

Demethylation of (Cytosine-5-C-methyl) DNA and regulation of transcription in the epigenetic pathways of cancer development

Samir Kumar Patra · Aditi Patra · Federica Rizzi ·
Tapash Chandra Ghosh · Saverio Bettuzzi

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Abstract Cancer cells and tissues exhibit genome wide hypomethylation and regional hypermethylation. CpG-methylation of DNA (Me CpG-DNA) is defined as the formation of a C–C covalent bond between the 5'-C of cytosine and the –CH₃ group of *S*-adenosylmethionine. Removal of the sole –CH₃ group from the methylated cytosine of DNA is one of the many ways of DNA-demethylation, which contributes to activation of transcription. The mechanism of demethylation, the candidate enzyme(s) exhibiting direct demethylase activity and associated cofactors are not firmly established. Genome-wide hypomethylation can be obtained in several ways by inactivation of DNMT enzyme activity, including covalent trapping of DNMT by cytosine base analogues. Removal of

methyl layer could also be occurred by excision of the 5-methyl cytosine base by DNA glycosylases. The importance of truly chemically defined direct demethylation of intact DNA in regulation of gene expression, development, cell differentiation and transformation are discussed in this contribution.

Keywords Cancer-epigenetics · –CpG– islands · (Cytosine-5C-methyl)-DNA-demethylation · MBD2 · DNA-demethylase · Transcription

1 Introduction

In the post-genomic era of cancer biology, it is becoming increasingly evident that epigenetic controls of gene expression play an important role in determining the phenotype of cancer cells, possibly suggesting a novel approach and strategy for epigenetically restoring normal phenotype in cancer cells. Histone modifications and DNA methylation–demethylation events are central to the epigenetic regulations of development [1–7]. The mechanisms controlling these events and their dynamic changes have important implications in developmental cell biology as well as carcinogenesis and tumor progression. Very little of the mammalian genome (2–3%) contains five carbon–methyl cytosine (Me C). The paternal genome undergoes preferential demethylation after fertilization, while the maternal genome remains largely unaffected in the mouse, rat, pig, bovine, and human zygote [1–7]. Later, the maternal genome is passively demethylated by a replication-dependent mechanism after the two-cell embryo stage [7]. A main key to understand development is to unravel the mechanism of control of the expression pattern of genes regulating cell proliferation and differentiation in the embryo

S. K. Patra (✉) · A. Patra
Cancer Epigenetics Research,
Kalyani (B-7/183),
Nadia, 741235 West Bengal, India
e-mail: skpatra_99@yahoo.com

A. Patra
Venkateshwara Hatcheries,
KB21, Sector III,
Salt Lake City, Kolkata 700 098, India

F. Rizzi · S. Bettuzzi
Department of Medicina Sperimentale, University of Parma,
Parma, Italy

F. Rizzi · S. Bettuzzi
Istituto Nazionale Biostrutture e Biosistemi (INBB),
Rome, Italy

T. C. Ghosh
Bioinformatics Centre, Bose Institute,
P 1/12, C. I. T. Scheme VII M,
Kolkata, 700 054 West Bengal, India

under the influence of many other epigenetic/environmental factors. Epigenetic/environmental factors may contribute signals to cells and tissues that can trigger completion of development of the whole body as final biological response [8], and deregulated signals may cause tumour development [9]. Hence, development requires constant negotiation with the environment. Studies in this field have not advanced so much, probably due to the difficulty of monitoring individual cells and cell-cell or cell-extracellular matrix interaction within the same organ *in vivo* [8–10]. Many decades ago (1957), C. H. Waddington suggested the idea of “epigenesis and development” [11], and Hotchkiss discovered the 5-carbon methylated cytosine ($^{\text{Me}}\text{C}$) from DNA and named it as epicytosine in 1949 ([12], reviewed in [13]).

The enzyme DNA methyltransferase (DNMT) has received convincing confirmation to be responsible for methylating DNA on cytosine at 5-carbon position, for creating additional layer of epigenetic information post-replicatively. The mechanism of action of DNMT has been characterized (reviewed in [13]). Addition of methyl groups to cytosines within the CpG-dinucleotide by DNMT is also implicated in regulating transcription, maintaining genome stability, imprinting, and X-chromosome inactivation [1–5, 13–16]. In mammalian cells, DNA-methylation is catalyzed by two important classes of DNMTs [14, 15]. DNMT1 (EC 2.1.1.37) resides at the replication fork and methylate CpG-dinucleotides in the newly synthesized strand. DNMT1 is essential for maintaining DNA-methylation patterns in proliferating cells [1–5, 13, 14]. Two members of the second class of methyltransferases, DNMT3a and DNMT3b are required for *de novo* methylation during embryonic development. DNMT3L cooperates with the DNMT3 family to establish maternal imprints in mice [4, 5, 14, 15]. However, the mechanism for direct removal of the methyl group(s), keeping the DNA strand intact is obscure, and the candidate protein, MBD2b, possibly responsible for that activity, is controversial [13–26]. Obviously, there are other possible modalities of achieving active demethylation, but these require DNA strand break and/or nucleoside excision repair of DNA [27–32]. Overexpression of DNMTs and hypermethylation of –CpG– islands at the regulatory regions of certain genes, global hypomethylation, as well as selective demethylation, are now well known to participate for regulation of transcription in close association with crucial biological events, including cancer development [15–17, 32–34]. The –CpG– sites present in specific regions of the promoter of these highly expressed genes are rarely methylated, or perhaps are very quickly demethylated in normal cells with the important exceptions of inactivated X-chromosome and imprinted genes. Recent data have shown that methylation of DNA and deacetylation of histones H3 and H4 leads to inactivation/repression, while selective acetylation of histones H1, H3, H4, methylation of

H3 K4, and DNA demethylation are associated with activation of nucleosomes and gene transcription. Repression of transcription may occur through different mechanisms, including recruitment of proteins (co-repressors) that prevents transcription factors to bind DNA regulatory elements [33–40]. Some methylated DNA binding proteins (MeCP2, MBD1, MBD2, MBD3 and MBD4) selectively bind –CpG– and/or methylated –CpG– sequences, contributing to remodeling of nucleosomes and chromatin structure. Under these conditions, chromatin would be inactivated as a consequence of histones deacetylation caused by recruitment of histone deacetylases (HDACs). These events precede and inhibit binding of transcription factors, including RNA polymerase [15–17, 24, 25, 33–41]. DNA demethylation causes a reduction of the repression potential of a gene. Because MBD proteins no longer remain bound to the respective demethylated CpG-regions, the activation energy required for nucleosomes to be promoted to the transition state complex formation will be lowered, by binding of co-activators and transcription factors. This event would allow RNA polymerase to enter and bind promoter for initiation of transcription. MBD2 and MBD4 were shown to cause DNA-demethylation through different mechanisms. It has been suggested that MBD2, probably together with other unknown proteins and cofactors, would be responsible for removal of the –CH₃ group from methylated cytosine ($^{\text{Me}}\text{C}$). MBD4 was originally isolated as a G/T mismatch glycosylase and is particularly active on $^{\text{Me}}\text{C}$ -deamination of the $^{\text{Me}}\text{CpG}$ dinucleotide. This enzyme is believed to remove the whole base (5C-methylated Cytosine), creating an abasic site which can be sealed with the help of mismatch/base/nucleotide excision repair enzymes and secondary ligases [27, 28, reviewed in ref. 30]. It is now clear that abnormal methylation, genome wide hypomethylation and site-selected hypermethylation of –CpG– islands are not restricted to cultured cells, but can also occur during ageing and tumor development. The specific mechanisms involving DNA-binding proteins, enzymes, co-repressors and co-activators participating to recruitment of an active transcriptional complex through interaction with specific response elements present in promoters are now only partially understood [15–22, 27–46].

During the early work on epigenetic control mechanisms of genes there were two main hypotheses concerning methylation/demethylation of DNA and its biological significance: the first hypothesis was that—the cytosine-5C–CH₃ covalent bond formation is an unusual event that may even be irreversible (reviewed in [13, 14]). An alternative hypothesis was suggesting the dynamic nature for these events in tissue specific gene expression. It is now clear that indeed there are enzymes that directly methylate and demethylate DNA, but the demethylases require further studies by purification and reconstitution (reviewed in [13,

[14, 30, 33]). Recent data on active demethylation and its role on development, as well as in cancer progression, have raised a considerable growth of interest in this field [6, 7, 14–23, 27–30, 32, 45–48]. The understanding of processes such as replication-coupled loss, local demethylation triggered by transcription factors, RNA mediated catalysis and DNA repair based mechanisms is in progress. At the beginning of the science of epigenetic DNA methylation, it was assumed that methylation may be a biochemical strategy positively selected by evolution for safe guard of microbial DNA from endonuclease digestion [49]. Now, we have many more useful information concerning chromatin remodeling and chemical modifications of DNA and histones that have greatly improved our understanding of preferential gene expression and its importance in biological processes such as differentiation of totipotent cells [1, 2, 6, 7, 32, 47].

