N6-Methyladenine: A Conserved and Dynamic DNA Mark

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Abstract

Chromatin, consisting of deoxyribonucleic acid (DNA) wrapped around histone proteins, facilitates DNA compaction and allows identical DNA codes to confer many different cellular phenotypes. This biological versatility is accomplished in large part by posttranslational modifications to histones and chemical modifications to DNA. These modifications direct the cellular machinery to expand or compact specific chromatin regions and mark 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. 2011), this chapter will focus on methylation of the sixth position on adenines (6mA), as this modification has been poorly characterized in recently evolved eukaryotes, but shows promise as a new conserved layer of epigenetic regulation. 6mA was previously thought to be restricted to unicellular organisms, but recent work has revealed its presence in metazoa. 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 enzymes that bind and regulate this mark and finally examine known and potential functions of 6mA in eukaryotes.

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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 141 different modifications identified to date (Machnicka et al. 2013; Grosjean 2015). The limited number of DNA modifications (relative to RNA) is presumably evolutionarily selected for to protect the DNA code from mutations and to enable formation of the double helix. Nevertheless, several DNA modifications occur 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 research.

Although 6mA was discovered soon after cytosine methylation (5mC), it 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, removed, and recognized. We examine 6mA's role in biology, discuss the possibility of 6mA maintaining epigenetic information across cell divisions and potentially across generations, and summarize exciting areas for future research.

