Reactive oxygen species in health and disease

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Local chromatin microenvironment determines DNMT activity: from DNA methyltransferase to DNA demethylase or DNA dehydroxy-methylase.

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Chapter 7

Abstract

Insights on active DNA demethylation disproved the original assumption that DNA methylation is a stable epigenetic modification. Interestingly, mammalian DNA methyltransferases 3A and 3B (DNMT-3A and -3B) have also been reported to induce active DNA demethylation, in addition to their well-known function in catalyzing methylation. In situations of extremely low levels of S-adenosyl methionine (SAM), DNMT-3A and -3B might demethylate C-5 methyl cytosine (5mC) via deamination to thymine, which is subsequently replaced by an unmodified cytosine through the base excision repair (BER) pathway. Alternatively, 5mC when converted to 5-hydroxymethylcytosine (5hmC) by TET enzymes, might be further modified to an unmodified cytosine by DNMT-3A and -3B under oxidized redox conditions, although exact pathways are yet to be elucidated. Interestingly, even direct conversion of 5mC to cytosine might be catalyzed by DNMTs. Here, we summarize the evidence on the DNA dehydroxymethylase and demethylase activity of DNMT-3A and -3B. Although physiological relevance needs to be demonstrated, the current indications on the 5mC- and 5hmC-modifying activities of de novo DNA C-5 methyltransferases shed a new light on these enzymes. Despite the extreme circumstances required for such unexpected reactions to occur, we here put forward that the chromatin microenvironment can be locally exposed to extreme conditions, and hypothesize that such waves of extremes allow enzymes to act in differential ways.

Introduction

DNA methylation is the first and best studied epigenetic modification and, as no mammalian enzymes were known to actively remove this mark, DNA methylation was classically considered to be a stable mark. Mammalian DNA methylation is regulated by three DNA (cytosine-5)-methyltransferases: DNMT1, -3A and -3B and underlies a wide variety of processes in the body including cognition. In general, DNA methylation takes place at CpG sites and, especially when around transcription start sites, is mostly associated with gene repression. Despite this generally accepted association, many studies have documented that increased DNMT levels correlate with increased gene expression.

Ageing-associated cognitive deficits, for example, were associated with a decreased expression of DNMT3A2 in the hippocampus in mice, and the deficits could be rescued by transfection of DNMT3A2 (1). Remarkably, the induced expression of DNMT3A2 in rescued mice was associated with an increased expression of early activity genes *arc* and *bdnf* (1). Another example of increased DNMT3 expression and increased expression of certain genes is "first-time-event" fear, e.g. a student appearing for his first *viva voce* exam. Subsequent exposures to similar events reduce the sensation of fear due to habitual conditioning (2). The molecular mechanisms of such fear conditioning have been investigated and differential DNA methylation is among the changes observed for hippocampal neurons: Increased expression of the *de novo* DNA methyltransferases DNMT3A/3B was observed, while the DNA methylation status of the *Reelin* promoter was lowered (3).

To explain such seemingly contradictory observations, recent reports have suggested a direct DNA demethylase and hydroxymethylase activity of DNMT3A and -3B *in vitro* (4, 5). We here examine cellular context requirements which would allow these enzymes to function in the process of active DNA demethylation.

Known players in DNA demethylation

Active DNA demethylation refers to the enzymatic removal or modification of the methyl group from 5mC, eventually resulting in an unmodified cytosine (C) (6). In this respect, ten-eleven translocation (TET) methylcytosine dioxygenases, activation-induced cytidine deaminase (AID), growth arrest and DNA-damage-inducible protein 45 alpha (Gadd45a) and thymine DNA glycosylase (TDG) are some of the factors described to play a role in this process of active DNA demethylation (7-15).

The enzymatic action of TET enzymes results in 5-hydroxymethylcytosine (5hmC) (13, 16), a modification that is observed in many tissues (17) and which can be further oxidized by TET to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (18, 19). 5caC can be further decarboxylated (20) or excised by TDG (19, 21, 22), which can also remove 5fC. In addition, 5hmC, 5fC and 5caC are also considered DNA demethylation intermediates as these can undergo replication-mediated dilution leading to passive DNA demethylation (23, 24). Alternatively, 5hmC can be deaminated to 5hmU by AID, followed by excision by TDG and replacement by an unmodified cytosine by the base excision and repair (BER) mechanism (25).

A role for DNMT3A and -3B in the active DNA demethylation process?