The presence of higher levels of regional CpG-island methylation, as well as the fact that increased rate of mutation due to global genomic hypomethylation correlates with tumor progression, made us clear that precise genetic alterations over time are a major driving force for neoplastic onset and progression for virtually every cancer type. But fortunately, mutation of specific genes is an inefficient process, because of the unceasing maintenance of genomic integrity by a complex array of enzymes continuously monitoring and repairing DNA. Thus, genome maintenance teams (made by many protein components working as active complexes) strive to ensure that DNA sequence information remains unchanged [32, 40, 49, 50]. We have now reached to a critical mass of information that will be very helpful for a better understanding of the chemical mechanism and the complexity of direct demethylation of intact DNA. This review is devoted to address the current status, and to discuss the possibility of future advancement on the specific issue of direct ($^{Me}C-5$) DNA demethylation, i.e. active demethylation without breaking the DNA strand, and roles of DNA-demethylation in development and cancer.

2 DNA methylation and chromatin structure

Nucleosome is the basic building block and the smallest functional unit of chromatin [51, 52]. Nucleosome core comprises 147 bp of DNA wrapped 1.59 to 1.7 times around the histone octamer that is made of two copies of each histone type (H2A, H2B, H3 and H4) in a left-handed super helix. Nucleosomes are connected by 10–60 bp of linker DNA that may be naked or in association with histone H1 (H5), which protect an additional ~20 bp of DNA from nuclease digestion at the core particle boundary. Each core histone contains two functional domains: one

histone-fold motif for histone–histone and histone–DNA contacts within the nucleosome, and the domain containing sites for posttranslational modifications (such as acetylation, methylation, phosphorylation and ubiquitination) with NH₂- and COOH-terminals. Although not related to the core histones, linker histone H1 also contain a globular domain flanked by NH₂- terminal tail sub-domains. Globular domain of linker histones is essential for binding to nucleosome. The tail domains are probably important for higher order chromatin folding [51–53].

2.1 Structural changes are primarily imposed at the DNA level

Cytosine methylation of the DNA sequence d(GGCGCC)₂ was shown to cause an extended eccentric double helix structure called E-DNA. Like B-DNA, E-DNA has a long helical rise and the base is perpendicular to the helix axis [53]. The 3'-*endo* sugar conformation origins the characteristic deep major groove and shallow minor groove of A-DNA. Also, if allowed to crystallize for a longer period of time with respect to that yielding E-DNA, the same methylated sequence originates standard A-DNA, suggesting that E-DNA is a kinetically trapped intermediate in the transition to A-DNA. Thus, the structures presented on Vargason et al. [54] report chart emphasize a crystallographic pathway from B-DNA to A-DNA through the E-DNA intermediate in a single sequence. The E-DNA surface is highly accessible to solvent, with water molecules interacting with the exposed surfaces of the stacked nucleotides in the major groove. It was suggested that the geometry of such interactions would promote the spontaneous deamination of ^{Me}C in the transition mutation of d(5- ^{Me}CpG) to d(TpA) base pairs [54], without requiring any deaminase activity [31].

The analysis of the hydration pattern around methylated CpG sites in crystal structures of A-DNA decamers in three high resolutions (1.7, 2.15 and 2.2 Å) reveals that the methyl groups of cytosine residues are well hydrated [55]. A comparative study of the native structure of two structurally distinct forms of the decamer d(CCGCCGG CGG), fully methylated at its CpG sites, showed that, under specific structural and sequence contexts, the methylated cytosine base is more hydrated than the unmodified one. These water molecules seem to be stabilized in front of the methyl group through the formation of C–H••O type of interactions. In addition, these structures provide the first observation of magnesium cations bound to the major groove of A-DNA, revealing two distinct modes of metal binding in methylated and native duplexes. These findings suggested that methylated cytosine bases could be recognized by protein on DNA polar residues through their tightly bound water molecules [55].

2.2 Structure at the level of nucleosome

DNA methylation inhibits gene expression in animal cells probably by affecting chromatin structure, since the presence of methyl groups on DNA affect the interaction of other proteins and enzymes with local nucleosomes [56, 57]. Methylation of DNA brings about a general deacetylation of histones H3 and H4 [17, 24, 25, 33, 34, 37, 39, 52, 53, 57], prevents methylation at H3 K4, and induce methylation of H3 K9 [56–60]. Histone H3 K4-methylation is associated with transcriptionally active nucleosomes of chromatin [58–65] in which K4 of H3 are tri-methylated [62–64]. Methylation of histones has been regarded as a stable modification defining the epigenetic program of the cell, which regulates chromatin structure and transcription. However, the recent discovery of histone demethylases has challenged the stable nature of histone methylation [42, 43, 65]. Christensen et al. have demonstrated that the JARID1 proteins RBP2, PLU1, and SMCX are histone demethylases specific for di- and trimethylated histone H3 K4. Shi et al. [42] have shown that LSD1 represses transcription through demethylation of H3 K4. Using patch-methylated stable episomes with methylated DNA patches in the promoter, in the luciferase reporter gene, Hsieh and colleagues have recently demonstrated how DNA methylations affect chromatin structure and gene expression [58, 59]. They observed that acetylated histones are associated with unmethylated DNA and are nearly absent from the methylated DNA regions. DNA methylation immediately downstream from the transcription start site has a dramatic impact on transcription, showing a more important effect on transcription elongation than initiation. Hsieh and colleagues also showed that dimethylated histone H3 at K4 (H3K4Me2) is depleted from regions with DNA methylation and that this effect is not linked to the transcriptional activity in the region. This is a local effect that does not extend more than 200 bp from the methylated DNA regions. Although depleted primarily from the methylated DNA regions, the presence of trimethylated histone H3 at K4 (H3K4Me3) may be affected by transcriptional activity as well. Their data suggested that DNA methylation at the junction of transcription initiation and elongation is most critical in transcription suppression and that this effect is mechanistically mediated through chromatin structure [58]. While some important implications were suggested in other studies [57, 60–64], the impact of the methylated DNA segments on transcription is difficult to interpret because the size and location of the methylated DNA region may differently affect transcription. While a methylated coding region has little impact on transcription initiation when it is about 1 kb downstream from the promoter, as observed by Lorincz et al. [66], it is conceivable that the same methylated sequence might have a much larger impact on

transcription initiation if it was immediately downstream from the promoter [58, 66, 67]. Okitsu and Hsieh [58] have observed a tight correlation between the depletion of H3K4Me2 in the regions of DNA methylation, regardless of the reporter gene activity. Conversely, the level of H3K4Me2 remains high in the unmethylated DNA regions regardless of the presence of Pol II. Furthermore, DNA methylation approximately 200 bp downstream does not affect the presence of H3K4Me2 in the unmethylated promoter on pMeLuc and pMe5'Luc. This distinct boundary effect strongly supports the view that DNA methylation affects the presence of H3K4Me2 directly [57, 58]. They proposed that DNA methylation dictates a closed chromatin structure that is devoid of H3K4Me2 and inhibits transcription, and that the presence of H3K4me2 marks an open chromatin structure that would permit transcription if all other conditions for active transcription are fulfilled [58]. In early development, genomic methylation is erased and the somatic methylation pattern is re-established at the time of implantation [1–7, 27–30, 32, 47, 57]. The most of our experimental information are from *in vitro* studies in which DNA containing short ^{Me}CpG-sequence is compared with unmodified CpG-sequence. Therefore, the effects of methyl layer on individual nucleosome and overall nuclear chromatin structure have to be inferred from those *in vitro* observations.

The initiation of DNA-dependent nuclear processes is highly dependent on chromatin structure. This implies that nucleosome position is biased by the DNA sequence to facilitate access to initiation factors. Activation of specialized domains by removal of loosely associated mobile proteins, like HMG, HP and H1, in conjunction with insulator action, regulates the expression of independent genes. It is now well known that special boundaries are present for regulation of chromatin domains. Boundary elements/insulators act to subdivide eukaryotic chromosomes into autonomous regulatory domains. Such boundaries represent structural/functional barriers preventing the processive spreading of “active” or “silenced” chromatin between domains. In addition, the partitioning into autonomous functional units is a consequence of an underlying structural subdivision of the chromosome into higher order “looped” domains. In this view, boundaries are thought to delimit structural domains by interacting with each other or with other nuclear structures. The insulators are DNA sequences that do not exert their action unless bound to form complexes with specific protein partners [68–74].

3 Cytosine 5 C-methyl-DNA demethylase and the demethylation reactions

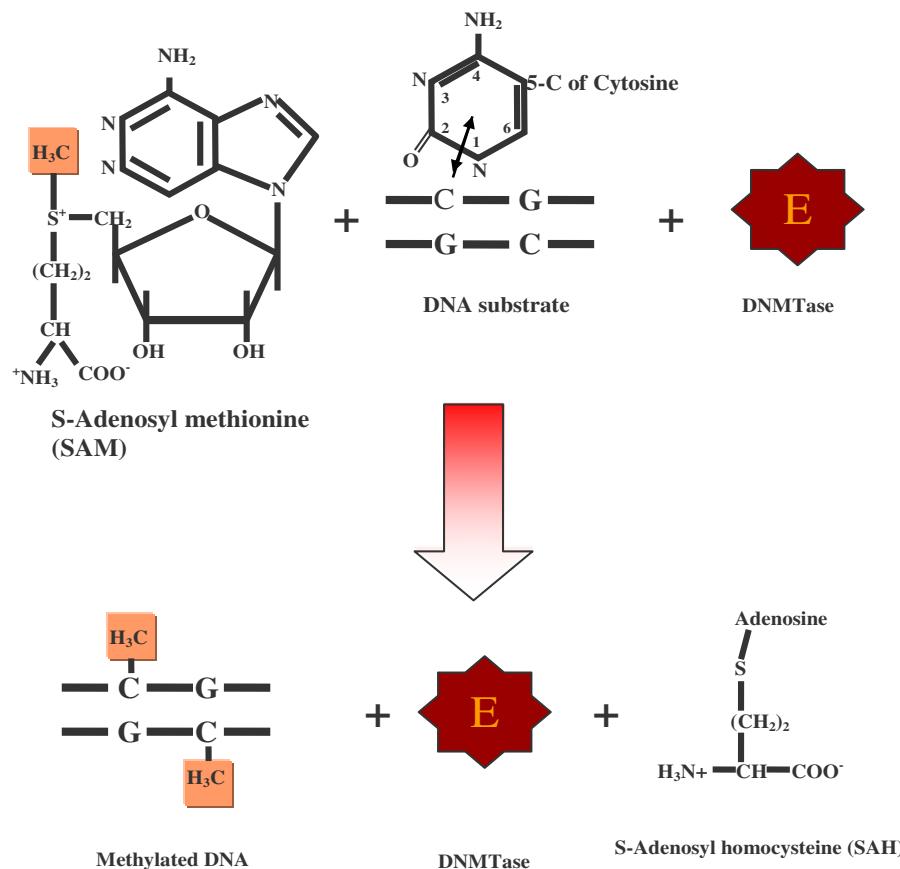
In biochemical terms, DNA cytosine-5-carbon methylation is the post-replicative addition of a methyl group to

