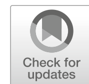

# N6-methyladenine: A Rare and Dynamic DNA Mark

8

Zach Klapholz O'Brown and Eric Lieberman Greer

**Abstract**

Chromatin, consisting of deoxyribonucleic acid (DNA) wrapped around histone proteins, facilitates DNA compaction and allows identical DNA code to confer many different cellular phenotypes. This biological versatility is accomplished in large part by post-translational modifications to histones and chemical modifications to DNA. These modifications direct the cellular machinery to expand or compact specific chromatin regions and mark certain regions of the DNA as important for cellular functions. While each of the four bases that make up DNA can be modified (Iyer et al., Prog Mol Biol Transl Sci. 101:25–104, 2011), this chapter will focus on methylation of the 6th position on adenines (6mA). 6mA is a prevalent modification in unicellular organisms and until recently was thought to be restricted to them. A flurry of conflicting studies have proposed that 6mA either does not exist, is present at low levels, or is present at relatively high levels and regulates complex processes in different multicellular eukaryotes. Here, we will briefly describe the history of 6mA, examine its evolutionary conservation, and evaluate the current methods for detecting 6mA. We will discuss the proteins that have been reported to bind and regulate 6mA and examine the known and potential functions of this modification in eukaryotes. Finally, we will close with a discussion of the ongoing debate about whether 6mA exists as a directed DNA modification in multicellular eukaryotes.

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**Keywords**

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## 8.1 Introduction

DNA must faithfully transmit the blueprints of life from generation to generation. However, it is also necessary that different cell types have access to different portions of the genome, and that specific cell types can respond appropriately to changes in the environment. Such dynamic responses are mediated in part by transcription factor complexes, and by chemical modifications to chromatin. DNA is not as heavily modified as RNA, which has over 170 different modifications identified to date (Frye et al. 2018). The limited number of DNA modifications (relative to RNA) is presumably evolutionarily selected to protect

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the DNA code from mutations, and to enable the formation of a stable double helix. Nevertheless, several DNA modifications occur in different species distributed across the tree of life and are important as both signals of DNA lesions and as epigenetic regulators of diverse biological processes. Importantly, DNA modifications increase the repertoire of cellular phenotypes that can be encoded by a single DNA sequence, without directly altering the integrity of the genetic code. Soon after DNA was discovered, variants of each base were identified. However, the role of DNA methylation in the context of normal biological processes and disease pathogenesis remains an active area of study.

Although 6mA was discovered in 1955 (Dunn and Smith 1955, 1958) soon after cytosine methylation (5mC) which was confirmed in 1950 (Johnson and Coghill 1925; Hotchkiss 1948; Wyatt 1950), 6mA was thought to exist predominantly in prokaryotes and was therefore not given the same amount of research attention in eukaryotes as 5mC. The discovery that 6mA exists in more recently evolved eukaryotes has revived interest in this DNA modification. To understand the dynamic regulation of and by adenine methylation, it is useful to view the role of 6mA across evolution. Here, we aim to provide a broad overview of the historical research on 6mA across the evolutionary spectrum and discuss the mechanisms by which N6-adenine methylation is established, reversed, and recognized. We examine the role of 6mA in biology, discuss the possibility of 6mA playing a functional role in multicellular eukaryotes as well as contradictory evidence regarding its existence, and summarize exciting areas for future research.

## 8.2 Types of DNA Modifications

Each DNA base is modified to varying degrees in different organisms. DNA methylation occurs either as non-enzymatic DNA damaging lesions or as directed modifications with signaling function, which are actively introduced by specific methyltransferase enzymes. DNA lesions include N1-methyladenine (1mA), N3-methyladenine (3mA), N7-methyladenine (7mA), N3-methylcytosine (3mC), N2-methylguanine (2mG), O6-methylguanine (6mG), N7-methylguanine (7mG), N3-methylthymine (3mT), and O4-methylthymine (4mT), while directed methylation includes N6-methyladenine (6mA), N4-methylcytosine (4mC), and C5-methylcytosine (5mC) (Sedgwick et al. 2007; Iyer et al. 2011; Grosjean 2009). Other DNA modifications include deaminated cytosines (Shapiro and Klein 1966; Lindahl and Nyberg 1974), oxidized derivatives of 5mC (5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC)) (Wyatt and Cohen 1952; Privat and Sowers 1996; Shen et al. 2014) and the hypermodified thymine base J (Gommers-Ampt et al. 1993). These modifications are discussed in greater detail in other reviews; we will focus on 6mA, a relatively uncharacterized DNA modification in eukaryotes with potential epigenetic function.

Of the directed DNA methylation events, 5mC is the most extensively studied. 5mC occurs at a higher frequency in more recently evolved organisms and its abundance in the genome ranges from 0.002% to 27% of cytosines, depending on the organism (Fig. 8.1). In mammals and plants, 5mC is the most abundant DNA modification (Iyer et al. 2011), and functions in the regulation of gene expression and maintenance of epigenetic memory (Bird 2002). 5mC in promoter regions typically leads to transcriptional gene silencing and therefore plays important roles in diverse cellular and developmental processes, including X-chromosome inactivation, genomic imprinting, stem cell pluripotency and differentiation (Bird 2002). Other directed DNA methylation events include 4mC and 6mA. 4mC has been identified mainly in thermophilic bacteria and archaea (Janulaitis et al. 1983; Ehrlich et al. 1985, 1987; Grosjean 2009; O'Brown et al. 2019). Until recently, 6mA was also thought to be restricted to bacteria, archaea, and protists. However, its recent identification in several eukaryotes raises the possibility that 6mA serves as an epigenetic

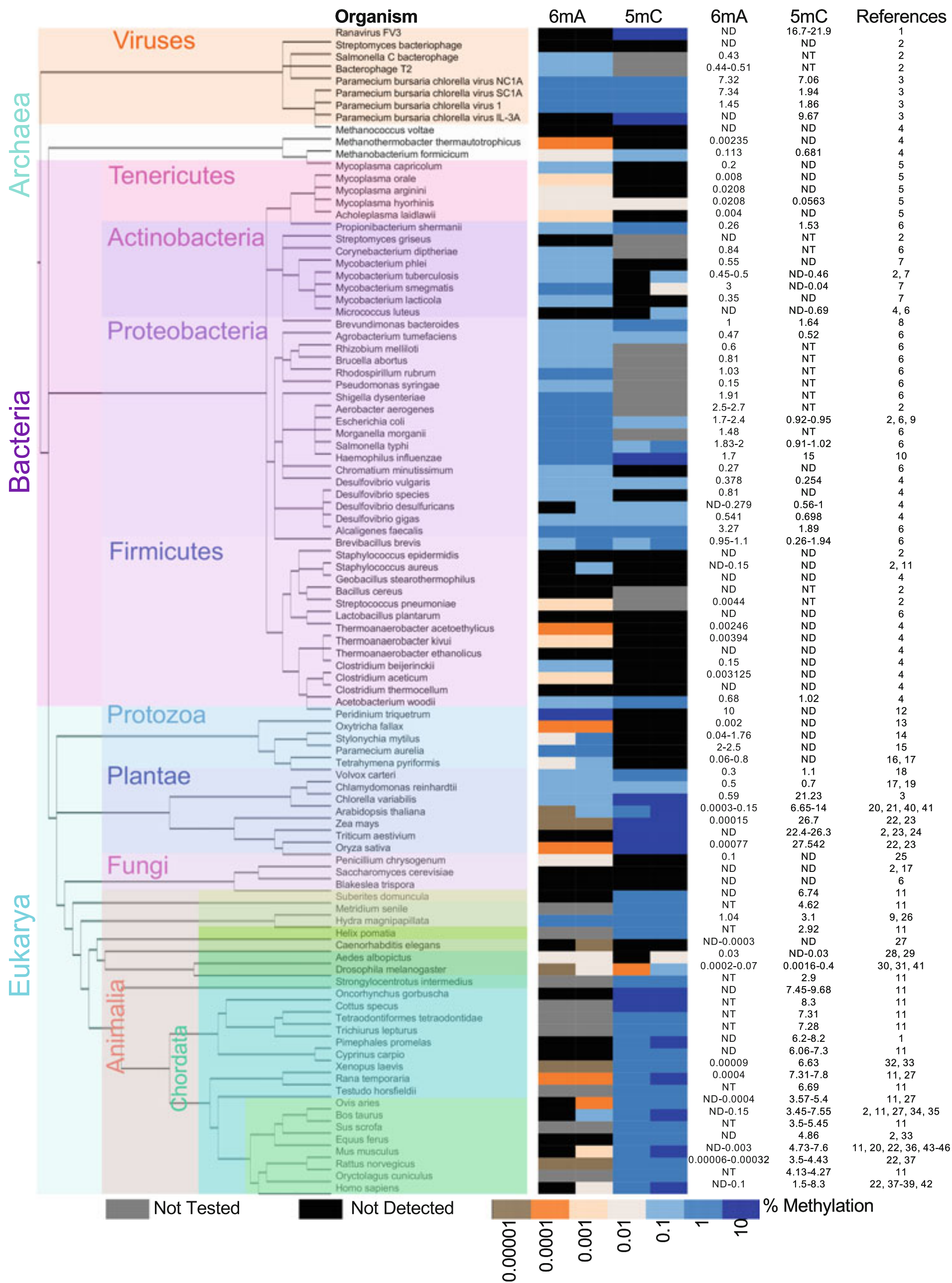


**Fig. 8.1** Abundance of 6mA and 5mC across the tree of life. The relative abundance of 6mA and 5mC are displayed in a heat map. The first column of the heat map displays the percentage of adenines that are N6-methylated (%6mA/A) and the second column displays the percentage of cytosines that are C5-methylated (%5mC/C) for the organism indicated in each row. Blue color represents lower 6mA or 5mC abundance and red color represents higher 6mA or 5mC abundance. Gray color indicates that the methylation mark was not tested in that organism. Dark blue color indicates that the methylation mark was not detected in that organism, and therefore may or may not be present at levels below the limit of detection for the technique used. For some organisms, the level of methylation has been shown to vary across multiple measurements, between different studies or between different cell types within the same organism. In such cases, a range is presented where the left half of the column reflects the lowest detected level (or not detected in some cases) and the right half of the column shows the highest detected level. Methylation values are presented on the right along with citations.

signaling modification within an organism and potentially across generations.