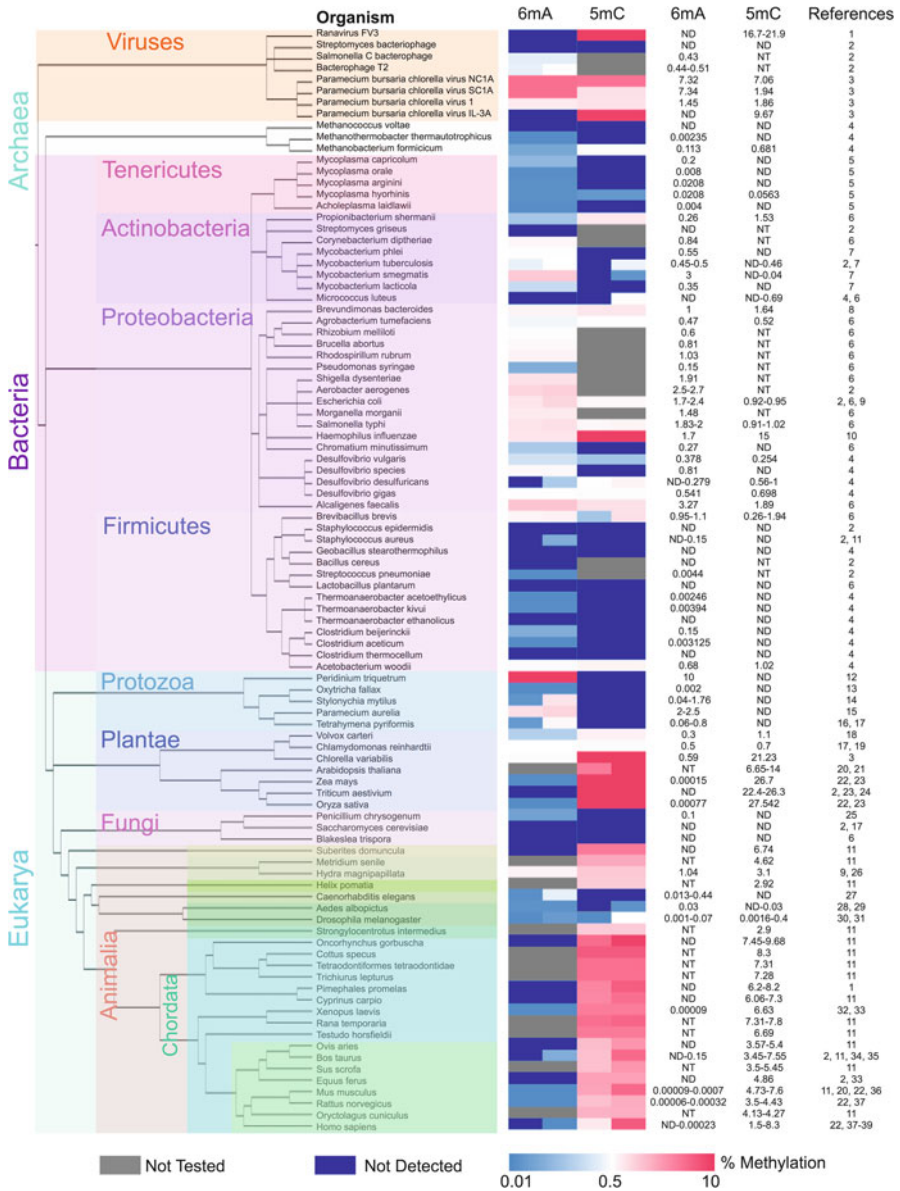# 2   Types of DNA Modifications

Each DNA base is modified to varying degrees in different organisms. DNA methylation occurs either as nonenzymatic 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 (5hmC, 5fC, and 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. 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). 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 signaling modification within these organisms and potentially across generations.

3   Discovery of 6mA Across 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). The discovery of 6mA started with the identification of an unknown base initially in *E. coli*. 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 maxima and minima 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;