In addition to the above described mechanisms of DNA demethylation, three possible alternative pathways of active DNA demethylation, involving DNMT3A and -3B, have been proposed (4, 5, 26), although their physiological relevance is under debate. Interestingly, two of these pathways concern BER-independent conversion of 5mC or 5hmC to unmodified cytosine (DNA demethylase and DNA dehydroxylase activity, respectively). BER-independent conversion of methylated cytosines would provide a safer alternative to BER-mediated replacement as the TDG/BER pathway acts via DNA cleavage: its localized action on multiple methylated cytosines in a given region likely causes instability of the particular locus (4, 5). In addition to DNMT3s, also DNMT1 was found to contain strong DNA demethylating activity (5), although its DNA dehydroxymethylase activity did not exceed background levels (4).

Role in deamination-induced DNA demethylation

Representative bacterial DNA methyltransferases, like M.*Hpa*II and M.*Sss*I, can convert C to uracil (U) in double stranded DNA in the absence of SAM (27, 28). During the process of methylation of C to 5mC in the presence of SAM, methyltransferases initiate a flipping of C out of the DNA helix, followed by the formation of a covalent bond between a cysteine in the methyltransferase and the C6 carbon of the flipped C (29). This covalent interaction with the C6 carbon forms the activated cytosine which is then converted into an intermediate, namely 5,6-dihydrocytidine (28-30). Dependent on the context, the cytosine intermediate can follow one of two possible routes: a) in the presence of SAM, it will accept the methyl group to form 5mC, and b) in the absence of SAM, the cytosine intermediate would be deaminated into U (28). In line with bacterial

methyltransferases, also mammalian DNMT3A and -3B could deaminate C to U and importantly, 5mC to thymine (T) (26). The resulting T:G mismatch recruits TDG and other BER proteins which induce completion of the demethylation process. These observations tempted researchers to speculate on dual roles of DNMT3A and -3B in living cells: as a demethylator at the beginning of the transcription cycle to induce expression of *pS2*, and as a methylator at the end of the cycle to stop the expression (26, 31). Indeed, the cyclical methylation of *pS2* is abolished upon inhibition of DNMT3A and -3B. In the above studies, however, it is possible that the, at that time unidentified, TET enzymes may have functioned as the active DNA demethylases as suggested by other studies (32, 33).

Role in redox-dependent DNA demethylation

In the absence of SAM, M.*Hpa*II and M.*Sss*I, as well as murine DNMT1 (albeit weakly), are able to catalyze the addition of formaldehyde to C forming 5hmC (34). Interestingly, the authors also demonstrated that formaldehyde can be released from 5hmC which is then reconverted to an unmodified C in a reverse reaction catalyzed by these bacterial DNA methyltransferases (34). DNA dehydroxylase activity was also shown for purified mammalian DNMT3A and -3B in the absence of SAM, although the exact mechanism has not been uncovered (4). Importantly, the DNMT3A/3B-mediated dehydroxylase activity depended on the redox state: an oxidizing agent (H_2O_2) enhanced dehydroxylase activity, while suppressing the DNA methylation activity; reducing agents (DTT, ß-mercaptoethanol) inhibited the dehydroxylase activity while slightly enhancing DNA methylation (4). When the cysteine residue in the methylation-active center of DNMT3A or -3B was replaced by a serine residue, both enzymes exhibited reduced 5hmC dehydroxymethylase activity. Interestingly, oxidative post-translational modifications of particular cysteine residues have the potential to modulate protein activity (35). Possibly, a similar mechanism might be in place for DNMT3A and -3B. Despite such observations, another study, although confirming a direct 5hmC dehydroxylase activity for human DNMT3A (when in combination with the catalytically inactive DNMT3L), did not observe an effect of H_2O_2 (36).

In the absence of SAM, DNMT3s could also directly convert 5mC to C in a non-reducing environment (5). However, supraphysiological concentrations of Ca^{2+} are required for this reaction to occur (1 mM Ca^{2+} is optimal for demethylation; the minimum concentration is 10 µM). Apart from a direct action on 5mC and 5hmC, again in the absence of SAM, DNMT3A and -3B can also directly convert 5caC, but not 5fC, into unmodified C (36). These results indicate

the intriguing possibility that active DNA demethylation can proceed via the reduction of oxidized products of 5mC without the need for BER, although these processes may serve as complementary pathways to BER.

