fig. 2C outlines several previously unreported examples of taxa which may have evolved unconventional epigenetic systems upon fusion with chromatin-interacting domains: the C-terminal Tudor domain in a putative N^6A-MTase from *Chrysochromulina parva*, a phytoplanktonic haptophyte, and the C-terminal SET domain in a clade of multicopy DNA transposons from red algae (*Chondrus crispus, Porphyra umbilicalis, Gracilariopsis chorda*). The latter clade may represent an interesting case of C5-specificity acquisition by amino-MTases, as the closest REBASE matches are C5-specific MTases recognizing CCTC, although the DPPY/F motif is typical of N6-MTases. With the development of more accurate methods permitting base-pair resolution of non-canonical DNA modifications,[56,66] application of such methods to eukaryotic genomes with unconventional MTase types may yield interesting insights into the epigenetics of understudied eukaryotes.

POTENTIAL ROLES OF 4mC IN EUKARYOTIC GENOMES

Shortly after the discovery of 4mC in bacteria, it was noted that, in contrast to 5mC, this base modification is highly resistant to deamination.[101,102] Cytosine deamination (C->U and especially 5mC->T) is highly mutagenic, and the prevalence of 4mC in thermophilic

bacteria is widely believed to serve as protection from heat-induced deamination, although examples to the contrary are also known.[103] Possibly, part of the reason why 5mC is not as prevalent in bacteria as it is in eukaryotes could be the higher degree of bacterial exposure to external environmental stresses and temperature shifts. Bdelloid rotifers are well-known for their extraordinary resistance to harsh environmental conditions, and are thriving in extreme environments from glaciers to acidic hot springs.[104–107] Thus, the avoidance of cytosine deamination, when combined with benefits from epigenetic TE silencing, may be beneficial in terms of lower mutagenicity. In the radioresistant bacterium *D. radiodurans*, 4mC is thought to contribute to genome stability, as its removal yielded higher spontaneous mutation frequencies and enhanced recombination and transformation efficiencies.[108] More esoteric effects of 4mC on DNA include preferential formation of pyrimidine(6–4)pyrimidone photoproducts resistant to hydrolysis, rather than cyclobutane pyrimidine dimers favored by 5mC, with the potential to considerably change DNA UV photochemistry.[109,110]

Not too many studies have investigated the direct influence of DNA modifications on physical properties of DNA, although in early works researchers reported destabilization of DNA double helix by either 6mA or 4mC, slightly lowering the T_m,[111–113] while more recent studies reported that 5mC reduces DNA flexibility, disfavoring nucleosomes.[114,115] Although these changes may affect nucleosome stability *in vitro*, the action of nucleosome remodelers may neutralize any such effects *in vivo*.[58] Thus, it is likely that recruitment of a non-canonical base is best realized *via* its potential to serve as a covalently attached mark, which is recognizable as a chemically distinct DNA alteration by host proteins.

Such recognition mechanism, however, if not pre-existing in the host cell or brought in externally, must evolve within the new host, implying that a horizontally transferred MTase should survive through the period of adaptive evolutionary response from the host. For efficient use as an epigenetic mark, one of the prerequisites is the addition of a chromatin-interacting domain *via* fusion to a resident eukaryotic gene. Different paths to this structural reorganization could be entertained: direct fusion as DNA, e.g. by introducing a double-strand break (DSB) with subsequent resection and non-homologous end-joining (NHEJ) to accomplish in-frame fusion, or *via* illegitimate recombination with unequal exchange; and/or through alternative splicing, with utilization of a cryptic splice site outside the MTase moiety and an existing splice site within a multi-domain protein, with subsequent re-insertion of a chimeric cDNA copy into the host genome. Bdelloids indeed undergo frequent DSB formation and NHEJ repair following repeated cycles of desiccation and rehydration, and harbor potentially active retrotransposable elements that could produce cDNA copies *in trans*.[116–119]

Initial MTase survival, however, may depend on a possible function not requiring incorporation into the host epigenetic system. This has apparently happened in the liverwort *Marchantia polymorpha*, where N4-MTase has somehow acquired a function critical for sperm maturation *via* an unknown mechanism not dependent on domain fusion and integration with the pre-existing 5mC methylation systems. In fact, the enzyme is uniformly labeling all sequences but TEs, which are already covered by dense 5mC marks, precluding 4mC addition to the same cytosine, which would be damaging to DNA.[34,120] It remains to

be established how recent the 4mC acquisition was in liverworts, although the shared intron was acquired prior to the split of the orders Marchantiales and Lunulariales (Fig. 2A,C). It remains possible that N4CMT in bdelloid rotifers was initially retained for its intrinsic affinity for certain motifs, and was recruited for epigenetic TE suppression at a later stage when the fusion to CHD occurred.

