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Point of View

The Chemistries and Consequences of DNA and RNA Methylation and Demethylation

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Since the discovery of (desoxy)adenine (dA, A), (desoxy)cytosine (dC, C), (desoxy)guanine (dG, G), (desoxy)thymine (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).[1, 2] Particularly in transfer-RNA (tRNA) but also in ribosomal RNA (rRNA), modified bases are central elements, needed to fine tune the translation of the genetic code.[3-6] In rRNA 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.[7] More recently it was discovered that also messenger RNA (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 deoxycytosine (5mdC). 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.[1, 2]

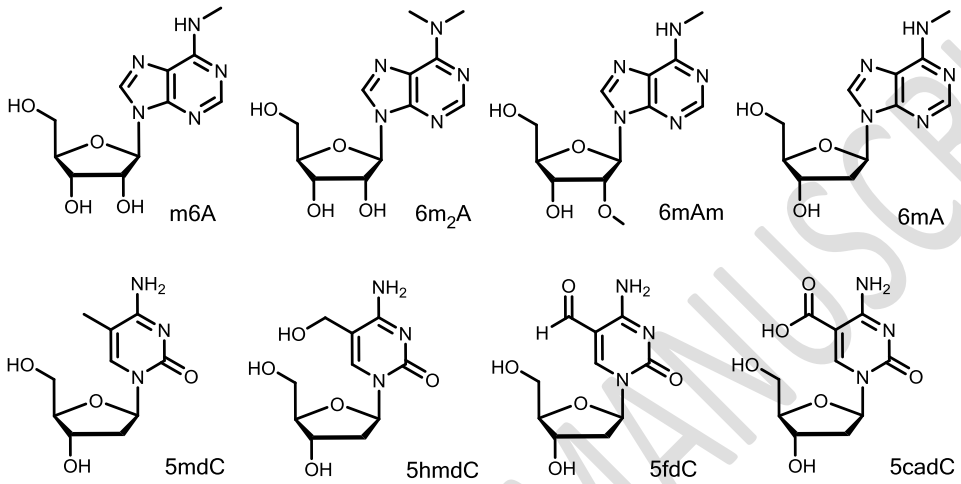

Fig. 1. Examples of methylated and oxidized bases found in RNA and DNA.

5mdC is the most abundant modified base in genomic DNA of eukaryotes and also present in the DNA of prokaryotes.[8-10] In mammals, 5mdC typically reaches global levels of between typically 1 and 5% in genomic DNA.[11, 12] 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[13, 14], its presence was only recently shown in several higher eukaryotic organisms. In *Caenorhabditis elegans*, where 5mdC is not detectable, 6mA is dynamically regulated and linked to other epigenetic marks[15] 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.[16] In the unicellular green alga *Chlamydomonas reinhardtii*, 6mA was discovered

in 84 % of the genes, where it is mainly located at transcription start sites.[17] Recently, it was reported that mammalian DNA, including human and mouse, also contains 6mA.[13, 18] There, 6mA seems to be distributed across the genome, but absent in gene exons,[19] and 6mA-demethylation in mouse embryonic stem cell (mESC) DNA was shown to correlate with ALKBH1 depletion.[18] 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.[20] 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.

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.[21-23] 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, three active DNA-methyltransferases (DNMTs: DNMT1, DNMT3a and DNMT3b) exist.[24, 25] DNMT3a and 3b are *de novo* DNMTs, which methylate canonical dC bases.[26] 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.[27] As such, DNMT1 converts the methylation of dC into an inheritable modification that can be transferred during reproduction.[28, 29]

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.[28] A global deletion of only one of the three DNMTs leads to severe cellular aberrations and is therefore lethal in early embryogenesis (DNMT1 and 3b) or postnatal (DNMT3a).[26, 30] During differentiation the "methylome" is highly dynamic and a celltype-characteristic 5mC pattern is established during this process.[31] 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.[32-34] 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.[35] 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.[36, 37] CGIs are regions of high CpG frequency over a length of at least 500 base pairs compared to the bulk genomic DNA and found in 40% of promoter regions in the mammalian genome, with even higher levels (60%) in the human genome.[38, 39] Symmetric methylation of CpG:GpC islands is consequently a hallmark of silenced genes.[40, 41]

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 $6m_2A$, 4mC or m6Am. SAM as natures' "methyl iodide" is hence reactive enough to methylate even weak nucleophilic centers such as the exocyclic amino groups of dA, which feature, as an sp^2-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-CH_3* system. This will be important in the context of active demethylation (*vide infra*).