Organism	6mA	5mC	6mA	5mC	References
Viruses					
Ranavirus FV3			ND	16.7-21.9	1
Streptomyces bacteriophage			ND	ND	2
Salmonella C bacteriophage			0.43	NT	2
Bacterophage T2			0.44-0.51	NT	2
Paramecium bursaria chlorella virus NC1A			7.32	7.06	3
Paramecium bursaria chlorella virus SC1A			7.34	1.94	3
Paramecium bursaria chlorella virus 1			1.45	1.86	3
Paramecium bursaria chlorella virus IL-3A			ND	9.67	3
Archaea					
Methanococcus voltae			ND	ND	4
Methanothermobacter thermautotrophicus			0.00235	ND	4
Methanobacterium formicicum			0.113	0.681	4
Tenericutes					
Mycoplasma capricolum			0.2	ND	5
Mycoplasma orale			0.008	ND	5
Mycoplasma arginini			0.0208	ND	5
Mycoplasma hyorhinis			0.0208	0.0563	5
Acholeplasma laidlawii			0.004	ND	5
Actinobacteria					
Propionibacterium shermanii			0.26	1.53	6
Streptomyces griseus			ND	NT	2
Corynebacterium diptheriae			0.84	NT	6
Mycobacterium phlei			0.55	ND	7
Mycobacterium tuberculosis			0.45-0.5	ND-0.46	2, 7
Mycobacterium smegmatis			3	ND-0.04	7
Mycobacterium lacticola			0.35	ND	7
Micrococcus luteus			ND	ND-0.69	4, 6
Proteobacteria					
Brevundimonas bacteroides			1	1.64	8
Agrobacterium tumefaciens			0.47	0.52	6
Rhizobium meliloti			0.6	NT	6
Brucella abortus			0.81	NT	6
Rhodospirillum rubrum			1.03	NT	6
Pseudomonas syringae			0.15	NT	6
Shigella dysenteriae			1.91	NT	6
Aerobacter aerogenes			2.5-2.7	NT	6
Escherichia coli			1.7-2.4	0.92-0.95	2, 6, 9
Morganella morganii			1.48	ND	6
Salmonella typhi			1.83-2	0.91-1.02	6
Haemophilus influenzae			1.7	15	10
Chromatium minutissimum			0.27	ND	6
Desulfovibrio vulgaris			0.378	0.254	4
Desulfovibrio species			0.81	ND	4
Desulfovibrio desulfuricans			ND-0.279	0.56-1	4
Desulfovibrio gigas			0.541	0.698	4
Alcaligenes faecalis			3.27	1.89	6
Firmicutes					
Brevibacillus brevis			0.95-1.1	0.26-1.94	6
Staphylococcus epidermidis			ND	ND	2
Staphylococcus aureus			ND-0.15	ND	2, 11
Geobacillus stearothermophilus			ND	ND	4
Bacillus cereus			ND	NT	2
Streptococcus pneumoniae			0.0044	NT	2
Lactobacillus plantarum			ND	ND	6
Thermoanaerobacter acetoethylicus			0.00246	ND	4
Thermoanaerobacter kivui			0.00394	ND	4
Thermoanaerobacter ethanolicus			ND	ND	4
Clostridium beijerinckii			0.15	ND	4
Clostridium aceticum			0.003125	ND	4
Clostridium thermocellum			ND	ND	4
Acetobacterium woodii			0.68	1.02	4
Protozoa					
Peridinium triquetrum			10	ND	12
Oxytricha fallax			0.002	ND	13
Stylonychia mytilus			0.04-1.76	ND	14
Paramecium aurelia			2-2.5	ND	15
Tetrahymena pyriformis			0.06-0.8	ND	16, 17
Plantae					
Volvox carteri			0.3	1.1	18
Chlamydomonas reinhardtii			0.5	0.7	17, 19
Chlorella variabilis			0.59	21.23	3
Arabidopsis thaliana			NT	6.65-14	20, 21
Zea mays			0.00015	26.7	22, 23
Triticum aestivium			ND	22.4-26.3	2, 23, 24
Oryza sativa			0.00077	27.542	22, 23
Fungi					
Penicillium chrysogenum			0.1	ND	25
Saccharomyces cerevisiae			ND	ND	2, 17
Blakeslea trispora			ND	ND	6
Animalia					
Suberites domuncula			ND	6.74	11
Metridium senile			NT	4.62	11
Hydra magnipapillata			1.04	3.1	9, 26
Helix pomatia			NT	2.92	11
Caenorhabditis elegans			0.013-0.44	ND	27
Aedes albopictus			0.03	ND-0.03	28, 29
Drosophila melanogaster			0.001-0.07	0.0016-0.4	30, 31
Strongylocentrotus intermedius			NT	2.9	11
Oncorhynchus gorbuscha			ND	7.45-9.68	11
Cottus species			NT	8.3	11
Tetraodontiformes tetraodontidae			NT	7.31	11
Trichiurus lepturus			NT	7.28	11
Pimephales promelas			ND	6.2-8.2	1
Cyprinus carpio			ND	6.06-7.3	11
Xenopus laevis			0.00009	6.63	32, 33
Rana temporaria			NT	7.31-7.8	11
Testudo horsfieldii			NT	6.69	11
Ovis aries			ND	3.57-5.4	11
Bos taurus			ND-0.15	3.45-7.55	2, 11, 34, 35
Sus scrofa			NT	3.5-5.45	11
Equus ferus			ND	4.86	2, 33
Mus musculus			0.00009-0.0007	4.73-7.6	11, 20, 22, 36
Rattus norvegicus			0.00006-0.00032	3.5-4.43	22, 37
Oryctolagus cuniculus			NT	4.13-4.27	11
Homo sapiens			ND-0.00023	1.5-8.3	22, 37-39

Not Tested   Not Detected   % Methylation

0.01   0.5   10

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 reinhardtii* (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, detection of 6mA in mosquitoes 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 recently been identified in multicellular eukaryotes including *Caenorhabditis elegans* and *Drosophila melanogaster* (Greer et al. 2015b; 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). A recent paper detected 6mA 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

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Fig. 1 Abundance of 6mA and 5mC across the tree of life. The relative abundance of 6mA and 5mC is 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. 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 (Greer et al. 2015b), 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), and 39 (Ehrlich et al. 1982)

human cells by high-performance liquid chromatography coupled with mass spectrometry (HPLC-ms/ms) (Huang et al. 2015). More recently, 6mA was found by dot blots, HPLC, and methyl DNA immunoprecipitation followed by sequencing (MeDIP-seq) in *Xenopus laevis* and mouse kidney (Koziol et al. 2016) and by dot blots, MeDIP-seq, HPLC, and SMRT-seq in mouse embryonic stem (ES) cells (Wu et al. 2016). 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. RNA m6A (discussed below) could also account for contaminating signal in dot blots and immunofluorescence if not properly removed. We must also recognize that the 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 biological conditions under which the modification changes. The recent studies suggest that 6mA might be a conserved DNA modification and 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 posttranscriptional modification of prokaryotic and eukaryotic mRNAs (Niu et al. 2013). In humans, there are over 18,000 m6A sites representing approximately 7000 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 phenotypic consequences of RNA m6A might provide clues to the roles of N6-adenine methylation on DNA.

