Prospects & Overviews

DNA adenine methylation in eukaryotes: Enzymatic mark or a form of DNA damage?

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Abstract
6-methyladenine (6mA) is fairly abundant in nuclear DNA of basal fungi, ciliates and green algae. In these organisms, 6mA is maintained near transcription start sites in ApT context by a parental-strand instruction dependent maintenance methyltransferase and is positively associated with transcription. In animals and plants, 6mA levels are high only in organellar DNA. The 6mA levels in nuclear DNA are very low. They are attributable to nucleotide salvage and the activity of otherwise mitochondrial METTL4, and may be considered as a price that cells pay for adenine methylation in RNA and/or organellar DNA. Cells minimize this price by sanitizing dNTP pools to limit 6mA incorporation, and by converting 6mA that has been incorporated into DNA back to adenine. Hence, 6mA in nuclear DNA should be described as an epigenetic mark only in basal fungi, ciliates and green algae, but not in animals and plants.

KEYWORDS
6mA, cancer, DNA damage, DNA modifications, epitranscriptome/epigenome, nucleotide salvage, transcription

BIOCHEMISTRY OF ADENINE METHYLATION

The adenine base can be methylated chemically or enzymatically, at diverse sites, especially in RNA. In DNA biology, only three types of methylated adenine bases are considered biologically relevant. These are 1-methyladenine (1mA) and 3-methyladenine (3mA), generally regarded as products of alkylation damage in DNA, and of interest in the context of genome stability and DNA repair. In contrast to 1mA and 3mA, 6-methyladenine (6mA) is hardly made at all by chemical methylation (because aromatic amines are poor nucleophiles). Therefore, the presence of this base is generally attributable to enzymatic activity. In this review, we focus exclusively on 6-methylation of adenine. When we refer to adenine methylation, 6-methylation is meant.

Abbreviations 6mA and m6A refer to methylated adenine in DNA and RNA, respectively.

The properties of the 6-methyladenine base have been extensively characterized (Figure 1). The 6-methyl group of adenine tends to be close to the plane of the nucleobase, and of interest in the context of genome stability and DNA repair. In contrast to 1mA and 3mA, 6-methyladenine (6mA) is hardly made at all by chemical methylation (because aromatic amines are poor nucleophiles). Therefore, the presence of this base is generally attributable to enzymatic activity. In this review, we focus exclusively on 6-methylation of adenine. When we refer to adenine methylation, 6-methylation is meant.

Abbreviations 6mA and m6A refer to methylated adenine in DNA and RNA, respectively.

The properties of the 6-methyladenine base have been extensively characterized (Figure 1). The 6-methyl group of adenine tends to be close to the plane of the nucleobase, because of electronic effects (Figure 1A). It therefore points either towards the Watson-Crick edge (Figure 1B), or away from it (Figure 1C). Only the conformation of the methyl group pointing away from the Watson-Crick edge, which is disfavored for the free nucleobase, is compatible with Watson-Crick
Figure 1  Biophysical properties of 6-methyladenine. (A) Small molecule structural studies show that the methyl group in 6-methyladenine is (almost) in the plane of the nucleobase. This can be understood as a consequence of the sp2 hybridization of the N6 nitrogen, which makes it possible for its p-orbital (shown in grey) to be conjugated to the aromatic π-electrons of the nucleobase (represented by the contributing p-orbital on the C6, in grey). (B-C) The 6-methyl group can point towards the Watson-Crick edge as in (B), or away from the Watson Crick edge as in (C). Outside the context of dsDNA, the conformation in (B) is preferred, because it avoids the steric conflict with the purine ring (small orange lightning symbol in C). However, in dsDNA this conformation is incompatible with Watson-Crick pairing (large orange lightning symbol in B), and therefore the adenine methyl group has to adopt the otherwise unfavorable conformation in (C). (D) As 6-methyl groups on adenines have to adopt an otherwise unfavorable conformation, their presence in dsDNA facilitates strand separation and lowers the melting point pairing. The requirement for the 6mA base to adopt an unfavorable conformation (relative to the 2′-deoxyribose) destabilizes double stranded DNA[3] (Figure 1D) (Box 1).

In ApT context, and only in this context, adenine methyl groups in the two strands of double-stranded DNA are in steric conflict for the canonical B-form.[4] This conflict has to be resolved by conformational change, which can be used for indirect methylation readout (Figure 2A). Once the steric clash has been accommodated, the methyl groups are either located apart or stacked favorably against each other. This may explain why adenine methyl groups in ApT context are believed to increase rigidity of DNA,[5] even though they do not necessary do so in general.[6] Proximity of adenine methyl groups is also relevant in a nucleosome context (Figure 2B). Together with increased rigidity and direct repulsion, it may explain the anti-correlation of 6mA with nucleosomes that is observed in cellular context in algae and ciliates[5,7] (Figure 2C).

The biophysical effects of adenine methylation on transcription, even in naked DNA are ambiguous. Upstream of transcriptional start sites (TSSs), adenine methylation is expected to favor transcription, by facilitating DNA strand separation,[3] and thus the opening of transcriptional bubbles. Downstream of TSSs, more facile DNA strand separation should generally favor transcription, but 6mA in the template strand could also cause polymerase stalling,[8] presumably due to the non-canonical shape of 6mA and the tendency of the methyl group to interfere with Watson-Crick pairing. Hence, even at the simplest biophysical level, both positive and negative associations

Box 1: Properties of 6-methyladenine

The properties of the 6-methyladenine base have been extensively characterized. In contrast to the 5-methyl group on cytosine, which is completely fixed, the 6-methyl group on the adenine base has some degree of conformational freedom. However, it is not freely rotatable around the C6-N6 bond as is often assumed. Conjugation of the N6-lone pair to the purine aromatic ring drives the methyl group close to the plane of the aromatic ring, by a “conjugation” force that is similar to the “conjugation” force that enforces peptide bond planarity.[110] Therefore, 6-methyladenine exists predominantly in two conformations, which differ by a 180° flip of the methyl group.[1] The barrier that separates the two forms has been estimated to be between 11 and 18 kcal/mol, which is sufficient to make the two forms semi-stable on biological timescales.[2] For the free 6-methyladenine base (or the corresponding 2′-deoxynucleoside) the methyl group points with a 20:1 preference towards the Watson-Crick edge and not the Hoogsteen edge of the base.[2] However, only the latter form is compatible with Watson-Crick pairing of bases in double stranded DNA. As the base pairing requires the less favored form of 6mA, the presence 6-methyl groups in adenine acts as a destabilizing factor for double stranded DNA.
of 6mA with transcription are expected. The biophysical effects are small, but could have been played upon by evolution to bring about the modern-day transcriptional outcomes of DNA adenine methylation.

