

New concepts in DNA methylation

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The widely-cited model of maintenance of DNA methylation at CpG sites implies that DNA methylation is introduced by the Dnmt3 *de novo* DNA methyltransferases during early development, and methylation at hemimethylated CpG sites is specifically maintained by the Dnmt1 maintenance methyltransferase. However, substantial experimental evidence from the past decade indicates that this simple model needs to be revised. DNA methylation can be described by a dynamic stochastic model, in which DNA methylation at each site is determined by the local activity of DNA methyltransferases (Dnmts), DNA demethylases, and the DNA replication rate. Through the targeting and regulation of these enzymes, DNA methylation is controlled by the network of chromatin marks.

Classical model of DNA methylation as a paradigm of epigenetic information transfer

Molecular epigenetics, including DNA methylation (see [Glossary](#)), has become a central subject of scientific research in various fields of biology and molecular medicine. For a long time DNA methylation at CpG sites in mammals had been considered a paradigm of epigenetic information transfer, because its molecular mechanism of inheritance was explained by a convincing model proposed now almost 40 years ago in two seminal papers Riggs [1] and Holiday and Pugh [2]. This classical maintenance methylation model was an insightful extension of the Watson–Crick principle that the complementary information encoded in the two DNA strands is the basis of biological inheritance. Basically, it implies that methylation is introduced in both DNA strands at palindromic CpG sites by *de novo* Dnmts, which create a pattern of fully methylated and unmethylated CpG sites. After each round of DNA replication, the methylation is still present in the parental strands creating a pattern of hemimethylated and unmethylated CpG sites. Therefore, the methylation pattern can be maintained by the specific activity of a maintenance methyltransferase that specifically remethylates hemimethylated CpG sites. In turn, the unmethylated CpG sites are kept in the unmethylated state by the absence of *de novo* methylation (Figure 1). The enzymatic and biochemical properties of the Dnmt1 DNA methyltransferase, which shows a preference for hemimethylated DNA [3,4], suggests that Dnmt1 plays the role of the maintenance enzyme. By

contrast, the Dnmt3a and Dnmt3b enzymes do not discriminate between unmethylated and hemimethylated substrates and have a role in *de novo* DNA methylation [4]. Genetic studies have revealed that the knockout of any of the three enzymes in mice is lethal and leads to a massive, genome-wide loss of DNA methylation [5,6], clearly demonstrating the importance of these enzymes and DNA methylation for mammalian development.

However, during the past decade compelling experimental evidence has accumulated that indicates that the site-specific maintenance methylation model needs to be revised and extended. Although concerns regarding the classical model were clearly expressed in seminal contributions from Riggs and Jones [7,8], the classical view of maintenance methylation still dominates parts of the field, probably because of its overwhelming conceptual elegance. In the following paragraphs of this review, we first summarize the various experimental observations that are not fully compatible with the original site-specific maintenance methylation model, briefly considering the process of *de novo* DNA methylation, and then discussing the maintenance of DNA methylation in a greater depth. Second, we present a modified stochastic DNA methylation model that explains all findings and provides a unified description of both the *de novo* and the maintenance methylation process at CpG and non-CpG sites. Finally, the biological implications of this conceptual overhaul are briefly discussed.

Dnmt1 has a role in *de novo* DNA methylation

The classical methylation model proposes a functional partitioning of Dnmt3 and Dnmt1 into *de novo* and maintenance enzymes, respectively. However, the concept of

Glossary

Hemimethylation: a state, in which a CpG site (or a DNA molecule) is methylated in one of the DNA strands.

Full methylation: a state, in which a CpG site (or a DNA molecule) is methylated in both DNA strands.

DNA methylation pattern: this term is used inconsistently in the literature. We use the phrase 'site-specific DNA methylation pattern' for a pattern, in which the methylation state of each individual CpG site is defined. The more general term 'DNA methylation pattern' is used to refer to the average methylation of several CpG sites at one DNA region.

Dnmt3: The Dnmt3 family comprises two active enzymes (Dnmt3a and Dnmt3b) and the inactive regulator Dnmt3L. All three proteins form higher homo- and heteromultimeric complexes [79]. We use the term Dnmt3 here to refer to any of these enzymes alone or in complex.

