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Mammalian DNA demethylation

Multiple faces and upstream regulation

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DNA cytosine methylation is a reversible epigenetic mark regulating gene expression. Aberrant methylation profiles are concomitant with developmental defects and cancer. Numerous studies in the past decade have identified enzymes and pathways responsible for active DNA demethylation both on a genome-wide as well as gene-specific scale. Recent findings have strengthened the idea that 5-methylcytosine oxidation catalyzed by members of the ten-eleven translocation (Tet1–3) oxygenases in conjunction with replication-coupled dilution of the conversion products causes the majority of genome-wide erasure of methylation marks during early development. In contrast, short and long patch DNA excision repair seems to be implicated mainly in gene-specific demethylation. Growth arrest and DNA damage-inducible protein 45 a (Gadd45a) regulates gene-specific demethylation within regulatory sequences of limited lengths raising the question of how such site specificity is achieved. A new study identified the protein inhibitor of growth 1 (Ing1) as a reader of the active chromatin mark histone H3 lysine 4 trimethylation (H3K4me3). Ing1 binds and directs Gadd45a to target sites, thus linking the histone code with DNA demethylation.

Keywords: 5-methylcytosine, DNA demethylation, reprogramming, Gadd45, Ing1, H3K4me3

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Introduction

In mammalian DNA, cytosines within a CpG dinucleotide context are commonly marked by a methyl group at carbon 5 of the pyrimidine ring. By influencing, typically silencing, gene expression, the resulting modification, 5-methylcytosine (5mC), has been implicated to bear pivotal roles during embryonic development, imprinting, X-chromosome inactivation and cancer.[1-3] DNA cytosine methylation permits organisms to gain an additional layer of genetic information on top of the primary DNA sequence and, hence, is classified as an epigenetic mark.

Methylation marks are commonly maintained during DNA replication by the action of the DNA methyltransferase DNMT1.[4] Consequently, once set, DNA methylation has been thought to be stable even through cell divisions. Additionally, loss of methylation marks observed in dividing cells was referred to, by default, as passive DNA demethylation after several rounds of replication in the absence of DNMT1. However, research in the last decade uncovered scenarios in which methylated DNA is demethylated in a replication-independent, active manner, both at the genome-wide level as well as at specific genomic loci.

After fertilization, DNA methylation marks of the paternal pronucleus in the mouse zygote are globally erased prior to the first cell cycle.[5,6] Similarly, in primordial germ cells (PGCs), the progenitor cells of gametes, methylation is lost genome-wide during their migration to the genital ridge between embryonic days E8.5–E11.5.[7]

A remarkable example of loci-specific active DNA demethylation in human cells was described at the estrogen receptor target gene *pS2*. After estrogen stimulation the *pS2*-promoter undergoes cycles of methylation and demethylation in less than 100 min indicating DNA methylation to be not only reversible but also highly dynamic.[8,9]