## 8.3 Discovery of 6mA in Various Eukaryotes

DNA N6-methyladenine (6mA) is a widespread modification in prokaryotes. Although 6mA is not necessary for viability in prokaryotes (Marinus and Morris 1973; Russell and Hirata 1989), it plays crucial roles in regulating DNA replication (Campbell and Kleckner 1990; Yamaki et al. 1988), repair (Pukkila et al. 1983), transposition (Roberts et al. 1985), transcription (Wallecha et al. 2002; Robbins-Manke et al. 2005), and cellular defense (Luria and Human 1952; Meselson and Yuan 1968; Linn and Arber 1968; Smith et al. 1972). For reviews on 6mA in prokaryotes, please see (Marinus and Lobner-Olesen 2014; Wion and Casadesus 2006; Murray 2002) and Chapter 2. At the time of 6mA discovery, an unknown base was initially identified in *E. coli* and, using several techniques, this base was compared to synthesized nucleotides to identify 6mA. Hydrolyzed bases were separated by two-dimensional paper chromatography in different solvents, ultraviolet absorption spectrum maximums and minimums were measured, and electrophoretic mobility of this unknown base all confirmed the detection of 6mA (Dunn and Smith 1955, 1958). The existence of 6mA was subsequently confirmed in a variety of different bacterial species (Vanyushin et al. 1968). These initial detection techniques were capable of detecting 6mA at ~0.01% of total adenines (Vanyushin et al. 1970). This detection limit, combined with the confounding presence of commensal symbionts, technical variability, tissue-specific differences, development/stage-specific variability, or subtle environmental effects on 6mA levels initially led to contradictory reports of the identification of 6mA in eukaryotes. Indeed, 6mA was reported by one group to occur in bull and human sperm (Unger and Venner 1966), but other groups were unable to replicate this result or detect 6mA in other metazoa (Dunn and Smith 1958; Vanyushin et al. 1970). 6mA was reported to occur in some unicellular eukaryotes including *Paramecium aurelia* (Cummings et al. 1974), *Stylonychia mytilus* (Ammermann et al. 1981), *Oxytricha fallax* (Rae and Spear 1978), *Chlorella variabilis* (Van Etten et al. 1985), *Tetrahymena pyriformis* (Gorovsky et al. 1973) and *Chlamydomonas reinhardi* (Hattman et al. 1978). Two reports also identified 6mA in multicellular eukaryotes, including the mosquito *Aedes albopictus* (Adams et al. 1979) and the sponge *Suberites domuncula* (Vanyushin et al. 1970). However, the detection

**Fig. 8.1** (continued) The phylogenetic tree was generated using the PhyloT web server (http://phylot.biobyte.de/index.html) and visualized using the Interactive Tree Of Life web server (http://itol.embl.de/). The phylogenetic tree ("rooted" setting) displays the inferred evolutionary relationships between the indicated genera based on their genetic similarity (Letunic and Bork 2011). The tree was created using FigTree v1.4.2. The different organisms are subdivided into different colored boxes to represent different kingdoms and phyla. For some phyla only one organism has been examined. 1: (Willis and Granoff 1980), 2: (Dunn and Smith 1958), 3: (Van Etten et al. 1985), 4: (Ehrlich et al. 1985), 5: (Razin and Razin 1980), 6: (Vanyushin et al. 1968), 7: (Srivastava et al. 1981), 8: (Degnen and Morris 1973), 9: (Yuki et al. 1979), 10: (Drozdz et al. 2012), 11: (Vanyushin et al. 1970), 12: (Rae 1976), 13: (Rae and Spear 1978), 14: (Ammermann et al. 1981), 15: (Cummings et al. 1974), 16: (Gorovsky et al. 1973), 17: (Hattman et al. 1978), 18: (Babinger et al. 2001), 19: (Fu et al. 2015), 20: (Capuano et al. 2014), 21: (Kakutani et al. 1999), 22: (Huang et al. 2015), 23: (Wagner and Capesius 1981), 24: (Montero et al. 1992), 25: (Rogers et al. 1986), 26: (Hassel et al. 2010), 27: (O'Brown et al. 2019), 28: (Adams et al. 1979), 29: (Proffitt et al. 1984), 30: (Zhang et al. 2015), 31: (Lyko et al. 2000), 32: (Koziol et al. 2016), 33: (Jabbari et al. 1997), 34: (Unger and Venner 1966), 35: (Romanov and Vanyushin 1981), 36: (Wu et al. 2016), 37: (Gama-Sosa et al. 1983), 38: (Tawa et al. 1992), 39: (Ehrlich et al. 1982), 40: (Liang et al. 2018), 41: (Kong et al. 2022), 42: (Xie et al. 2018), 43: (Yao et al. 2017), 44: (Douvlataniotis et al. 2020), 45: (Hao et al. 2020), 46: (Schiffers et al. 2017)

of 6mA in mosquitos was not reproduced (Proffitt et al. 1984), and its detection in the sponge was dismissed as potentially coming from symbiotic prokaryotes or algae (Vanyushin et al. 1970). Therefore, until recently, 6mA was thought to be restricted to prokaryotes and unicellular eukaryotes (Casadesus and Low 2006).

With the advent of more sensitive detection techniques (discussed below), 6mA has been identified in multicellular eukaryotes including *Caenorhabditis elegans* and *Drosophila melanogaster* (Greer et al. 2015; Zhang et al. 2015). Several other papers reported low levels of 6mA in more recently evolved eukaryotes, but each of these has caveats that we must acknowledge. 6mA was detected in *Drosophila,* calf thymus, and human placental samples by dot blots (Achwal et al. 1983). 6mA was also detected by immunofluorescence in mouse heart tissues (Sun et al. 2015). Another group identified 6mA in the plants *Oryza sativa* and *Zea mays,* rat tissues, and human cells by high-performance liquid chromatography coupled with mass spectrometry (HPLC-ms/ms) (Huang et al. 2015). Furthermore, 6mA was found by dot blots, HPLC, and methyl DNA immunoprecipitation followed by sequencing (MeDIPseq) in *Xenopus laevis* and mouse kidney (Koziol et al. 2016), and by dot blots, MeDIPseq, HPLC and SMRT-seq in mouse embryonic stem (ES) cells (Wu et al. 2016). A number of studies have also reported 6mA occurring in human cell lines as well as in human tissues (Xiao et al. 2018; Xie et al. 2018; Pacini et al. 2019; Hao et al. 2020). While these papers raise the exciting possibility that 6mA may indeed be present across the tree of life, it is difficult to discount potential contaminating microbiota and to confirm that the detection of 6mA is real when the reported levels of 6mA are at the limit of detection. In fact, several studies have reported that detected 6mA in each of these multicellular eukaryotes is the consequence of artifacts introduced during tissue or genomic DNA (gDNA) sample preparation, or methodological flaws in 6mA detection or mapping techniques (O'Brown et al. 2019; Schiffers et al. 2017; Liu et al. 2017; Douvlataniotis et al. 2020; Musheev et al. 2020; Lentini et al. 2018). RNA m6A (discussed below) could also account for contaminating signal in dot blots and immunofluorescence if not properly removed. It has been proposed that the presence of 6mA in genomic DNA is unlikely because injection of N6-adenine methylated oligos into mice induces a greater immune response than unmethylated oligos, as measured by the production of IL-12 (Tsuchiya et al. 2005). But this does not necessarily confirm that 6mA is a foreign base in mice, as unmethylated CpG motifs also induce a more substantial immune response (Tsuchiya et al. 2005). These results raise the possibility that 6mA is either not present in mammals, or present in sufficiently small quantities to keep it as an immunogenic species in the mammalian repertoire. To confirm the existence of 6mA across eukaryotes, it will be necessary to identify the enzymes that regulate 6mA and specific biological conditions under which the modification changes.

The studies suggesting that 6mA might be a conserved DNA modification raise several fundamental and largely unexplored questions about the evolutionary importance of 6mA across the tree of life. From an evolutionary perspective, why did higher eukaryotes shift from 6mA (the most pervasive DNA modification in prokaryotes), toward using 5mC as the more dominant DNA modification? To what extent are the ancient functions of 6mA and its modifying enzymes conserved from prokaryotes to more recent eukaryotes?

In contrast to DNA adenine methylation, RNA adenine methylation (m6A) has long been recognized as the most abundant post-transcriptional modification of prokaryotic and eukaryotic mRNAs (Niu et al. 2013). In humans, there are over 18,000 m6A sites representing approximately 7,000 unique mRNA transcripts (Jia et al. 2011; Meyer et al. 2012; Dominissini et al. 2012). Furthermore, m6A is enriched in 3'UTRs in highly conserved regions (Meyer et al. 2012; Dominissini et al. 2012; Deng et al. 2015), suggesting a shared function for m6A in evolutionarily distant species. N6-methyladenosine regulates multiple aspects of RNA metabolism, including mRNA stability/

decay, translation, splicing, and localization (Wang et al. 2014, 2015; Zhou et al. 2015; Niu et al. 2013), and participates in diverse cellular and biological processes including meiosis and embryonic stem cell differentiation (Yue et al. 2015; Batista et al. 2014; Hongay and Orr-Weaver 2011; Bodi et al. 2012). The prevalence of RNA m6A raises the possibility that DNA adenine methylation could be a consequence of methylated adenines in RNA recycled via the nucleotide salvage pathway. Another possibility is that DNA adenine methylation is catalyzed by RNA methyltransferases, either as an off-target effect of these enzymes or as a biologically regulated process. Unlike the better-characterized RNA m6A, relatively little is known about the functional importance of DNA 6mA in metazoan genomes, and whether 6mA plays a similarly conserved role in the dynamic regulation of biological processes. The effects that RNA m6A have on RNA structure and function might provide clues to the roles of N6-adenine methylation on DNA.

## 8.4 Abundance of 6mA

The relative genomic abundance of 6mA can provide clues to its biological function across evolutionarily distinct organisms. 6mA and 5mC appear to have a large range of abundance in the genomes of different organisms across evolution (Gommers-Ampt and Borst 1995). 5mC is undetectable in many bacterial species, as well as the genome of *S. cerevisiae,* and ranges from 0.0016% of cytosines in *D. melanogaster* to as high as 10% in some mammals and 30% in certain plant species (Gommers-Ampt and Borst 1995; Capuano et al. 2014; Wagner and Capesius 1981). If we accept that published literature documenting the presence of 6mA in different organisms is in fact detecting 6mA in the reported organism (rather than in contaminating symbionts or technical artifacts), the genomic abundance of 6mA varies by several orders of magnitude across the tree of life as well (Fig. 8.1). Generally, organisms with higher levels of 6mA such as bacteria and single-celled eukaryotes tend to have lower levels of 5mC, while organisms with higher levels of 5mC such as plants and mammals tend to have lower levels of 6mA. The detected level of 6mA ranges from ~0.0001 to 0.0003% of adenines in plants and mammals to as high as 3% of adenines in some species of bacteria, and up to 10% of adenines in the dinoflagellate *Peridinium triquetrum* (Rae 1976). Early studies of nucleic acid composition in the 1950s examined the base composition of DNA in different strains of bacteria using 2D paper chromatography (Dunn and Smith 1958). It was found that 6mA comprised 1.75% of all adenines in *E. coli* and 2.5% of adenines in *Aerobacter aerogenes* (Dunn and Smith 1958). Subsequent studies examined the content of 6mA in the DNA of unicellular eukaryotes, such as the ciliate *Tetrahymena pyriformis* (0.65–0.8% of adenines) (Gorovsky et al. 1973), *Paramecium aurelia* (2.5%) (Cummings et al. 1974), and *Stylonychia mytilus* (0.176%) (Ammermann et al. 1981). The level of 6mA in these unicellular eukaryotes is comparable to the 6mA abundance in many species of bacteria. Interestingly *Tetrahymena* and *Stylonychia mytilus* have 4–13 fold lower 6mA levels in their micronucleus than their macronucleus (Gorovsky et al. 1973; Ammermann et al. 1981), suggesting that this modification plays an important role in determining the differences between the two nuclei in these species, which are separated by ~1159 million years of evolution (Parfrey et al. 2011).