4   Abundance of 6mA

The relative genomic abundance of 6mA can provide clues to its biological relevance 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 species is in fact detecting 6mA in the reported organism (rather than in contaminating symbionts), the genomic abundance of 6mA varies by several orders of magnitude across the tree of life (Fig. 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).

Recently, 6mA was 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 ultrahigh-performance liquid chromatography followed by mass spectrometry (UHPLC-ms/ms) (Greer et al. 2015b). Based on the UHPLC-ms/ms data, the levels of 6mA ranged from 0.013 to 0.39 % of adenines, representing a 30-fold variation in the global level of adenine methylation between different batches of wild-type *C. elegans*. The observation that 6mA abundance can vary by more than an order of magnitude within an isogenic population of animals is interesting, as it suggests that the levels of 6mA in these organisms might be particularly sensitive to subtle changes in the environment (e.g., stress stimuli).

A recent study quantified the genomic abundance of 6mA 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 293 T cells, respectively). Another group identified 6mA in 0.00009 % of adenines in *Xenopus laevis* by HPLC and MeDIP-seq (Koziol et al. 2016). More recently 6mA was identified in mouse ES cells at 0.0006–0.0007 % of adenines (Wu et al. 2016). These finding suggests that 6mA in plants and mammalian genomes is ~1000-40,000-fold lower in abundance 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 Sect. 9.6 below).

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 the analysis of ultraviolet absorption spectra (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 the 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 unmethylated, hemimethylated, 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, ultrahigh-performance liquid chromatography coupled with mass spectrometry (UHPLC-ms/ms) has been used to detect concentrations of 6mA on the order of 0.00001 % (Huang et al. 2015). 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-cross-linking, 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. 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 which provides accurate sequence reads and measures the rates of nucleotide incorporation and polymerase pause times 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. However, when coupled with UHPLC-ms/ms (which can distinguish 1mA from 6mA), this technique can give rather unambiguous confirmation of both the presence and genomic location of 6mA in a specific organism (Greer et al. 2015b). Methylated residues can be confirmed by restriction digest coupled with quantitative 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 complementary techniques is ideal, since each technique has its own set of limitations (Table 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.

Table 1 Recent methods for detecting and quantifying 6mA

Detection/Quantitation Method	Description/Limitations	6 mA Specificity	Sensitivity (lower limit of detection)	Genomic sequence information	Reference(s)
6 mA-sensitive restriction enzymes	Restriction endonuclease cleavage of methylated motifs. Cannot detect 6 mA 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 hemi-methylated or dually methylated depending on the enzyme	None normally Can be used, in combination with real-time RT PCR, to validate sites identifies by sequencing methods	Bird and Southern (1978), Geier and Modrich (1979)
6mA Dot Blotting	Antibody-dependent semi-quantitative detection of 6 mA levels in genomic DNA samples	High (antibody-dependent) It DNA is not single stranded 6 mA antibodies generally recognize 1 mA as well	Moderate (can not distinguish lowly methylated samples from each other)	None	Achwal et al. (1983)
Immunofluorescence	Antibody-dependent method for detecting 6 mA in whole animals or tissues at cell-level resolution. Very difficult to validate that signal is coming from 6 mA rather than background (ideally need to manipulate the 6 mA regulating enzymes)	High (antibodydependent)	Moderate. Immunofluorescence is not good for assessing relative changes in 6 mA. (antibody-dependent)	None normally Could be used in combination with DNA probes to confirm 6 mA localization in specific genomic regions	Greer et al. (2015 b), Sun et al. (2015)
MeDIP-seq	Antibody-dependent method for identifying genomic regions harboring 6 mA	High (antibody-dependent)	High (antibody- and organism-dependent (C. elegans and D. melanogaster gave very low coverage depth))	Genome-wide 6 mA localization at near base pair resolution	Pomraning et al. (2009), Chen et al. (2015)