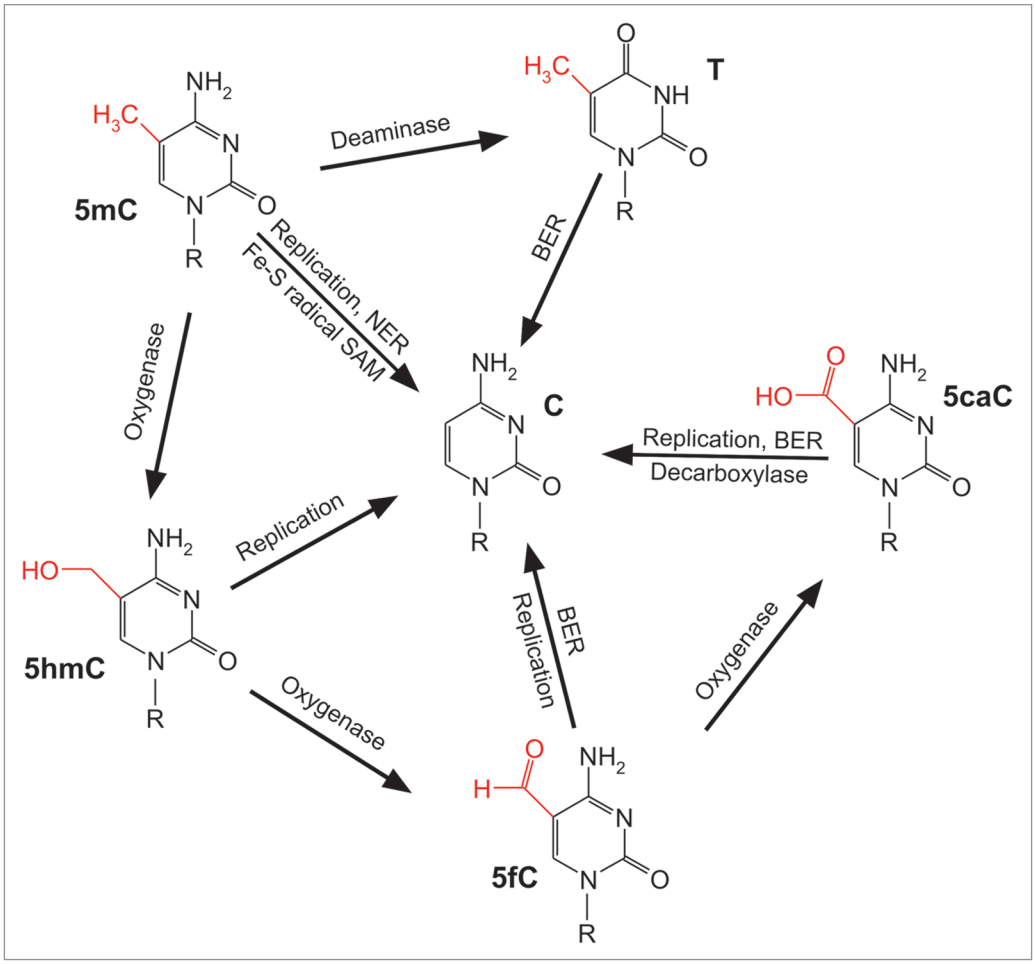

Figure 1. The multiple faces of mammalian DNA demethylation. Schematic representation of the enzymology implicated in 5-methylcytosine (5mC) demethylation. The exocyclic group at carbon 5 of each cytosine derivative is highlighted in red. C, cytosine; T, thymine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxylcytosine; BER: base excision repair; NER, nucleotide excision repair. Note: Direct base excision repair of 5mC and potential deamination of 5hmC to 5-hydroxymethyluracil has not been considered due to lack of experimental confirmation. For details see main text.

DNA demethylation is of particular importance for the generation of induced pluripotent stem cells (iPSCs), the artificial reprogramming of somatic cells to their pluripotent ground state. Genes for key transcription factors like *Oct4* and *Nanog* are fully demethylated during reprogramming and this demethylation, in turn, drives their expression.[10,11]

Enzymes of Active DNA Demethylation

While the enzymes responsible for cytosine methylation are confined and well characterized,[12] the enzymes responsible for mammalian active DNA demethylation remain rather numerous and controversial (**Fig. 1**).[13] A single enzymatic reaction that releases the methyl group from 5mC but keeps the DNA backbone untouched (a DNA "demethylase" reaction) seems difficult to conceive. Thermodynamically it is challenging to break unpolar carbon-carbon bonds. Nevertheless, the Fe-S radical S-adenosylmethionine (SAM) domain of elongator complex protein 3 (Elp3) was shown to be involved in paternal DNA demethylation in the mouse zygote leading to the hypothesis of a direct radical SAM-mediated demethylation mechanism.[14]

On the other hand, DNA repair mechanisms that exchange complete nucleotides by canonical (unmethylated) DNA building blocks attracted attention in regard to active DNA demethylation. In fact, DNA glycosylases from the Demeter/ROS1 family initiate base excision repair (BER) of methylated cytosines in plants leading to DNA demethylation.[15-17] In mammals, however, a 5mC-specific DNA glycosylase could not be confirmed.[18-21] An elegant loophole was provided by the idea that 5mC is first deaminated to thymine resulting in a T/G mismatch which, in turn, is processed by thymine-specific DNA glycosylases such as TDG. PGCs deficient in activation-induced (DNA-cytosine) deaminase (AID) show a significant albeit far from complete impairment of global demethylation.[22] In line with this, using an interspecies heterokaryon technology for reprogramming it was shown that AID is required to demethylate the critical genes *OCT4* and *NANOG* in human fibroblasts.[23] Moreover, reprogramming of mouse embryonic fibroblasts (MEFs) to

iPSCs by the four Yamanaka factors Oct4, Sox2, c-Myc and Klf4[11] was demonstrated to depend on a catalytically active AID.[24] Interestingly, in absence of the cofactor SAM the de novo DNA methyltransferases Dnmt3a and Dnmt3b are able to deaminate 5mC, thereby, potentially enabling dynamical methylation/demethylation cylces in short periods of time on specific genes.[9]

The paternal pronucleus in the mouse zygote exhibits high amounts of DNA strand breaks within the critical time frame of global demethylation indicative for an involvement of DNA repair in this process.[25] Strikingly, DNA strand breaks in direct vicinity of 5mCs were found in an enhancer region of the *tyrosine aminotransferase* gene that is demethylated after hormone stimulation.[26] The result supports short patch base excision repair as being part of the demethylation machinery.