CpG depletion: Methylation of cytosine is slightly mutagenic, which has led to the loss of CpG sites in mammalian genomes during evolution. In the human genome CpG sites are globally found 3–4 times less often than statistically expected. The frequency of CpGs is not reduced in so called CpG islands, which are often found in promoter regions of genes and typically are unmethylated in the germ line.

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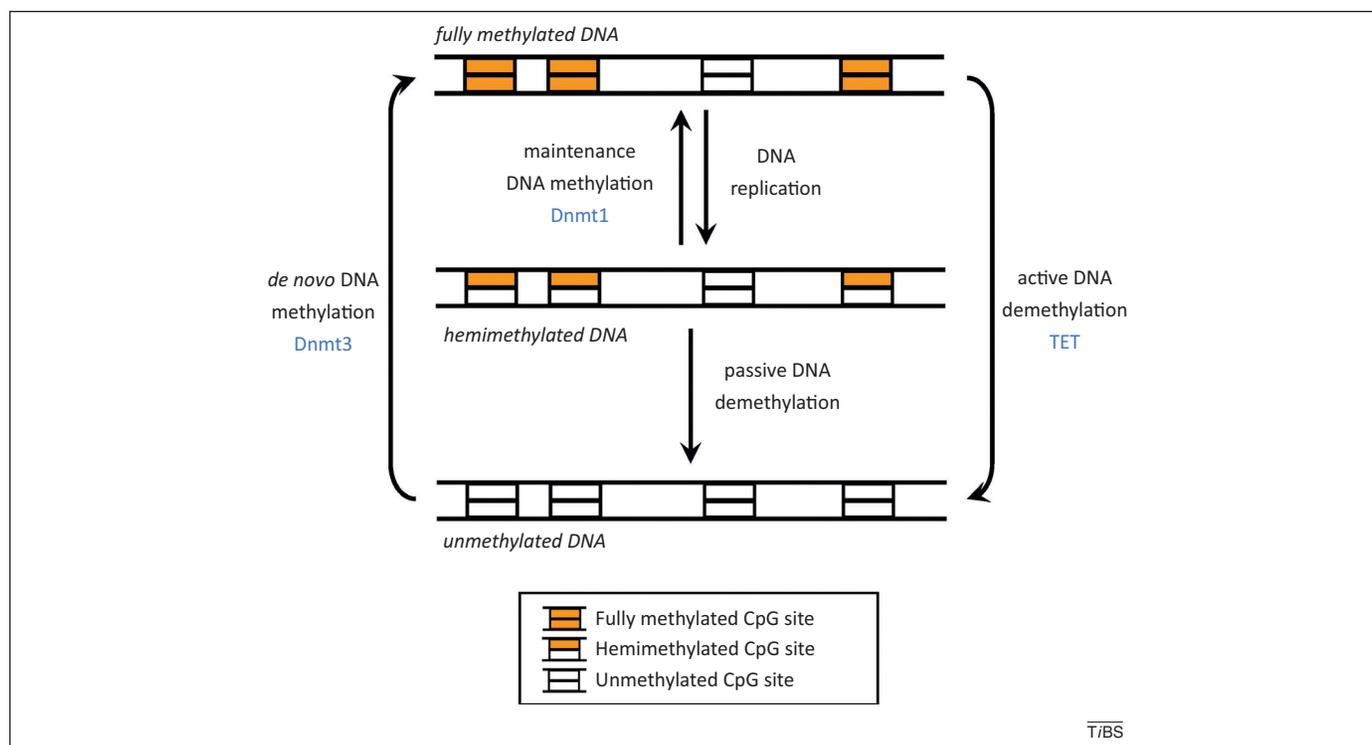


Figure 1. Schematic drawing of the classical site-specific DNA maintenance methylation model (based on [4]). Initially, the DNA methylation pattern consisting of unmethylated and fully methylated CpG sites is generated by *de novo* methyltransferases. Later it is maintained through DNA replication by the maintenance methylation, but can be lost through passive or active demethylation and the new methylation can be introduced as well. Typically, Dnmt1 is considered to be a maintenance enzyme, whereas Dnmt3a and Dnmt3b are regarded as *de novo* methyltransferases. Abbreviations: Dnmt, DNA methyltransferase; TET, ten eleven translocation enzyme.