CONCLUSIONS AND PROSPECTS

DNA methylation is a heritable alteration that can be superimposed onto the underlying genetic code without changing it. For a long time, studies of eukaryotic DNA methylation were focused on 5mC and the corresponding C5-MTases. After recent detection of 6mA in multiple eukaryotes, a flurry of publications started to investigate 6mA and potential N6-MTases. Here, we are adding the third methyl mark, 4mC, to the eukaryotic repertoire, and begin to explore 4mC/N4-MTase occurrence throughout eukaryotes and its potential to shape epigenetic landscapes and to participate in TE silencing. While TE suppression has always been an underlying theme in most epigenetic studies,[121–123] a newly acquired MTase would not specifically target TEs without undergoing evolutionary adaptation.

HGT is mostly thought to involve the “operational” genes,[32] and is not ordinarily expected to contribute to the complex regulatory systems of the host. Early horizontal recruitments of C5-MTases into the eukaryotic epigenetic system predated the divergence of the eukaryotic crown group into animal, fungal and plant kingdoms, but post-dated the histone modification system, which evolved in the last eukaryotic common ancestor.[27,124] Almost invariably, MTase recruitment implies acquisition of additional protein domains required for interaction with nuclear DNA which is organized into chromatin. It is fascinating to observe how convergent evolution operated on relatively recent time scales when establishing connections of an introduced bacterial MTase with other epigenetic layers involved in TE silencing. Another prerequisite to this paradigm-shifting HGT role in re-shaping eukaryotic regulatory networks is the availability of gene duplications to facilitate evolution of regulatory loops, which can stimulate cross-talk in different directions to form multi-layered epigenetic systems.

Finally, it is worth re-emphasizing that future improvements of base modification detection techniques should rely on high-confidence training datasets that would not include data from species in which the presence of a given modification is in doubt. More exotic DNA modifications in addition to the bacterial “big three” are being reported, although the enzymatic basis for their deposition has not always been elucidated.[125–127] Identification of the corresponding enzymes capable of adding such modifications should achieve highest priority in future studies exploring unconventional epigenetic systems across the eukaryotic tree of life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The data supporting the findings of this study were obtained from the following public databases: Genbank, https://www.ncbi.mlm.nih.gov; Pfam, at http://pfam-legacy.xfam.org until early 2023 and now integrated with InterPro https://www.ebi.ac.uk/interpro/; and REBASE, http://rebase.neb.com/rebase/; and are available as supplementary material to this article.

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Box 1.

Methylated bases in DNA.

Together with histone modification, chromatin remodeling, and small RNAs, DNA modification constitutes one the cornerstones of epigenetics. In eukaryotes, the predominant form of DNA base methylation is C5-methylcytosine (5mC, m^5dC), which represents the primary epigenetic modification involved in control of crucial biological processes such as repression of transposable elements (TEs) and certain genes, genomic imprinting, X-chromosome inactivation, and early embryonic development.[7,8] For a long time, investigations of epigenetic functions of methylated bases in eukaryotes were focused on 5mC, aptly named “the fifth base” of eukaryotic DNA due to its prevalence in plants and animals, especially in mammals. Besides the C5 position in the pyrimidine ring, the methyl group can also be added to the exocyclic amino group of adenine or cytosine (Fig. 1, top). N6-methyladenine (6mA, m^6dA, N^6mA) represents the most abundant DNA modification in bacteria[9,10] and was also reported in selected eukaryotes.[11–13] In contrast, N4-methylcytosine (4mC, m^4dC, N^4mC) is relatively rare and was previously thought to occur exclusively in bacteria.[11,14,15] Note that the corresponding nucleoside modifications in RNA are typically denoted as m^6A and m^4C, to facilitate discrimination from DNA. Superscripts correspond to the numbering of atoms; however, the use of superscripts is optional. No DNA MTases have yet been reported to act on RNA, and vice versa.

Box 2.

Methodological advantages and drawbacks in detecting non-canonical DNA base modifications.