Long patch excision repair has also been attributed to DNA demethylation. Notably, in *Xenopus laevis* oocytes demethylation of an *oct4*-reporter was shown to critically depend on the xeroderma pigmentosum complementation group G protein (XPG), the 3'-endonuclease of nucleotide excision repair (NER).[27] Demethylation of the same reporter was accompanied by BrdU incorporation, indicating long patch repair synthesis instead of a one nucleotide exchange.[27] Demethylation of the human rDNA promoter requires the endonuclease activity of XPG[28] and the *RARβ2* promoter is occupied by NER factors and exhibits DNA strand breaks, triggering DNA demethylation upon retinoic acid stimulation.[29,30]

Research in the field was revolutionized by two breakthrough papers in 2009 describing the (re)discovery of 5-hydroxymethylcytosine (5hmC) in human cells.[31,32] 5hmC is the oxidation product of 5mC catalyzed by the ten-eleven translocation (Tet1–3) family of enzymes.[32,33] Tet enzymes can oxidize 5hmC further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC).[34,35] All three oxidation products have been detected in genomic DNA[35-37] and their role as potential DNA demethylation intermediates has been extensively analyzed since then. Indeed, 5fC and 5caC are substrates for TDG[34,38] and the embryonic lethal phenotype of *Tdg* null mice was explained in part by aberrant methylation during early development.[39]

Recently, the distribution of 5fC and 5caC in the genome of mouse ES cells (mESCs) has been achieved by high-throughput sequencing of (1) immunoprecipitated DNA using modification-specific antibodies and (2) streptavidin-captured DNA after chemical reduction and specific biotin-labeling of 5fC.[40,41] Both studies demonstrated a significant accumulation of 5fC (and 5caC) residues at distal regulatory regions (enhancers) of genes across the genome in TDG-deficient mESCs, as compared with wild-type cells. Hence, 5fC and 5caC serve as intermediates of active DNA demethylation triggered by the Tet-family and TDG/BER at least at distinct genomic loci.

5hmC was postulated to be deaminated by members of the AID/APOBEC family of deaminases with the resulting 5-hydroxymethyluracil (5hmU) being a substrate for 5hmU-glycosylases such as SMUG1.[42] However, neither AID nor APOBEC enzymes are able to deaminate 5hmC in vitro[43,44] and 5hmU could not be detected in genomic DNA.[43]

The importance of Tet-mediated DNA demethylation was demonstrated in a recent study describing a modified protocol for the generation of iPSCs. One of the Yamanaka factors, Oct4, could be replaced by Tet1, highlighting the significance of the conversion of 5mC to 5hmC during reprogramming.[45]

Genomic stability is challenged by strand breaks, base-free sites or nucleotide gaps that necessarily occur during base or nucleotide excision repair. If coping with such toxic intermediates during demethylation at single loci might be a solvable task for cells, the same problem, likely, is of a different nature during global DNA demethylation in a limited time frame. As a matter of fact, in the paternal pronucleus of the mouse zygote, 5hmC signals appear concomitant with the loss of 5mC signals.[46-48] A major fraction of 5mCs was, obviously, oxidized, and this was dependent on Tet3. Later, it was also shown that 5fC and 5caC signals occur during late pronuclear stages.[36] Strikingly, the signals for 5hmC, 5fC and 5caC diminished subsequently after each round of replication by a factor of two.[36,49] The majority of genome-wide DNA demethylation in the paternal pronucleus is therefore a mixture of active enzymatic conversion of 5mCs and passive, replication-coupled dilution of the oxidation products, avoiding any threat for genomic instability.[50] Moreover, a similar strategy for global DNA demethylation holds true for mouse PGC reprogramming. In contrast to what happens in the zygote, Tet1 and Tet2 are responsible for the conversion of a majority of 5mCs to 5hmCs in PGCs.[51]

The three-step oxidation of a methylated pyrimidine, as it is the case for Tet-mediated conversion of 5mC to 5caC, has a precedent. In the thymidine salvage pathway, thymine is converted to uracil, in other words, 5-methyluracil is demethylated. Beside a consecutive three-step oxidation to iso-orotate (5-carboxyluracil), the pathway includes a final decarboxylation to gain uracil.[52] In search for a parallel pathway for 5mC demethylation, a decarboxylation activity toward 5caC was indeed demonstrated in a cell-free system using nuclear extracts of mESCs.[53] The result opens up a new avenue for non-toxic DNA demethylation.