de novo generation of DNA methylation patterns by the Dnmt3 enzymes during embryonic development is undermined by the fact that the Dnmt3 enzymes show strong flanking sequence preferences [9–11]. Therefore, they preferentially introduce methylation in only one of the two DNA strands at most CpG sites, leading to the massive generation of hemimethylated sites, which then represent ideal substrates for Dnmt1. Furthermore, the Dnmt3a complex binds to DNA in a tilted fashion, so that its two active sites can introduce hemimethylation in adjacent CpG sites, but they cannot methylate both DNA strands of one CpG in one binding event [12,13]. Because Dnmt1 preferentially methylates hemimethylated CpG sites, methylation of both strands of unmethylated DNA can be achieved more efficiently by the cooperation of Dnmt3 enzymes with Dnmt1 [14,15], because Dnmt1 can perform the methylation of the second strand. In addition, Dnmt1 shows considerable *de novo* methylation activity on unmethylated DNA *in vitro* [16–18], and several studies have indicated that this activity can also be detected *in vivo*. Indeed, residual DNA methylation has been observed in Dnmt3a/Dnmt3b double knockout embryos [6] and unmethylated reporter DNA introduced into Dnmt3a/Dnmt3b double knockout cells acquired some *de novo* methylation [19]. In addition, overexpression of Dnmt1 in cells results in the *de novo* methylation of some previously unmethylated sequences [20–22]. A recent genome-wide DNA methylation analysis in wild-type and Dnmt knockout cells providing an estimation of the relative contributions of DNA methyltransferases for *de novo* and maintenance DNA methylation, also showed that Dnmt1 has a considerable *de novo* methylation activity

at certain repetitive elements and single copy sequences [23].

Experimental evidence in favor of a modified view on maintenance methylation

There are several major lines of experimental observations that are not compatible with the classical site-specific DNA maintenance methylation model which include the nature of the experimental methylation patterns, the change in DNA methylation observed in Dnmt knockout cells, non-CpG methylation, the phylogeny of DNA methylation systems, and the discovery of enzymes involved in DNA demethylation. Additionally, recent research in the field documented sophisticated processes for regulation and targeting of Dnmts controlling their access to DNA, which indicates that the overall process of DNA methylation is more complicated than anticipated by the classical maintenance model.

Key prerequisites of the classical maintenance methylation model are not fulfilled

The classical site-specific maintenance methylation model has two critical prerequisites: it assumes that the methylation states of single CpG sites are stably inherited, and that a perfect maintenance enzyme for this task exists. A stable inheritance of CpG-specific methylation patterns would imply that all cells of one tissue have the same methylation pattern. However, numerous DNA methylation analyses conducted by bisulfite conversion, followed by cloning and DNA sequencing of individual clones, have shown that is not the case. Instead, average methylation densities of DNA regions are maintained, but not exact

CpG site-specific methylation patterns [8,24], and changes in methylation levels were shown to occur through stochastic processes [25]. The incomplete fidelity of the DNA methylation maintenance process has been observed experimentally, because studies using premethylated proviral reporter constructs showed that initial methylation was maintained at most, but not all CpG sites [19]. The general observation of a lack of CpG site-specific methylation patterns is in agreement with the enzymatic properties of Dnmt1. Various studies have determined that Dnmt1 has a 10–40-fold preference for hemimethylated over unmethylated CpG sites [3,26–29]. Although of vital importance for the DNA methylation process in cells, this preference is by far not enough to copy faithfully and site specifically the methylation state of the ~56 million CpG sites in the human genome over several rounds of DNA replication. Thus, the two main cornerstones of the classical model were not found as predicted: (i) DNA methylation patterns are not site-specific in general; and (ii) there is no maintenance enzyme with sufficient specificity to copy genome-wide methylation patterns.