SMRT-seq	Provides base modifications at single-nucleotide resolution 6 mA and 1 mA are indistinguishible and quite expensive.	Medium (1 mA and 6 mA are indistinguishible)	Can identify single methylated adenine with sufficient coverage	Single base resolution 6 mA detection genome-wide	Flusberg et al. (2010)
UHPLC-MS/MS	Chemical separation and detection by mass spectrometry.	Highest	High (As low as 0.0001 % 6 mA/A)	None	Yuki et al. (1979), Zhang et al. (2015), Fu et al. (2015), Greer et al. (2015a, b), Huang et al. (2015)
6mA-specific probes	DNA probe containing a formyl group on the O6 position of a G base discriminates between adenine and 6 mA via formation of an interstrand cross-link (ICL). 6 mA can not form ICL. ICLs detected by PAGE or HPLC	Untested for other modifications (such as 1 mA)	Detection by electrophoresis (6 mA has no ICLs and will be single-stranded on the gel)	Can confirm 6 mA 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 1 mA	Moderate (0.01 % limit)	None	Krais et al. (2010)

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* ultrahigh-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

6 Enzymes Introducing 6mA

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 pre-made 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 poly-nucleotides rather than pre-methylated nucleotides being incorporated during the biosynthesis of polynucleotide (Kornberg et al. 1959, 1961; Fleissner and Borek 1962). These findings led to the hypothesis that methylation occurs after DNA syn-thesis (Theil and Zamenhof 1963), rather than on unincorporated nucleotides, and spurred attempts to identify the DNA methylating enzymes. The first studies were conducted in *E. coli* by fractionation of total protein lysates followed by methyl-ation assays with each fraction. Early studies identified a single fraction that methyl-ated 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. 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, 1981).

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 orthology to other members of the MT-A70 family of methyltransferases, the candi-date DNA adenine methyltransferase enzymes in multicellular organisms likely evolved from a 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. 2015b), 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. 2015b). 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 (personal communications C. He), suggesting that the same enzymes are capable of methylat-ing 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. At the structural level, all of these 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 (AdoMet) 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 restriction-modification systems 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 do not show sequence specificity (Drozdz et al. 2012). Similarly, 6mA sites in *C. elegans* only appeared modestly enriched in specific sequence contexts (Greer et al. 2015b), suggesting that targeted adenines might be selected by more complicated metrics than simple sequence codes. It remains to be seen whether other multicellular eukaryotes, which possess 6mA, show a sequence-specific pattern of adenine methylation (similar to bacteria and unicellular eukaryotes) or whether these organisms show little to no sequence specificity in their adenine methylation pattern, as observed for *C. elegans*. It remains to be seen whether methyltransferases that do not recognize specific DNA sequences are recruited to specific locations of the genome by other DNA-binding proteins or other epigenetic chromatin features.

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 endogenous 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, 2006).

Early reports identifying that DNA was methylated suggested that AdoMet was the primary methyl donor (Gold et al. 1963), and future work has shown that this cofactor is the predominant methyl donor for not only DNA and RNA methylation but also for methylation of 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.

DNA methylation rates have been measured for the T4 bacteriophage DNA adenine methyltransferase, T4Dam (Malygin et al. 2000), and the EcoRI adenine methyltransferase (Reich and Mashhoon 1991). For T4Dam 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 this holds true for M.MunI-like methyltransferases remains to be determined. Reducing the double-stranded 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-AdoMet complexes, suggesting that the enzyme first binds to AdoMet before methylating its substrates (Reich and Mashhoon 1991). This is opposite to what has been observed with EcoDam and the bacterial 5mC methyltransferase HhaI, where the methyltransferase first binds to DNA, followed by AdoMet (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 prokaryote 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.

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 (Chen et al. 2015; Kamat et al. 2011). 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 (Marinus and Lobner-Oleson 2014). 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. Whether NMAD-1 can also demethylate m6A on RNA remains to be tested. Recently ALKBH1 was also shown to demethylate 6mA in single-stranded DNA in vitro (Wu et al. 2016). Additionally ALKBH1 knockout causes an increase in global 6mA levels in mouse embryonic stem cells, and this increase can be rescued by a wild-type but not a catalytic domain mutant of ALKBH1 (Wu et al. 2016), suggesting that ALKBH1 functions as a 6mA demethylase in mammals.