In conclusion, the mechanisms of active DNA demethylation in mammals not only tend to be highly multifaceted but also offer the promise of surprising us in future research.

Gadd45 Proteins are Regulators of DNA Demethylation

Knowledge about the core enzymes acting on 5mC and its derivatives is just one side of the coin. Non-enzymatic mediators of DNA demethylation shed light on the regulation of the process upstream. Growth arrest and DNA damage-inducible protein 45 a (Gadd45a) were shown to promote DNA demethylation by DNA repair.[27,54,55] Gadd45a is an 18 kDa acidic protein without obvious enzymatic activity. Gadd45a is located in the nucleus and associated with ribonucleoprotein speckles.[56] Together with Gadd45b and Gadd45g, it is part of a family of histone fold stress-response proteins that modulate diverse cellular processes, one of them being DNA repair.[57,58] Epigenetically, Gadd45 proteins act as scaffold proteins for downstream components of the repair machinery, thus

directly regulating DNA demethylation. Gadd45 seems to promote gene-specific demethylation only.[59-61] Typically, specific external stimuli lead to the upregulation of Gadd45 proteins, which, in turn, drive the demethylation and transcriptional activation of certain target genes. For instance, after electroconvulsive treatment of adult mice neurons Gadd45b is strongly upregulated leading to demethylation and expression of key genes for adult neurogenesis.[62]

Together with components of the NER machinery (see above) Gadd45a has been demonstrated to be required to demethylate reporter plasmids in *Xenopus* oocytes as well as the human rDNA and *RARβ2* promoter.[27,28,30] Gadd45 proteins also promote BER-mediated DNA demethylation, as has been first demonstrated for foreign methylated DNA and endogenous target genes in zebrafish embryos.[63] A T/G mismatch intermediate accompanied the demethylation, suggesting deamination of 5mC prior to BER.[63] GADD45a was also found to physically interact with AID and TDG in human cells.[64] The protein, thus, might regulate pivotal processes during development. However, in contrast to *Tdg*$^{-/-}$ mutants, mice lacking *Gadd45a* (but not simultaneously *Gadd45b* and *Gadd45g*) are viable and do not show significant alterations in global DNA methylation.[59] A potential involvement of Gadd45 in Tet-mediated DNA demethylation remains to be investigated (see below).

Ing1 Directs DNA Demethylation to H3K4me3

Gene-specific DNA demethylation primarily affects methylated CpGs within regulatory regions of limited lengths, whereas 5mCs within the gene body or in intergenic regions are often left untouched. This site specificity suggests a targeting mechanism that guides general demethylation factors to certain CpGs.

Mammalian DNA is wrapped around histone proteins organized in nucleosomes that consist of two H2A-H2B dimers and a H3-H4 tetramer each. Histone proteins are posttranslationally covalently modified by, e.g., methylation, acetylation or phosphorylation of distinct amino acids at the N-terminal tails, constituting the so-called histone code.[65] The histone code is an important epigenetic feature regulating gene expression and DNA repair, among other processes. Trimethylation of H3 lysine 4 (H3K4me3) is typically found at promoter regions of genes that are transcriptionally active.[66] Thus, the occurrence of H3K4me3 strikingly resembles hypomethylation of promoter regions after gene-specific DNA demethylation and, hence, gene activation. Are both epigenetic marks linked to each other to determine the transcriptional status of the corresponding gene? And, if yes, what is the cause and what is the effect?