In contrast to the formation of *het-CH_3* 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-CH$_3$* connectivities which feature a strong and stable C-C single bond.

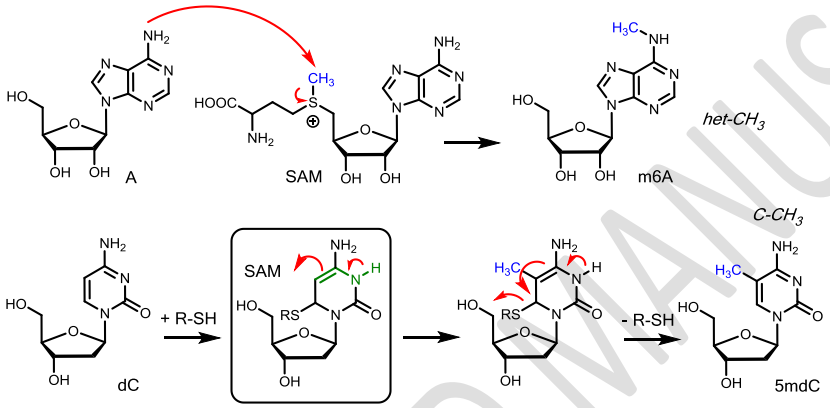

Fig 2: Mechanism of methylation leading to the formation of *het-CH$_3$* and *C-CH$_3$* connectivities in RNA and DNA.

Chemistry of Demethylation

In order to establish the reversibility needed for switching biochemical processes, nature requires to remove the attached methyl groups. Removal of het-CH_3 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).[42] The Fe(IV)=O species is able to abstract a

H-atom from the het-CH_3 group to form a het-stabilized het-CH_2• radical, which reacts with the Fe-bound hydroxylradical to form a *het-CH_2-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 two 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.[43] Alkbh5-deficient mice had a similar effect as FTO knock-down in human cells and resulted in increased m6A levels of the mRNA.[44] 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.[42, 44]

In 2009 it was found that also 5mdC is further enzymatically oxidized in a stepwise fashion to give first 5-hydroxymethyldesoxycytosine (5hmdC), followed by 5-formyldesoxycytosine (5fdC) and 5-carboxydesoxycytosine (5cadC). "Ten-eleven translocation" (TET) enzymes, which are Fe^{2+}/α-KG dependent dioxygenases, were discovered to catalyze this iterative 5mdC oxidation reaction.[45, 46] Regarding the first oxidation step that transforms 5mdC to 5hmdC, the Fe^{2+}/α-KG catalyzed reaction generates a stable *C-CH_2-OH* connectivity, which is as a primary alcohol stable in water (Fig. 3). 5hmdC is consequently a stable DNA base modification and it was suggested that the base has indeed epigenetic functions. For example, 5hmdC 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 5hmdC levels in fully differentiated tissues were found in the brain with up to 1% of all cytosines.[48, 49] Evidence accumulates that 5hmdC in a given gene is able to

accelerate transcription and it is not surprising that 5hmdC 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 three different types (TET1 – TET 3) 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 5hmdC in mammalian DNA was described first already in 1972.[56] It took more than 30 years to confirm that 5hmdC is really present in substantial amounts that are highly depending on the cell.[57]

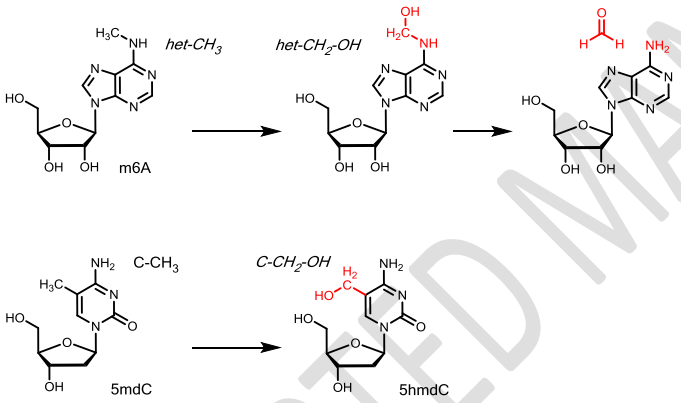

Fig. 3: Oxidation of m6A followed by decomposition of the hemiaminal to A and oxidation if 5mdC to stable 5hmdC.