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 formaldehyde, regenerating the unmodified adenine base (Fig. 2) (Fedeles et al. 2015). Whether the same mechanism holds true for the demethylation of 6mA in eukaryotes and, if so, whether 6hmA has any additional function remains to be seen. 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 h (Fu et al. 2013), suggesting that if 6hmA does have additional functions, they would require a 6hmA specific binding protein which could stabilize the intermediate. 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. 2). If hypoxanthine is not removed, it can cause a transition mutation (an AT pair would be converted to a GC pair), 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
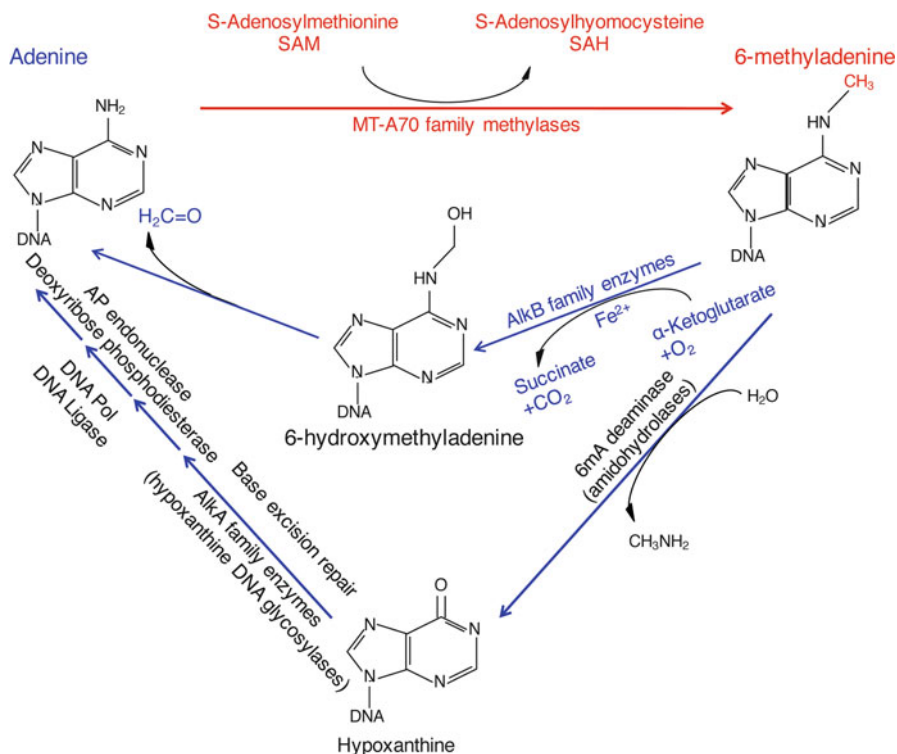
Fig. 2 Mechanisms of N6-adenine methylation and demethylation. MT-A70 family methylases catalyze the methylation of adenine at the sixth position of the purine ring. MT-A70 methylases use S-adenosylmethionine (SAM) as their methyl donor to generate 6-methyladenine and S-adenosylhomocysteine (SAH). Adenine 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

deamination. However, 6mA deaminases in *Neisseria meningitidis* have not yet been identified. Similarly to 6mA deamination, 5mC is converted to thymine when deaminated, which leads to a transition mutation (Lindahl and Nyberg 1974; Heindell et al. 1978). Deamination of adenine, 6mA, or cytosine all leads to nonnatural bases, which can easily be identified by specific glycosylases. Deamination of 5mC, in contrast, 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 the induction by different alkylating agents is cell-type specific and may only occur in certain cell types. In *C. elegans*, deletion of NMAD-1, a member of the AlkB family, causes a global increase in 6mA, and purified NMAD-1 is capable of demethylating N6-adenine methylated oligonucleotides *in vitro* (Greer et al. 2015b). Importantly, mutation of the NMAD-1 catalytic domain abolished the *in vitro* demethylase activity of NMAD-1, identifying NMAD-1 as a 6mA demethylase in *C. elegans* and highlighting the mechanistic conservation of AlkB enzymes from bacteria to metazoa (Greer et al. 2015b).