Several methods for 5mC detection have been successfully adapted for detection of other methylated bases, and novel approaches based on long-read technologies can be used to detect a broad range of modifications. However, it is important to highlight potential pitfalls associated with each of these methods, and the need to apply orthogonal methods.

DNA immunoprecipitation sequencing

DNA immunoprecipitation sequencing (DIP-seq or MeDIP-seq) is a common method for methylation detection and genome-wide mapping. Modern high-throughput sequencing techniques provide sufficient depth, identifying most of immunoprecipitated methylated DNA at relatively low cost. However, the specificity is limited by fragment size and by potential cross-reactivity of antibodies raised against specific methylated bases (anti-5mC, anti-6mA, anti-4mC) with other similar methylated bases, such as m^6A or m^1A modifications in RNA, or with certain simple unmethylated motifs or secondary structures promoting off-target binding.[46,47] Considering the limitations of antibody conjugation, one can address these problems by including input and IgG controls, as well as by using orthogonal non-antibody-based techniques. Bioinformatic curation in assembled reference genomes should be performed routinely to remove contaminants (e.g., DNA of commensal bacteria).[48,49]

Chromatography-based techniques

Chromatography-based techniques, such as LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry), HPLC- (High-Performance-) and UHPLC-MS/MS (Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry), are highly specific and sensitive methods for bulk detection of non-canonical base modifications. Unlike DIP-seq, HPLC-MS/MS can precisely quantify each base signal (methylated/unmethylated ratio), and distinguish from methylated RNA products.[14] Yet, HPLC-MS/MS results can be misinterpreted due to contaminated samples. Therefore, mass-spectrometry is a highly useful method that must be combined with other, sequence-based, methods when searching for rare base modifications. Commercial enzymes used to digest DNA may also carry contaminating bases, necessitating background measurements.[50]

Bisulfite sequencing

Bisulfite sequencing (BS-seq) provides a quantitative assay for methylated cytosine detection at base-resolution.[51] Known as the gold standard for characterizing 5mC in eukaryotic genomes, it is based on deamination of unmethylated cytosines to uracil, leaving methylated cytosines intact. After treatment, DNA is PCR-amplified with a uracil-tolerant polymerase, causing uracil to convert to thymine. Sequences can be read directly by traditional Sanger or Illumina short-read sequencing through comparison to a reference or untreated sequence. However, bisulfite treatment can damage DNA, leading to degradation and PCR problems. Treating genomic DNA with TET (ten–eleven

translocation) enzymes before bisulfite treatment makes it possible to characterize both 5mC and 4mC in bacterial genomes, although the differences are not always easily distinguishable.[52]

PacBio SMRT and Oxford Nanopore

PacBio SMRT and Oxford Nanopore single-molecule sequencing technologies can detect DNA methylation at base resolution, relying on changes in fluorescent pulse intervals or in electrolytic current signals, respectively.[41–43] In contrast to BS-seq, single-molecule methods do not require base conversion to detect modifications. In SMRT-seq, base addition kinetics is measured during polymerization, and IPD (interpulse duration) ratio-based methylation profiling detects 6mA, 4mC, or other modifications such as 5-hydroxymethylcytosine (5-hmC) by measuring changes in the rate of polymerase processivity caused by modified bases. Initially applied to bacteria,[10] the SMRT technology also boosted detection of non-canonical base modifications in eukaryotes, [24,26,34] although it cannot distinguish between 6mA and 1mA, or reliably call modifications near cytosines.[46,53,54]

The biggest SMRT-seq drawback is a high false discovery rate (FDR) at low modified/unmodified base ratios.[46,50] At borderline values (under 1%), deeper coverage is needed to reduce the FDR: as read coverage increases, the variance of normal distributions of IPDs at the single-molecule level decreases, providing increased power for separation between methylated and non-methylated bases.[55] Contaminating DNA in reference genomes was shown to inflate 6mA/A ratios in fruit flies, plants and humans, and a recent quantitative metagenomic approach applies machine learning to minimize artifacts.[56,57] The most reliable way to reduce FDR is to use unmethylated DNA (*via* demethylation or PCR amplification) as a control.[58,59] Although far from cost-effective, this approach demonstrated that multiple IPD shifts in *C. elegans* were caused by organism-specific idiosyncratic sequence contexts,[60] in addition to previously known obstacles such as non-B DNA motifs.[61]