cytosine at $-CpG$ -dinucleotide sequences of DNA by DNMTs, where *S*-adenosylmethionine (SAM) is the donor of methyl group (Fig. 1(a, b)). Figure 1(a) shows how DNA methyltransferases (DNMT), 1, 3a and 3b catalytically removes the $-CH_3$ group (square block) from *S*-Adenosyl methionine, and puts it at the 5-carbon of cytosine at $-CpG-$ sequences. SAM changes to *S*-Adenosyl homocysteine, SAH (for further details see, [14–16, 75, 76]). Figure 1b shows the detailed mechanism for mammalian DNMT3a mediated catalysis, as proposed by Reither et al. [75]. ^{Me}C -DNA demethylation (Fig. 2(a)) is the removal of such methyl group from DNA [15–30, 77]. Removal of methyl group (or

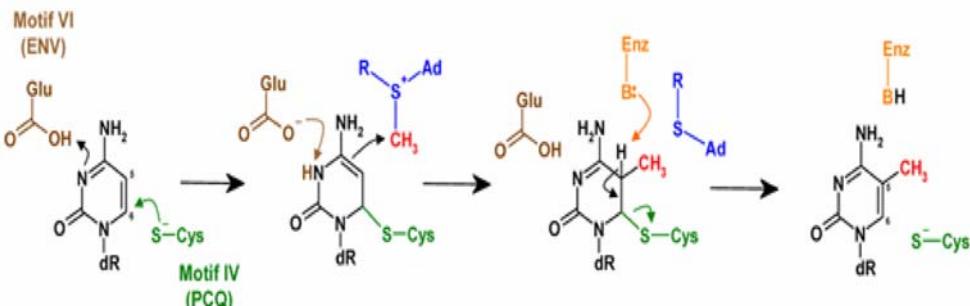
Fig. 1 (a) DNA methyltransferases (DNMT), 1, 3a and 3b catalytically removes the $-CH_3$ group (square block) from *S*-Adenosyl methionine and puts it at the 5-C (arrow pointing up) of Cytosine at $-CpG-$ sequences. SAM changes to *S*-Adenosyl homocysteine, SAH, while the enzyme remains intact after the reaction (See also [14, 15, 76, 110–112, 114] for more details). (b) Detailed mechanism as proposed by Reither et al. [75] for mammalian DNMT3a mediated catalysis, see [75] for further details

demethylation) is essential for transcription of many genes, which could be obtained following three possible paths: (1) Direct active process—demethylation, (2) Indirect active process—mismatch/excision repair, (3) Passive loss of the methyl group due to failure of maintenance of methylation after replication or mechanical inhibition/DNMT-trapping by 5-Aza-2'-deoxycytidine (AzdC) incorporated into DNA in cultured cells. MBD2 itself does not demethylate DNA *in vitro*. In some *in vivo* systems MBD2 represses or activates transcriptions of genes by binding to $-CpG-$ sequences. Indirect removal of $-CH_3$ could occur by removal of whole cytosine base. Blocking the DNMT enzyme activity

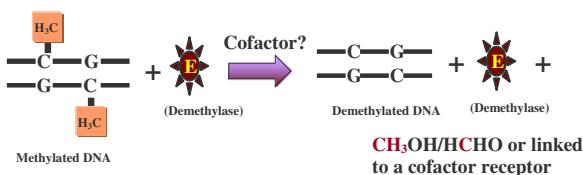
(a) The DNA (cytosine-5-carbon) methylation reaction



(b)

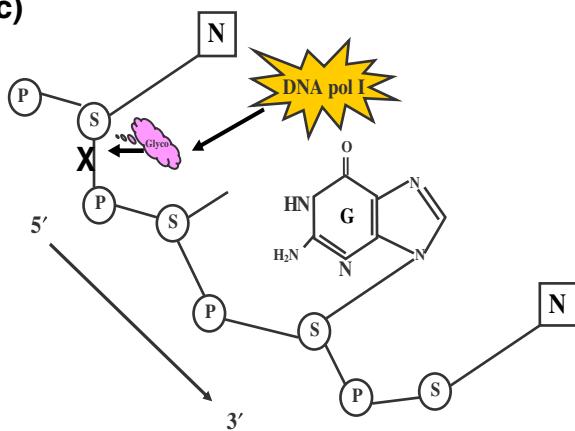


(a) The DNA (5-methyl cytosine) demethylation reaction

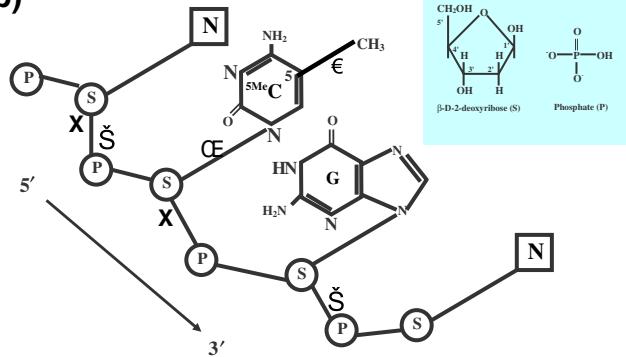


1. Direct active process: demethylation
2. Indirect active process: mismatch /excision repair
3. Passive loss of methyl group: failure of maintenance of methylation status after replication or DNMT-trapping by 5-Aza-2'-deoxycytidine incorporated into DNA.

(c)



(b)



(d)