Interestingly, a different family of enzymes, the so-called 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 structures of the TET family catalytic domains are not suitable for accommodating flipped out purines (Aravind et al. 2015) suggesting that they cannot act on mA. Moreover, the TET family has a good phyletic correlation with DNA cytosine methylases, but not with DAMT-1 or other Dam family methylases (Aravind et al. 2015). Additionally in bacteria, there is little evidence that TET is 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 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 role as 6mA demethylases as well.

8 Proteins Binding 6mA

Beyond the machinery that catalyzes 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 below, Sect. 9). We will discuss later in this chapter the direct chemical consequences of adenine methylation, but 6mA can be recognized by specific effector molecules that alter chromatin architecture and/or transcriptional

states directly, or indirectly, via recruitment of other DNA-binding proteins. Alternatively, methylation could function by preventing binding of chromatin factors. 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 DNA 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 of 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. Thus far, these binding proteins have only been identified in prokaryotes, but an important next step to fully understand the possible biological roles of 6mA will be to identify eukaryotic 6mA-binding proteins.

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. Whether 6mA plays a conserved functional role in eukaryotes remains to be seen, but discussing its functional effects in prokaryotes raises several interesting potential functions which will need to be further explored in eukaryotes.

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. 5mC causes an increase in helix stability, while adenine methylation destabilizes the DNA,

as measured by denaturing gradient gel electrophoresis (Collins and Myers 1987). 6mA within GATC sequences causes slight DNA unwinding of 0.5°/methyl group (Cheng et al. 1985). Consistent with these observations, two-dimensional NMR studies suggest that, in almost all cases, 6mA has only minor effects on helix conformation, as it retains the canonical B-form (Fazakerley et al. 1985, 1987; Quignard et al. 1985). 6mA occurring directly after thymines, on the other hand, causes severe unwinding and bending of the DNA helix relative to the canonical B conformation (Fazakerley et al. 1989). However, 6mA lowers melting temperatures and slows the rate of helix formation, as demonstrated by enthalpy of dissociation studies (Quignard et al. 1985; Fazakerley et al. 1985). 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 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 hypothesized to be an activator. 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)). Thus, the effects of 6mA on gene transcription may depend on its location in the genome as well.

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). 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).

9.2 Restriction-Modification Systems

In prokaryotes, DNA N6-adenine methylation is 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). This system does not appear to be conserved in eukaryotes that have evolved more complex immune systems.

9.3 DNA Damage Control

Early reports suggested 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).

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 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 resynthesizes the mismatch region of single-stranded DNA using the methylated parental strand as a template (Pukkila et al. 1983).

9.4   Effect on Transcription

Several studies have suggested that N6-adenine methylation correlates with increased gene expression. 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 as to 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 fivefold (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 two- to sixfold 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.

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. Similarly, recent data suggest that 6mA may play a role in transcriptional regulation in the single-celled eukaryote *Chlamydomonas reinhardtii*, 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, and 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). Future studies will be required to confirm the existence of 6mA in human DNA using independent detection methods. Despite recent progress in defining the potential functions of 6mA in different organisms, the roles of 6mA in more recently evolved eukaryotes, including its possible roles in human health and disease, remain unknown. Given the high degree of evolutionary conservation of MT-A70 family methyltransferases and Alkb family demethylases, along with the recent discovery of 6mA in eukaryotes, we propose that 6mA is likely to play an important role in the regulation of diverse biological processes in metazoa.

9.5   Nucleosome Positioning

In the protists *Tetrahymena* and *Chlamydomonas*, 6mA is preferentially located in the linker regions between nucleosomes (Karrer and VanNuland 2002; Fu et al. 2015; Pratt and Hattman 1983), 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. Enrichment for 6mA in specific genomic regions was not observed in *C. elegans* (Greer et al. 2015b), but the analysis was performed on mixed tissue samples, which could have obscured any positional bias that may exist in specific cell types. 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.

