The chemistries and consequences of DNA and RNA methylation and demethylation

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ABSTRACT

Chemical modification of nucleobases plays an important role for the control of gene expression on different levels. That includes the modulation of translation by modified rRNA-bases or silencing and reactivation of genes by methylation and demethylation of cytosine in promoter regions. Especially dynamic methylation of adenine and cytosine is essential for cells to adapt to their environment or for the development of complex organisms from a single cell. Errors in the cytosine methylation pattern are associated with most types of cancer and bacteria use methylated nucleobases to resist antibiotics. This Point of View wants to shed light on the known and potential chemistry of DNA and RNA methylation and demethylation. Understanding the chemistry of these processes on a molecular level is the first step towards a deeper knowledge about their regulation and function and will help us to find ways how nucleobase methylation can be manipulated to treat diseases.

Since the discovery of (deoxy)adenosine (dA, A), (deoxy)cytidine (dC, C), (deoxy)guanosine (dG, G), (deoxy)thymidine (dT, T), and uracil (U) in the early 20th century as the information carrying building blocks, which form the basis for RNA and DNA, various modifications of these nucleosides were discovered (Fig. 1). Particularly in transfer-RNA (tRNA) but also in rRNA (rRNA), modified bases are central elements, needed to fine tune the translation of the genetic code. In RNA of bacterial pathogens, many methylated bases are present to block binding of small molecules that work as translation inhibitors, resulting in a resistance against antibiotics such as aminoglycosides. More recently it was discovered that also mRNA (mRNA) contains modified bases. Although it is not yet fully understood what the function of these bases are, it was revealed that the modification chemistry is to some extent reversible. This suggests that the modification and de-modification chemistry has a novel and yet unexplored regulatory function. In this regard N6-methylated adenine (m6A) is the best analyzed modification, but most recently also the reversible formation of N6, C2'-dimethyl adenine (m6Am) was discovered. According to current knowledge, reversible chemistry on modified RNA bases is limited to methyl groups, which are introduced by methyltransferases and removed by demethylases. DNA, in contrast, as the prime carrier of genetic information in the biosphere, is structurally less complex and only few modified bases are known. Most prominent is the methylated base 5-methyl deoxy-cytosine (5mC). Ideas about the potential chemistry of methylations and demethylation are the focus of this review. For other aspects, the following excellent reviews can be consulted. In mammals, 5mC typically reaches global levels between 1 and 5% in genomic DNA. Methylated adenine (6mA), which is the DNA equivalent to m6A in RNA, is another DNA modification that is under intensive investigation at the moment. Whereas 6mA is a well-characterized modification in bacterial DNA, its presence was only recently shown in several higher eukaryotic organisms. In Caenorhabditis elegans, where 5mC is not detectable, 6mA is dynamically regulated and linked to other epigenetic marks and in early embryos of Drosophila melanogaster. 6mA levels are high, but decrease fast during development, resulting in very low 6mA levels in adult tissue. In the unicellular green alga Chlamydomonas reinhardtii, 6mA was discovered in 84% of the genes, where it is mainly located at transcription start sites. Recently, it was reported that mammalian DNA, including human and mouse, also contains 6mA. There, 6mA seems to be distributed across the genome, but absent in gene exons, and 6mA-demethylation in mouse embryonic stem cell (mESC) DNA was shown to correlate with ALKBH1 depletion. These findings question the previous paradigm that DNA modifications in mammalian genome are limited to cytosine residues. However, when our group tried to confirm these results by a novel ultrasensitive UHPLC-MS method, we were not able to detect 6mA in mESC DNA or DNA from mouse tissue, whereas Chlamydomonas DNA, which served as a positive control, delivered the expected positive result. These observations suggest that 6mA might be present at defined time points in mammalian DNA, but is not an epigenetic mark. In the coming years, the question whether 6mA is a relevant modification in mammalian DNA or not will thus certainly be under intensive investigation.