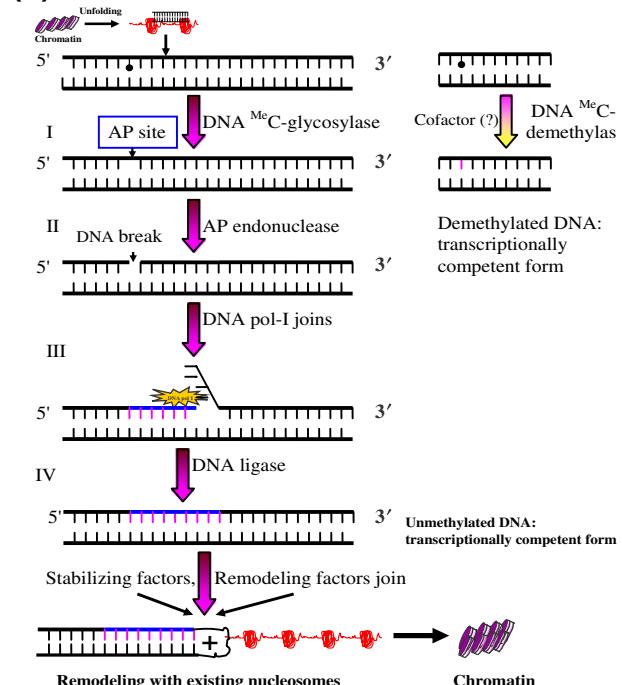


Fig. 2 (a) DNA demethylase protein(s) or complexes of biomolecules and cofactor(s) are yet to be discovered. MBD2 itself does not demethylate DNA *in vitro*. In some *in vivo* systems it represses or activates transcriptions of genes by binding to -CpG- sequences. Indirect removal of -CH₃ could occur by removal of whole cytosine base. Blocking the DNMT enzyme activity could artificially create hypomethylation status. But these types 2 and 3 are not true demethylation reactions. An enzymatic removal of -CH₃ group from 5-carbon of cytosine in the context of -CpG- island containing promoters is necessary for transcriptional activation of many genes. The fate of the expelled -CH₃ group would be in the form of CH₃OH or HCHO, or most likely trapped by scavengers or taken by a cofactors (See [15, 16, 18, 19, 21, 22, 24, 25, 30, 33, 77]). (b) Possible levels of action for the removal of methyl group(s): (1) Methyl excision by demethylase (E), (2) 5-methylcytosine base excision (E), (3) ^{Me}Cp-mononucleotide excision (X), and (4) ^{Me}CpG-dinucleotide excision (S). Inset shows the structures of 2-deoxyribose sugar and phosphate group. (c) The DNA glycosylase break the β-N glycosidic bond to create an AP site. AP endonuclease recognizes this site and nicks the damaged DNA on the 5' side (upstream) of the AP site creating a free 3'-OH. DNA polymerase, Pol I, extends the DNA from the free 3'-OH using its exonuclease activity to replace the nucleotide of the damaged base, as well as a few downstream, followed by sealing of the new DNA strand by DNA ligase activity (see the steps in d). (d) Schematic representation of DNA glycosylase mediated removal of methyl layer from gene needs to be activated (left panel), an atypical BER/NER pathway; demethylation of DNA by direct excision of the methyl group (right panel). When signals are transmitted to the nuclear chromatin, the specific nucleosomes (of the respective genes) become activated. Step I: attack by a glycosylase specific for ^{Me}C, ●, (for example, TDG and MBD4 as proposed by Jost et al.), which breaks the β-N glycosidic bond to create an AP site. Step II: AP endonuclease recognizes this site and nicks the damaged DNA on the 5' side (upstream) of the AP site creating a free 3'-OH. Step II may be the first step for ^{Me}Cp-mononucleotide, and ^{Me}CpG-dinucleotide excision repair (NER) based mechanisms specifically for hemimethylated DNA, as proposed by Weiss et al. [84], who suggested the involvement of an enzyme and small RNA. Step III: DNA polymerase, Pol I, extends the DNA from the free 3'-OH using its exonuclease activity to replace the nucleotide of the damaged base, as well as a few downstream, followed by sealing of the new DNA strand by DNA ligase (Step IV). Finally, chromatin remodeling factors fold the DNA into its normal nucleosome fold, which may further be stabilized by stabilizing factors. Kress et al. [27] suggested a mechanism, which involves ^{Me}C mononucleotide or ^{Me}CpG-dinucleotide excision from the Tat gene which then follow the common steps of the NER/BER pathway—Step III onwards. One step direct demethylation of intact DNA (Right panel)

the steps in d). (d) Schematic representation of DNA glycosylase mediated removal of methyl layer from gene needs to be activated (left panel), an atypical BER/NER pathway; demethylation of DNA by direct excision of the methyl group (right panel). When signals are transmitted to the nuclear chromatin, the specific nucleosomes (of the respective genes) become activated. Step I: attack by a glycosylase specific for ^{Me}C, ●, (for example, TDG and MBD4 as proposed by Jost et al.), which breaks the β-N glycosidic bond to create an AP site. Step II: AP endonuclease recognizes this site and nicks the damaged DNA on the 5' side (upstream) of the AP site creating a free 3'-OH. Step II may be the first step for ^{Me}Cp-mononucleotide, and ^{Me}CpG-dinucleotide excision repair (NER) based mechanisms specifically for hemimethylated DNA, as proposed by Weiss et al. [84], who suggested the involvement of an enzyme and small RNA. Step III: DNA polymerase, Pol I, extends the DNA from the free 3'-OH using its exonuclease activity to replace the nucleotide of the damaged base, as well as a few downstream, followed by sealing of the new DNA strand by DNA ligase (Step IV). Finally, chromatin remodeling factors fold the DNA into its normal nucleosome fold, which may further be stabilized by stabilizing factors. Kress et al. [27] suggested a mechanism, which involves ^{Me}C mononucleotide or ^{Me}CpG-dinucleotide excision from the Tat gene which then follow the common steps of the NER/BER pathway—Step III onwards. One step direct demethylation of intact DNA (Right panel)

could artificially create hypomethylation status. But these types 2 and 3 are demethylation reactions that are not completely chemically defined. An enzymatic removal of $-CH_3$ group from 5-carbon of cytosine in the context of $-CpG-$ island containing promoters is necessary for transcriptional activation of many genes. The fate of the expelled $-CH_3$ group would be in the form of CH_3OH or $HCHO$, or most likely trapped by scavengers or taken by cofactors (See [13–15, 30, 33]). The hypothesized mechanism of demethylation with MBD2 protein requires a cofactor, which is not known to date. The proposed mechanism [18, 77] forms a cytosine Michael adduct [13, 14, 76]. The prevailing view on Michael adduct chemistry is that in these types of complexes SN2 mechanism would not occur. In general, water added across the 5C–4C double bond with the hydroxyl group attacking carbon-4, followed by elimination of ammonia, will yield thymidine. As of yet to think in terms of SN2 mechanism without the involvement of cofactors, the leaving group is the activated cytosine ring complexes with the enzyme nucleophile and not the methanol (reviewed in [13, 30, 76]). Alternative assumption is that cofactors, such as P450 monooxygenase, may mediate attack by molecular oxygen in presence of NADPH, converting the methyl group to a hydroxymethyl group and then to a formyl group, which would be expelled as formic acid from the intermediate 5-formyl cytosine, but experimental evidences of this type of reaction-based hypothesis are lacking. Another possible way looks very similar to Michael Retrogression type DNA demethylation for the removal of methyl group from methyl cytosine, keeping the DNA strand intact. We have been working on DNA methylation and epigenetics for years in search of DNA-demethylase and associated factors, including investigations with DNMTs and MBD proteins [9, 15–17, 33]. Preliminary data have convinced us that purified MBD2 itself is unable to demethylate ^{Me}C -5-DNA, ^{Me}C -5-poly-d(CpG)-, or ^{Me}I -5 poly-d(IpG)-duplexes; only the crude tumor or cellular extracts/fractions containing MBD2, together with some (but not all) elution fractions containing MBD2, showed demethylase activity *in vitro* on fully ^{Me}C -5-DNA substrates [15–17, 33]. There could be a possibility that the demethylation machinery is a huge protein complex, while MBD2 only function as a chaperon-component. Although it is apparent from the works of others that MBD2 may not be a demethylase, the inertness of some fractions rich in MBD2 to demethylation reaction [24, 25] can be argued as there might be different sets of MBD2 inhibitor in cells [78, 79].

3.1 Indirect active mechanisms accounting the loss of methyl groups/layers from DNA

Nucleotide excision repair and base excision repair (NER and BER) are cellular mechanisms, which can repair

damaged DNA during DNA replication. Repairing DNA sequence errors is necessary so that mutations are not conserved during replication. Single bases in DNA can be chemically mutated, for example by deamination or alkylation, resulting in incorrect base-pairing and mutations in the DNA. Of all repair systems, NER is the most versatile in terms of damage recognition. Two NER-sub pathways exist with partly distinct substrate specificity: global genome NER surveys the entire genome for distorting injuries, and transcription-coupled repair (TCR) focuses on damage that blocks elongating RNA polymerases (For a detailed review see [50]). BER involves flipping of the mutated base out of the DNA helix and repairing the base alone [50, 80]. There are two main enzymes involved in BER pathway: DNA glycosylases and apurinic/apyrimidinic (AP) endonucleases. The DNA glycosylase is used to break the β -N glycosidic bond to create an AP site. AP endonuclease recognizes this site and nicks the damaged DNA on the 5' side (upstream) of the AP site creating a free 3'-OH (compare Fig. 2(b, c and d)). DNA polymerase, Pol I, extends the DNA from the free 3'-OH using its exonuclease activity to replace the nucleotide of the damaged base, as well as a few downstream bases, followed by sealing of the new DNA strand by DNA ligase (Fig. 2(d), left panel). DNA glycosylases are typically low molecular weight (200–300 aa) monomeric enzymes responsible for recognizing base lesions in the genome and initiating the DNA BER pathway [30, 50, 81].

The proposed active mechanisms for ^{Me}C DNA demethylation, other than the excision of the $-CH_3$ group alone, actually damages the DNA and cell has to pay a price for recruiting costly DNA repair team (Fig. 2(b, c and d)). Hence, removal of the $-CH_3$ group along with the total ^{Me}C -base, total ^{Me}C -nucleotide or the ^{Me}CpG dinucleotide is obviously active, but it can't be conceived as a really chemically defined DNA-demethylation. Also, in terms of cellular economy BER/NER reactions may not account for genome wide loss of methyl groups, but certainly it is observed in cellular systems. Cells may use these repair processes as buffer, as additional localized demethylation pathways for gene transcription [27, 29, 30]. Moreover, if the cellular physiology and chemistry normally permits removal of ^{Me}C -base by BER/NER pathway, then the genome should not be so heavily methylated. This rationale suggests that other factors are certainly responsible for reversible regulation of DNA methylation. Possible levels of action for the removal of methyl group(s) as shown in Fig. 2(b) are: (1) Methyl excision by demethylase (ϵ), (2) 5-methylcytosine (^{Me}C) base excision (ϵE), (3) $-^{Me}Cp$ -mononucleotide excision (X), and (4) $-^{Me}CpG$ -dinucleotide excision (\dot{S}). Single ^{Me}C base excision and ^{Me}C mononucleotide or ^{Me}CpG -dinucleotide excision from the genome are followed by BER. The first of the DNA BER repair

processes call for a role for DNA glycosylases, which cleave the bond between the ^{Me}C base and the deoxyribose moiety in DNA (Fig. 2(c, d) Step I). The abasic site is then repaired by resident BER repair activity resulting in replacement of the ^{Me}C with a cytosine [82, 83]. According to the second mechanism, the ^{Me}C -mononucleotide is removed by nucleotide excision, which directly enters into the Step II of Fig. 2(d) and is then replaced by a cytosine [79, 83] (Fig. 2(b), possibility (3)), possibly mimicking NER pathway [50]. Using extracts from tissue culture cells, Weiss et al. [84] observed an *in vitro* loss of methyl group through excision of the ^{Me}CpG -dinucleotide (Fig. 2(b), (4)). Several experimental observations supported their suggestion that the reaction was mediated through the participation of RNA molecules. This proposed active role of RNA in the nucleotide excision repair reaction was later re-evaluated [85].