In this review, we discuss biophysics and enzymology of adenine methylation, the distribution of 6mA in the genome, its links with transcription, and the potential use of nuclear 6mA levels as a diagnostic marker in human cancers. We argue that concepts from cytosine methylation are useful as a framework to understand adenine methylation in basal fungi, ciliates and algae, despite the opposite transcriptional outcomes. We also argue that the same concepts can be misleading when applied to nuclear adenine DNA methylation in animals and plants, even in situations when adenine methylation, like cytosine methylation, may be negatively correlated with transcription.

6mA in DNA of BASAL FUNGI, CILIATES AND ALGAE

6mA is abundant and concentrated in ApT context in nuclear DNA

Nuclear 6mA levels are high in several phylogenetic branches of the lower eukaryotes. Levels as high as 2.8% have been reported for the “early diverging” fungi, and appear to be shared feature for this group of eukaryotes. High methylation levels have also been reported for the ciliates Oxytrichia and Tetrahymena, and for the single celled green algae Chlamydomonas. High 6mA levels are not, however, a shared feature of the simpler eukaryotes in general. Methylation levels are low, for example, in Dikarya including Ascomycota and Basidiomycota, and in the metazoan lineage.
**Figure 3** Phylogeny of 6mA DNA methyltransferases. (A) Phylogenetic tree of DNA adenine methyltransferases, based on the tree of Iyer and colleagues.[14] Note that all RNA methyltransferases branches have been omitted. (B) Enrichment of 6mA near, but not directly at the transcriptional start sites (TSSs) for the different clades. Note that the figure shows a low resolution picture of adenine methylation near TSSs. The oscillations with ∼150 bp periodicity that arise from anti-correlation of 6mA with nucleosomes are not shown. Green arrows indicate positive associations of 6mA (in light blue) with transcription. In animals and plants, both negative and positive associations of 6mA with transcription have been reported (not shown).

In all studied lower eukaryotes that have high levels of adenine methylation, the modification occurs predominantly in ApT sequence context.[7,9,10] The ApT dinucleotide sequence is special. Apart from TpA, it is the simplest sequence that contains adenine bases in both strands, so that methylation can be maintained in semi-conservative DNA replication, by parental strand instruction of daughter strand methylation. In principle, the ApT and TpA palindromes are equally suited for this task, but only ApT appears to be used. This choice may not be accidental, and may be due to the unique effects that methylation in this context has on structure and dynamics of DNA, that can be exploited for indirect readout by reader proteins and histones (Figure 2).

**Dedicated ApT maintenance methyltransferases have been identified in fungi, ciliates and algae**

The association with functional elements in the genome (TSSs) and the palindromic context of adenine methylation (ApT) strongly suggest that methylation is maintained by parental-strand instruction of daughter strand methylation. The idea is supported by much other circumstantial evidence as well. In Chlamydomonas, which has served as model for much of the early 6mA research in basal eukaryotes, 6mA levels drop by 40% after a few rapid cell divisions, and then rebound.[7] The best evidence for a dedicated ApT methyltransferase, however, has so far been obtained in ciliates. In the ciliate Oxytrichia, a biochemical fractionation approach traced the methyltransferase activity to a protein complex termed MTA1c. This complex consists of two MT-A70 methyltransferase subunits (MTA1 and MTA9), and two homeobox subunits and targets double stranded DNA. As expected for a maintenance adenine methyltransferase, activity is highest when the substrate DNA is hemi-methylated.[10] In the ciliate Tetrahymena, 6mA is only found in the macronucleus, but is absent from the micronucleus,[12] again suggesting that the mark is enzymatically placed. The methyltransferase could be identified in Tetrahymena as AMT1 or TAMT-1, a member of the MTA1 family.[5] It has the properties of a maintenance methyltransferase: it specifically maintains methylation in ApT context under parental strand instruction.[13] As the identification was based on genetic data, it remains possible that AMT1 or TAMT-1 acts in a complex with other proteins.

The methyltransferases that have been implicated in eukaryotic DNA adenine methylation occur in different clades of the phylogenetic tree (Figure 3A). According to the classification by Iyer and colleagues,[14] the deepest split is between the methyltransferases in the basal fungi in clade 4 of the Group-I methyltransferases on the one hand and all other DNA adenine methyltransferases in clade 1 of Group-I, known as the MT-A70 clade. Within the MT-A70 clade, the ciliate methyltransferases belong to eukaryotic subclade 4, whereas the algae MT-A70 enzymes have subunits from eukaryotic subclade 1 (also termed the METTL3 clan) and eukaryotic subclade 5. It is possible that differences between the methyltransferases explain the different distribution of 6mA around TSSs (bimodal around the TSS...
in algae, mono-modal downstream of the TSS in ciliates and basal fungi).

To the extent that they have been characterized in more detail, the DNA adenine methyltransferases in basal fungi, ciliates and algae have the properties of maintenance methyltransferases. It is not clear whether dedicated de novo methyltransferases should also exist, or whether the already identified methyltransferases have sufficient de novo activity as well to make them unnecessary.

6mA is highly enriched around, but not directly at transcriptional start sites (TSSs)

6mA in the nuclear DNA of basal eukaryotes (that have high levels of DNA adenine methylation) exhibits a very characteristic genomic distribution. The modification is highly enriched in the vicinity of TSSs, but absent from the TSSs themselves. The precise distribution depends on the organism. Low resolution methods suggest that in basal fungi and in the macronucleus of the protist Tetrahymena, 6mA is enriched in a region up to about 1 kb downstream of the TSS, but not upstream of the TSS.[5,9] In the green algae Chlamydomonas, an additional (slightly) weaker 6mA enrichment occurs also upstream of the TSS,[7] so that the overall distribution of 6mA is bimodal, with a local minimum in between the upstream and downstream peaks at the TSS (Figure 3B).