6mA was initially identified in the DNA of *C. elegans,* using both antibody-based approaches and antibody-independent methods of quantitation, including single molecule real time (SMRT) sequencing and ultra-high performance liquid chromatography followed by mass spectrometry (UHPLC-ms/ms) (Greer et al. 2015). Based on the UHPLC-ms/ms data, the levels of 6mA ranged from 0.013% to 0.39% of adenines. However, more recent measurements in *C. elegans* have quantified that 6mA is either undetectable or only occurs at 0.0003% of adenines (O'Brown et al. 2019). The initial higher quantifications appear to be due to artifacts introduced because of the presence of bacteria in the guts of *C. elegans*, exogenous methylated

adenines introduced to the samples by recombinant bacterial enzymes used to digest gDNA samples prior to UHPLC-ms/ms analysis, as well as by limitations of 6mA sequencing techniques (O'Brown et al. 2019).

6mA abundance was quantified in plants, rat tissues, and human cells using HPLC-ms/ms (Huang et al. 2015). These data must be viewed with caution, as there was no independent validation that the 6mA modification was occurring in the reported organisms, rather than contaminating symbionts. In that study, the abundance of 6mA in plant and mammalian genomes ranged from 0.00008% of adenines in rat lung DNA to as high as 0.0007% of adenines in plant DNA. The human cell lines had 0.0017% and 0.0023% 6mA (in Jurkat and 293T cells, respectively). Another group identified 6mA in 0.00009% of adenines in *Xenopus laevis* by HPLC and MeDIPseq (Koziol et al. 2016). More recently 6mA was identified in mouse ES cells at 0.0006–0.0007% (or 6–7 parts per million) of adenines (Wu et al. 2016). However, each of these quantifications has been called into question by conflicting reports which have questioned whether the modification exists at all in mammals (Schiffers et al. 2017; Douvlataniotis et al. 2020). The large range of reported 6mA levels in mammals, either not occurring (Schiffers et al. 2017; Douvlataniotis et al. 2020), occurring at the lower range of around 0.1–1 part per million bases (Huang et al. 2015) to the higher range of ~400 parts per million in mitochondrial DNA (Hao et al. 2020) or even as high as ~1000 parts per million in human glioblastoma derived stem cells (Xie et al. 2018), suggests that these differences are not biological but rather methodological. It will be important in future studies to ensure that when making direct comparisons culturing conditions as well as the methods used for detecting and quantifying 6mA are comparable. In summary, these findings suggest that if 6mA occurs in plants and mammalian genomes it is ~1,000–40,000-fold lower than in some bacteria and single-celled eukaryotes. The large degree of variability in 6mA abundance between eukaryotes motivates further exploration into the environmental factors and evolutionary pressures that led to a decline in 6mA levels and an increase in 5mC levels during eukaryotic evolution. These differences could also indicate that at very low 6mA levels, 6mA is at the limit of detection. Therefore, quantitative differences between different samples could be attributed to technical errors, rather than true biological variability. Moreover, these modifications are typically detected under basal conditions. It is possible that 6mA levels are dramatically altered under specific environmental conditions. Finally, we should note that even if a relatively rare percentage of adenines are methylated, the presence of a single methylated adenine at a critical genomic location could have dramatic phenotypic consequences by affecting the binding of specific regulatory proteins (see cell cycle regulation below).

## 8.5 Methods of Detecting 6mA

Detection of DNA methylation has evolved over the years to become increasingly sensitive and accurate. Detecting different DNA modifications started with a technique of combining the cytosine fraction with picric acid to form crystalline picrate. After purification by crystallization, salt crystals were compared to synthetic pyrimidines of known structure. By this method, the authors reported the identification of 5mC in *Mycobacterium tuberculosis* in 1925 (Johnson and Coghill 1925). Detection techniques shifted to paper chromatography (Hotchkiss 1948), which had a limit of detection of 1%, and was used to compare synthetically generated 5mC to the content of 5mC in animal, plant, viral, and bacterial DNA (Wyatt 1950). By the time, 6mA was first identified in 1955, its presence was confirmed by a combination of ultraviolet absorption spectrum (Mason 1954), electrophoretic mobility, and its paper chromatographic movement in different solvents (Dunn and Smith 1955). Because these early methods were relatively insensitive, the presence of 6mA in a number of animal species was undetectable. Researchers quickly realized that they could take advantage of restriction enzymes to identify methylated residues (Bird and Southern 1978; Geier and Modrich 1979). A

limitation of this approach is that detection of methylation sites is dependent on the methylated residue occurring in the appropriate restriction enzyme target motif, and whether the restriction enzyme preferentially recognizes un-, hemi-, or fully-methylated substrates. Therefore, not all sequence contexts can be addressed with this method.

High-performance liquid chromatography was subsequently used to determine that *E. coli* has 1.4% 6mA (Yuki et al. 1979). Liquid chromatography has become increasingly sensitive and, recently, ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-ms/ms) has been used to detect concentrations of 6mA in the order of 0.00001% (Huang et al. 2015). However, UHPLC-ms/ms and other quantitative techniques cannot discriminate from which species the genomic DNA originates. This can cause problems if the gDNA is contaminated with microbiota or other species which could have substantially higher levels of 6mA than the species being queried. If the levels of 6mA are low, the contaminating prokaryotic DNA could cause an artificially elevated signal. Additionally, the enzymes used to digest DNA for UHPLC-ms/ms could be contaminated with methylated DNA from their recombinant production and therefore add abundant 6mA into the sample, which must be avoided if possible or subtracted from final concentrations when quantifying 6mA levels (O'Brown et al. 2019; Boulias and Greer 2021; Douvlataniotis et al. 2020). An alternative technique, called capillary electrophoresis and laser-induced fluorescence (CE-LIF), uses the fluorescent dye boron-dipyrromethene (BODIPY), to specifically bind to 6mA, followed by capillary electrophoresis combined with laser-induced fluorescence to detect 6mA levels (Krais et al. 2010). This technique has a lower limit of detection of 0.01% 6mA and was used to confirm the presence of 6mA in Bacteriophage λ, *E. coli,* and to identify 6mA's presence in *Hydra magnipapillata* (1.04% of adenines) (Krais et al. 2010). At this limit of detection, the authors could not detect 6mA in calf thymus or human kidney samples.

While the aforementioned techniques have proven useful for detecting whether 6mA is present in a particular organism, they do not provide information on the genomic location of this modification. To determine the genomic locations of 6mA, several methylation-sensitive sequencing techniques have been developed. Methylated DNA immunoprecipitation (MeDIP) coupled with microarray analysis (Weber et al. 2005) has evolved into MeDIP sequencing (MeDIP-seq) (Pomraning et al. 2009). MeDIP-seq has been optimized by a combination of photo-crosslinking, exonuclease digestion, and restriction enzyme digestion to achieve near single-nucleotide resolution of 6mA (Chen et al. 2015; Fu et al. 2015). MeDIP-seq, however, is dependent on the antibody specifically recognizing 6mA. While the most commonly used 6mA antibody displays a greater than 1000-fold affinity for methylated adenines relative to unmethylated adenines (Greer et al. 2015), if 6mA is rare, as is the case in most multicellular eukaryotes, non-specific binding can still confound analyses. Sequencing methods have an inherent error rate which can be further exacerbated by the non-specific binding of IgG to unmodified repetitive DNA sequences (Lentini et al. 2018; Douvlataniotis et al. 2020). Alternative techniques have also been developed to identify where throughout the genome 6mA occurs. One such technique consists of radioactive methylation of DNA followed by restriction digest, electrophoresis, and sequencing (Posfai and Szybalski 1988). Single-molecule real-time sequencing (SMRT-seq) is a next-generation sequencing technique that provides accurate sequence reads and measures the kinetic rate of nucleotide incorporation during sequencing (Flusberg et al. 2010). Since different DNA modifications result in different kinetic signatures, SMRT-seq can identify every DNA modification at single-base resolution. This technology, however, does have troubles distinguishing several closely related modifications from each other, including 1mA from 6mA. While SMRT-seq provides an antibody-independent manner of detecting, at nucleoside resolution, every different DNA

modification which produces a unique kinetic signature, this method requires high sequence depth and loses accuracy when 6mA levels are lower than 10 parts per million (Mondo et al. 2017; Ye et al. 2017; O'Brown et al. 2019; Douvlataniotis et al. 2020; Zhu et al. 2018). The earliest SMRT-seq analyses were performed using mapping algorithms that were designed for bacterial species where 6mA occurs at high abundance in specific motifs (Zhang et al. 2018), it is important to confirm with higher sequencing depth that detected methylated bases are not false positives (Zhu et al. 2018; O'Brown et al. 2019; Kong et al. 2022). Oxford Nanopore sequencing is an alternative long read sequencing technology that reads out disruption of ionic current as a DNA molecule passes through a nanopore present in a lipid bilayer (Bayley 2015). This sequencing method has been used to examine 6mA (McIntyre et al. 2019; Shah et al. 2019) but is subject to many of the same limitations as SMRT-seq. One of the most promising new 6mA sequencing technologies, nitrite sequencing, uses sodium nitrite under acidic conditions to selectively deaminate unmethylated adenines while not affecting N6-methylated adenines. This deamination converts unmethylated adenines to hypoxanthines, which pairs with cytosine rather than thymine. Therefore when sodium nitrite treated DNA is subjected to polymerase chain reactions all unmethylated adenines are converted to guanines during sequencing (Mahdavi-Amiri et al. 2020). While it still remains to be determined what the limit of detection of nitrite sequencing is, and whether it can accurately detect 6mA at lower concentrations than 10 parts per million, this chemical-based sequencing method will be a powerful tool for accurate mapping of 6mA in genomic DNA. Methylated residues can be confirmed by restriction digest coupled with real-time RT PCR to determine the methylation at a specific locus (Fu et al. 2015). Alternatively, sequence-specific probes have been developed that can selectively bind to 6mA or unmodified adenines in specific sequence contexts (Dohno et al. 2010).