DNA methylation changes in Dnmt knockout cells

Genetic studies in mouse and mammalian cell lines argue against a strict functional categorization of Dnmt1 and Dnmt3 enzymes into maintenance and *de novo* enzymes, respectively. In 2002, Liang *et al.* reported in a groundbreaking study that, in Dnmt knockout cell lines, Dnmt1 alone could only maintain DNA methylation at CpG-poor regions, but both Dnmt1 and Dnmt3a and/or Dnmt3b were required for methylation of repeat elements, including the murine long interspersed nuclear element-1 promoter [30]. Based on these observations, the authors concluded that cooperation between Dnmts is needed for the maintenance of DNA methylation. This finding was soon confirmed by Chen *et al.* (2003), who observed that deletion of Dnmt3a and Dnmt3b led to a loss of DNA methylation at repetitive elements, despite the presence of functional Dnmt1 [31]. Similarly, loss of Dnmt3b alone also led to a reduction in DNA methylation [32]. Studies with human cell lines indicated that cells containing a Dnmt1 variant with reduced activity have reduced DNA methylation. However, cells in which Dnmt3b was lost as well showed a further reduction in DNA methylation levels [33]. These results clearly demonstrate that, in addition to Dnmt1, the Dnmt3 enzymes are needed for maintenance of DNA methylation, particularly at repeat elements (Box 1). This conclusion was confirmed recently in a genome-wide DNA methylation analysis in wild-type and Dnmt knockout cells [23]. Thus, DNA methylation observed in Dnmt knockout cells showed that Dnmt3 enzymes have a role in the maintenance of DNA methylation.

Non-CpG methylation in human cells and other organisms

In addition to CpG-directed DNA methylation, plants also contain methylation in palindromic CHG sequences (H = A, T, or C), where maintenance methylation would be possible in principle. However, this methylation is propagated by the CHROMOMETHYLASE 3, which has no detectable preference for hemimethylated target sites, but is directed

Box 1. DNA methylation at CpG rich regions

As described in the main text, the contribution of Dnmt3 enzymes to the maintenance of DNA methylation is particularly required at repeats, indicating that they constitute the greatest challenge to the maintenance methylation system. On average, the genome is methylated at CpG sites, but the density of such sites is low due to CpG depletion. Methylation at these sites can, therefore, be maintained relatively easily. Regions of higher CpG density, so called CpG islands, exist, but they are mostly unmethylated, such that maintenance methylation is not needed there. However, repeats in general are CpG rich and also highly methylated, and therefore require high maintenance methylation activity for efficient maintenance of the methylated status, which depends on the dedicated targeting of Dnmts to these sites.

by histone H3 K9 methylation [34]. Furthermore, numerous organisms, including plants, many lower eukaryotes, and insects also show cytosine-C5 methylation at asymmetric sequences in one DNA strand [35], which in principle cannot be maintained using information from the second DNA strand. This methylation is often propagated by RNA-based mechanisms [34]. Therefore, patterns of non-CpG DNA methylation can be generated and maintained by other epigenetic processes, not following the principles of the classical maintenance model. Notably, non-CpG methylation is not restricted to plants and lower eukaryotes, because human embryonic stem cells, germ cells, and differentiated neuronal cells also contain relatively high levels of non-CpG methylation, especially at CpA sites [36–38]. Its introduction is associated with the Dnmt3a enzyme [23,39], which shows relatively high catalytic activity also at non-CpG sites [40,41]. The stable presence of non-CpG methylation in the human genome is a clear indication of a permanent *de novo* activity of Dnmt3a, which is not in agreement with the classical methylation model. In general, non-CpG methylation in human cells and in other species is preserved by processes that do not use information from the second DNA strand.

Role of DNA demethylation

DNA methylation patterns are also influenced by DNA demethylation, which can occur passively by replication, if remethylation does not take place, or through an active process. In the past few years the family of ten eleven translocation (TET) dioxygenases was discovered, which oxidize the methyl groups of methylcytosine and play a main role in active DNA demethylation [42–44]. TET enzymes are mainly expressed in early development and germ lines, but they are also present during later stages, indicating that DNA demethylation occurs permanently and needs to be counteracted by the methylation activity of Dnmts. Active DNA demethylation adds another powerful layer to the regulation of DNA methylation, and the targeting and regulation of demethylases is equally important in the establishment and maintenance of DNA methylation patterns as the targeting and regulation of Dnmts. For example, there is increasing evidence of dynamically regulated DNA methylation at specific loci [45,46] and hydroxymethylation of DNA by TET enzymes has been proposed to keep CpG islands in unmethylated state by counteracting stochastic DNA methylation [47].