How the pattern for 6mA in the vicinity of transcriptional start sites is laid down is presently unclear and subject to speculation. The 6mA peak downstream of the TSS could be due to physical association of the methyltransferases with RNA polymerase. In such a scenario, the DNA methyltransferase could exploit the slow starting phase of transcription to deposit more adenine methyl groups than in the later, faster phase of transcriptional elongation. The model would explain the occurrence of a 6mA peak downstream of the TSS, its positive skew, and also the width of the 6mA peak of about 1 kb, which is consistent with the region of slowest transcriptional elongation.[15] According to this point of view, the 6mA peak upstream of TSSs in algae would have to be attributed to pervasive bidirectional transcription.[16] However, it is not clear why bidirectional transcription should play a larger role in algae than in basal fungi or ciliates. Apart from relying on the RNA polymerases, the adenine methyltransferases of algae, basal fungi and ciliates could also take instructive cues from chromatin marks that are known to be enriched near TSSs in higher[17] and probably all eukaryotes. This model is particularly likely for the fungi, because their DNA methyltransferases contain multiple chromatin binding domains in addition to the catalytic domain.[14]

6mA is anti-correlated with nucleosome position

Higher resolution mapping shows that the peaks detected with low resolution methods are an average of an oscillating 6mA distribution with a periodicity of 140–150 bp.[5,7] The oscillations arise, because DNA adenine methylation is predominantly located in the linker region between nucleosomes, but not in the DNA regions that are actually wrapped around histones. The simplest explanation for this pattern is that nucleosomes interfere with enzymatic DNA methylation, and thus direct adenine DNA methylation primarily towards linker DNA. The anti-correlation between 6mA and nucleosomes has also been attributed to the exclusion of histones from 6mA containing regions of DNA. Repulsion of histones by 6mA is supported by in vitro reconstitution experiments, and by genetic experiments that show perturbed nucleosome positions when 6mA levels are altered.[5] Repulsion could arise from steric conflicts, since nucleosome wrapped DNA periodically faces the histone surface with the major groove side, where the methyl groups are exposed. Alternatively, it has been suggested based on computational experiments, that the presence of adenine methyl groups increases DNA rigidity and therefore complicates wrapping on histones.[15] Modelling supports the idea that properties of ApT methylated DNA disfavor wrapping on histones. In a computational model of a poly(6mApT) nucleosome, most 6mApT dinucleotide steps have steric conflicts between the methyl groups in the two strands (Figure 2B).

6mA is positively correlated with transcription

In basal fungi, ciliates and algae that have high levels of adenine methylation, the presence of 6mA in the vicinity of TSSs is positively correlated with transcription rates (Figure 3B). So how does adenine methylation around TSSs promote transcription? For the 6mA upstream of TSS, reported for Chlamydomonas, facilitated DNA strand separation[3] could help to open transcriptional bubbles. For 6mA downstream of TSSs, reported for Chlamydomonas and other basal eukaryotes with high 6mA levels, the positive correlation suggests that facilitated strand separation is more important than the stalling by 6mA in the template strand. Alternatively, or additionally, the positive correlation with transcription may have to do with the effects of 6mA on chromatin structure. It has been noted that nucleosome positioning is better defined in the vicinity of TSSs, and hypothesized—albeit without clear molecular mechanism—that this effect may be linked to adenine methylation. If indeed adenine methylation controls nucleosome positioning, then it may prevent sliding or drift of nucleosomes towards the actual TSS, and by keeping it nucleosome free, contribute positively to transcription.[18]

A dedicated 6mA DNA demethylase remains to be identified in fungi, ciliates and algae

Most enzymatically introduced chromatin marks are reversible. It would therefore be surprising if this was not also the case for 6mA in the DNA of basal fungi, ciliates and algae, despite the alternative option to reduce 6mA levels in DNA passively by a replication-coupled dilution. In contrast to 5-methylation of cytosine, 6-methylation of adenine could be reversed directly, without the risk to DNA strand integrity. As already reported for the RNA context, 6-methyladenine could be hydroxylated to 6-hydroxymethyladenine, and then 6-formyladenine, which revert spontaneously to adenine with loss of formaldehyde or
formic acid on a time scale of hours.[19] Hydroxylation could either be direct, or a two-step process, by dehydrogenation and subsequent hydration.[20] However, specific enzymes with a role in the reversal of enzymatic adenine methylation in the DNA of lower eukaryotes have not been reported, and it is unclear whether they "should" exist. In the special case of Tetrahymena, adenine methylation is exclusively present in the DNA of the somatic macronucleus, which is degraded upon conjugation and formation of a new germline macronucleus.[12] Other basal eukaryotes may be able to eliminate their adenine DNA methylation passively by DNA replication.

6mA IN THE DNA OF ANIMALS AND PLANTS

6mA in mitochondria and chloroplasts

In animals and plants, 6mA levels are much higher in organelar than in nuclear DNA.[21,22] In animal mitochondria, the ratio of 6mA to A in DNA was estimated as roughly 400 ppm (parts per million).[21] For plant mitochondria, Vanyushin and colleagues reported an even higher figure of about 5000 ppm (=0.5%).[23] The reported estimates differ by more than a factor of 10. However, because the estimates were established using very different methodologies, a direct side-by-side-comparison is still needed before definitive conclusions on relative levels are drawn. Comparative studies suggest that adenine methylation levels in plant chloroplasts are similar or even higher than in plant mitochondria.[22,24]

At least in mitochondria, and probably also in plant chloroplasts, DNA adenine methylation is enzymatically maintained. A candidate adenine methyltransferase, was first purified from wheat,[25] but its identity has never been fully established. In animals, the relevant methyltransferase was recently identified as METTL4.[21] Activity of METTL4 towards DNA fits prior bioinformatics analysis, which had determined that this particular methyltransferase clusters with DNA methyltransferases, rather than with other RNA methyltransferases.[14] However, activity in mitochondria was unexpected, because the enzyme lacks a dedicated mitochondrial targeting sequence, and already has an established role in the nucleus in m6A metabolism.[26] Other basal eukaryotes may be able to eliminate their adenine DNA methylation passively by DNA replication.