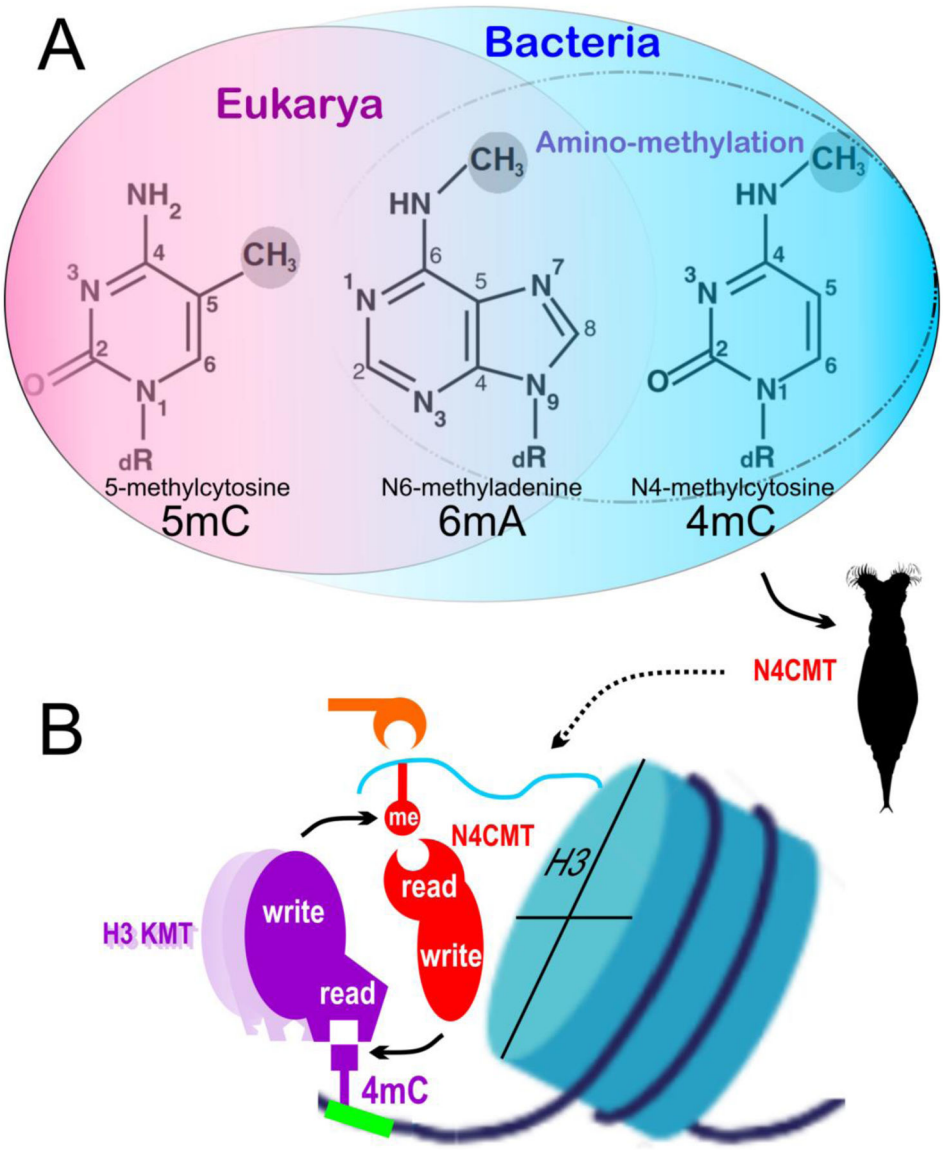

Figure 1.

DNA methyl marks from bacteria to eukaryotes. (**A**) Three major types of DNA methylation. The atoms in the purine and pyrimidine rings are numbered. The methyl groups added to cytosine and adenine are circled. dR, deoxyribose. Coloring denotes the prevalence of modifications in different domains of life, with blue for bacteria, pink for Eukarya, and the overlap between them as blended color. The bottom panel (**B**), adapted from [26], exemplifies recruitment of a horizontally transferred bacterial N4C-MTase into a eukaryotic epigenetic silencing system based on histone modifications. Both DNA and histone MTases (H3KMT) are composed of catalytic (“write”) and recognition (“read”) domains, which exist in several variants in H3KMT paralogs.