To convincingly identify rare modifications, such as 6mA, a combination of multiple complimentary techniques is ideal since each technique has its own set of limitations (Table 8.1). UHPLC-ms/ms can be complemented by restriction enzyme digestion confirmation (as long as 6mA occurs in the appropriate motif), dot blots and MeDIP with a 6mA-specific antibody, and SMRT-seq. For a complementary discussion of the methods for detection of 5mC see chapter 16.

## 8.6 6mA Regulating Enzymes

### 8.6.1 DNA Methyltransferases

An important step in the confirmation of 6mA as a regulated mark of biological significance has been the identification of enzymes that deposit and remove this mark. It was previously thought that methylated adenines were incorporated premade into genomic DNA. This assumption likely hampered initial efforts to identify 6mA in eukaryotes. A study in the early 1970s concluded that 6mA did not exist in eukaryotes, because radioactively labeled adenines, but not methylated adenines were incorporated into DNA when added exogenously (Vanyushin et al. 1970). However, several groups demonstrated that DNA could be glycosylated and RNA could be methylated at the N6 position of adenines after incorporation into polynucleotides, rather than pre-methylated nucleotides being incorporated during the biosynthesis of polynucleotide (Kornberg et al. 1959; Kornberg et al. 1961; Fleissner and Borek 1962). These findings led to the hypothesis that methylation occurs after DNA synthesis (Theil and Zamenhof 1963), rather than on unincorporated nucleotides, and spurred attempts to identify the DNA methylating enzymes. The first biochemical studies aiming to identify DNA methyltransferases were conducted in *E. coli* by fractionation of total protein lysates followed by methylation assays with each fraction. Early studies identified a single fraction that methylated DNA at the C5 position of cytosines and the N6 position of adenines, but this fraction was only efficient at methylating foreign DNA (Gold et al.

**Table 8.1** Recent methods for detecting and quantifying 6mA

| Detection/ Quantitation Method | Description/Limitations | 6mA Specificity | Sensitivity(lower limit of detection) | Genomic sequence information | Reference(s) |
|---|---|---|---|---|---|
| 6mA-sensitive restriction enzymes | Restriction endonuclease cleavage of methylated motifs.<br>Cannot detect 6mA outside of restriction recognition sites. | High (enzyme-dependent) | Can identify single methylated adenine so long as it occurs within specific recognition motifs (e.g., GATC) Preference for hemimethylated or dually methylated depending on the enzyme | None normally<br>Can be used, in combination with real-time RT PCR, to validate sites identified by sequencing methods | Bird and Southern (1978), Geier and Modrich (1979) |
| 6mA Dot Blotting | Antibody-dependent semi-quantitative detection of 6mA levels in genomic DNA samples. If samples have contamination with other nucleosides can be mistaken for 6mA | High (antibody-dependent)<br>If DNA is not single-stranded 6mA antibodies generally recognize 1mA as well | Moderate (can not distinguish lowly methylated samples from each other) | None | Achwal et al. (1983) |
| Immunofluorescence | Antibody-dependent method for detecting 6mA in whole animals or tissues at cell-level resolution.<br>Very difficult to validate that signal is coming from 6mA rather than background (ideally need to manipulate the 6mA regulating enzymes) | Moderate (antibody-dependent). Can recognize RNA or RNA:DNA or 1mA<br>Necessary to eliminate all RNA and RNA:DNA hybrids by treatment with RNAses | Moderate.<br>Immunofluorescence is not good for assessing relative changes in 6mA. (antibody-dependent) | None<br>Could be used in combination with DNA probes to confirm 6mA localization in specific genomic regions | Greer et al. (2015), Sun et al. (2015), Liu et al. (2016b) |
| MeDIP-seq | Antibody-dependent method for identifying genomic regions harboring 6mA. Typical problems of antibody pull downs including IgG affinity for non-methylation regions | Moderate (antibody-dependent) | Moderate (antibody- and organism-dependent) | Genome-wide 6mA localization at near base pair resolution | Pomraning et al. (2009), Chen et al. (2015), Lentini et al. (2018) |

| SMRT-seq | Long-read sequencing, kinetics of sequencing is altered when bases are methylated. Provides base modifications at single-nucleotide resolution 6mA and 1mA are indistinguishable and quite expensive. | Single nucleotide (but 1mA and 6mA are indistinguishable) | Can identify single methylated adenine with sufficient coverage (if 6mA is > 10 ppm) | Single base resolution 6mA detection genome-wide | Flusberg et al. (2010), O'Brown et al. (2019) |
|---|---|---|---|---|---|
| UHPLC-MS/MS | Chemical separation and detection by mass spectrometry. Mycoplasma and digestion enzymes mixes can contaminate samples with methylated bases and cause artificially high readings | Highest | High (As low as 0.0001% 6mA/A) | None | Yuki et al. (1979), Zhang et al. (2015), Fu et al. (2015), Greer et al. (2015), Huang W et al. (2015), O'Brown et al. (2019) |
| 6mA-specific probes | DNA probe containing a formyl group on the O6 position of a G base discriminates between adenine and 6mA via formation of an interstrand cross-link (ICL). 6mA can not form ICL. ICLs detected by PAGE or HPLC | Single site. Untested for other modifications (such as 1mA) | Detection by electrophoresis (6mA has no ICLs and will be single-stranded on the gel) | Can confirm 6mA in specific genomic locations, not a discovery tool | Dohno et al. (2010) |
| CE-LIF | BODIPY FL EDA binds covalently to the phosphate group of deoxyribonucleotide after activated by carbodiimide reagent. Run by CE-LIF which distinguishes different bases from each other and methylated bases based on migration time. | Can distinguish from 5mC or other bases but untested with 1mA | Moderate (0.01% limit) | None | Krais et al. (2010) |

(continued)

**Table 8.1** (continued)

| Detection/ Quantitation Method | Description/Limitations | 6mA Specificity | Sensitivity(lower limit of detection) | Genomic sequence information | Reference(s) |
|---|---|---|---|---|---|
| Nitrite sequencing | Treatment of DNA with sodium nitrite under acidic conditions selectively deaminates unmethylated As which are read out as guanosines after OCR amplification. This chemical-based conversion should be extremely specific but lower limits of sensitivity have not been tested. | Single nucleotide (it is unclear how sodium nitrite treatment behaves with other methylated adenines) | Undetermined | Single base resolution 6mA detection genome-wide | Mahdavi-Amiri et al. (2020) |

[en]
Recent methods for detecting and quantifying 6mA are summarized in this table. Relative limitations and sensitivity of each method are discussed and references to the primary papers are cited. Abbreviations: *MeDIP-seq* Methylated DNA immunoprecipitation followed by high throughput DNA-sequencing, *SMRT-seq* Single-Molecule Real-Time sequencing, *UHPLC-MS/MS* Ultra-high performance liquid chromatography coupled to tandem mass spectrometry, *BODIPY FL EDA* fluorescent dye 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl ethylene diamine hydrochloride, *CE-LIF* Capillary electrophoresis with laser-induced fluorescence

1963; Gold and Hurwitz 1964). Subsequent studies using increasingly subdivided fractions were able to identify multiple adenine and cytosine methyltransferases in *E. coli* (Nikolskaya et al. 1976; Nikolskaya et al. 1981). However, the identification and characterization of active DNA methyltransferases does not preclude that premethylated RNA or DNA nucleosides could be incorporated through the nucleotide salvage pathway or DNA polymerases. Several groups have demonstrated that administering exogenous premethylated adenines to mammalian cells leads to the incorporation of these N6-adenine methylated bases into the mammalian DNA (Schiffers et al. 2017; Charles et al. 2004; O'Brown et al. 2019; Musheev et al. 2020; Liu et al. 2021). Using exogenous heavy isotopes has revealed that exogenous DNA N6-methyladenine and even RNA N6-methyladenosine can both be incorporated into mammalian DNA (Schiffers et al. 2017; Musheev et al. 2020; Liu et al. 2021).

Additional evidence for the widespread presence and functional importance of 6mA in eukaryotic genomes comes from the observation that members of the MT-A70 family of known or putative N6-adenine methyltransferases exist in most organisms, ranging from bacteria to humans (Luo et al. 2015). Based on structural similarity to other members of the MT-A70 family of methyltransferases, the candidate DNA adenine methyltransferase enzymes in multicellular organisms likely evolved from the bacterial M. MunI-like 6mA methyltransferase, which functions in the host restriction modification system (Iyer et al. 2011). The MT-A70 family includes both RNA and DNA methyltransferases, including IME4 (also called SPO8) in *S. cerevisiae* (Clancy et al. 2002), DAMT-1 in *C. elegans* (Greer et al. 2015), and members of the methyltransferase-like (METTL) family in mammals, including METTL3 (an N6-adenosine RNA methyltransferase) (Liu et al. 2014), and METTL4 (a homolog of DAMT-1) (Greer et al. 2015). Whether the same enzymes catalyze both RNA and DNA adenine methylation in different organisms remains an open question. Notably, biochemical in vitro studies have suggested that the mammalian RNA methyltransferase METTL3 also methylates DNA (Woodcock et al. 2019), suggesting that the same enzymes can be capable of methylating both RNA and DNA in certain contexts, but the substrate specificity (i.e. RNA, DNA or both) for each member of the different MT-A70 family members remains incompletely characterized. Recent research has suggested that METTL4 is present in the mitochondria and is necessary for 6mA (Hao et al. 2020) which is highly enriched on mitochondrial DNA in humans (Koh et al. 2018; Hao et al. 2020). Knock-down of METTL4 caused an increase in the expression of mitochondrial DNA genes and an increase in mtDNA copy number (Hao et al. 2020). It was suggested that these effects were mediated by 6mA repelling the mitochondrial transcription factor TFAM (Hao et al. 2020). METTL4 was shown to be active in vitro against mitochondrial DNA (Hao et al. 2020), raising the possibility that 6mA could be a directed active epigenetic modification. However, METTL4 has also been reported to catalyze $m^6$Am on U2 snRNAs (Goh et al. 2020; Chen et al. 2020; Gu et al. 2020). It will be important for future experiments to determine the physiologically relevant substrate of METTL4. Moreover, several groups have also been unable to detect 6mA in appreciable levels in mitochondrial DNA (Ratel et al. 2006; Chen et al. 2020). It will be important for future experiments to determine whether techniques to isolate mitochondrial DNA could explain differences in detecting 6mA, whether 6mA is only present on mammalian mitochondrial DNA under specific stress conditions, or whether 6mA is absent from mammalian mitochondrial DNA. At the structural level, all of the MT-A70 containing enzymes are characterized by a 7-ß-strand methyltransferase domain at their C-terminus, fused to a predicted alpha-helical domain at their N-terminus and require S-adenosyl-L-methionine (SAM) as a methyl donor (Iyer et al. 2011). The high degree of amino acid sequence conservation among the predicted N6-adenine methyltransferases motivates further exploration into their potential functional conservation.