The above evidence can be summarized in the following scheme depicting how and when DNMT3A and -3B could contribute to active DNA demethylation (**Fig. 1**). In the presence of SAM, DNMT3A and -3B transfer a methyl group to the C5 of the activated cytosine to form 5mC (**1**). Whereas in the absence of SAM, DNMT1 (DNMT3s have not been analyzed) can couple a formaldehyde to the activated C to form 5hmC directly (**2**). When 5mC is formed, DNMT-mediated active DNA demethylation can, depending on the environment, proceed via three different pathways: Independently of the DNMTs, TET enzymes can directly convert 5mC to 5hmC (**3**). When SAM levels are low or completely depleted, DNMT3A and -3B can contribute to the active DNA demethylation process. In the case of low SAM levels, DNMT3A and -3B catalyze the deamination of 5mC to T (**4)**. T is in turn replaced by an unmodified C via the BER pathway (**5**). In the case of absence of SAM combined with high levels of Ca^{2+} and an oxidized redox environment, DNMT3A and -3B can convert 5mC to an unmodified C (**6**). Also the conversion of 5hmC to an unmethylated C might be catalyzed by DNMT3A and -3B. This reaction can take place when SAM depletion is combined with an oxidizing redox environment (**7**).

DNMT3A and -3B as demethylating enzymes: Implications

Aberrant DNA hypomethylation is associated with various clinical phenotypes and can be explained by low SAM substrate availability or increased active DNA demethylation. Increased active DNA demethylation can be caused by deregulated activity of enzymes known to be involved in the demethylation pathway, including TET family members. As an alternative mechanism, we are here concerned with the processes of active DNA demethylation that involve DNMTs. However, specific circumstances are required for these activities to occur. In this respect, the calcium-dependent character of DNMT-induced 5mC demethylation requires extremely high levels of Ca^{2+} (> 10 μM), which prevented this mechanism from reaching broad acceptance for physiological relevance (5, 37). Also the oxidizing conditions, potentially required for both the DNA demethylase as well as the DNA dehydroxylase activity of DNMT3, are unlikely to occur on a cellular level: 1 mM H_2O_2 is extremely toxic to cells. Below, we advocate that it is conceivable that substrate availability and redox conditions are not steady throughout the nucleus (38); Extreme microenvironmental chromatin

conditions might enable the cell to locally generate conditions that are required for DNMTs to act in the process of active DNA demethylation without the high cytotoxicity that is associated with total cell exposure. Here, we point out mechanisms that enable the cell to generate such local chromatin microenvironments, enabling the DNMTs to act differently on the local level.

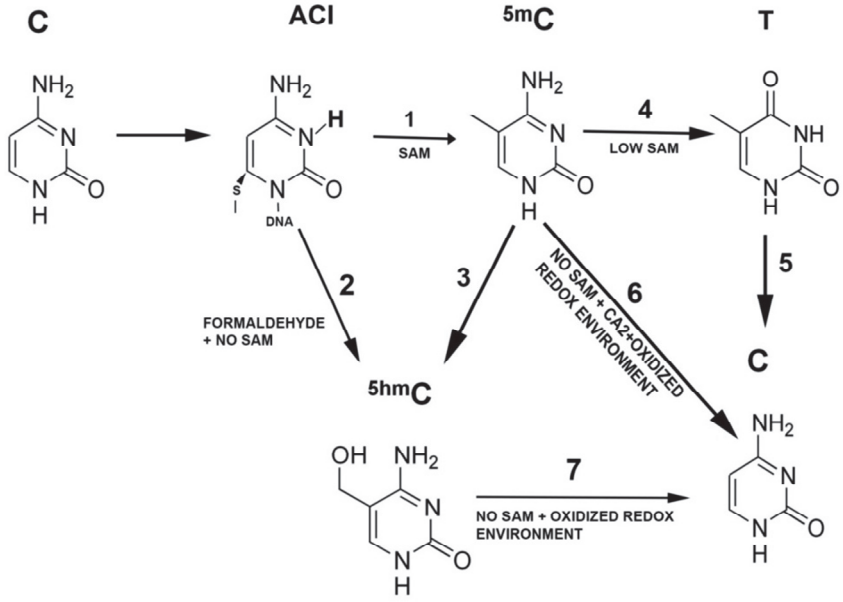

Figure 1. Involvement of DNMTs in the active DNA demethylation process. In the presence of SAM, DNMT3A and -3B transfer a methyl group to the C5 of the activated cytosine to form 5mC (**1**). Whereas in the absence of SAM, DNMT1 (DNMT3s have not been analyzed) can couple a formaldehyde to the activated C to form 5hmC directly (**2**). When 5mC is formed, DNMT-mediated active DNA demethylation can, depending on the environment, proceed via three different pathways: Independently of the DNMTs, TET enzymes can directly convert 5mC to 5hmC (**3**). When SAM levels are low or completely depleted, DNMT3A and -3B can contribute to the active DNA demethylation process. In the case of low SAM levels, DNMT3A and -3B catalyze the deamination of 5mC to T (**4**). T is in turn replaced by an unmodified C via the BER pathway (**5**). In the case of absence of SAM combined with high levels of Ca^{2+} and an oxidized redox environment, DNMT3A and -3B can convert 5mC to an unmodified C (**6**). Also the conversion of 5hmC to an unmethylated C might be catalyzed by DNMT3A and -3B. This reaction can take place when SAM depletion is combined with an oxidizing redox environment (**7**).