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. 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 methyl-ated 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, pre-sumably 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 the *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 essen-tial 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). Whether 6mA plays a similar role in controlling the cell cycle in eukaryotes remains to be seen.

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 semiconservative 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 mainte-nance 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; Martin and Zhang 2007). Whether adenine methylation propagates nongenetic information through cell divisions or from parents to their offspring remains to be seen. However, there are some hints that 6mA could transmit nonge-netic 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 methyla-tion is tightly coordinated with cell division Casadesus and Low (2006)) (see Sect. 9.6 above), enabling inheritance of parental methylation patterns. Thus, a key unanswered question is whether there exists a mode of inheritance of adenine meth-ylation in eukaryotes or whether different organisms have evolved different mecha-nisms 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 nonreplicating DNA contained hemimethylated sites and that the vegetatively growing macronucleus contained a combination of partially methylated sites and fully methylated sites (Capowski et al. 1989). These findings are inconsistent with a simple semiconservative 6mA inheritance mechanism and suggest that inheritance of 6mA in some organisms may rely on hemimethylation-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 life span (Greer et al. 2016). This is accompanied by a progressive decline in H3K9me3 and accumulation of H3K4me2 and 6mA (Greer et al. 2014, 2015b). Deletion of the 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. 2015b; Greer et al. 2016), raising the possibility that 6mA might transmit epigenetic information across generations. It remains to be seen whether methylated adenines themselves are transmitted across generations as they are transmitted across cell divisions, or whether 6mA is erased in the germ line and established de novo during somatic development (see Sect. 9.6), or if 6mA is more indirectly involved in these processes. Future studies will also reveal whether 6mA can regulate transgenerational inheritance in other species.

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 plants. 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 H3K4 methylation in *C. elegans*, as described in the previous paragraph (Greer et al. 2015b). It remains to be seen whether this reciprocal cross talk is real and whether other species show a similar co-association between 6mA levels and H3Kme2 levels. Future work should reveal whether 6mA methyltransferases can bind to specific methylated histones to direct DNA methylation to particular loci.

10   Conclusions and Future Directions

As detection techniques are becoming increasingly sensitive, 6mA has begun to be convincingly observed in several metazoa. The conservation of 6mA methyltransferases and demethylases along with the initial detection of 6mA in several metazoa suggests that N6-adenine methylation might be 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). 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 (Niu et al. 2013; Meyer et al. 2012; Dominissini et al. 2012; Deng et al. 2015; Wang et al. 2014, 2015; Zhou et al. 2015; Yue et al. 2015; Batista et al. 2014). 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.

The inheritance of 6mA methylation during bacterial cell division (Wion and Casadesus 2006) raises the question of whether 6mA can be inherited in eukaryotes. Is 6mA passed on through successive generations or erased in the germ line? Recently described paradigms of transgenerational inheritance in *C. elegans* have raised the possibility that 6mA itself might carry epigenetic information across generations (Greer et al. 2015b). Alternatively, 6mA might communicate with other heritable epigenetic marks that reciprocally regulate the levels of 6mA. Future studies should reveal whether 6mA is incompletely erased in the germ line and inherited in subsequent generations. In mice, 5mC is mostly erased by passive demethylation during the expansion of primordial germ cells preceding the formation of gametes (Seisenberger et al. 2012); methylation patterns are then reestablished during early embryonic development by the de novo methyltransferases Dnmt3a and Dnmt3b (Okano et al. 1999). However, some regions of 5mC, such as those near imprinted genes, escape the typical erasure and can therefore carry nongenetic information across generations (Breiling and Lyko 2015). Whether a similar situation exists for 6mA remains to be seen.

Given the dynamic nature of 5mC in mammalian development and cell differentiation (Okano et al. 1999), 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. Future studies should also reveal the environmental factors that regulate the levels of 6mA and its modifying enzymes in metazoa, which should provide clues to its evolutionary conservation and biological relevance. The diversity of methods for detection of 6mA in DNA will allow for 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, open an exciting new chapter of discovery in the field of adenine methylation.

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