6mA levels in nuclear DNA are very low

There is a growing consensus that adenine methylation in nuclear DNA is either undetectable or very low. An isotope controlled LC/MS study of murine nuclear DNA concluded that 6mA levels were below the detection limit of 1.2 ppm 6mA/A.[28] A meta-analysis of earlier mass spectrometry data also concluded that 6mA levels were too little correlated with tissue source and too much with experimental protocol to provide reliable evidence for 6mA in nuclear DNA.[29] Other careful mass spectrometry studies found typical 6mA/A ratios of 6–7 ppm,[30] below 8 ppm,[31] or around 1 ppm.[32] However, the latter study also found an exceptional cell line (3T3 cells) with a 6mA/A ratio as high as 500 ppm. Exempting this special case, 6mA levels in nuclear DNA are at least an order of magnitude, and possibly two orders of magnitude lower than in mitochondrial DNA. Compared to cytosine methylation (5mC/C ~40,000 ppm), the difference in abundance is even more drastic and more than 1000-fold. In the following, we will assume nuclear 6mA levels of 10 ppm, which is likely an overestimate in most cases. Reported higher 6mA levels, especially in earlier studies, are likely attributable to experimental artifacts.[29]

Due to the extremely low levels of 6mA in the nuclear genomes of animals and plants, the detection and quantification of 6mA in nuclear DNA is very prone to such artifacts. For methods that do not analyze the sequence context of the methylation signal, contamination is a major concern. Mitochondrial genomes, despite their higher copy number and adenine methylation, are too small to be relevant. By contrast, bacterial DNA rich in 6mA, due to Dam and other methyltransferases, is a serious concern. In cultured cells, it has been shown that 6mA levels can be inflated by mycoplasmal contamination. In zebrafish, adherent bacteria are now known[33,34] to have inflated the signal for "nuclear" 6mA.[35] For LC/MS based methods, DNA has to be degraded and dephosphorylated to 2'-deoxynucleosides. It is now known that the nucleases and phosphatases that are used in these experiments are contaminated to varying extents with 6mA containing DNA.[33] For antibody based methods, specificity is another major concern.[36] To detect 6mA reliably at the 10 ppm level, antibodies have to have a 1,000,000-fold preference for 6mA over A. This is at odds with experiments that suggest that the 6mA specific antibodies have only about 300-fold preference for 6mA over A,[29] which agrees much better with a rough biophysical estimate than the 1,000,000-fold preference that is implicitly assumed in studies that use 6mA antibodies to detect 6mA in nuclear DNA at the 10 ppm level (Box 2). The interpretation of antibody based mapping studies is additionally complicated by bias from the intrinsic affinity of IgG for short unmodified repeats.[37] Antibody based studies may also falsely report high 6mA levels in nuclear DNA because they were done before it became clear that adenine methylation is also present in the RNA component of R-loops in the nucleus,[38] and in persistently chromosome associated regulatory RNAs, termed carRNAs.[39]

Sequencing technologies that can call DNA modifications[40,41] safeguard against contamination problems with unrelated DNA. However, those that rely on 6mA DNA immunoprecipitation (6mA-DIP) and do not call the modification directly in the sequenced DNA are still plagued by potential cross-contamination of the signal by m6A in RNA,[42] unless stringent controls exclude this possibility. Surprisingly, even real-time sequencing, that calls modified bases directly, does not safeguard against this problem completely, as shown by the presence of reads with exon-exon junctions.[29] This is of major concern, even though it is unclear how the reads can be generated by the DNA polymerase used for sequencing. The most serious problem for sequence based 6mA calling is the scarcity of 6mA. Assume for simplicity that a
Box 2: Problematic detection of 6-methyladenine in DNA by antibodies

Antibodies against modified nucleotides and 2′-deoxynucleotides are typically raised with the nucleotide, that has been ring-opened at the vicinal 2′ and 3′ OH groups to generate reactive aldehyde groups as anchors for groups that enhance antigenic properties. Specificity ratios R depend on ΔΔG differences of binding free energies according to R = \exp(\Delta \Delta G/kT), where kT is the Boltzmann energy, which is about 0.6 kcal/mol at room temperature. Hence, preferences by 3.0 and 5.5 kcal/mol correspond to 150- and 9500-fold preferences, respectively. The latter preference is likely near the biophysical limit. The main driving force for methyl binding is the hydrophobic effect. A methyl group adds 20 Å^2 (our calculations for 6mA) to 30 Å^2, respectively. The latter preference is likely near the biophysical limit. A methyl group adds 20 Å^2 (our calculations for 6mA) to 30 Å^2, respectively. The latter preference is likely near the biophysical limit.

Multiple reads for the same genomic region improve accuracy but not sufficiently, and are altogether not helpful when methylation is distributed stochastically. Currently, single molecule real time (SMRT, PacBio) sequencing is the most sensitive sequencing based method for 6mA detection. With simulated samples, a modification in a single DNA molecule can be revisited multiple times to improve calling accuracy. However, with standard inter-pulse delay (IPD) thresholds, the false positive rate is still too high to achieve even 50% FDR assuming a 10 ppm 6mA incidence. Moreover, reanalysis of published data shows that the false positive rate for 6mA identification is higher in regions of high cytosine methylation, suggesting a confounding influence of cytosine methylation on 6mA identification. Given the many possible sources of false positives, it is not surprising that sequence based studies overestimate 6mA levels.

DNA methyltransferases of the METTL4 family contribute to nuclear 6mA levels

An early study of 6mA in the human nuclear attributed the presence of 6mA to the activity of N6AMT1 (also known as HemK2/KMT9). N6AMT1 is a promiscuous methyltransferase and may well affect 6mA levels in DNA indirectly. However, direct activity of the enzyme (in complex with partner protein Trm112) on DNA was subsequently excluded. Other methyltransferases that have been implicated in adenine DNA methylation belong to the METTL4/DAMT1 family of methyltransferases, that is eukaryotic subclade 3 of the MT-A70 clade of the group I methyltransferases. This places the candidate methyltransferases in a separate eukaryotic subclade, within the MT-A70 clade that also harbors the adenine DNA methyltransferases of ciliates and algae. Experimental reports are available for the mouse orthologue Mettl4 and the Caenorhabditis DAMT-1 homologue. Doubts about the classification of the METTL4/DAMT-1 family as nuclear DNA methyltransferases arise because human METTL4 is also responsible for mitochondrial DNA methylation, which is far denser than nuclear adenine DNA methylation. Complicating the picture further, it has recently also been shown that METTL4 is also involved in m6Am methylation of U2 small nuclear RNA (snRNA) and in splicing. Note also that Caenorhabditis DAMT-1 proved biochemically intractable and could only be genetically linked to DNA adenine methylation, leaving open the possibility that its effects on adenine methylation in nuclear DNA could be due to either promiscuous activity, or salvage of RNA nucleotides (Figure 4A).