Two mismatch-repair glycosylases, the G/T mismatch repair enzyme [86, 87] and the methylated binding protein MBD4 [87] were shown *in vitro* to possess ^{Me}C DNA-glycosylase (5-Mcdg) activity. Chicken embryo nuclear extracts that can promote active loss of 5C-methyl group associated with the cytosine base were used by Jost et al. [83, 87, 89] to purify an enzyme. The enzyme is a DNA glycosylase that acts preferentially on hemimethylated CpGs and initiates removal of the methyl-base by breaking the glycosidic bond of ^{Me}C , thus leaving an abasic site that can be further processed by an AP-endonuclease and other DNA repair enzymes, which would finally follow BER pathway (Fig. 2(b, c and d)). Cloning and characterization of the enzyme proved that the corresponding gene encodes a G/T mismatch repair DNA glycosylase [86]. MBD4, a human homolog of the chicken enzyme, also has ^{Me}C DNA glycosylase activity [86]. The authors also reported evidences that RNAs and an RNA helicase, p68, are components of the enzyme complex and are involved in the demethylation activity [87, 90]. Overexpression of a human 5-Mcdg in human embryonic kidney cells led to demethylation of the promoter of a hormone-regulated reporter gene [88]. The specific demethylation of the transgene promoter but not genome-wide demethylation was attributed to the physical association of the 5-Mcdg with retinoid X receptor that has binding sites in the transgene promoter [88]. A major concern about the function of MBD4 and other G/T mismatch repair DNA glycosylase as demethylases is that the activities of these enzymes towards 5-methylcytosine DNA substrates are very weak compared to their activities towards G/T mismatch DNA substrates [86, 87]. Thus, it would be possible that these enzymes have strong ^{Me}C DNA glycosylase activity *in vivo*, possibly requiring other cofactors. In support of a DNA glycosylase mechanism of active loss of methyl group, Vairapandi et al., independently,

also found that HeLa nuclear extracts can cause loss of methyl group of DNA through a DNA glycosylase mechanism. They partially purified this enzyme activity [91, 92]. However, the identity of this protein has not been identified to date.

Kress et al. [27] have recently added one more example of BER/NER mediated mechanism of local targeted DNA demethylation in living cells. They discovered an enhancer-specific demethylation event at an endogenous gene, triggered by a transcriptional activator during development. They analyzed the demethylation occurring at a glucocorticoid-responsive unit (GRU) located 2.5 kb upstream of the transcription start site of the tyrosine aminotransferase (Tat) gene upon activation by the glucocorticoid receptor. The event took place in the liver before birth and was involved in the memorization of the first stimulation of the gene by glucocorticoids [29]. They demonstrated that demethylation of cytosines at this gene under physiological conditions results from an active mechanism that involves the creation of nicks in the DNA 3' to the ^{Me}C nucleotide. This event indicates the involvement of a mechanism producing DNA strand breaks, and suggests that the methylated base is processed by a base lesion repair pathway. Nucleotide excision repair of the ^{Me}C would be compatible with the cleavage observed, but there is no evidence for such a mechanism, because the only demethylating nuclease activity reported before excises the dinucleotide ^{Me}CpG [84]. Some key experimental data using Ligation-mediated-PCR techniques suggested that both mono- and bi-functional DNA glycosylases are compatible with the cleavage products. Overall, the data produced by Kress et al. [27] are convincing and suggest a mechanism involving ^{Me}C mononucleotide or ^{Me}CpG -dinucleotide excision from the Tat gene which then follows the common steps of the NER/BER pathway (compare Fig. 2(b, c and d)).

3.2 Passive loss of the methyl groups/layer from the genome

^{Me}CpG dinucleotides display a symmetrical configuration that allows conservation of the methylation pattern following replication. The replication machinery incorporates unmethylated cytosine in the newly synthesized strand. The resulting hemimethylated sites are converted back to fully methylated sites by the maintenance methyltransferase DNMT1 that acts preferentially on hemimethylated CpGs. DNMT1 acts soon after replication because the enzyme is targeted to replication foci through its interaction with the DNA polymerase clamp PCNA [27, 92, 93]. The observed non-methylated-CpG, and genome wide hypomethylation could simply result from an absence of maintenance methylation after replication, which is known as passive

demethylation (reviewed and discussed in [30, 32, 81, 94, 95]). This would be a passive mechanism, since it does not involve any demethylating enzyme. The passive loss must take place during several DNA replication cycles to ensure that a significant part of the daughter molecules are effectively demethylated: three rounds of replication demethylate only 87.5% of the CpG. This statistics is consistent with the kinetics of demethylation of the maternal genome that is observed during early mouse development [84], and that are observed for the local glucocorticoid-regulated demethylation of the *Tat* enhancer [27]. More on the genome wide passive loss of methyl group is available in past and recent reviews and articles [30, 81].

4 Many facets of the methyl-CpG-binding protein—MBD2

The discovery of MBD2 gene can be envisaged as an important landmark in the science of epigenetics in development and cancer. This is not only a ^{Me}CpG-sequence containing genes suppressor, but it also binds to unmethylated promoter CpG-sequences to act as an activator. Fujita et al. [26] have investigated the regulation of transcriptional repression on methylated cyclic AMP (cAMP)-responsive element (CRE). They found that MBD2a [25], but not MBD2b [18], promoted activation of the unmethylated cAMP-responsive genes. An *in vivo* binding assay revealed that MBD2a selectively interacted with RNA helicase A (RHA), a component of CREB transcriptional coactivator complexes. MBD2a and RHA cooperatively enhanced CREB-dependent gene expression. Co-immunoprecipitation assays demonstrated that MBD2a binding to RHA was not associated with HDAC1 [17, 26]. These results indicate a novel role for MBD2a in gene regulation. The cloning, mutational as well as conditional knock out and covalent trapping of DNMT1 showed that there are various regulatory mechanisms acting on this enzyme that is fundamental for embryonic development [48] and induction of cell transformation [96]. The presence of demethylation activity was elegantly showed by Gjerset and Martin [97] several years ago and by Patra et al. [15, 16]; however, the active protein and other cofactor was unknown. Recently, it has been showed that MBD2b is associated with demethylase activity ([13, 15, 18, 19, 21, 22], discussed in [27, 30]).

Many laboratories studied the demethylase activity of MBD2 [24–26, 31, 98–100]. Two research groups [20, 23] have shown, by transfection of the MBD2b construct [18], that genes like uPA (urokinase type plasminogen activator) and hexokinase type II are activated by demethylation of hypermethylated promoters leading to overexpression of their protein products. Recently, Jacob et al. [98] have

confirmed predominant association of MBD2 with the endogenous methylated rRNA promoter by MBD2b transfection and chromatin immunoprecipitation analyses of the cells lysates, which suggests a selective role for MBD2 in the methylation-mediated inhibition of ribosomal RNA gene expression. These results implicate that MBD2 may mediate gene and promoter/enhancer specific binding and repression, or may activate transcription by DNA demethylation reaction associated with specific demethylase machinery.

In animal model, knockout of MBD2 showed that lack of this protein prevents the growth of tumours that are known to be associated with hypermethylation of a few important genes [101]. Three genes have been tested (p16, Integrin- α 4 (Int α 4) and Timp3). Integrin- α 4 (Int α 4) and tissue inhibitor of metalloproteinase-3 (Timp-3) were found almost completely methylated in the knock out animals. It could be possible that the demethylation mechanisms of these genes are dependent by MBD2 protein association. The fact that MBD2-deficient mice are viable and fertile does not mean that MBD2 is not associated with demethylase function. It suggests that other proteins might exist as component of the demethylase machinery. The studies with the knock out mice were extended further recently by Berger et al. [102] on colon cancer in mice lacking MBD2 and human colon cancer cell line. They suggested that modulation of MBD2 during gut development establishes a region-specific gene expression pattern that is essential for establishing correct segmental character [102]. Galetzka et al. demonstrated that the sex-specific time windows for concomitant upregulation of DNMT1, DNMT3A, MBD2, and MBD4 are associated with prenatal re-methylation of the human male and female germ line. This may provide an important clue for the molecular mechanisms to parent-specific epigenetic modifications in human male and female germ cells [103].