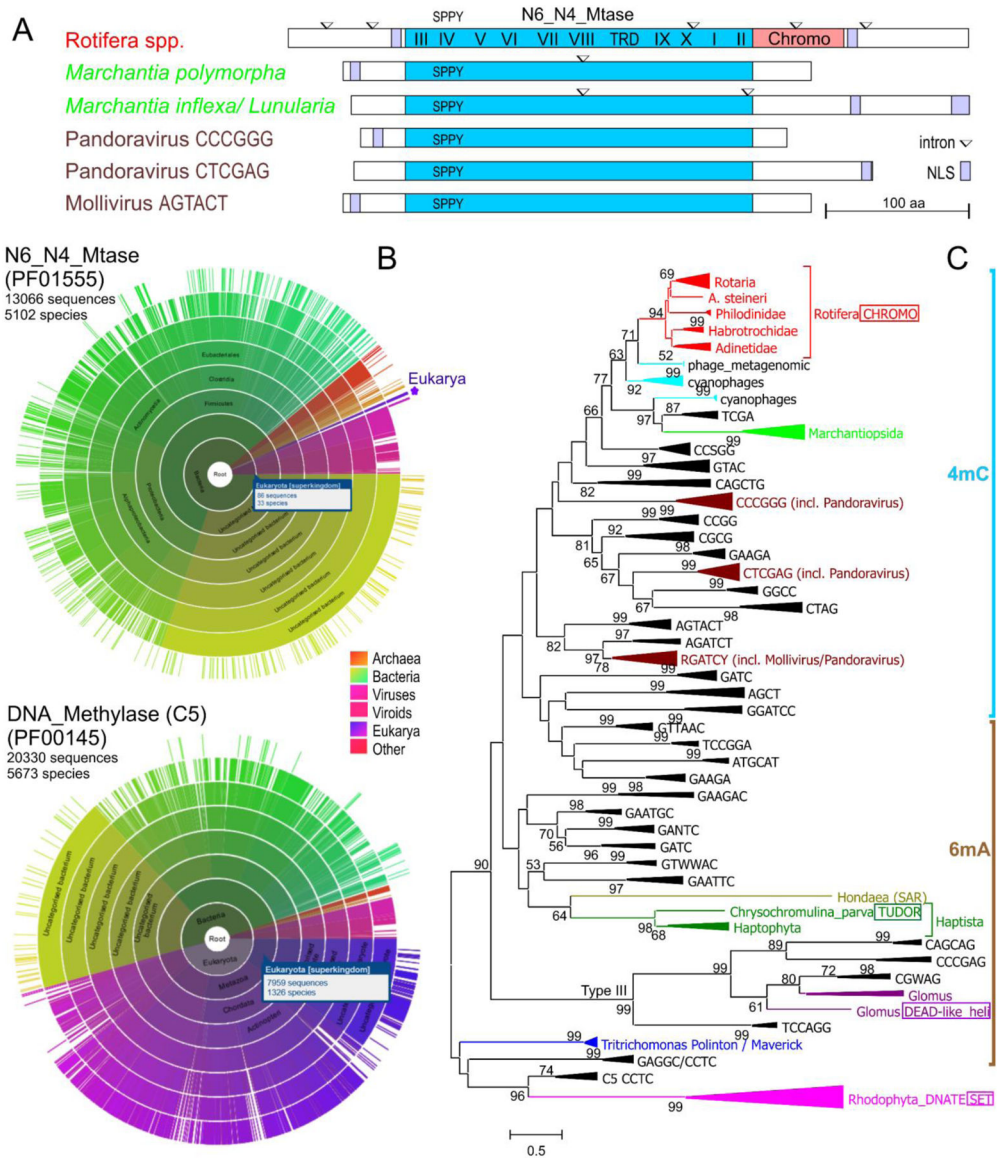

Figure 2.