RNA methyltransferases contribute to nuclear 6mA levels, mostly indirectly

As m6A methylation of mRNA is co-transcriptional, the proximity of RNA methyltransferases to the transcriptional machinery could occasionally lead to erroneous DNA methylation. This model predicts...
Figure 4  DNA adenine methylation in animals. (A) Mitochondrial DNA is enzymatically methylated. Methylation in nuclear DNA may be due to a nuclear METTL4 pool, but stems most likely predominantly from salvage of nucleotides that were originally adenine methylated in RNA context, and made their way into DNA by salvage. (B) The salvage model for nuclear DNA methylation\(^{[32,52]}\) suggests a speculative model for the coordination between maternal RNA degradation and the onset of embryonic transcription at the maternal-to-zygotic transition (MZT). As a consequence of maternal RNA degradation, an increase in nuclear DNA adenine methylation can be expected (but has not yet been clearly demonstrated). The model could be applicable to any animal with a late onset of embryonic transcription. At present, there is only circumstantial evidence for it, and only in the fruit fly. In this organism, nuclear DNA adenine methylation is known to recruit Jumu, which in turn recruits the pioneer transcription factor Zelda\(^{[78]}\). (C) Possible explanation for the report\(^{[56]}\) that low 6mA levels are a negative prognostic factor in cancer from the perspective of the salvage model. According to this model, low 6mA levels should correlate with low m6A levels, which are favorable for the tumor.

that adenine methylation should be concentrated in genic regions, in agreement with some\(^{[50]}\) but not other\(^{[30]}\) studies on the distribution of 6mA in the nuclear genome of higher eukaryotes. As many RNA adenine methyltransferases target single stranded RNA, promiscuous RNA methyltransferase activity is also a good candidate to explain the enrichment of 6mA in regions of stress-induced double helix destabilization (SIDD).\(^{[51]}\)

Adenine methylation in the nuclear DNA of animals and plants could be a side product of the activity of RNA adenine methyltransferases, even if these discriminate strictly between RNA and DNA. When RNAs are degraded, the building blocks can be recycled into both DNA and RNA, by the salvage pathway. The salvage pathway is quantitatively important. In some conditions, it is fully sufficient to satisfy cellular dNTP needs. Both feeding\(^{[33]}\) and isotope labelling studies\(^{[32,52]}\) demonstrate clearly that the enzymes of the salvage pathway tolerate adenine methylation, and at least one DNA polymerase, Pol λ, can incorporate recycled d6mATP. Hence, the abundant presence of m6A in RNA should lead to a contamination of the 2-deoxynucleotide pool with the 2′-deoxy-6-methyladenosine triphosphate. It has long been known that cells sanitize the dNTP pool by hydrolyzing damaged dNTPs.\(^{[53]}\) One of the NUDIX hydrolases involved in this process, MTH1 (MutT homologue 1), which occurs in animals, but not plants, cleaves 2′-deoxy-6-methyladenosine triphosphate ~20-fold more efficiently than 2′-deoxy-adenosine triphosphate,\(^{[54]}\) suggesting that cells attempt to minimize the nuclear 6mA levels from salvage. Despite this scrutiny against methylation carry-over from RNA, the salvage
pathway is the dominant source of 6mA in DNA according to the isotope labeling studies.\textsuperscript{[32,52]} From this perspective, the lack of a clear sequence motif for 6mA in nuclear DNA of animals and plants is no longer surprising. Nucleotide salvage as the main source of nuclear 6mA may also explain seemingly contradictory results on methylation levels, since the activity of the salvage pathway could have varied and was not monitored (Figure 4A).

**ALKBH/TET type dioxygenases demethylate 6mA in nuclear DNA and other substrates**

From a chemical perspective, adenine methylation is directly reversible by a single or two sequential hydroxylation reactions. Mechanistically, the reaction is much better characterized in RNA context. In RNA, the products of single and double hydroxylation of 6-methyladenine, 6-hydroxymethyladenine and 6-formyladenine, are semi-stable,\textsuperscript{[19]} and decay back to adenine on a timescale of hours with loss of formaldehyde and formic acid, respectively. In genomic DNA, only the product of a single hydroxylation round, 6-hydroxymethyladenine, has been reported so far,\textsuperscript{[55]} but it is likely that the chemistry is similar. As the conversion of the oxidized 6mA bases back to adenine is spontaneous, there is no need for a base excision repair step, as required for the reversal of cytosine methylation. The known 6-methyladenine demethylases all belong to the group of 2-oxoglutarate (2OG)-dependent dioxygenases. Somewhat surprisingly, enzymes of both the ALKBH and TET clades of 2OG-dependent dioxygenases have been implicated in the reaction.

Members of the ALKBH clade linked to adenine demethylation in animals are C. elegans NMAD-1 (N-methyladenine demethylase)\textsuperscript{[47]} and the mammalian ALKBH1\textsuperscript{[55,56]} and ALKBH4.\textsuperscript{[46]} ALKBH1 has very low activity on 6mA in a double stranded DNA context and is primarily active on bubbled or bulged DNAs, but not on regular double stranded DNA.\textsuperscript{[57]} ALKBH4 was reported to associate with proteins associated with transcription.\textsuperscript{[58]} Both properties predict that actively transcribed regions should be more effectively pruned of 6mA than non-transcribed regions, and would be consistent with reports that 6mA in ES cells is less scarce in intergenic than genic regions.\textsuperscript{[50]} However, both ALKBH1 and ALKBH4 also have other protein and RNA substrates.\textsuperscript{[61,62]} Recently, it has even been shown that ALKBH5 and FTO (ALKBH9) previously only considered as RNA m6A demethylases, can also act on DNA, at least in vitro.\textsuperscript{[63]} In plants, the ALKBH1 orthologue has been implicated in 6mA demethylation.\textsuperscript{[64]} Taken together, current evidence supports the view that the biology of 6mA demethylation in DNA is dominated by ALKBH1, with possible smaller contributions from several other ALKBH paralogues. All candidate ALKBH 6mA demethylases have other substrates as well. Therefore, phenotypes that result from a deficiency of an ALKBH parologue should not be interpreted in terms of defective reversal of adenine methylation in nuclear DNA alone.