After fertilization the sperm and egg genomes are remodeled into pronuclei before the first embryonic mitosis [6, 7, 32, 47, 57, 104–106]. Nonetheless, the highly compact sperm chromatin requires extensive nuclear remodeling and protamine-histone exchange, which is not required for nucleosomal maternal chromatin. The discovery of demethylation of the murine paternal pro-nucleus four hours after fertilization, before DNA replication have been observed, which demonstrated the existence of an active demethylation process in mammalian cells, except sheep and rabbit [7, 47, 104, 105]. By using interspecies intracytoplasmic sperm injection Beaujean et al. [106] have demonstrated that sheep sperm DNA can be demethylated in mouse oocytes. Amazingly, mouse sperm can also be demethylated to a limited extent in sheep oocytes. These findings [106] provide evidence that embryonic murine demethylation process is mainly paternally driven, although the nature of the activity is unknown. It is noteworthy that

mutant MBD2 mice that lack a functional methyl-CpG binding domain showed the same demethylation profile of the paternal pro-nucleus as in wild-type controls [47]. Although a testis specific truncated MBD2 transcript has been identified, there are no reports of an oocyte-specific form of MBD2 to date that could possess demethylase activity [106]. This suggests that there may be proteins with demethylase activity other than MBD2. Of interest is the finding that in bovine somatic cell nuclear transfer embryos, the donor nucleus showed more efficient satellite demethylation in the presence of the oocyte nucleus than in enucleated oocytes, suggesting that much of the demethylating activity is associated with the maternal nucleus [107]. Because enucleated sheep oocytes are also able to demethylate serum-starved fibroblast nuclei by ~50%, either sheep ooplasm has considerable residual demethylating activity or the sheep fibroblast DNA is particularly prone to demethylation ([106] and references therein). Wischnewski et al. [108] have elucidated the mechanistic roles of MBD proteins on methylation-dependent restricted expression of the tumor-associated MAGE antigens, including the possibility that MBD2 protein may play a role on silencing of MAGE-genes. Their results displayed that the transcriptional repression domains of MBD2 had no inhibiting effect on the promoter activity. Furthermore, cotransfections of Mbd1-deficient mouse fibroblasts and MCF-7 cells with MBDs showed that strong methylation-dependent repression of the MAGE-A promoters could not be further down-regulated by the MBD proteins, MBD1 or MeCP2. Interestingly, however, they observed that the two MBD1 splice variants, 1v1 and 1v3, were able to repress the basal activity of unmethylated MAGE-A promoters, and the transcription factor Ets-1 could not abrogate the MBD1-mediated suppression. In contrast with the repressive effect mediated by MBD1, MBD2a was found to up-regulate the basal activity of the promoters.

A very recent report shows that the MBD2 protein is controlled by arginine methylation [36]. The enzyme arginine methyltransferase has been identified. It catalyzes the methylation of MBD2 arginine residues, inhibiting the repressive function of MBD2 and reducing MBD2-methyl-DNA and MBD2-HDAC repression complexes formation. This report provides a molecular description of a potential regulatory mechanism for an MBD protein family member. This work was the first demonstration that arginine methyltransferases participate in the epigenetic DNA methylation system controlling chromatin functions [36].

To further clarify these issues, more studies on inactivation of DNMTs or MBDs are needed. But yet the results may simply show a correlation of methylation with gene repression, or will only suggest the virtual existence of other proteins with demethylase activity. The fundamental biochemical approach to solve this problem is to identify the

cognate component(s) by purification followed by reconstitution of active cofactor/enzyme–substrate complex.

5 Switching activation of transcription

Bruniquel and Schwartz [46] have recently defined a small region in the promoter-enhancer of the interleukin-2 [112] gene that gets demethylated in T lymphocytes following activation, and remains demethylated thereafter. This demethylation process started as early as twenty minutes after stimulation and was not prevented by G₁- to S-phase cell cycle inhibitors that block DNA replication. This finding implies that the demethylation process is dependent on an enzymatic mechanism [46, 109]. DNA methylation emerges as one of the central switches that allows interconversion between permissive and repressive chromatin domains. These principles are not only at the heart of transcriptional regulation, but they are also likely to govern other processes involving chromatin substrates. As indicated elsewhere, domain-wide and site-specific histone acetylation are necessary for transcription [17, 21, 78], but are not sufficient to establish full chromatin accessibility. Histone lysine methylation is one of the most robust epigenetic marks, essential for the regulation of multiple cellular processes. H3 K4 methylation perhaps has larger impact on nucleosome activation and transcription [56–67]. However, the switch to a permissive chromatin structure is required for subsequent synergistic action of nucleosome remodelling complexes containing ATPase of the SWI/SNF family ([17] and references therein). These enzymes increase the accessibility of nucleosomal DNA by weakening histone–DNA interactions leading to signal cascade dependent demethylation, activation of promoters and transcription of the specific promoter-driven gene follows.

5.1 5-Aza-2'-deoxycytidine induced transcription: the mechanistic dilemma

The catalytic mechanism of DNMT involves the formation of a covalent bond between a cysteine residue in the active site of the enzyme and carbon 6 (C6) of cytosine DNA. This event increases the electronic flow to carbon 5 (C5), with subsequent attack on the methyl group of SAM. Abstraction of a proton from C5 followed by β-elimination allows reformation of the 5C–6C double bond and release of the enzyme and substrate DNA with a methylated cytosine. Elaborated mechanisms are described in recent articles and reviews [13–15, 75, 76, 80, 110–116]. 5-Aza-2'-deoxycytidine (AzdC) has long been suggested for clinical use [113]. Trials for assessment of safety/toxicity and efficacy for treatment of various tumours had been done and are still ongoing [117–120]. Molecular analyses

after AzdC treatment confirmed re-expression of genes, which were under the repression condition in association with higher CpG-island methylation [119, 121–126]. As a possible mechanism, it has been suggested that AzdC inhibits DNA methylation only when incorporated into DNA by formation of a covalent bond between the enzyme and AzdC [112, 113, 126]. The DNMT–AzdC adduct is considered to be stable. It is thus assumed that trapping of DNMTs would generate hypomethylation, causing activation of transcription. DNMT–AzdC complexes may be tethered to distinct regulatory sites via modified DNA sequences that drive the interactions with the nucleosome itself. Hence, sections of DNA incorporating AzdC won't be properly packaged to chromatin, even when histone deacetylases are hyper active, unless repair enzymes operated [17, 34, 50, 127]. This assumption requires that the DNMT itself avidly binds to unusual structures formed in AzdC damaged genes that were to be packed into heterochromatin. In this model, transition state analogs formed in these unusual structures would prevent the enzyme from completing its catalytic cycle. Thus, once trapped, the DNMT would target appropriate repair, recombination or condensation complexes to the site ([15] and refs there in, [112, 113, 126, 127], reviewed in [13, 30, 76]).

Transcriptional activation within a permissive domain frequently correlates with additional, targeted demethylation of DNA at promoter nucleosomes [34, 76, 120] although notable exceptions exist [13, 26, 32]. The most intriguing question is how transcription machinery binds and rolls if AzdC entraps DNMTs? Maybe following the path of base excision repair [28, 30, 50]. Whereas tethering of DNMTs to defined sites via AzdC explains local hypomethylation, it is less obvious how the demethylation of large domains is achieved. Potential mechanisms include the recruitment of histone acetyltransferases (HATs) to distinct ‘entry sites’ from which they ‘spread’ throughout a domain [17, 34, 78], possibly by attachment to a tracking protein associated with RNA polymerase II transcription complex [15, 128]. Alternatively, the residence of a particular chromosomal domain within a demethylation-competent nuclear compartment may ensure relative uniform modification. If transcription activation complex itself were to generate high-affinity binding sites for demethylase, schemes for explaining propagation could be envisaged.

5.2 Interpreting demethylation signals in cells

An important issue in studying epigenetically mediated re-expression of genes silenced in cancer by CpG-island hypermethylation is the understanding of how demethylation of promoter–enhancer participates in the re-gain of gene transcription. In experimental systems, it seems that methylation of promoters does not lead to silenced

transcription until chromatin proteins are recruited to the specific region which mediate gene silencing [13–16, 20, 30, 34, 37]. Similarly, CpG-islands of enhancer region, when methylated, may inhibit structural changes and function of enhanceosome. Alternatively, demethylation of enhancer/promoters may require signals that remove proteins bound to chromatin for activation and gene re-expression. But how precisely or specifically the methyl-CpG-binding proteins are removed? In this setting, acetylation of one or more lysine (K) of H3 N-terminal at positions 9, 14, 18, and 23, and methylation of H3 K4 seems to initiate the process that results in regain of transcription [34, 42, 57–62, 65]. However, in some cellular settings, including abnormal gene-expression typical of cancer, it is unclear whether demethylation is the initial event for expression or whether it is targeted to the region by earlier chromatin remodeling events. In some systems, the action of an inhibitor is required to initiate gene re-expression before promoters in the region become densely methylated.

Whatever the sequence of events is at a given promoter, it seems that promoter demethylation (or histone hyperacetylation) must be integral to the gain of gene function because drugs that block DNA methylation, such as 5-AzdC, procannamide and Green Tea Catechins (EGCG), or drugs that block histone deacetylation, such as Trichostatin, SAHA and valproic acid salts, can partially reactivate silenced genes in cancer cells and restore their function ([17], reviewed in [34, 120–126, 129, 130]). 5-AzadC can induce expression of a broad spectrum of genes, whereas in prostate cancer cells procannamide induce expression of a P16 transcript, π -GSTP1, when the cells grow in nude mice. EGCG induces clusterin (SGP2/ApoJ) gene in prostate cancer cells [130]. Clusterin gene is known to be regulated through promoter hypermethylation [130]. If chromatin is crucial to aberrant gene silencing/expression in cancer, either primarily or secondarily, which processes are involved? Most of the vertebrate genome is normally packaged as transcriptionally repressive chromatin of the same kind found in “Pericentromeric Heterochromatin” regions. This type of chromatin is heavily methylated, and the identities of the chromatin associated protein complexes that might link DNA-methylation to transcriptional silencing have been discovered over the past few years (reviewed in [34, 39, 40, 57, 103, 120–127]). In these transcriptionally silent regions, DNA is packaged into compacted nucleosomes that contain deacetylated histones, deacetylated H3 in particular. These histones are extensively deacetylated through the actions of HDACs, maintaining nucleosomes in a compacted and transcriptionally silent state. The transcriptional repression status is also stabilized by the participation of key proteins, such as CBX5, the human homologue of *Drosophila* Chromodomain protein Pp1 α

(also known as suppressor of variegation 205, Su(Var)205) [34, 68–74]. DNA methylation itself also seems to be involved crucially in the transcriptionally silent state of pericentromeric heterochromatin. Methyl-CpG-domain binding proteins (MBPs) associate with methylated cytosine and also with various chromatin remodeling complexes. Also, they have been shown to act as transcriptional repressor *in vitro*. Importantly, these MBPs are also found in complexes that contain HDACs, such as Sin3 corepressor harbour MeCP2, MBD1 and MBD2 [17, 24, 25, 34, 127].