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Chemistry of RNA and DNA base methylation

The addition of the methyl-group to DNA and RNA bases (Fig. 2) is catalyzed by DNA- and RNA-methyltransferases that use S-adenosyl-methionine (SAM) as an active methyl-group donor. While the methyltransferases that methylate RNA bases are now under extensive investigations, the enzymes that catalyze the methylation of dC in DNA are well characterized. In mammalian cells, 3 active DNA-methyltransferases (DNMTs: DNMT1, DNMT3a and DNMT3b) exist. DNMT3a and 3b are de novo DNMTs, which methylate canonical dC bases. In contrast, DNMT1 maintains the methylation status during cell division. DNMT1 operates on hemi-methylated DNA during replication, where the template strand is already methylated, but the newly synthesized strand is lacking methylation. As such, DNMT1 converts the methylation of dC into an inheritable modification that can be transferred during reproduction.

DNMTs and thus cytosine methylation is essential in those multicellular organisms, where it exists. The presence or absence of 5mdC is associated with various important cellular functions, such as transcription control, X-chromosome silencing and genomic imprinting. A global deletion of only one of the 3 DNMTs leads to severe cellular aberrations and is therefore lethal in early embryogenesis (DNMT1 and 3b) or postnatal (DNMT3a). During differentiation the "methylome" is highly dynamic and a celltype-characteristic 5mdC pattern is established during this process. While 5mdC is located to a CpG-dinucleotide context in the majority of somatic cells, non-CpG methylation is also present in embryonic stem cells, many pluripotent progenitor cells and adult brain. However, CpG-methylation is also dominating here. Cytosine-methylation in vertebrates occurs in all types of DNA sequence contexts, including repetitive and regulatory sequences, genes and transposable elements; in contrast to invertebrates, where mostly repetitive sequences are methylated. The majority of cytosines in a CpG-context, depending on the cell type up to 80%, are methylated, leaving so-called CpG islands (CGI) of actively transcribed genes as unmethylated patterns in a CpG-context.
CGIs are regions of high CpG frequency over a length of at least 500 base pairs compared with the bulk genomic DNA and found in 40% of promoter regions in the mammalian genome, with even higher levels (60%) in the human genome. Symmetric methylation of CpG:GpC islands is consequently a hallmark of silenced genes.

The enzymatic mechanism of how methyltransferases methylate DNA and RNA bases is shown in Fig. 2. Centers with a certain nucleophilicity like the amino group of the RNA base A can attack the SAM coenzyme directly leading to immediate methylation. This type of direct methylation is certainly operating for the formation of 6m2A, 4mC or m6Am. SAM as nature’s ‘methyl iodide’ is hence reactive enough to methylate even weak nucleophilic centers such as the exocyclic amino groups of A, which feature, as an sp2-hybridized N-atom only a very weak nucleophilic lone pair at the N-atom. This type of direct methylation creates bases, which possess the methyl group attached to a heteroatom establishing a het-CH3 system. This will be important in the context of active demethylation (vide infra).

In contrast to the formation of het-CH3 connections, methylation of the dC base in DNA at position C5 is far more complex. The C5-center features no nucleophilicity at all, making direct methylation impossible. Nature solves this problem by exploiting a helper nucleophile (R-SH, Fig. 2). The DNMT enzymes attack the dC base first with a nucleophilic thiol in a 1,6 addition reaction. This establishes a nucleophilic enamine substructure (green in Fig. 2), which can subsequently be methylated with the SAM cofactor. Importantly, the helper nucleophile is subsequently eliminated, thereby re-establishing the aromatic system. This more complex enzymatic transformation allows nature to methylate non-nucleophilic carbon atoms to create C-CH3 connectivities which feature a strong and stable C-C single bond.

Chemistry of demethylation

To establish the reversibility needed for switching biochemical processes, nature requires to remove the attached methyl groups. Removal of het-CH3 groups found predominantly in RNA was found to occur with the help of α-ketoglutarate (α-KG) dependent oxidases. These proteins contain a reactive Fe(II) center, which reacts to a strongly oxidizing Fe(IV) = O species with oxygen under concomitant decarboxylation of α-KG to succinate (Fig. 3). The Fe(IV) = O species is able to abstract a H-atom from the het-CH3 group to form a het-stabilized het-CH2• radical, which reacts with the Fe-bound hydroxylradical to form a het-CH2-OH hemiaminal/acetal functionality.