A member of the TET clade linked to adenine demethylation is the D. melanogaster enzyme DMAD (DNA methyladenine demethylase).\textsuperscript{[65]} Activity of the enzyme towards 6mA was surprising, because the TET orthologue from the honeybee, A. mellifera, had previously been shown to have the canonical activity towards 5-methylcytosine\textsuperscript{[66]} that is typical for the very thoroughly studied mammalian orthologues.\textsuperscript{[67]} As the insect enzymes have only been tested on one or the other substrate, it is unclear whether biological roles of insect TETs are clade-specific (diptera vs. hymenoptera) or whether the enzymes have a shared broader specificity.

**6mA in nuclear DNA is broadly distributed and not limited to the ApT context**

In higher eukaryotes, nuclear DNA adenine methylation is not enriched in ApT context. Unrelated and more complicated motifs have recently been suggested based on a hard-to-control machine learning approach.\textsuperscript{[68]} However, these motifs are not conserved between species, and are curiously enriched for cytosine bases in Arabidopsis that has DNA cytosine methylation, but not Drosophila that lacks cytosine methylation, suggesting that unaccounted cytosine methylation could have influenced the analysis. Unlike in basal fungi, ciliates and green algae, 6mA in the nuclear DNA of animals and plants is not concentrated near TSSs.

Mapping studies of nuclear 6mA in animals and plants are exceedingly difficult and fraught with systematic problems that are mostly due to the scarcity of 6mA. Moreover, even consistent findings from early influential studies carried out in independent laboratories have been questioned on methodological grounds. Many of the confounding influences that are now known to affect and distort 6mA mapping results were not appreciated when the original studies were carried out. Even when studies from different laboratories are in agreement, the conclusions may nonetheless be problematic because of common methodological limitations.

In animals, 6mA enrichment has been reported in intergenic, but not gene-coding regions, and in young LINE elements,\textsuperscript{[130]} or in the promoters of young LINE elements in mammalian nuclear DNA.\textsuperscript{[69]} Another study found 6mA enrichment in pericentromeric heterochromatin, and in the coding regions (gene bodies) of actively transcribed genes.\textsuperscript{[56]} Very recently, 6mA was reported to be upregulated during the development of mouse trophoblast stem cells, and to be specifically enriched in SIDD regions and at boundaries between eu- and heterochromatin.\textsuperscript{[51]}

In plants, 6mA is reported to be associated with pericentromeric heterochromatin, but published studies conflict on whether 6mA levels are higher in genic or intergenic regions.\textsuperscript{[50,64,70]} Remarkably, one report found pronounced 6mA peaks at transcription termination sites, but not at transcription start sites.\textsuperscript{[64]} Nearly all the mapping studies can be, and in some cases have been challenged for a range of methodological problems that include issues of antibody specificity and RNA contamination.\textsuperscript{[29,33]} Therefore, even mapping results that are consistent between different studies, but could suffer from the same systematic errors, should be treated with caution.

A non-uniform distribution of 6mA in the nuclear genome, if it indeed exists, is not necessarily at odds with the concept that most 6mA stems from the salvage pathway.\textsuperscript{[32]} It is conceivable, but to our knowl-
edge not experimentally confirmed, that the d6mATP/dATP ratio could rise during S-phase due to preferential consumption of unmodified A in the early S-phase. If so, this could lead to higher 6mA levels in late replicating heterochromatin compared to early replicating euchromatin. Alternatively, or additionally, the preference of ALKBH1 for bulged or bubbled substrates over regular double stranded DNA could cause preferential pruning of 6mA from transcriptionally active regions, which would also cause enrichment in transcriptionally silent regions, in agreement with the majority of the mapping studies.

### Nuclear 6mA is generally too scarce to control transcription in animals and plants

Typical mammalian genes range in length from 1000 to 100,000 bp. Low steady state levels of 6mA in the nuclear DNA of animals and plants (several 10 ppm 6mA/A) imply very low 6mA counts per gene. Assuming a uniform 6mA distribution in the nuclear genome, the scarcity of 6mA implies that long genes (~100 kb) have of the order of one 6mA mark per gene, and that the vast majority of short genes (~1 kb) do not have a single methylated adenine base. Taking into account the preferential accumulation of 6mA in intergenic and repetitive regions of the genome, even lower estimates can also be justified. This scarcity makes it very unlikely that 6mA acts as an epigenetic mark. Reports of negative and positive associations of 6mA in promoters and gene bodies in animals and plants are therefore at best valid on average. Clear-cut transcriptional control by 6mA cannot be expected on a per gene basis, despite concrete mechanistic proposals for such control in special cases. As 6mA is too scarce to have significant influence on transcription, other mechanisms must explain the reported (mild) enrichment of 6mA in heterochromatic and intergenic regions. Among various possible explanations, we favor two speculative models that both rely on the conclusion that most nuclear 6mA is derived from salvage.

In contrast to 6mA in DNA, 6mA in co-transcriptionally methylated RNA is more abundant, and more likely to influence transcription. To better understand this influence, it is useful to distinguish between messenger RNAs, which are only transiently chromosome associated, and the carRNAs that remain persistently chromosome associated. Adenine methylation in these two types of RNA transcripts has opposite effects on transcription, perhaps because m6A effects on RNA lifetime can only affect transcription directly when the RNAs remain chromatin-associated.

For the mRNAs, it has been shown that adenine methylation deposited by the RNA methyltransferase METTL3/14 attracts the histone demethylase KDM3B. This enzyme then demethylates H3K9me2 and thus removes a repressive histone mark. This mechanism may be considered as a positive feed-back loop that helps to maintain transcription. For the carRNAs, which remain chromatin-associated, the effect of RNA methylation on lifetime may be dominant. carRNAs, which are transcribed from promoters and enhancers, are expected to be positively correlated with transcription, and to be destabilized by adenine methylation. The two effects may explain the observed negative effect of carRNA adenine methylation on transcription. Curiously, reported genomic distributions for m6A in carRNAs and 6mA in nuclear DNA are similar. The most parsimonious explanation for this co-occurrence is contamination of the 6mA signal from the more abundant m6A in carRNAs. Of course, it also remains possible that 6mA in nuclear DNA cooperates with the more abundant m6A in carRNAs. However, any such model would have to explain how 6mA is targeted to the “correct” loci, even though most 6mA stems from salvage.