In contrast only a small fraction of the genome is transcriptionally competent. The state of chromatin in these regions must be dynamic to meet the changing transcriptional requirements of a cell. This balance between euchromatin and heterochromatin ensures that the gene-expression pattern of a given cell type is stably maintained in daughter cells as a heritable state. In terms of DNA methylation, there are two types of gene promoters [1, 3, 4, 30, 33, 34, 58, 131]. One type accounts for ~50% of the genes in the mammalian genome and contains unmethylated CpG-islands. The other promoter type is CpG-poor in composition, as is the rest of the genome. Among the genes that have CpG-poor promoters, it is not known for how many of them CpG-methylation might have a modulatory role in their transcription. However, there are examples in which the methylation of individual CpG-sites in such promoters can determine the transcriptional status of a gene by blocking the access of certain transcription factors that are sensitive to the methylation of CpG sites [34, 37, 39, 40]. Alternatively, the methylation of sites in these regions might also silence gene by helping to recruit chromatin protein complexes that repress transcription [24, 25, 30, 127]. CpG islands are generally assumed to be unmethylated in the germ line, except imprinted loci [3, 32, 34, 56, 131]. However, several research teams have attempted to predict cytosine methylation patterns in different cells based on DNA sequences. In an attempt to discriminate CpG islands that are prone to methylation in human lymphocytes from those that remain unmethylated Bock et al. [132] found that CpG islands that remain unmethylated are particularly GpC- and CpG-rich. Conversely, islands that are prone to methylation in the lymphocytes are enriched for segmental duplications, tandem repeats, and sequences with multiple self-alignments (reviewed in [60, 132]). In an another attempt to identify general sequence predictors of DNA methylation in brain tissues, Das and colleagues, as expected, have found that unmethylated sequences are enriched for CpG islands, and depleted of Alu elements [133]. These investigators also identified short sequence motifs that help discriminate between methylated and unmethylated DNA fractions from brain tissue, though their functional signification remain unclear (reviewed in [60], see also [133]).

The mammalian genome depends on patterns of methylated cytosines for normal function, but the relationship between genomic methylation patterns and the underlying sequence is unclear. Rollins et al. [134] have characterized the methylation landscape of the human genome by global analysis of patterns of CpG depletion and by direct sequencing of 3,073 unmethylated domains and 2,565 methylated domains from human brain DNA. The genome was found to consist of short (<4 kb) unmethylated domains embedded in a matrix of long methylated domains. Unmethylated domains were enriched in promoters, CpG islands, and first exons, while methylated domains comprised interspersed and tandem-repeated sequences, exons other than first exons, and non-annotated single-copy sequences that are depleted in the CpG dinucleotide. The enrichment of regulatory sequences in the relatively small unmethylated compartment suggests that cytosine methylation constrains the effective size of the genome through the selective exposure of regulatory sequences. This buffers regulatory networks against changes in total genome size and provides an explanation for the C value paradox, which concerns the wide variations in genome size that scale independently of gene number. This suggestion is compatible with the finding that cytosine methylation is universal among large-genome eukaryotes, while many eukaryotes with genome sizes $<5 \times 10^8$ bp do not methylate their DNA. In comparison with CpG poor promoters, methylation does not normally participate in regulating the transcription of genes with promoters that contain CpG islands, whether such genes are being actively transcribed or not. These islands reside, especially in active promoters, in chromatin that is composed of widely and irregularly spaced nucleosomes, which contain highly acetylated histone. Such a chromatin conformation and histone acetylation state is thought to facilitate the accessibility of the enhancer and promoter to enhanceosome-forming and transcription-activating complexes respectively. Unmethylated CpG-island regions are generally flanked by regions of less-CpG-rich DNA, which is heavily methylated in a chromatin conformation which, presumably, resembles that of transcriptionally silent pericentromeric heterochromatin. When the transcription of these CpG-island promoters is down regulated, as required by a given cellular state, the island remains free of methylation. It is likely in such inactive states, that the promoter is associated with transiently positioned repressor complexes that do not involve methylated DNA or MBD protein complexes. The separation of unmethylated promoter CpG-island regions from immediately flanking areas of methylation seems to involve a functional boundary. Much remains to be determined about how these boundaries for transcriptionally active versus inactive chromatin are established. However recent exciting discoveries have begun to link a specific histone code to the targeting of methylation to DNA. Specific methylation marks on histone

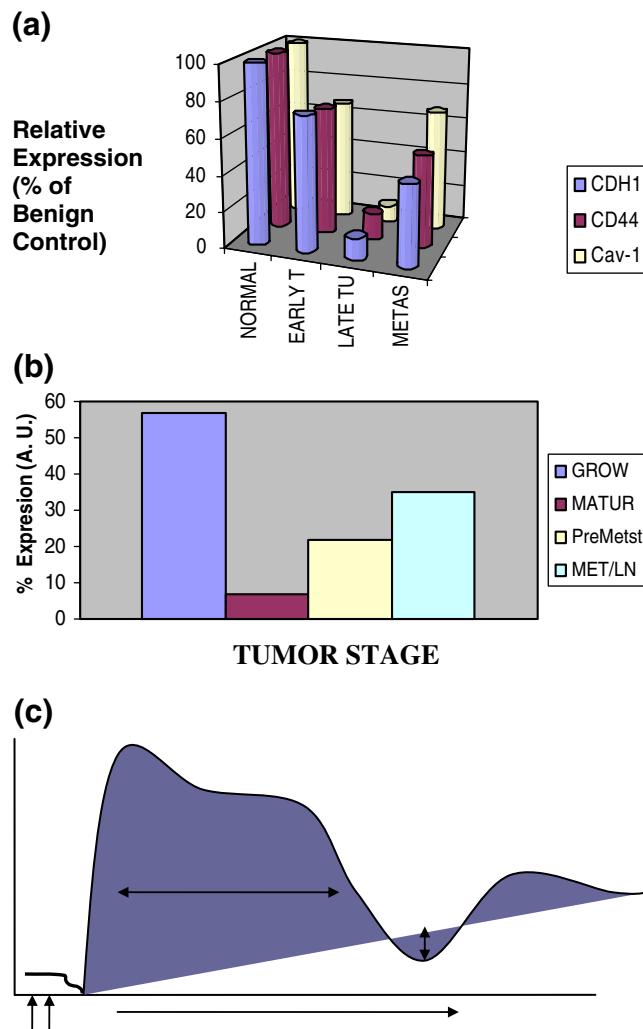
H3 seem to provide a signal that separates regions of transcriptionally inactive from regions of transcriptionally active chromatin [34, 56–67, 135, 136].

6 Molecular analyses of genes from clinical tissue specimens and test tube studies support active reversal of DNA methylation

In T-lymphocyte, a section of promoter-enhancer of the IL2 gene has been earmarked to be demethylated followed by activation and remains demethylated thereafter [46]. Local demethylation of transfected DNA fragments that do not replicate has also been experimentally proven [137, 138]. Active demethylation of fully methylated-CpGs to produce hemimethylated molecules, and demethylation of hemimethylated sites both have been proven to operate with transfected DNA fragments ([139, 138] reviewed in [30, 81]). In many cancers it has been shown that the level of expression of cell adhesion molecules and metastasis protein factors are differentially regulated in growing, progressing and metastatic diffusion. The promoters of the various cell surface marker genes, including Caveolin-1, E-cadherin and CD44 are inactivated in association with

Fig. 3 CD44, E-cadherin and Cav-1 are primarily function as proteins which maintain rigid cellular architecture and keep tissue integrity; and which are also important in various signaling pathways. Potential uses of hypermethylated genes as biomarkers also suggest that these known cell surface markers, tumor suppressor or metastasis suppressor would be of vital importance. (a) Shows the approximate abundance of these proteins in cells and tissues (e.g., breast, prostate, bladder, kidney, liver and lung etc.), normal, transformed/early tumour, late/mature tumour and at distance metastases/lymph nodes (These data are manipulated from the data of protein expression profiles available in the literature [15–17, 139–141, 144–160, 163, 166–168]). (b) This shows, more precisely, the approximate expression profile of E-cadherin in Bladder cancer: Normal—Tumor transition in early growth stage (*GROW*), Mature/Full blown (*MATUR*), the cells that are, ready to/or already detached from the tumor lump (*PreMetst*), and metastasized (*MET/LN*) (See also [15–17, 152–159]). (c) Cadh-1, Cav-1 and CD44 promoters are hypermethylated in bladder, prostate, breast, lung and colorectal cancer. Initial cell transformation occurs at this mark (*double arrow pointing up*); tumor progression and metastatic diffusion phase (*arrow pointing right*). The approximate methylation density of CpG-islands is represented by the shaded area and the red arrow (*horizontal double-headed arrow*). Just before detachment and re-implantation phase, methylation of the Cadh1, Cav-1 and CD44 decreases (*vertical double-headed arrow*). In the last phase of progression, the genes may be again inactivated by methylation, but the underlying biochemical mechanisms are not, or very poorly, understood. Last phase data available are virtually limited to very few case-control studies. Under these conditions, patients undergo chemotherapy and methylation density could be affected by treatment. Graph is the approximate result of inferring data on DNA-methylation available in the literature [refs 15–17, 139–141, 143–160]. *Double arrow pointing up* indicates the start point, where cells are transformed from normal to tumor. *Arrow pointing right* mark indicates that cells may be advancing through tumor maturation to metastasis