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.[60-63] 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.[64-66] 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.[67] Interestingly, 6mA demethylation in *Drosophila* is catalyzed by *Drosophila's* TET homologue (DMAD or dTet). DMAD depletion results in higher 6mA levels, but unchanged 5mdC 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 5hmdC 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 5hmdC, converting 5hmdC directly to dC, therefore supporting these ideas.[69]

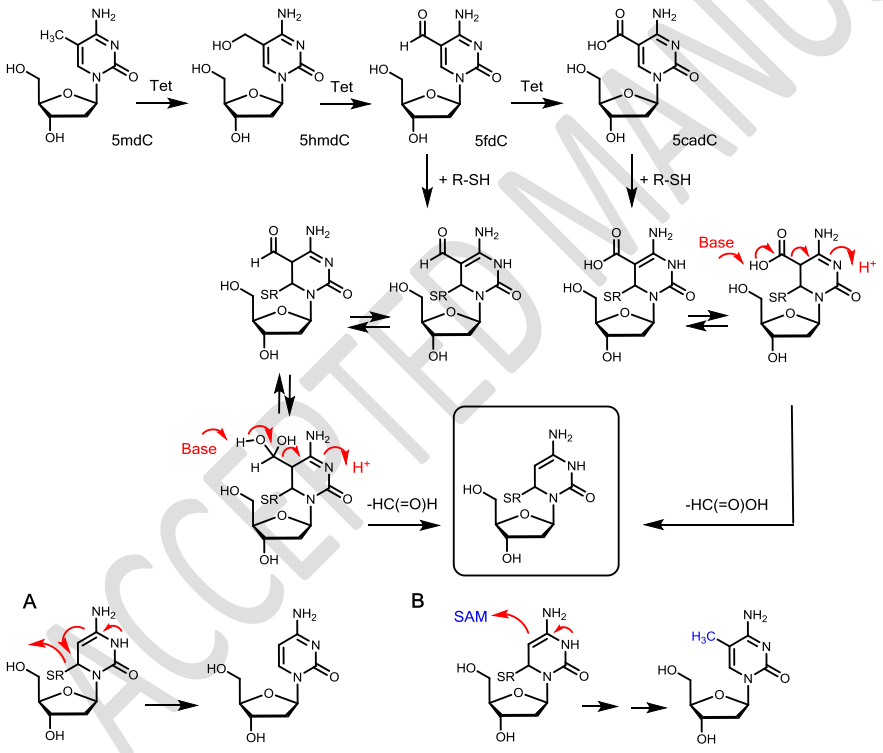

Fig. 4: Potential mechanism of chemically induced active demethylation with a potential immediate re-methylation.

In this context, it is interesting to note that 5hmC and 5fC were also discovered in RNA. In human cells at tRNA position C34, the oxidation of the corresponding RNA base 5mrC to 5frC is catalyzed by the Fe^{2+}/α-KG dependent enzyme ALKBH1, which is also responsible for m1A demethylation in mammalian tRNA.[70, 71] Interestingly, 5hmrC was not detected as an intermediate in the ALKBH1-dependent 5mrC oxidation.[70] In *Drosophila*, 5hmrC was discovered in polyadenylated RNA and is associated with enhanced mRNA-translation efficiency back to normal level, when 5mrC has lowered the efficiency.[72] Surprisingly, the oxidation reaction is catalyzed by *Drosophila's* TET homologue 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 5mrC oxidation[73, 74], but at the moment it is not clear whether TET-mediated 5hmrC 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 5dfC ore 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 to 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]