### A 6mA surge may link cytoplasmic RNA degradation with the onset of zygotic transcription

The salvage model predicts that 6mA levels in nuclear DNA should peak when bulk RNA degradation takes place. Organisms with a late onset of embryonic transcription, such as the fruit fly or zebrafish, rely on maternal RNAs for the first phase of their development. At the maternal-to-zygotic transition (MZT), maternal RNAs are rapidly degraded in part under the control of m6A as an RNA degradation signal. Both the higher adenine m6A level, and the increased RNA turnover, predict a surge in 6mA levels in nuclear DNA according to the salvage model.

In the fruit fly, 6mA levels have not been monitored with sufficient time-resolution to test the prediction of a spike at the MZT, at about 1.5 h post-fertilization. However, it is known that 6mA levels are much higher before the MZT (at 0.75 h, when some maternal RNAs are already degraded) than after the MZT (at 2.0 h and several later time points, when the maternal RNAs are already gone), and that levels of the 6mA demethylase DMAD are very low at the MZT, and only raise well after the MZT. The 6mA in the nuclear genome is sensed by the protein Jumu which then cooperates with the pioneer transcription factor Zelda to initiate transcription from the zygotic genome. Zelda is responsible for the transcription of many early transcribed genes, and also activates components of the machinery for degradation of maternally expressed transcripts. Together, the data suggest that nuclear 6mA may contribute to the coordination between maternal RNA degradation and the onset of zygotic transcription at the MZT. Although nuclear 6mA is more abundant at the MZT than at other time points, it is nevertheless still scarce, and therefore the general doubts about nuclear 6mA as an epigenetic mark in animals apply too, albeit to a lesser extent. Therefore, it is likely that a surge of 6mA levels at the MZT contributes, but is not sufficient to initiate embryonic transcription. This could be tested by artificially raising nuclear 6mA levels before the MZT and monitoring transcription, but to our knowledge this experiment has not yet been carried out.

In zebrafish, an initial report suggested high nuclear 6mA levels throughout early development. A later reanalysis attributed high 6mA levels at least in part to chorion adherent contaminating bacteria, and suggested instead a sharp peak of 6mA levels (~100 ppm) at the 64 cell stage. The 64 cell stage is reached at approximately 2 h post fertilization, considerably before the representative 3.5 h time point for the MZT. Conflicting reports on the timing of RNA degradation before...
the MZT (summarized in[77]) make it difficult to judge whether the timing of the 6mA peak is consistent with the salvage model, but it remains an intriguing possibility. It is currently unknown whether the transient rise in nuclear 6mA levels contributes to the activation of the zygotic genome in zebrafish, as has been suggested for Drosophila.

6mA levels may have prognostic value in cancer

It is now clear that adenine methylation in RNA has many links with cancer.[81,82] A common explanation is that m6A promotes RNA degradation,[75] which in turn affects the transcriptome. A frequent point of view is that 6mA facilitates transcriptome remodeling,[83] and therefore the resolution of naïve pluripotency towards differentiation.[84,85] This conceptually attractive, but over-simplified point of view suggests three predictions: low 6mA levels should be favorable for the tumor, mRNA adenine methyltransferases should act as tumor suppressors, and RNA demethylases should be oncogenes. Little is known about the correctness of the first prediction. The second prediction is true in some, and false in other cases. RNA adenine methyltransferases have been ascribed anti-oncogenic and oncogenic properties, depending on enzyme and malignancy (for a review of the complex literature, see[81]). The third prediction agrees very well with the experimental evidence. An oncogenic role for m6A demethylases has been reported in acute myelogenic leukemia,[86,87] cervical cancer,[88] lung adenocarcinoma,[89,90] glioblastoma[91] and squamous cell carcinoma.[92] Confusingly, however, both the RNA methyltransferases and their demethylase counter-players have been described as oncogenes in some malignancies (such as AML) suggesting transcript-specific effects.[81] Irrespective of these complexities, it is clear that perturbations of adenine methylation in RNA play an important role in cancer.

At least two recent publications have suggested that 6mA levels in nuclear DNA may have prognostic value in cancer.[56,93] The very scarce 6mA bases are unlikely to play any causal role in the development of malignancies. Instead, it is more plausible that the 6mA levels are informative, because they correlate with m6A levels (Figure 4C). Correlations could be erroneously inferred because of imperfect discrimination between very rare DNA and much more abundant RNA adenine methylation. Alternatively, they could be a genuine, and a consequence of salvage of RNA bases. The extent of genuine correlation between m6A and 6mA levels remains to be investigated. A report on decreased nuclear 6mA levels in response to FTO overexpression supports the idea that 6mA levels can indeed be informative about the activity of RNA adenine methyltransferases and demethylases.[31] According to the concept that low 6mA levels favor the tumor, low nuclear 6mA levels should be associated with more aggressive malignancy, and a poorer prognosis, as indeed reported in one study of gastric and liver cancer.[56] Confusingly, however, the other study found high, not low 6mA levels in glioblastoma.[93] The glioblastoma study also suggested that ALKBH1 depletion reduced tumor proliferation and extended the life-span of tumor bearing mice.[93] and attributed this effect to altered transcriptional regulation of hypoxia-related genes.[93] If the latter explanation was confirmed, it would indicate that nuclear adenine methylation, like nuclear cytosine methylation may be subject to therapeutic manipulation. However, since ALKBH1 also targets mitochondrial and cytoplasmic tRNA, the effect is more readily explained by the expected strong reduction of mitochondrial translation and reduced activity of the respiratory complex.[62]

COMPARISON WITH CYTOSINE METHYLATION

In this review, we make the case that nuclear DNA adenine methylation is very different in basal fungi, ciliates, and algae on the one hand, and animals and plants on the other hand. Both distinct adenine methylation systems have similarities with cytosine methylation, at different levels. For the comparison, it is useful to briefly recall key features of cytosine methylation in animals and plants.