CpG-island hypermethylation at the onset of tumor development and remains thereafter methylated in full blown tumors but were found to be expressed in metastatic foci and lymph nodes (reviewed in [9, 33]). Since these genes are mostly inactivated by DNA-methylation; their reactivations certainly need demethylation activity (Fig. 3(a)) [9, 33]. For instance, mRNA and protein expression of caveolin-1, a major component of caveolae proteins, is frequently lost in multiple cancers. Caveolin-1 is known to be a tumor suppressor gene. Current results are consistent with the dual function of caveolin-1 both as tumor suppressor gene and metastasis-promoting gene. At the cancer onset, Caveolin-1 gene is repressed by DNA-methylation, while re-expression occurs just before metastasis (reviewed in [9, 32, 139–141]). CD44 is known to be involved in re-organization of highly dynamic structures of cytoskeleton when cells respond to extracellular stimuli by division and/or changes in shape or activity [142]. mRNA and protein expression of CD44 is frequently lost in association with DNA hypermethylation in multiple cancers at the early stage of cell transformation and tumor progression [33, 143–148].



Again, re-expression of CD44 gene was found necessary for metastatic diffusion of many tumors [32, 148–152]. E-Cadherin, a transmembrane glycoprotein, is also a calcium-dependent cell–cell adhesion molecule, known to play a key role in the maintenance of tissue integrity by forming complex with catenin. E-Cadherin is eventually tagged to actin cytoskeleton through catenins. Because loss of E-cadherin expression results in disruption of cellular clusters, it has been postulated that E-cadherin functions as tumor suppressor gene. mRNA and protein expression of E-cadherin is frequently lost on DNA methylation in multiple cancers at the early stage of tumor progression [15, 33, 153–159]. Also in this case, re-expression of E-cadherin had been shown to be clinically significant at the metastatic foci of many cancers [141, 148, 154, 156, 157]. Figure 3b shows more precisely the approximate expression profile of E-cadherin in Bladder cancer (See also [15–17] and [152–158, 160]). There are exceptions also, e.g., persistent expression of E-cadherin was observed in inflammatory breast cancer [158, 161]. Cavalli et al. [160] have studied the epigenetic and genetic changes and alterations in breast sentinel lymph nodes (SLN) compared to matched primary tumors using comparative genomic hybridization and bisulfite polymerase chain reaction analyses for CpG island hypermethylation of the following genes: TP16, THBS2, E-Cadherin (E-Cad), RARbeta2, MINT1, MINT2, and MINT31. The study was done in six paired primary breast tumors and their matched SLN. They noticed that an overall 71% (30 of 42) of the methylation was identical between the primary tumors and the SLN. Of the six cases, two showed no differences between the primary tumors and SLN, one tumor with four out of seven genes hypermethylated in the primary tumor exhibited loss of all four hypermethylation events in the SLN, and the remaining three tumors showed loss of one methylation event and simultaneous gain of one to two methylation changes in the SLN. Their observation is the first study reporting genetic and epigenetic alterations in breast SLN compared to their corresponding primary tumors. Characterization of such alterations may lead to identification of initial events associated with the metastatic dissemination process. Even though it is clear so far that active demethylation of intact DNA occurs in some instances, the type and nature of the enzyme(s) that are responsible for this has not been unambiguously established [27–30, 33, 46, 57, 162]. Active DNA-demethylation is a complex event. Researchers are actively involved to clarify why there is coexistence of regional hypermethylation and genome wide hypomethylation in cancer and embryonic development? Why gene specific local demethylation is necessary for development? [7, 27–30, 33, 36, 44, 46, 81, 103–109, 162–165]. Circulating tumor cells in peripheral blood have been demonstrated to reflect the biological

characteristics of tumors, including the potential of metastasis development and tumor recurrence [163].

Cadh-1, Cav-1 and CD44 promoters are hypermethylated in bladder, prostate, breast, lung and colorectal cancer. Figure 3(c) shows what may happen in initial cell transformation (mark ↑↑), tumor progression and metastatic diffusion phase (→). The approximate methylation density of CpG-islands is represented by the shaded area and the red arrow (↔). Just before detachment and re-implantation phase, methylation of the Cadh1, Cav-1 and CD44 decreases (↓). In the last phase of progression, these genes may again be inactivated by methylation, but the underlying biochemical mechanisms are not, or very poorly, understood. Last phase data available are virtually limited to very few case-control studies. Under these conditions, patients undergo chemotherapy and methylation density could be affected by treatment. Graph is the result of inferring data on DNA-methylation available in the literature [15, 139–141, 143–158]. Of significance is that, DNA hypomethylation of multiple genes successfully detected in circulating tumor cells from cancer patients may prove valuable for disease monitoring [33, 34, 38, 44, 163–165]. A number of epigenetic markers may feasibly enable the detection of circulating tumor cells from patients with different cancer types [33, 34, 117, 119–126, 163–172]. Among the possible therapeutic implications of aberrant DNA methylation is the implementation of methods that could eventually provide useful information for assessing final outcome of cancer patients. Future advances in understanding cancer and development epigenetics will certainly be of paramount importance for diagnosis, monitoring and possibly for the development of novel or alternative therapeutic strategies [33, 34, 117, 120–124, 163–170].

7 Conclusion and perspectives

Aberrant promoter methylation has emerged as a fundamental molecular abnormality leading to transcriptional silencing of tumor suppressor, DNA repair and metastasis inhibitor genes. This is closely linked to the predisposition of genetic alterations of other cancer-associated genes [30, 33, 34, 164, 165]. This epigenetic inheritance has significant biological implications for cancer progression and metastasis formation [120]. The current knowledge of DNA (cytosine-5-carbon) methylation has now provided us a critical mass of information by the work of many scientists (For further reviews and books see [164–168, 171–176]).

Although the importance of epigenetic alterations as a consequence of reversible DNA methylation for cancer and development has been widely recognized during the past several years, the concept of epigenesis and development,

an old paradigm [11], with potentially significant implications for mechanistic insights into histone modification in development, tumor progression and therapy, has gained a new momentum. Epigenetic mechanisms are known to be crucial for embryogenesis and cellular differentiation. Thus, one of the key issues for development and cancer research in the immediate future will be not only to define the genetic changes of the cancer cell genome but also to understand the epigenetic alterations that affect the differentiation potential of cancer cells.

Here, we have given an overview of how DNA methylation/^{Me}C DNA demethylation are involved in regulation of cell differentiation or proliferation, in tissue development and transformation. One of the signals for chromatin inactivation is transduced via CpG-DNA methylation and complex formation with methylated CpG-binding MBD proteins. Although it seems convincing that one of the signals for chromatin activation is transduced through ^{Me}C DNA demethylation, the chemistry and mechanism of demethylation is still a matter of debate. Nucleosomes containing methylation associated genes and their complexes with MBD proteins in the promoters and enhancers produce diverse effects on their regulation. In this context, the possible contribution of MBD2 as one of the active components for cytosine demethylation deserves serious considerations for further investigations. It is now experimentally evidenced from various laboratories world wide that, that MBD2 may either repress or activate the basal activity of promoters. In our laboratory, as well as in many others, we collected evidences suggesting that MBD2 is one of the components of a complex that can catalytically activate promoters, but more work has to be done to fully understand the reaction mechanism and to discover the cofactors involved. MBD2 protein is a novel area of research in the field of reversible DNA-methylation and epigenetics. A better understanding of key reprogramming factors and their chemistry, in particular the demethylase activity in the fertilized egg is crucial for improving human infertility treatment and the efficiency of mammalian embryo cloning. The single most notable feature of DNA methylation chemistry is its reversibility, and that of DNA methylation biology is its participation in so many aspects of cellular biology, ranging from the fundamental (for example, developmental reprogramming) to the highly specialized (for example, imprinting). Future studies will certainly provide more examples of gene control by DNA (cytosine-5-carbon) methylation/demethylation, paying necessary tributes to R. D. Hotchkiss, who discovered the “miracle switch,” 5-methyl cytosine, in DNA, and to C. H. Waddington, for conceiving the link between cell differentiation and epigenesis.

The picture emerging from these studies is that, DNA methylation function in a network of epigenetic signaling

for chromatin (DNA and histones) modifications, nucleosome stability and activation. These networks enable DNA methylation to organize the rigid structure of heterochromatin, and DNA demethylation to a relaxed euchromatin, allowing it to participate in transcription. Understanding DNA methylation/demethylation and histone codes in more detail will shed new light on development, as well as on a variety of pathologic states, including cancer, and will greatly help to understanding the intrinsic robustness of cellular homeostasis.

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