However, these structures are unstable. In water, they decompose in a spontaneous reaction under loss of formaldehyde to give the unmethylated compound. It is interesting that formaldehyde is formed as a byproduct of this reaction because it is typically a rather toxic compound. It needs to be seen how this molecule is detoxified in the context of the demethylation reaction. Particularly well studied is the removal of the N6-methyl group from m6A to revert into the canonical RNA base A. So far 2 α-KG dependent oxidases were found to catalyze the oxidation. One is the fat mass and obesity-associated protein (FTO) protein and the second is ALKBH5. It was shown, that knockdown of FTO led to increased amounts of m6A and in turn overexpression of FTO resulted in decreased m6A levels. Alkbh-deficient mice had a similar effect as FTO knockdown in human cells and resulted in increased m6A levels of the mRNA. The demethylation activity of both proteins is comparable, although ALKBH5 shows direct demethylation, whereas FTO-mediated demethylation is supposed to create hm6A and f6A as intermediates.

In 2009 it was found that also 5mdC is further enzymatically oxidized in a stepwise fashion to give first 5-hydroxymethyldeoxytrosine (5hmC), followed by 5-formyldeoxytrosine (5fdC) and 5-carboxydeoxytrosine (5cadC). “Ten-11 translocation” (TET) enzymes, which are Fe2+/α-KG dependent dioxygenases, were discovered to catalyze this iterative 5mdC oxidation reaction. Regarding the first oxidation step that transforms 5mdC to 5hmC, the Fe2+/α-KG catalyzed reaction generates a stable C-CH2-OH connectivity, which is as a primary alcohol stable in water (Fig. 3). 5hmC is consequently a
stable DNA base modification and it was suggested that the base has indeed epigenetic functions. For example, 5hmCdC constitutes 0.6% of all nucleotides in Purkinje neurons, a special neural cell type of the cerebellum, and 0.032% of all nucleotides in embryonic stem (ES) cells.45,47 The highest 5hmCdC levels in fully differentiated tissues were found in the brain with up to 1% of all cytosines.48,49 Evidence accumulates that 5hmCdC in a given gene is able to accelerate transcription and it is not surprising that 5hmCdC is mainly present in the promoter of actively transcribed genes.50,51

TET enzymes are in this sense required to orchestrate the transcriptional activity of genes. In vertebrates, TET proteins exist in 3 different types (TET1 – TET3) that do not differ regarding their chemistry, but seem to have different spatio-temporal activity. Whereas TET1 is mostly expressed in stem cells, TET3 is upregulated during differentiation and the most abundant TET enzyme in fully differentiated cells.52-54 A global TET3-knockout is lethal in embryogenesis, because it prevents epigenetic reprogramming during differentiation.55 It is interesting, that the presence of 5hmCdC in mammalian DNA was described first already in 1972.56 It took more than 30 y to confirm that 5hmCdC is really present in substantial amounts that are highly depending on the cell and tissue type.57

The further oxidized bases 5fdC and especially 5cadC (Fig. 4) could not be associated yet with distinct cellular functions, but for 5fdC it was reported that it might have regulatory purposes and is also a stable epigenetic mark.58 In accordance with these previous findings, a recently reported single-cell 5fdC-sequencing method called CLEVER-seq revealed that the generation of 5fdC in promoter regions precedes the upregulation of gene expression.59 Despite this faint evidence for epigenetic functions, 5fdC and 5cadC are currently mainly considered to be intermediates on the way of an active DNA demethylation process. DNA demethylation is a crucial process of cell development. Especially during fertilization (paternal part of the genome), early embryogenesis (maternal part of the genome) and the development of germ cells, DNA demethylation takes place in a genome-wide manner, allowing a broad reprogramming of the fertilized oocyte and the cells in the early embryo.60-62 But not only during development, also in fully differentiated cells, it occurs at specific sites of the genome. In brain, for example, locus-specific DNA demethylation and de novo methylation is induced by neural activation, arguing that DNA demethylation is important for normal brain function, including memory formation and learning.63-65 DNA demethylation can take place either actively, which means replication-independent, or passively when DNMT1 does not methylate the nascent DNA strand in hemi-methylated DNA after replication. Passive demethylation occurs, when DNMT1 is absent or blocked during the replication process, which happens for example during early embryogenesis to ensure the demethylation of the maternal genome.65 Interestingly, 6mA demethylation in Drosophila is catalyzed by Drosophila’s TET homolog (DMAD or dTet). DMAD depletion results in higher 6mA levels, but unchanged 5mCdC patterns, and is lethal at pupa stage or shortly after.16 DMAD and TET possess similar catalytic active Cys-rich and DSBH domains, however, 6mA-demethylation activity was not observed yet for mammalian TET enzymes.16