The “reference” case of nuclear cytosine methylation

Cytosine methylation in animals and plants is enzymatic, largely limited to CpG context, enzymatically maintained by a combination of de novo and maintenance methyltransferases, and abundant enough to affect transcription.[94,95] Functionally, cytosine methylation is associated negatively with transcription in promoters, and positively in gene bodies. Mechanistically, both effects can be understood as transcriptional repression, since positive transcriptional effects of gene body methylation on transcription from the promoter of a gene can be attributed to suppression of competing transcription from ectopic start sites.[96] From an evolutionary point of view, the transcription repressing effects of cytosine methylation are likely related to the stabilizing effect on the double stranded form of DNA, which should make it harder to initiate transcription. However, the biophysical effects are too weak to explain the clear-cut modern-day outcomes of cytosine methylation. Steric hindrance of transcription factor binding, sometimes cited as a reason for repressive effects, can play a role in special cases. However, it is unlikely to explain the general negative association, since many transcription factors are indifferent to cytosine methylation, and since the proportions of transcription factors that respond positively and negatively to cytosine methylation are similar.[97] Instead, repressive effects of cytosine methylation are more likely due to the recruitment and assembly of repressive nucleoprotein complexes[98] by 5mC reader proteins,[99] and the cross-talk of cytosine methylation with other chromatin marks.[100] Whether cytosine methylation affects DNA affinity to nucleosomes and nucleosome packing is controversial,[101] but reports of DNA methylation attracting histones[102,103] and increasing nucleosome compaction and rigidity[104] are consistent with a repressive role. Transcription helps to establish histone marks of silent and active chromatin, which in turn attract and repel DNA methyltransferases, respectively.[105–107] Taken together, the data for cytosine
methylation thus indicate that cytosine methylation and transcription mutually influence each other, to bring about the overall negative association.

**Nuclear adenine methylation in basal fungi, ciliates and green algae**

In basal fungi, ciliates and green algae, the enzymology of adenine methylation is strikingly similar to the enzymology of cytosine methylation in animals. Selective methylation in a specific, palindromic dinucleotide context makes it possible to methylate a daughter strand under parental strand instruction, by a dedicated maintenance methyltransferase. As in cytosine methylation, adenine methylation controls transcription mostly from regions proximal to TSSs. There is a notable difference however. While cytosine methylation influences transcription primarily from the promoter, that is, a region upstream of the TSS, adenine methylation appears to be placed primarily in the region immediately downstream of the TSS in basal fungi and ciliates, and downstream and upstream in green algae. In contrast to cytosine methylation, adenine methylation in basal fungi, ciliates and green algae has a transcription activating effect. As in the case of cytosine methylation, this may be a result of evolution building on intrinsic biophysical tendencies. As adenine methylation destabilizes the double stranded form of DNA, transcriptional activation is the evolutionarily expected outcome. However, the modern-day correlation of 6mA with transcription is far too strong to be due to biophysical effects of adenine methylation on dsDNA stability alone. So far, it is primarily attributed to a repellence of nucleosomes by 6mA. It remains to be seen whether DNA adenine methylation in basal fungi, ciliates and green algae is also read by a set of dedicated 6mA reader proteins, and whether these proteins recruit transcription-promoting factors. As the methylation is mostly downstream of the TSS, such hypothetical factors would be more likely involved in facilitating the acceleration of RNA polymerase, than in transcriptional initiation itself.

**Nuclear adenine methylation in animals and plants**

In animals and plants, the enzymology of nuclear adenine methylation is very different from the reference enzymology of cytosine methylation. Most adenine methylation in nuclear DNA stems from nucleotide salvage, possibly with an additional contribution from METTL4, now primarily considered as a mitochondrial DNA methyltransferase, and possibly also from promiscuous RNA methyltransferases. Despite these enzymatic contributions, there is no evidence for parental strand instructed daughter strand methylation. Nuclear 6mA in animals and plants is far too scarce and too uniformly distributed to serve as a general epigenetic mark. To act as an epigenetic mark even in special cases, 6mA would have to be read by dedicated reader proteins. Based on computational analysis, RAMA, HARE-HTH and PUA-superfamily proteins have been suggested as potential 6mA readers, but biochemical support for specific binding of 6mA is scarce for the eukaryotic members of these groups. Experimentally, histone deubiquitinases ASXL1 and MPND have been identified as 6mA sensors, but their mode of action is unusual, because they are proteolytically degraded upon 6mA binding. So far, the fruit fly protein Jumu is the most clear-cut case of a reader of 6mA in nuclear DNA. However, if the protein senses a general rise of 6mA at the MZT, as suggested in this review, the presence of this protein also does not support a role of 6mA as a locus specific mark. Together, the lack of dedicated methylation machinery for nuclear DNA, and the lack or scarcity of “typical” reader proteins that could mediate transcriptional outcomes speak against 6mA as genomic mark. By contrast, there is much support for the view that nuclear 6mA is a price that cells pay for the adenine methylation in RNA and mitochondrial DNA. It seems that animal and plant cells try to minimize this price, by removing d6mATP from the dNTP pool, and by converting nuclear 6mA back to A by the dioxygenase activity of ALKBH1 and possibly other ALKBH paralogues. From a biophysical point of view, it is not surprising that cells, especially those with large nuclear genomes, may want to limit 6mA levels. In naked DNA, without the confounding influence of nucleosomes, 6mA in the template strand causes (slight) transcriptional stalling. Moreover, template strand 6mA is also detrimental for DNA replication judging from its effect on model polymerases and some eukaryotic DNA polymerases.

**CONCLUSION**

The 6mA in nuclear DNA of basal fungi, ciliates and green algae clearly acts as an epigenetic mark. Therefore, concepts from the well-established field of cytosine methylation are useful as a framework for studies of 6mA in these organisms, despite opposite transcriptional outcomes, that likely reflect opposite biophysical effects. By contrast, the widespread view of animal and plant nuclear 6mA as an epigenetic mark may be misleading. In these organisms, the nuclear 6mA may be better viewed as an inevitable consequence of adenine methylation of RNA. If this view is correct, then future studies of 6mA in animal and plant nuclear DNA would be better guided by DNA repair concepts than by epigenetics concepts.

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CONFLICT OF INTEREST
The author declares no conflict of interest.

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