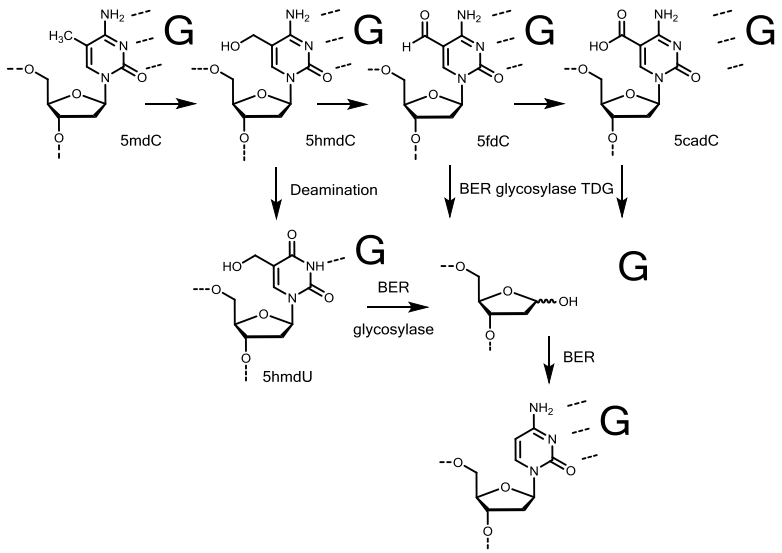

Fig. 5: Active demethylation via base excision repair. Two possibilities are discussed: A direct removal of 5fdC and 5cadC in xdC:dG base pairs or removal of a deaminated 5hmdU in a 5hmdU:dG mismatch by BER glycosylase.

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 5hmdC levels increase after 5mdC levels have dropped and global demethylation can be detected in Tet3-deficient zygotes.[80] It might be that deamination of genomic 5mdC to dT and subsequent dT/dG mismatch repair are the mechanism behind this observation.[81] However, this would also impair genomic stability.

Implication of misguided methylation and demethylation

Whereas the distribution of 5mdC and 5hmdC 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 hydroxyl-methylation pattern.[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.[84] One example is the hypermethylation of the promoter region of HIC1, which is a transcriptional repressor of SIRT1, a survival protein (proto-oncogene) that is consequently upregulated.[85] It was also shown that certain CGIs are coordinately methylated in some tumor cells, which is called "CpG island methylator phenotype".[86-88] Since TET1 was initially discovered in 2002 as a fusion protein to MLL1 H3K4 methyltransferase in patients with acute lymphoblastic leukemia, which is characterized by mutations in the MLL1 protein, it was considered to be an oncogene.[89] Only when the biological function of the TET enzymes was elucidated in 2009, it was proven a few years later that TET enzymes are actually tumor suppressors that are silenced in various types of tumors. Decreased levels and activity of TET1 and therefore reduced hmdC levels are associated with hematopoietic malignancies, colon, breast, prostate, liver and lung cancers, which show greater levels of proliferation and for breast cancer increased invasion rates as a direct consequence of TET1 downregulation.[90-93] TET2 mutations and consequently decreased 5hmdC levels occur in various myeloid malignancies, including chronic myelomonocytic leukemia, myeloid proliferative neoplasm and acute myeloid leukemia.[82, 94, 95] Mutations in the genes of isocitrate dehydrogenase (IDH) 1 and 2 lead to the production of D-2-hydroxyglutarate (D-2HG), a metabolite that inhibits TET2 activity for example in AML and MPN, but also in malignant gliomas, resulting in a dramatic decrease of 5hmdC levels.[96] Interestingly, IDH1/2 and TET2 mutations seem to be mutually exclusive in these types of tumor, with IDH1/2 mutations being the ones with higher oncogenic potential.[97, 98] Recent results show that not only mutations in TET genes or their inhibition by cancer metabolites are important for tumorigenesis, but also tumor hypoxia is responsible for reduced TET activity.[99]

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.[100, 101] As an intermediate in the tri-citric acid (TCA) cycle and part of nitrogen catabolism through deamination of glutamate, α-KG is a 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 α-KG are rate limiting for TET activity and higher α-KG levels result in higher TET activity with direct impact on differentiation processes.[102] Depending on the cell type and status, α-KG can either promote self-renewal or induce differentiation.[103] In brown adipose tissue (BAT) development, for example, TET3 mediates cell commitment to brown adipose tissue by demethylating the Prdm16 promoter. AMP activated protein kinase α1 (AMPKα1) influences α-KG levels positively. This increases TET activity and therefore mediates cell commitment to brown adipose tissue.[104] Glutamate and glutamine metabolism increases α-KG levels, leading to self-renewal in pluripotent mouse embryonic stem cells, while succinate supply leads to differentiation.[102] Additionally, succinate and also fumarate, another two intermediates of TCA cycle, show an inhibitory effect on TET enzymes *in vitro*.[105]

In the future, it will be challenging not only to prove the existence, but to reveal the distinct biological 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 5hmdC in regulatory and learning processes in brain, but also during development and in cancer cells are of great interest.

Acknowledgements

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