How adenine methyltransferases of recently evolved eukaryotes recognize their substrates

still remains to be determined. The utilization of adenine methylation by the restriction-modification system suggests that bacterial 6mA methyltransferases evolved to recognize specific sequences for methylation. In bacteria and the unicellular eukaryote *Tetrahymena,* DNA adenine methylation occurs in a palindromic sequence-specific manner in vitro and in vivo (Geier and Modrich 1979; Zelinkova et al. 1990; Bromberg et al. 1982). However, sequence-specific adenine methylation is not observed in all organisms and some bacterial DNA adenine methyltransferases show no sequence specificity (Drozdz et al. 2012). Similarly, 6mA sites in multicellular eukaryotes appear modestly enriched in specific sequence contexts (Greer et al. 2015; O'Brown et al. 2019; Pacini et al. 2019; Wu et al. 2016; Yao et al. 2017; Koh et al. 2018; He et al. 2019; Zhu et al. 2018; Li et al. 2019; Xiao et al. 2018) suggesting that targeted adenines might be selected by more complicated metrics than simply sequence codes. 6mA has been reported to correlate with chromatin boundaries (Li et al. 2020), the histone variant H2A.X (Wu et al. 2016), and various histone modifications (including histone H3 lysine 4 dimethylation (H3K4me2) (Greer et al. 2015), H3K9me3 and H3K27me3 (Xie et al. 2018; Yao et al. 2018)), leading to the supposition that these modifications could communicate with 6mA to help direct 6mA to specific locations beyond a sequence-specific pattern. However, since methods used to map 6mA can be prone to false positives (Lentini et al. 2018; Douvlataniotis et al. 2020; Zhu et al. 2018; O'Brown et al. 2019), accurate mapping and the existence of 6mA in multicellular eukaryotes must be confirmed before conclusions can be drawn on how this modification is localized.

### 8.6.2 Mechanism of 6mA Methyltransferases

Substantial work in prokaryotes has identified the mechanism of action, the preferred methyl donor, and the kinetics of 6mA methyltransferases. Whether these regulatory principles are conserved in eukaryotes remains to be seen. There was an initial debate as to whether N6 was directly methylated, or if adenines were first methylated on the N1 position and then, following a Dimroth rearrangement, the methyl group would be transferred to the N6 position. However, the enzyme *EcoRI* had been shown to methylate N6 directly rather than through an initial N1 methylation (Pogolotti et al. 1988). This result, combined with the slow rate of Dimroth reactions at physiological pH (Macon and Wolfenden 1968), suggests that N6 is the direct target of methyltransferases. This conclusion has been confirmed by the structures of different adenine-N6 methyltransferases in complex with DNA, showing a direct approximation of the N6 atom toward the methyl-donor (Goedecke et al. 2001; Horton et al. 2005; Horton et al. 2006).

Early reports identifying that DNA was methylated suggested that S-adenosyl-L-methionine (SAM) was the primary methyl donor (Gold et al. 1963), and subsequent work has confirmed that SAM is the predominant methyl donor for not only DNA and RNA methylation, but also for proteins and lipids (Chiang et al. 1996). However, 5,10-methylene tetrahydrofolate has been identified as the methyl donor for tRNAs in *Streptococcus faecalis* and *Bacillus subtilis* (Delk and Rabinowitz 1975; Delk et al. 1976; Urbonavicius et al. 2005). While the enzyme that utilizes 5,10-methylene tetrahydrofolate in *B. subtilis*, *GidA*, is absent in eukaryotes (Urbonavicius et al. 2005), this finding raises the possibility that some DNA methyltransferases might use alternative methyl donors.

Kinetic rates have been measured for the T4 bacteriophage DNA adenine methyltransferase, Dam (Malygin et al. 2000) and the EcoRI adenine methyltransferase (Reich and Mashhoon 1991). For Dam the methylation rate constant ($k_{meth}$) was significantly faster than the overall reaction rate constant ($k_{cat}$) (0.56 and 0.47 $s^{-1}$ vs 0.023 $s^{-1}$), suggesting that product dissociation is the rate-limiting step. Similar, but faster results were observed with EcoRI (Reich and Mashhoon 1991). These enzymes function by binding, flipping out the adenine, methylating, and restacking of the modified base (Allan et al. 1998). Whether

these hold true for M.MunI-like methyltransferases remains to be determined. Reducing the double strand duplex stability did not alter the $k_{meth}$, suggesting that base-flipping is not a rate limiting step in the methylation reaction (Malygin et al. 2000). Additionally, EcoRI enzyme-DNA complexes were less efficient compared to enzyme-SAM complexes, suggesting that the enzyme first binds SAM before methylating its substrates (Reich and Mashhoon 1991). This is opposite to what has been observed with *Dam* and the bacterial 5mC methyltransferase HhaI, where the methyltransferase first binds DNA, followed by SAM (Urig et al. 2002; Wu and Santi 1987), suggesting that the sequence of binding events in the DNA methylation reaction is enzyme-dependent.

An important step for the confirmation of the presence and role of 6mA in more recently evolved eukaryotes will be the identification of genuine 6mA methyltransferases. The conservation of MT-A70 domain-containing proteins in conjunction with the identification of 6mA in many eukaryotes suggests that this modification is conserved. Whether eukaryotic DNA methyltransferases function in a similar manner to prokaryotic methyltransferases remains to be seen. Interestingly, the RNA m6A methyltransferase, METTL3, functions in complex with METTL14 (Liu et al. 2014), raising the possibility that DNA methyltransferase enzymes, like many other chromatin regulating enzymes, function in multi-protein complexes. These multi-protein complexes could help the enzymes achieve their specificity.

## 8.7 DNA Adenine Demethylation

The identification of the enzymes that catalyze the removal of 6mA from DNA strongly suggests that 6mA is a regulated and dynamic epigenetic mark. Examination of the enzymes responsible for the removal of DNA base damage fostered the identification and characterization of the DNA demethylation processes. DNA base damage, in the form of 1mA and 3mC, was shown to be removed by the Fe(II)- and α-ketoglutarate-dependent dioxygenase AlkB in *E. coli* (Trewick et al. 2002). The AlkB family of dealkylating enzymes is highly conserved from bacteria to humans (Fedeles et al. 2015; Wei et al. 1996). AlkB enzymes can demethylate many DNA substrates, including the DNA lesions 1mA, 3mC, and 3mT (Kamat et al. 2011; Chen et al. 2015). Notably, humans have nine AlkB family members (ALKBH1-8 and FTO). Like *E. coli* AlkB enzymes, the mammalian enzymes ALKBH2 and ALKBH3 function in the repair of DNA alkylation damage (Duncan et al. 2002). In addition to their DNA demethylase activity, AlkB members catalyze oxidative demethylation of RNA (Aas et al. 2003). Interestingly, AlkB enzymes in RNA viruses preferentially demethylate RNA substrates, suggesting these AlkBs are necessary for maintaining the integrity of the viral RNA genome (van den Born et al. 2008). More recently, it was found that AlkB family members function in the oxidative demethylation of N6-methyladenosine in RNA, catalyzed by ALKBH5 and FTO in mammals (Jia et al. 2011; Zheng et al. 2013), and that the AlkB family member NMAD-1 in *C. elegans* demethylates 6mA in DNA (Greer et al. 2015), although whether alternative substrates are more physiologically relevant remains to be determined (Wang et al. 2019). FTO was also shown to demethylate 6mA in single-stranded DNA in vitro (Jia et al. 2011), raising the possibility that these enzymes might regulate both DNA and RNA 6mA. ALKBH1 and ALKBH4 have also been proposed to demethylate 6mA (Wu et al. 2016; Xie et al. 2018; Xiao et al. 2018). ALKBH4 demethylates 6mA in in vitro demethylation assays (Kweon et al. 2019) and ALKBH1 was also shown to demethylate 6mA in single-stranded DNA in vitro (Wu et al. 2016). Additionally, ALKBH1 knockout was reported to cause an increase in global 6mA levels in mouse embryonic stem cells and this increase can be rescued by a wildtype, but not a catalytic domain mutant of ALKBH1 (Wu et al. 2016), suggesting that ALKBH1 functions as a 6mA demethylase in mammals. *Alkbh1* knockout leads to embryonic

lethality and significantly more males born than females due to ALKBH1 regulating gene expression during spermatogenesis (Nordstrand et al. 2010). However, in vitro assays with ALKBH1 and a variety of potential substrates reveals that it preferentially demethylates m1A on tRNAs (Liu et al. 2016a) or m5C on tRNAs (Haag et al. 2016), again suggesting that determination of the physiologically relevant substrates of ALKBH1 and ALKBH4 must be determined.

Several studies have begun to dissect the mechanism of action of AlkB demethylases. In the presence of their essential cofactors α-ketoglutarate and Fe(II), AlkB demethylases use molecular oxygen to oxidize the methyl group of 6mA, forming the unstable intermediate 6-hydroxymethyladenine (6hmA), which spontaneously releases its aldehyde group, regenerating the unmodified adenine base (Fig. 8.2) (Fedeles et al. 2015). Whether the same mechanism occurs for the demethylation of 6mA in eukaryotes and if so, whether 6hmA has any additional function remains to be seen. 6hmA was detected in both rat tissues and human cell lines (Xiong et al. 2019). In mammals, FTO was recently shown to oxidize m6A on RNA to N6-hydroxymethyladenosine (hm6A) and N6-formyladenosine (f6A) (Fu et al. 2013). These mRNA derivatives have half-lives of ~3 hours (Fu et al. 2013), suggesting that if 6hmA does have additional functions, they would require a 6hmA-specific binding protein that could stabilize the intermediate. ALKBH1 was shown to generate 6hmA in vitro and ex vivo (Xiong et al. 2019), raising the possibility that this mechanism of 6mA demethylation is conserved. The same oxidation reaction mechanism is used by AlkB enzymes to demethylate 1mA and 3mC during the cellular response to DNA alkylation damage (Falnes et al. 2002; Trewick et al. 2002).