Although oxidation of 5hmCdC to 5fdC and 5cadC creates stable molecules due to the lack of a het-atom in β-position, it is discussed that both could be turned into unstable structures.
upon further chemical manipulation. A chemically attractive mechanism requires that 5fdC and 5cadC are attacked by a helper nucleophile, preferentially a thiol group at the C6 position, in a Michael-type reaction (Fig. 4). Hydratization of 5fdC and tautomerization of the reacted 5fdC and 5cadC allows us to formulate a ‘β-imino-type’ substructure that is prone to deformylation and decarboxylation (red arrows in Fig. 4). Indeed, we could show that reaction of 5fdC and 5cadC with a thiol-nucleophile leads to spontaneous deformylation and decarboxylation showing that the suggested chemistry is feasible. There is currently no evidence that this type of chemistry occurs in vivo but we could show that stem cell lysates feature a decarboxylating activity.68 Interesting is the observation that deformylation and decarboxylation of 5fdC and 5cadC after reaction with a thiol nucleophile leads to a reaction intermediate (boxed in Fig. 2 and 4) that is the key intermediate observed already during methylation of dC to 5mdC by the DNMTs. It is therefore tempting to speculate that DNMT enzymes are involved in the deformylation and decarboxylation maybe followed by immediate re-methylation. Although this reaction sequence would follow chemical logic, it needs to clarified in the near future, if such reactions occur indeed in nature. It was, however, shown that C5-DNA-methyltransferases are indeed able to remove formaldehyde from 5hmC, converting 5hmC directly to dC, therefore supporting these ideas.69

In this context, it is interesting to note that 5hmC and 5fC were also discovered in RNA. In human cells at rRNA position C34, the oxidation of the corresponding RNA base 5mC to 5frC is catalyzed by the Fe²⁺/α-KG dependent enzyme ALKBH1, which is also responsible for m1 A demethylation in mammalian rRNA.70,71 Interestingly, 5hmR was not detected as an intermediate in the ALKBH1-dependent 5mrC oxidation.70 In Drosophila, 5hmR was discovered in polyadenylated RNA and is associated with enhanced mRNA-translation efficiency back to normal level, when 5mR has lowered the efficiency.72 Surprisingly, the oxidation reaction is catalyzed by Drosophila’s TET homolog dTet that is also responsible for 6mA demethylation, but does not oxidize 5mdC.16,72 Moreover, there is evidence that TET enzymes are also responsible for 5mR oxidation,73,74 but at the moment it is not clear whether TET-mediated 5hmR or 5frC formation are stable or rather transient modifications.

In contrast to the chemical mechanism of active demethylation discussed above, strong evidence exists that active demethylation via formation of 5fdC and 5cadC is also linked to base excision repair (BER), which repairs also mismatches caused by deamination of 5hmdC to 5hmdU (Fig. 5). This mechanism includes excision of 5fdC or 5cadC and subsequent activation of BER. The dG/dT mismatch specific thymine DNA glycosylase (TDG) recognizes dG/dT mismatches, but with an even higher activity it excises 5fdC or 5cadC, but not 5mdC and 5hmdC, in vitro.75 This reactivity was not observed for other DNA glycosylases. Evidence that TDG excises 5fdC and 5cadC also in vivo is given by the fact that 5fdC and 5cadC levels are 5–10 times increased in TDG-deficient ES cells compared with the wildtype.76 However, TET/TDG-mediated demethylation is very unlikely to be the only demethylation mechanism. It rather occurs at defined promoter regions in the genome than in a genome-wide manner. First, TDG-activity causes abasic sites.77 If this happened genome-wide, it may impair genomic stability, which is crucial for correct development. Second, TDG knockout starts to be lethal not before embryonic day 12.5 and TDG levels are very low in the zygote, where the paternal genome is demethylated.78,79