The answers to those questions were provided recently by the identification of an H3K4me3 reader that directs Gadd45a and the demethylation machinery to their target 5mCs.[67] The protein inhibitor of growth 1 (human ING1b, mouse Ing1) is a known interactor of Gadd45a and exhibits similar properties as both are stress-response proteins influencing the cell cycle and promoting DNA repair.[68] Schäfer et al.[67] at first confirmed the physical interaction of GADD45a and ING1b in the human cell line HEK293T. They identified that ING1b cooperates with and is required for GADD45a to demethylate and reactivate methylation-silenced reporter plasmids. ING1b consists of an N-terminal PCNA interacting protein domain, a partial bromodomain and a C-terminal plant homeodomain (PHD finger), enabling binding to H3K4me3.[69] Importantly, the PHD finger domain was essential for GADD45a-mediated demethylation suggesting a link between the histone mark H3K4me3 and 5mC demethylation. Indeed, in RKO cells, the *MAGEB2* promoter, an endogenous target of GADD45a demethylation, was occupied with H3K4me3 marks; GADD45a and ING1b were bound to the same region simultaneously. MEFs lacking *Ing1* or *Gadd45a* had a reduced, double knockouts an abolished potential to induce expression of the *Mageb1–3* genes and demethylation of the *Mageb2* promoter upon UV-stimulation. Strikingly, knockdown of Wdr5, an essential subunit of the H3K4 methyltransferase complex MLL,[70] prevented *Mageb1–3* expression and *Mageb2* demethylation induced by either UV-irradiation or GADD45a and ING1b overexpression in wild-type MEFs. Moreover, loss of H3K4me3 marks impaired GADD45a and ING1b binding to the *Mageb2* locus. Global gene expression profiling of HEK293T cells overexpressing GADD45a, ING1b or both proteins as well as MEFs lacking *Gadd45a*, *Ing1* or both genes revealed roughly a hundred additional endogenous target genes in the human and mouse genome, respectively, potentially regulated by the same mechanism, as exemplified for the *Mage* genes above. Of note, this does not apply for all genes affected in their gene expression by Gadd45a and Ing1, since a comparable number of genes was, against expectations, also upregulated in double knockout MEFs. However, it should not be neglected that DNA demethylation is just one of many cellular processes influenced by Gadd45a and Ing1.

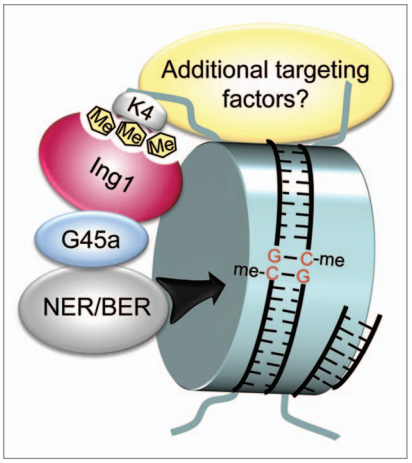

Figure 2. Ing1 directs Gadd45a and the demethylation machinery to H3K4me3. Proposed model for site-specific demethylation by Gadd45a. At a given gene promoter histone H3 trimethylated at lysine 4 (H3K4me3) is specifically recognized by Ing1. Gadd45a and the repair machinery are recruited through Ing1 binding. Subsequently, 5mCs are excised by DNA repair leading to DNA demethylation. Additional targeting factors may be required for the process. G45a, Gadd45a; NER, nucleotide excision repair; BER, base excision repair. Reproduced with permission from reference 67.

The study uncovered an unknown player in mammalian gene-specific DNA demethylation upstream of the enzymatic reactions on 5mC and links the histone code with DNA demethylation by two factors: Ing1, an H3K4me3 reader, and Gadd45a, a DNA demethylation regulator. The data favors a model by which H3K4me3 serves as a determinant

for DNA demethylation via Ing1 and Gadd45a (**Fig. 2**). Interestingly, in a pull-down assay of mESC nuclear extracts using modified oligonucleotides as bait, Ing1 was found to bind 5fC residues in vitro.[71] The result hints at an involvement of Ing1 in Tet-mediated DNA demethylation. In line with this, and as already stated, Gadd45a was shown to directly interact with TDG.[64] Thus, Ing1 might target Gadd45a/TDG to oxidized derivatives of 5mC. This assumption has to be clarified in upcoming studies. Additionally, future work has now to decipher the physiological relevance of the targeting process in development and disease, as well as to identify additional cofactors required for regulating demethylation. Finally, the chromatin context should be considered when analyzing the modus operandi of known or yet-to-be-identified demethylation factors.

Summary

The complex world of mammalian DNA demethylation emerges from studies identifying numerous enzymes acting on 5mC and its derivatives. Unequivocally, DNA repair mechanisms are involved in gene-specific DNA demethylation whereas cells presumably avoid hazardous intermediates of DNA repair in genome-wide erasure of methylation marks during development. Gadd45 proteins are regulators of gene-specific and repair-mediated demethylation in different contexts. Site-specific demethylation by Gadd45a is ensured by a targeting mechanism involving the histone mark H3K4me3 as determinant and the histone code reader Ing1 as transducer. The mechanism seems to be valid for the epigenetic regulation of a substantial amount of genes in humans and mice.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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