Domain structure and distribution of N6_N4_MTases. (**A**) Structural organization of eukaryotic N4C-MTases from rotifers, liverworts and giant viruses. Shown are conserved motifs I-X in the permuted MTase domain, intron positions (triangles), and nuclear localization signals (NLS) predicted *via* HMM.[128] (**B**) Visualization of PF01555 distribution across species as a “sunburst” diagram from PFAM, with each ring (from inside to outside) showing the number of taxa within superkingdom, kingdom, phylum, class, order, family, genus, and species, respectively. The eukaryotic sector is marked with a purple asterisk. For comparison, the analogous distribution of PFAM family members for PF00145 (C5 DNA_methylase) is shown below. HMM search details are outlined in Supplementary Table S1. (**C**) Clustering of eukaryotic MTases (in color) with their prokaryotic counterparts (in black) by the neighbor-joining algorithm. Prokaryotic MTases with their recognition sequences are from REBASE. N4C-MTases from bdelloid rotifer

families, red; from liverworts, lime green; giant virus N4-MTases (in brown) are nested within the respective bacterial groups; cyanophages are highlighted in cyan. N6A-MTases from SAR, Haptista and Fungi are in olive, green, and purple, respectively. Putative parabasalid N6A-MTases (blue) are located within Polinton/Maverick transposons; SET-domain-containing MTases from red algae (magenta), with the closest match to C5-MTases, also belong to multicopy DNA transposons (Plavaka). Fusions of chromatin-associated domains to MTases are boxed. All MTases are classified by REBASE as permuted type II, except for the Glomus-containing group, which belongs to type III. Scale bar, amino acid substitutions per site. The tree format does not imply phylogenetic relationships between taxa. The uncollapsed version with the complete set of taxa is shown in Supplementary Fig. S1, and accession numbers are in Supplementary Table S1.