Most recently it was suggested that nature may not need to oxidize 5mdC to 5fdC and 5cadC for demethylation and that a third TET-independent pathway has to exist. In the zygote, the most drastic demethylation occurs when 5mdC is globally erased from the paternal part of the genome, while the maternal part is shielded from demethylation. DNA-demethylation of
the paternal pro-nuclei is replication- and TET-independent, since 5hmCd levels increase after 5mCd levels have dropped and global demethylation can be detected in Tet3-deficient zygotites.\textsuperscript{80} It might be that deamination of genomic 5mCd to dT and subsequent dT/dG mismatch repair are the mechanism behind this observation.\textsuperscript{81} However, this would also impair genomic stability.

**Implication of misguided methylation and demethylation**

Whereas the distribution of 5mCd and 5hmCd is tightly regulated to ensure the anticipated functionality of a cell and its response to DNA damage, one hallmark of cancer cells is their completely different methylation and hydroxymethyl-methylation pattern.\textsuperscript{82,83} In many cancer types, the global methylation levels are decreased, while promoter regions of important regulatory and tumor suppressor genes are hypermethylated and therefore silenced.\textsuperscript{84} One example is the hypermethylation of the promoter region of HIC1, which is a transcriptional repressor of cell genesis, but also tumor hypoxia is responsible for reduced TET activity.\textsuperscript{99} Recent results show that not only mutations in TET genes or 5mCd are completely different methylation and hydroxymethyl-methylation related to ensure the anticipated functionality of a cell and its role in regulatory and learning processes in brain, but also during investigation and for DNA, especially the functions of 5hmCd levels positively and therefore increase TET3 activity.\textsuperscript{104} Glutamate and glutamine metabolism increases 5mCd levels, leading to self-renewal in pluripotent mouse embryonic stem cells, while succinate supply leads to differentiation.\textsuperscript{105} Additionally, succinate and also fumarate, another 2 intermediates of TCA cycle, show an inhibitory effect on TET enzymes in vitro.\textsuperscript{105}

In the future, it will be challenging not only to prove the existence, but to reveal the distinct biologic functions of the various DNA and RNA modifications that exist. The role of the modified bases in mRNA are currently under extensive investigation and for DNA, especially the functions of 5hmCd in regulatory and learning processes in brain, but also during development and in cancer cells are of great interest.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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There is more and more evidence that epigenetics and metabolism are closely connected not only via D-2HG in cancer metabolism, but also in normal cells.\textsuperscript{100,101} As an intermediate in the tricarboxylic acid (TCA) cycle and part of nitrogen catabolism through deamination of glutamate, \(\alpha\)-KG is one of the key metabolites. Since it is the co-substrate of TET enzymes and other dioxygenases involved in epigenetic regulation, such as histone lysine demethylase, it links epigenetics directly to metabolism. Levels of \(\alpha\)-KG are rate limiting for TET activity and higher \(\alpha\)-KG levels result in higher TET activity with direct impact on differentiation processes.\textsuperscript{102} Depending on the cell type and status, \(\alpha\)-KG can either promote self-renewal or induce differentiation.\textsuperscript{103} In brown adipose tissue (BAT) development, for example, TET3 mediates commitment to BAT by demethylating the Prdm16 promoter. AMP activated protein kinase \(\alpha1\) (AMPK\(\alpha1\)) influences \(\alpha\)-KG levels positively and therefore increase TET3 activity.\textsuperscript{104} Glutamate and glutamine metabolism increases \(\alpha\)-KG levels, leading to self-renewal in pluripotent mouse embryonic stem cells, while succinate supply leads to differentiation.\textsuperscript{105} Additionally, succinate and also fumarate, another 2 intermediates of TCA cycle, show an inhibitory effect on TET enzymes in vitro.\textsuperscript{105}
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