In addition to demethylation of 6mA by the AlkB demethylase family, 6mA can also be converted to hypoxanthine by a 6mA deaminase (Kamat et al. 2011). This modified base can then undergo base excision repair by hypoxanthine DNA glycosylases of the AlkA family (Saparbaev and Laval 1994) (Fig. 8.2). If hypoxanthine is not removed, it can cause a transition mutation (AT pairs would be converted to GC pairs), since hypoxanthine pairs with cytosine instead of thymine. Recently, 6mA was found to be correlated with increased point mutations in *Neisseria meningitidis* (Sater et al. 2015), suggesting that this modified base might be mutagenic, potentially as a consequence of unrepaired 6mA deamination events. However, 6mA deaminases in *Neisseria meningiditis* have not yet been identified. In contrast to 6mA deamination, which is only mutagenic if not removed, 5mC is converted to thymine when deaminated, which leads to a transition mutation in a single step (Lindahl and Nyberg 1974; Heindell et al. 1978). Deamination of adenine, 6mA, or cytosine all leads to non-natural bases, which can easily be identified by specific glycosylases. Deamination of 5mC, on the other hand, leads to thymine which requires a more complicated repair process. This more direct mutational path might explain why 5mC is more prone to mutation than 6mA. This divergence begs the question as to why evolution has selected for a higher prevalence of the more mutagenic DNA modification in more recently evolved species.

In *E. coli,* AlkB expression is induced by DNA damage and the enzyme functions in DNA repair via direct removal of base alkylation damage (Trewick et al. 2002). *AlkB* mutant *E. coli* are sensitized to cell death induced by the alkylating agent methyl methanesulfonate (MMS), and the predicted human ortholog of AlkB is sufficient to partially rescue the MMS-induced cytotoxicity seen in AlkB mutants (Wei et al. 1996). Interestingly, MMS treatment of human skin fibroblasts did not result in the same induction of AlkB seen in *E. coli*, suggesting that the regulation of AlkB expression may have diverged during the evolution of more recent eukaryotes (Wei et al. 1996), or that one of the other 8 AlkB family members in humans has taken on this role or that the induction by different alkylating agents is cell-type specific, and may only occur in certain cell types. In *Penicillium chrysogenum* mutants lacking DNA adenine methyltransferase mutate more readily and display increased sensitivity to DNA damaging agents, suggesting that 6mA could regulate DNA

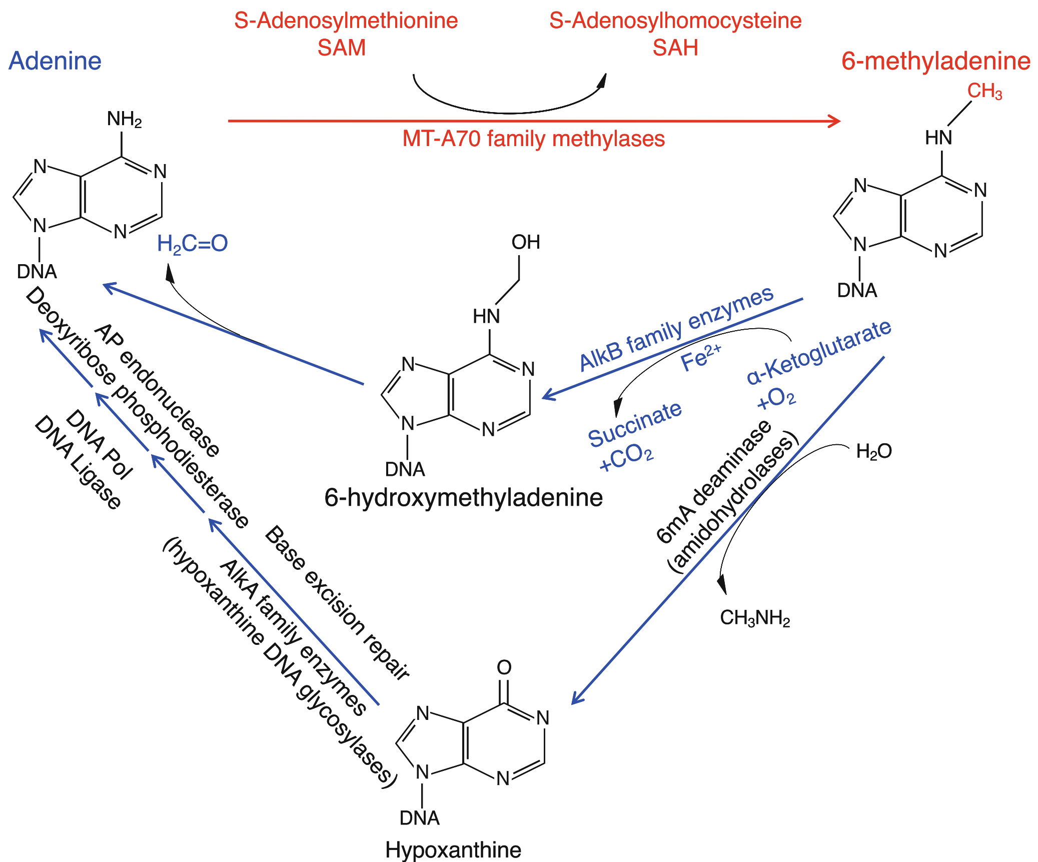


**Fig. 8.2** Mechanisms of N6-adenine methylation and demethylation. MT-A70 family methylases catalyze the methylation of adenine at the sixth position of the purine ring. MT-A70 methylases use S-adenosylmethionine (SAM) as their methyl donor to generate 6-methyladenine and S-adenosylhomocysteine (SAH). Adenine could be regenerated from 6mA by several different enzymatic mechanisms: AlkB family enzymes catalyze the oxidative demethylation of 6mA. AlkB enzymes require α-ketoglutarate and $Fe^{2+}$ and use oxygen to oxidize the methyl group. This oxidative demethylation reaction first generates 6-hydroxymethyladenine, which releases its formaldehyde group to generate adenine. Alternatively, 6mA can be deaminated and subsequently removed via the base excision repair pathway. First, 6mA deaminase hydrolyzes the methylamine to generate hypoxanthine. Hypoxanthine is recognized as a damaged base by AlkA family enzymes, which cleave the glycosyl bond to remove the base. Apurinic (AP) endonuclease cleaves the phosphodiester backbone at the abasic site, exposing the residual 5′ deoxyribose phosphate group, which is then removed by deoxyribose phosphodiesterase. Finally, DNA polymerase I incorporates the unmodified adenine and DNA ligase catalyzes the formation of the phosphodiester bond

damage or DNA repair in fungus as well (Rogers et al. 1986). In *C. elegans,* mutation of the putative 6mA demethylase *nmad-1*, causes increased DNA damage and defective expression of DNA repair genes (Wang et al. 2019), raising the possibility that some aspects of the prokaryotic DNA repair function of 6mA could be conserved in eukaryotes. However, NMAD-1 could function by demethylating other residues; therefore, NMAD-1's physiologically relevant substrates need to be identified before broader conclusions can be drawn about a conservation of 6mA's role in multicellular eukaryotes.

Interestingly, a different family of enzymes, ten-eleven translocation (Tet) proteins, has been shown to demethylate 5mC in many organisms

(Tahiliani et al. 2009; Ito et al. 2010, 2011). Unlike AlkB proteins, whose crystal structures have revealed that the enzymes flip out the base to facilitate demethylation (Yang et al. 2008; Sundheim et al. 2008), crystal structure of the TET enzymes demonstrated that TET catalytic domains are not suitable for accommodating flipped out purines (Aravind et al. 2015), suggesting that they cannot act on 6mA. The TET family has a good phyletic correlation with DNA cytosine methyltransferases, but not with DAMT-1 or other Dam family methylases (Aravind et al. 2015). Additionally in bacteria, there is little evidence that TET-related enzymes are capable of demethylating purines (Aravind et al. 2015). Given these findings, it is surprising that the *D. melanogaster* ortholog of Tet (named DMAD) was reported to function as a 6mA demethylase on DNA (Zhang et al. 2015). Nuclear extracts from DMAD mutant flies showed reduced in vitro demethylation activity compared to nuclear extracts from wild-type flies, while the addition of purified DMAD was sufficient to increase adenine demethylation in these assays (Zhang et al. 2015). It remains to be seen whether this 6mA demethylase activity can be biochemically confirmed using purified DMAD, and whether Tet proteins play a conserved role as 6mA demethylases.

## 8.8 6mA Binding Proteins

Beyond the machinery that catalyzes the addition and removal of 6mA, cells have evolved mechanisms to recognize 6mA as a regulatory signal that can be translated into different biological consequences (see Biological functions of 6mA). We will discuss later in this chapter the direct chemical consequences of adenine methylation, but 6mA can be recognized by specific effector molecules or complexes that alter chromatin architecture and/or transcriptional states. Alternatively, methylation could function by preventing the binding of proteins. Methyladenine-binding proteins have evolved to recognize and transduce 6mA signals into specific biological outcomes. For example, in *E. coli* the MutS enzyme binds to mismatch base pairs as a homodimer, facilitating recruitment of the MutL protein, which binds MutS. The MutS-MutL-DNA complex then loops out until it finds the nearest hemimethylated GATC site, which is bound by the endonuclease MutH. Upon binding of MutL-MutS to the MutH-DNA complex, MutH is activated and nicks the unmethylated daughter strand, allowing helicase and exonucleases to excise the single-stranded mismatch region (Su and Modrich 1986). Thus, hemimethylated GATC sites are used to specifically direct mismatch repair of the daughter strand (Lahue et al. 1987) Similarly, the *oriC* region of *E. coli* is hemimethylated to prevent premature replication before the cell has divided. These hemimethylated adenine sites are recognized and bound by the SeqA protein (Brendler et al. 1995; Slater et al. 1995), which prevents assembly of the DNA replication machinery at this region (von Freiesleben et al. 1994; Wold et al. 1998). The crystal structure for SeqA has revealed why SeqA binds preferentially to hemimethylated over fully methylated DNA (Guarne et al. 2002; Fujikawa et al. 2004), highlighting the importance of determining the crystal structure of 6mA binding proteins for deciphering the chemical and biological consequences of their binding. Several eukaryotic 6mA binding proteins have also been identified. The *D. melanogaster* transcription factor Jumu has a slight preference for binding to N6-adenine methylated DNA and might play a role in regulating the maternal-to-zygotic transition through binding to and transcribing N6-adenine methylated genes (He et al. 2019). The mitochondrial single-stranded DNA binding protein 1 (SSBP1) displays a ~2.5-fold higher affinity for N6-adenine methylated DNA (Koh et al. 2018). To fully understand the potential biological roles of 6mA it will be important to further identify and characterize 6mA binding proteins in eukaryotes.

## 8.9 Biological Functions of 6mA

The direct effects of adenine methylation on the structure of DNA and its roles in prokaryote biology have been well characterized (see also chapter 2). The functional role that 6mA plays in eukaryotes is actively being deciphered. Discussing 6mAs functional effects in prokaryotes raises several interesting potential functions which will need to be further explored in eukaryotes.