The epigenetic landscape is continuously maintained/adjusted by re-inforcing epigenetic enzymes acting on histones and on the DNA molecule. The enzymatic reactions are associated with transient local changes in the microenvironment, as described for e.g. histone demethylases. Currently, there are two known classes

of histone demethylases: the Flavin-dependent histone demethylases and the Jumonji-containing histone demethylases. The first class, containing lysine-specific histone demethylase 1 and 2 (LSD1, 2), is involved in modulating gene expression through demethylation of either mono- or di-methyllysine residues of H3K4 (38-41) or H3K9 (42-44) resulting in gene repression or gene activation, respectively (45). During this histone demethylation process, H_2O_2 is produced (46), which has been proposed to be involved in signaling processes (47). In the case of H3K9 demethylase activity, and thus gene activation, the H_2O_2 produced by LSDs might modulate the microenvironmental chromatin conditions in such a way that DNMT3s can re-inforce re-expression by acting in the process of active DNA demethylation.

The mechanism as proposed above might be especially relevant to rapidly "ultradian" oscillitating genes, in which gene expression is regulated in a rhytmic pattern of cyclical demethylation-remethylation events, with time periods of the order of hours (48). It is plausible that the cell uses the ability of DNMT3s to act both in the process of DNA methylation and DNA demethylation, in order to enable a quick switch from demethylation to remethylation that is required to regulate the fast oscillitation of these genes. Promoters that follow such a fast oscillitating behavior are for example, the estrogen-sensitive *pS2* promoter (26, 31) and promoters containing a retinoic acid response element (RARE) (49). The latter study by Zuchegna et al. revealed that the dual action of the histone demethylases LSD1 and JMJD2A resulted in histone demethylation, local DNA oxidation, attraction of base (BER) and nucleotide excision repair (NER) enzymes and chromatin looping, and all this together was essential for and directly contributed to RA-induced transcription (49). Moreover, this study clearly showed that the observed effects were strictly localized to the chromatin microenvironment of the RA-dependent genes, as the chromatin of neighboring RA-independent genes was unaffected. Similarly, LSD1 is also involved in the demethylation-remethylation cycles of the *pS2* promoter (50) and therefore, the above described mechanism could be a more general feature of "ultradian" oscillitating genes.

If such described local changes in redox state would also occur for Ca^{2+} and/or SAM levels, such fluctuations might thus alter the function of DNMT3A and -3B. To identify such situations in physiological situations, the targeting of DNMTs to pre-determined genomic loci by epigenetic editing, as described by us and others previously (51-55) provides an interesting tool to test and to eventually exploit DNA demethylation activities of DNMTs in vivo.

Apart from such extreme microenvironmental chromatin conditions, clinical situations exist where substrate conditions might allow DNMTs to act as demethylators: excess of formaldehyde is associated with ageing, and has been put forward to explain memory deficits (56). Moreover, in certain congenital diseases like Down's syndrome, SAM levels are found to be decreased while oxidation status is increased (57). Methionine adenosyl transferases (MATs), the enzymes that catalyze the conversion of methionine into SAM, are decreased in cirrhosis which is associated with genomic instability and an increased susceptibility of hepatocytes to oxidative stress-induced carcinogenesis (presumably due to impaired glutathione metabolism) (58). Preneoplastic changes seen in the liver of rats fed on a folate-deficient diet, resulting in decreased levels of SAM, are associated with global hypomethylation despite an increase in DNMT1 and DNMT3A (59). These conditions exemplify situations where DNMT3A could be actually functioning as a DNA demethylase in the context of SAM deficiency.

Conclusion

In vitro studies have suggested that DNMT3A and -3B, known to methylate DNA *de novo*, can function as players in the active DNA demethylation pathway in certain situations. In conditions of low SAM concentrations, DNMT3A and -3B can catalyze the deamination of 5mC to T, which could then be replaced by an unmodified C by the BER pathway. In an oxidizing redox chromatin microenvironment and in the absence of SAM, DNMT3A and -3B can even catalyze the direct conversion of 5mC or 5hmC to an unmodified C, although the exact pathways are yet to be defined. Further cellular studies are now required to investigate the physiological relevance of DNMT3A and -3B as players in active DNA demethylation. Epigenetic editing tools (50-54) offer exciting possibilities to address differential effects of epigenetic enzymes in various chromatin contexts in living cells (60).

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