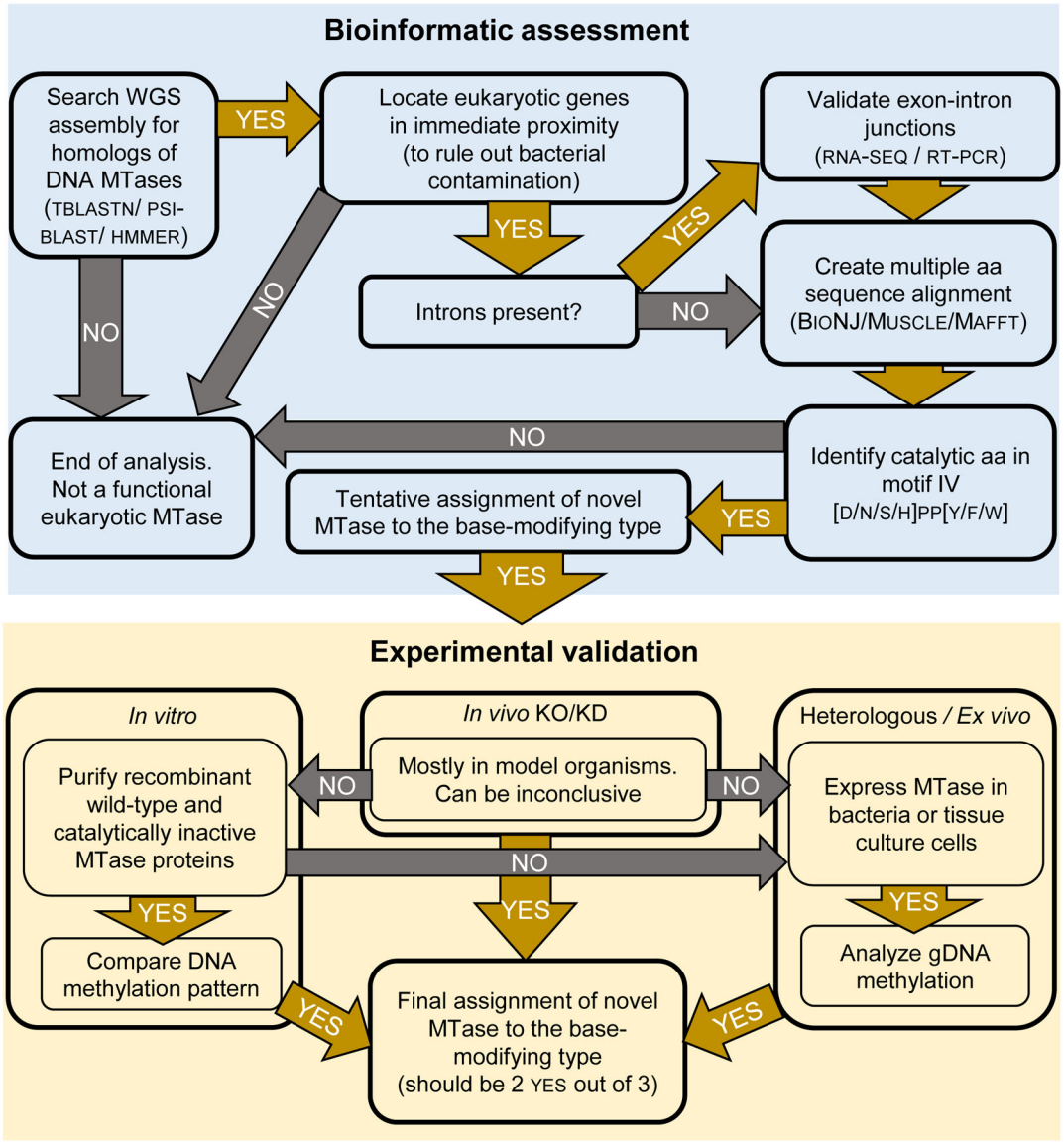

Figure 3.

A flowchart outlining the required steps in identification and validation of novel DNA amino-MTases in eukaryotic genomes. WGS, whole-genome sequence; aa, amino acid. The [DNSH]PP[YFW] amino acid string in motif IV can serve as an initial indicator: most N4C-MTases have the SPPY catalytic string, while most N6A-MTases feature DPPY that distinguishes them from eukaryotic N6AMT1 (NPPY, N^5-Gln), N6AMT2 (DPPY/F, N^5-Lys) or METTL4-like enzymes (DPPW, also seen in METTL3/IME4-like m^6A-RNA MTases).

Table 1.

Eukaryotic species containing the N6_N4_MTase domain with intact catalytic residues. Shown are the species analyzed in Fig. 2B. For complete information, see Supplementary Table 1. nd, not detected by HMM.

Species	Taxonomy	Catalytic motif	Introns	NLS	Fused domain
Adineta vaga	Rotifera; Bdelloidea; Adinetidae	SPPY	+	+	Chromo
Adineta ricciae	Rotifera; Bdelloidea; Adinetidae	SPPY	+	+	Chromo
Adineta steineri	Rotifera; Bdelloidea; Adinetidae	SPPY	+		Chromo
Macrotrachela quadricornifera	Rotifera; Bdelloidea; Philodinidae	SPPY	+	+	Chromo
Rotaria macrura	Rotifera; Bdelloidea; Rotaria	SPPY	+	+	Chromo
Rotaria magnacalcarata	Rotifera; Bdelloidea; Rotaria	SPPY	+		Chromo
Rotaria sordida	Rotifera; Bdelloidea; Rotaria	SPPY	+	+	Chromo
Rotaria sp. Silwood-1	Rotifera; Bdelloidea; Rotaria	SPPY	+	+	Chromo
Rotaria sp. Silwood-2	Rotifera; Bdelloidea; Rotaria	SPPY	+		Chromo
Habrotrocha rosa	Rotifera; Bdelloidea; Habrotrochidae	SPPY	+	+	Chromo
Marchantia polymorpha	Streptophyta; Marchantiopsida; Marchantiales	SPPY	+	+	nd
Marchantia paleacea	Streptophyta; Marchantiopsida; Marchantiales	SPPY	+	+	nd
Marchantia inflecta	Streptophyta; Marchantiopsida; Marchantiales	SPPY	+	+	nd
Lunularia cruciata	Streptophyta; Marchantiopsida; Lunulariales	SPPY	+		nd
Hondaea fermentalgiana	Sar; Stramenopiles; Bigyra; Thraustochytrida	DAPY	+		TPR
Chrysochromulina tobinii	Haptista; Haptophyta; Prymnesiales	DPPY	+	+	nd
Chrysochromulina parva	Haptista; Haptophyta; Prymnesiales	DPPY	-		Tudor
Diacronema lutheri	Haptista; Haptophyta; Pavlovales	DPPY	-	+	AP2
Emiliania huxleyi	Haptista; Haptophyta; Isochrysidales	DPPY	+		nd
Glomus cerebriforme	Fungi; Mucoromycota; Glomeromycetes	DPPY	+		DEAD
Tritrichomonas foetus (TE)	Metamonada; Parabasalia; Trichomonadida	DPPY	-		nd
Porphyra umbilicalis (TE)	Rhodophyta; Bangiophyceae; Bangiales	DPPY	-	+	SET
Chondrus crispus (TE)	Rhodophyta; Florideophyceae; Gigartinales	DPPF/Y	-		SET
Gracilariopsis chorda (TE)	Rhodophyta; Florideophyceae; Gracilariales	DPPY	-	+	SET