### 8.9.1 Effects of Adenine Methylation on DNA Structure

One possible role for adenine methylation, beyond providing a binding site for effector proteins, is to directly alter the overall structure of DNA. An early crystal structure suggested that 6mA might alter the secondary structure of DNA (Sternglanz and Bugg 1973). Adenine methylation is thought to affect DNA double helix formation through altering both base pair stability and base stacking. Ultraviolet photoelectron studies suggested that adenine methylation would lower the ionization potentials and cause the destabilization of valence electrons to increase base stacking in methylated adenines (Peng et al. 1976). This increased base stacking would be offset by a slight destabilization of base pairing ranging from ~0.35 to 0.95 kcal/mol (Engel and von Hippel 1978b). Interestingly, 5mC behaves oppositely to 6mA in these regards. Hence, 5mC causes an increase in helix stability, while adenine methylation destabilizes the DNA, as measured by denaturing gradient gel electrophoresis (Collins and Myers 1987). Moreover, 6mA within GATC sequences causes slight DNA unwinding of 0.5°/methyl group (Cheng et al. 1985), but two-dimensional NMR studies revealed that, in almost all cases, 6mA has only minor effects on the overall helix conformation, as it retains the canonical B-form (Fazakerley et al. 1985; Quignard et al. 1985; Fazakerley et al. 1987). The effects of 6mA on the thermodynamic stability and folding of DNA appear to be sequence-specific (Fazakerley et al. 1987). Indeed, when 6mA occurs directly after a T this can cause a highly altered structure that is overwound and bent (Fazakerley et al. 1989). However, 6mA lowers melting temperatures and slows the rate of helix formation, as demonstrated by the enthalpy of dissociation studies (Quignard et al. 1985; Fazakerley et al. 1985). While 6mA does not dramatically alter helix rigidity (Hagerman and Hagerman 1996; Mills and Hagerman 2004), it can increase DNA curvature to variable degrees, depending on sequence context (Diekmann 1987). These studies suggest that methylated adenines are associated with DNA regions that spend prolonged periods in the open state. These effects were confirmed by cruciform extrusion assays where 5mC inhibits extrusion and 6mA facilitates the initial opening of DNA (Murchie and Lilley 1989). These consequences seem to be in line with the reported effects of 5mC and 6mA on gene transcription; 5mC is generally believed to be a repressor of gene transcription when it occurs at promoters, while 6mA is generally associated with gene activation (Rogers and Rogers 1995; Graham and Larkin 1995; Allamane et al. 2000; Liu et al. 2016b; Zhang et al. 2018; Liang et al. 2018; Shah et al. 2019). However, the correlation between 5mC and gene transcription is dependent on the genomic context in which it occurs. When 5mC occurs within gene bodies, rather than promoters, it is correlated with gene transcription (Reviewed in (Jones 2012)). Similarly, 6mA has also been correlated with repression of gene expression (Zhang et al. 2015; Yao et al. 2018; Lizarraga et al. 2020) as well as repression of transposons (Wu et al. 2016; Yao et al. 2017; Koh et al. 2018; He et al. 2019). Thus, the effects of 6mA on gene transcription may depend on its location in the genome.

### 8.9.2 Restriction-Modification Systems

In prokaryotes, DNA N6-adenine methylation is oftentimes used to discriminate self from foreign DNA, as part of restriction modification systems;

a bacterial immune system by which pathogenic DNA from bacteriophages is recognized by endonucleases that selectively cleave unmethylated DNA at specific restriction sites that are methylated in the host's genome, and thus protected from endonuclease digestion (Low et al. 2001; Iyer et al. 2011). Interestingly, enterobacteriophages appear to have evolved to contain fewer GATCs to avoid the GATC R-M system of their hosts (McClelland 1984). However, GATC methylation is not always involved in the R-M system as discussed in more detail below (Marinus and Lobner-Olesen 2014). This system does not appear to be conserved in eukaryotes that have evolved more complex immune systems. However, 6mA has been suggested to correlate with long interspersed element (LINE) retrotransposons suppression (Wu et al. 2016; Yao et al. 2017; Koh et al. 2018; He et al. 2019; Zhu et al. 2018), raising the possibility that 6mA could recognize and inhibit foreign DNA through an independent mechanism when it is integrated into the host genome. But since the enrichment of 6mA at LINE elements is not always observed (Li et al. 2019; Xiao et al. 2018), further studies using alternative 6mA mapping methods are required to determine whether 6mA could play a role in suppressing foreign DNA in eukaryotes.

### 8.9.3 DNA Damage Control

Early reports indicated that *dam* mutant *E. coli* had higher mutation rates and were more sensitive to UV and mitomycin C, suggesting that 6mA could protect against DNA damage (Marinus and Morris 1974). It was subsequently suggested that 6mA could help to distinguish the parental DNA strand from the mutated daughter strand (Glickman et al. 1978; Glickman 1979). Similarly, *Penicillium chrysogenum* mutants deficient in 6mA had higher sensitivity to mutagenic agents without changes in the number of mutations (Rogers et al. 1986). Additionally, mutation of the putative DNA demethylase, *nmad-1,* in *C. elegans* leads to elevated levels of DNA damage (Wang et al. 2019). However, as stated above, NMAD-1 could regulate DNA damage through the regulation of substrates other than 6mA. Since deletion of *nmad-1* is correlated with defects in the expression of DNA repair genes (Wang et al. 2019), NMAD-1 could also regulate DNA damage repair in eukaryotes through indirect mechanisms.

In *E. coli* and other Gram-negative bacteria, DNA adenine methylation plays an important role in the DNA mismatch repair pathway, a strand-specific repair pathway that relies on the transient post-replicative hemimethylation of DNA. The DNA adenine methylase, Dam, binds selectively to hemimethylated DNA substrates and methylates GATC sites after DNA replication. The delay between DNA synthesis and methylation of the newly synthesized daughter strand is crucial for the fidelity of DNA mismatch repair (Pukkila et al. 1983). When DNA replication errors lead to base pair mismatches, the DNA repair machinery uses adenine methylation to distinguish the already methylated template strand from the newly synthesized unmethylated daughter strand. As described above (6mA binding proteins) hemimethylated DNA allows MutL, MutS, and MutH to identify and specifically cleave the daughter strand, allowing helicase and exonucleases to excise the single-stranded mismatch region. Subsequently, DNA polymerase III re-synthesizes the mismatch region of single-stranded DNA using the methylated parental strand as a template (Pukkila et al. 1983). However, mechanisms of DNA mismatch repair appear to be different in eukaryotes (Fukui 2010).

### 8.9.4 Effect on Transcription

Several studies listed below have suggested that N6-adenine methylation correlates with increased gene expression in different more recently evolved eukaryotes. Whether this is due to the direct effect on relaxing DNA structure (as discussed above), recruitment of 6mA-specific binding proteins, or both, remains unknown. It is still also unclear whether this phenomenon is conserved across all organisms that contain 6mA. While 5mC CpG methylation

had little effect on transcription in barley, 6mA methylation increased transcription two to five-fold (Rogers and Rogers 1995). Similarly, 6mA but not 5mC methylation increased gene expression by 3–50 fold of reporter constructs in tobacco or wheat protoplast, or intact wheat tissues (Graham and Larkin 1995). Luciferase reporter constructs purified from dam+dcm+ bacteria (with 5mC and 6mA methylation) had 2–6 fold increased luciferase production compared to constructs purified from dam-dcm- bacteria in rat or mouse cell lines, or when electroporated into mice (Allamane et al. 2000). Together, these results suggest that 6mA promotes gene expression.

6mA can also directly affect binding of transcription factors. Methylation of a HNF1 binding site reduces HNF1 binding affinity, but this only causes a minor reduction in gene transcription (Tronche et al. 1989; Lichtsteiner and Schibler 1989). Conversely, 6mA increases binding affinity for the transcription factor AGP1 in tobacco (Sugimoto et al. 2003). These results suggest that the effects of adenine methylation on transcription will be sequence- and transcription factor specific. Interestingly, 6mA was shown to reduce the incorporation rate of uridines by inducing a stalling of RNA polymerase II in *S. cerevisiae* in vitro experiments (Wang et al. 2017). This finding suggests that an increase in transcription would have to overcome a physical pausing of the polymerase, however, it is important to perform directed adenine methylation to determine what 6mA's causal effects are on transcription.

Similar to DNA cytosine methylation in metazoa, bacterial DNA adenine methylation regulates gene expression programs, including those related to virulence and phase variation (Low et al. 2001; Wallecha et al. 2002; Zaleski et al. 2005; Sarnacki et al. 2013), suggesting that 6mA levels might be sensitive to changes in environmental conditions. By directed manipulation of the Dam methyltransferase it was shown that 6mA in *Salmonella enterica* predominantly leads to activation of transcription (Sanchez-Romero et al. 2020). Similarly, recent data suggest that 6mA may play a role in transcriptional regulation in the single-celled eukaryote *Chlamydomonas reinhardti,* where 6mA occurs preferentially near actively transcribed genes (Fu et al. 2015). As preliminary evidence that 6mA levels might be relevant to human physiology and disease, it was reported that human patients with type 2 diabetes have reduced levels of m6A on RNA and 6mA on DNA, as measured by HPLC-ms/ms. It was proposed that these differences might be regulated by the cellular fat mass and obesity associated protein (FTO) (Huang et al. 2015), which was shown to function as an RNA m6A and single-stranded DNA 6mA demethylase (Jia et al. 2011) and DNA 3mT demethylase (Gerken et al. 2007). 6mA was found to be significantly enriched in the mitochondria where it was demonstrated that the mitochondrial transcription factor TFAM was repelled by N6-adenine methylated DNA and 6mA suppressed in vitro transcription of mitochondrial DNA (Hao et al. 2020). Future studies will be required to definitively determine whether 6mA exists in human DNA using independent detection methods.

### 8.9.5 Nucleosome Positioning

In the protists *Tetrahymena thermophilia*, *Chlamydomonas reinhardtii,* and *Oxytricha trifallax,* 6mA is preferentially located in the linker regions between nucleosomes (Karrer and VanNuland 2002; Fu et al. 2015; Pratt and Hattman 1983; Beh et al. 2019), raising the possibility that 6mA could help to direct nucleosome positioning. Alternatively, enrichment of 6mA in linker regions may reflect increased accessibility, or recruitment of the methyltransferase at regions of open chromatin. Interestingly, in rice, deletion of the nucleosome remodeler, DDM1, causes a 2.5-fold reduction in 6mA (Zhang et al. 2018). In future studies, it will be interesting to examine whether 6mA directs nucleosome positioning and whether it does so in a conserved manner, or whether other open chromatin modifications can direct N6-adenine methylation at those sites.

### 8.9.6 Cell Cycle Regulation

N6-adenine methylation marks regions for DNA replication initiation in prokaryotes and has been shown to alter the rate of cell cycle progression (see chapter 2). In *E. coli*, the Dam methyltransferase is necessary for precise timing between DNA replication events (Bakker and Smith 1989; Boye and Lobner-Olesen 1990). The hemimethylation of DNA plays an important role in modulating the initiation of DNA replication. The SeqA protein binds to hemimethylated DNA adjacent to the origin of replication *OriC*, preventing its methylation by Dam, and leading to a delay in DNA replication before the cell has divided, which is only initiated from a fully methylated promoter (Low et al. 2001; Lu et al. 1994). When DNA replication is desired, adenine methylation at the oriC region lowers the thermal melting temperature which could facilitate the unwinding at the origin of replication (Yamaki et al. 1988). Interestingly, 6mA also slows the rate of DNA polymerase I catalysis, presumably due to the effects of 6mA on base pairing (discussed above) (Engel and von Hippel 1978a).

In *Caulobacter crescentus,* the cell cycle-regulated DNA adenine methylase (CcrM) controls the timing of DNA replication and progression through the cell cycle (Collier et al. 2007). In contrast to *E. Coli* Dam methylase, which does not have a preference for hemimethylated sites, *C. crescentus* CcrM preferentially methylates hemimethylated DNA after replication (Berdis et al. 1998) and is essential for cell viability (Stephens et al. 1996). In *C. crescentus,* 6mA levels change throughout the cell cycle from fully to hemimethylated as the replication forks progress (Kozdon et al. 2013). The promoter of the replication initiation factor DnaA is preferentially activated when its promoter is fully methylated, leading to DnaA accumulation and progression through the cell cycle (Collier et al. 2007). In *C. elegans* deletion of *nmad-1* causes delayed DNA replication (Wang et al. 2019). Whether this change in DNA replication is due to a misregulation of cell cycle gene expression or through a direct consequence to DNA methylation remains to be seen. Mitochondrial DNA replication in humans could also be regulated by 6mA, as SSBP1, a mitochondrial DNA replication factor, is a 6mA binding protein (Koh et al. 2018). In vitro kinetic experiments with the human DNA polymerase $\eta$ suggest that 6mA directly decreases replication efficiency (Du et al. 2019). To determine a definitive role for 6mA in cell cycle regulation in eukaryotes it will be necessary to perform directed N6-adenine methylation or demethylation and measure the consequences on cell cycle progression.

### 8.9.7 Transgenerational Inheritance

DNA methylation at palindromic sites provides the most parsimonious method by which epigenetic information could be transmitted across generations. Because of the semi-conservative nature of DNA replication, methylation events on the parental strand can be replicated on the newly synthesized daughter strand. In mammals, 5mC methylation patterns are established by the *de novo* methyltransferases Dnmt3a and Dnmt3b during early embryonic development (Okano et al. 1999). Inheritance of cytosine methylation patterns through cell division is mediated by the maintenance methyltransferase Dnmt1 (Bestor et al. 1988). Dnmt1 preferentially binds hemimethylated DNA at the replication fork and copies parental-strand methylation patterns onto the unmethylated daughter strand (Stein et al. 1982; Yoder et al. 1997; Bestor 2000; Bashtrykov and Jeltsch 2018). Whether adenine methylation propagates non-genetic information through cell divisions, or from parents to their offspring remains to be seen. However, there are some hints that 6mA could transmit non-genetic information. Labeling experiments showed that newly synthesized *E. coli* DNA in Okazaki fragments were quickly N6-adenine methylated (Marinus 1976), consistent with the idea that parental methylation patterns might be passed on to their descendants during DNA replication. In some bacteria, DNA adenine methylation is tightly coordinated with cell division (Casadesus and

Low 2006)(see cell cycle regulation above), enabling the inheritance of parental methylation patterns. Thus, a key unanswered question is whether there exists a mode of inheritance of adenine methylation in eukaryotes, or whether different organisms have evolved different mechanisms for the inheritance of parental DNA methylation through somatic nuclear divisions and across generations. In the ciliate *Tetrahymena thermophila* macronucleus, analysis of methylation patterns using methylation-sensitive restriction enzymes showed that both actively replicating and non-replicating DNA contained hemimethylated sites, and that the vegetatively growing macronucleus contained a combination of partially methylated and fully methylated sites (Capowski et al. 1989). These findings are inconsistent with a simple semi-conservative 6mA inheritance mechanism and suggest that inheritance of 6mA in some organisms may rely on hemi-methylation-independent mechanisms of 6mA maintenance through cell division (Capowski et al. 1989).

In *C. elegans*, loss of the histone H3 lysine 4 dimethyl (H3K4me2) demethylase *spr-5* causes a progressive transgenerational loss of fertility (Katz et al. 2009) and a transgenerational extension in lifespan (Greer et al. 2016). This is accompanied by a progressive decline in H3K9me3 and accumulation of H3K4me2 and 6mA (Greer et al. 2014; Greer et al. 2015). Deletion of the putative 6mA demethylase, *nmad-1*, accelerates the progressive fertility decline, while deletion of the putative 6mA methyltransferase, *damt-1*, suppresses the transgenerational H3K4me2 accumulation, fertility, and longevity phenotypes (Greer et al. 2015; Greer et al. 2016), raising the possibility that 6mA might transmit epigenetic information across generations. 6mA also increases transgenerationally in response to electron transport chain stress, and deletion of *damt-1* eliminates the transgenerationally phenotype (Ma et al. 2019). However, the physiologically relevant substrates of NMAD-1 and DAMT-1 must be identified before it can be determined whether these transgenerational phenotypes are truly regulated by 6mA or some other modification. Future studies will be needed to reveal whether 6mA can regulate transgenerational inheritance in multicellular eukaryotes.

Many years of research have shown that chromatin modifications do not occur in isolation, but rather actively communicate with each other. For example, 5mC and H3K9me3 are coordinately regulated in mammals and plants (see chapters 11). The H3K9 methyltransferase binds to 5mC methylated DNA (Jackson et al. 2002; Johnson et al. 2007; Malagnac et al. 2002) and the DNA methyltransferase binds to H3K9me-containing nucleosomes (Du et al. 2012). It is possible that a similar reciprocal cross-talk occurs between 6mA and chromatin modifications. 6mA correlates with chromatin modifications in several eukaryotic species (Li et al. 2020; Wu et al. 2016; Greer et al. 2015; Xie et al. 2018; Yao et al. 2018). In *D. melanogaster,* Dmad binds to Wds, an H3K4 trimethyltransferase complex component, and deletion of Dmad causes a decrease in H3K4me3 (Yao et al. 2018). Future work should reveal whether 6mA methyltransferases can bind to specific methylated histones to direct DNA methylation to particular loci. However, this coordinate cross-talk between 6mA and chromatin modifications is predicated on accurate mapping of 6mA. The correlation between 6mA and chromatin modifications must first be confirmed in eukaryotes by alternative mapping techniques (Lentini et al. 2018; Douvlataniotis et al. 2020; Zhu et al. 2018; O'Brown et al. 2019), before any conclusions about cross-talk between 6mA and other modifications can be drawn.

## 8.10 Conclusions and Future Directions

As detection techniques are becoming increasingly sensitive, 6mA has begun to be convincingly observed in several metazoa. However, several groups have pointed to errors in these methods which could lead to high false positives (Schiffers et al. 2017; Liu et al. 2017; O'Brown et al. 2019; Douvlataniotis et al. 2020; Zhu et al. 2018; Lentini et al. 2018). Due to the relative paucity of 6mA in multicellular eukaryotes, at or near the limit of detection for multiple techniques,

changes in 6mA could help confirm or negate the conserved presence of this modification. 6mA might only occur under specific conditions of stress or in the mitochondria, which could be difficult to detect under basal conditions (Zhang et al. 2018; Ma et al. 2019; Yao et al. 2017; Li et al. 2019). The conservation of active 6mA methyltransferases, demethylases, and binding proteins, coupled with alternative detection techniques could confirm that N6-adenine methylation is a conserved signaling modification. However, it will be important to rigorously examine whether 6mA is present across the tree of life using a combination of rapidly evolving detection techniques (discussed in this review and others that are actively being developed). For metazoa that are confirmed to have 6mA in their DNA, it will be important to define the biological functions of 6mA and its genomic localization patterns in different cell types. A fundamental question is whether the biological functions of 6mA in bacteria are conserved in higher eukaryotes or whether 6mA has evolved new biological functions in these organisms. As 6mA occurs less frequently in more recently evolved organisms, this might reflect a more specialized functional role.

A growing body of work has revealed an important role for m6A on mRNAs in the regulation of gene expression and cellular differentiation in eukaryotes (Peer et al. 2017; He and He 2021; Zaccara et al. 2019). Therefore, another open question is whether N6-adenine methylation of DNA is coordinately regulated with N6-adenine methylation on RNA. Given that substrates of the AlkB family of demethylases and MT-A70 family of methyltransferases can include both RNA and DNA, it will be of interest to better characterize the substrate specificity of these enzymes in different organisms and to examine whether the same enzymes regulate both RNA and DNA N6-adenine methylation in different organisms. Moreover, it will be relevant to find out if in cases of overlapping substrate specificities, whether methylation of DNA or RNA (or both) is the biologically relevant signal under different physiological conditions. Additionally, RNA m6A, or methylated DNA from foreign organisms, could be incorporated into genomic DNA through the nucleotide salvage pathway (Schiffers et al. 2017; Charles et al. 2004; O'Brown et al. 2019; Musheev et al. 2020; Liu et al. 2021). While this indirect incorporation of 6mA into eukaryotic DNA would be less directed, it could still have an effect on biological processes in multicellular eukaryotes.

Given the dynamic nature of 5mC in mammalian development and cell differentiation (Okano et al. 1999; Chen and Zhang 2020) (see chapter 1 + 5), it will be of interest to define the dynamics and potential functions of 6mA during mammalian development, if its presence in mammals can be rigorously confirmed. 6mA has been proposed to change in development in *D. melanogaster, A. thaliana, D. rerio, M. musculus,* and *S. domesticus* (Liu et al. 2016b; Fernandes et al. 2021; Liang et al. 2018; Shah et al. 2019) however, it will be important to confirm that these changes are not due to changes in the relative contribution of foreign DNA with high levels of 6mA (O'Brown et al. 2019; Kong et al. 2022). Future studies should also reveal the environmental factors that regulate the levels of 6mA and its modifying enzymes in eukaryotes, which should provide clues to its evolutionary conservation and biological relevance. The diversity of methods for detection of 6mA in DNA will allow for a comprehensive and detailed examination of 6mA's presence, localization patterns, and potential functions in the genomes of diverse organisms. All in all, the newly developed and more sensitive tools for detection, along with the recent discovery of 6mA in metazoa tentatively open an exciting new chapter of discovery in the field of adenine methylation.

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