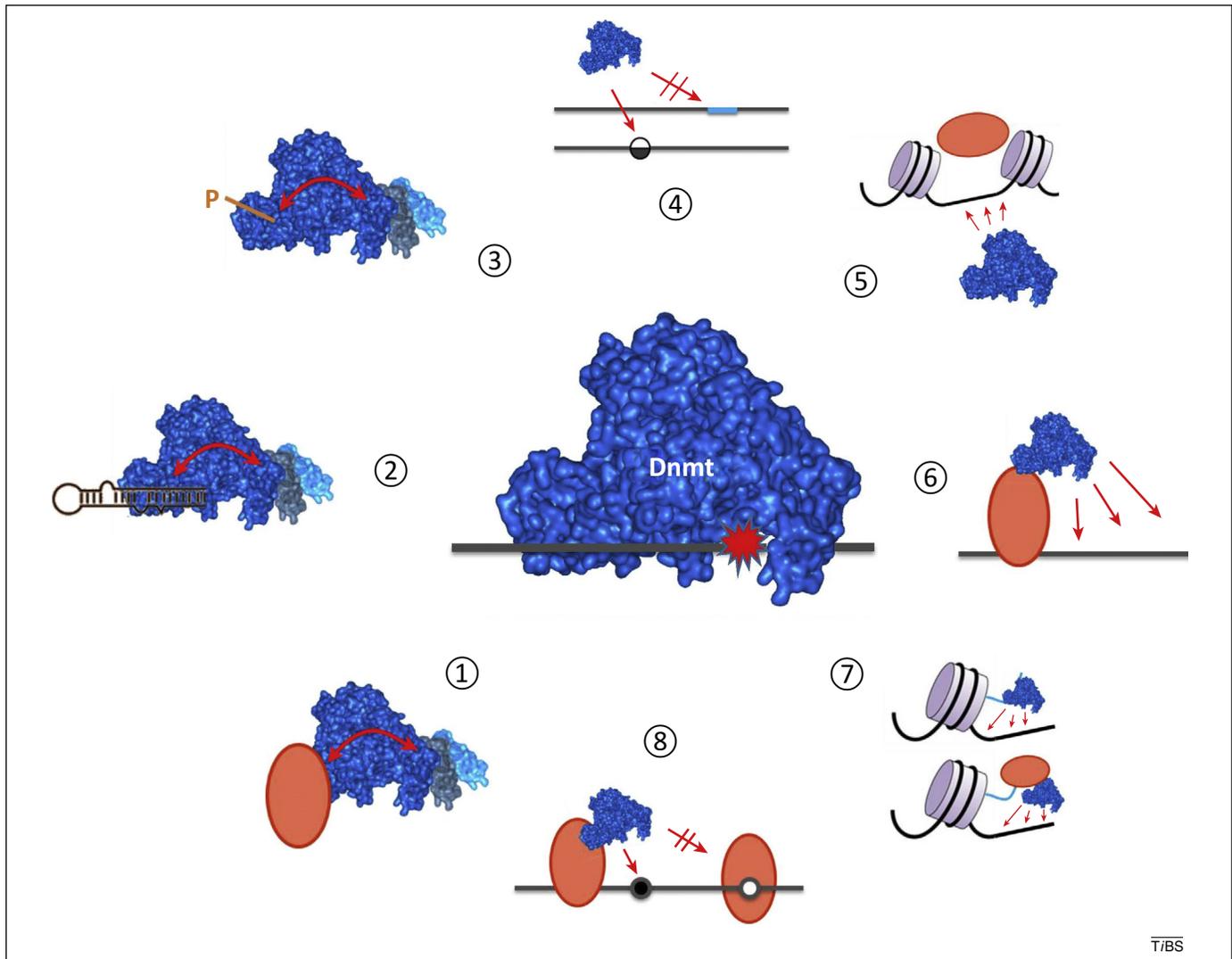


Figure 2. Processes controlling the recruitment and activity of Dnmts at specific target sequences (indicated by the red star). Dnmts are displayed in blue, other proteins are shown as orange ellipses. DNA is drawn as a grey line, circles represent CpG sites (unmethylated in white, methylated in black, hemimethylated in half white and half black). P denotes phosphorylation. The catalytic activity of the enzyme can be regulated by conformational changes induced by bound proteins (1), RNA molecules (2), or post-translational modifications, such as phosphorylation (P) (3). The sequence context of the target site can influence Dnmt activity, as well as the methylation state of the DNA (in particular in the case of Dnmt1) (4). The accessibility of the target region for enzymatic methylation is controlled by chromatin remodeling (5). Targeting of Dnmts to particular DNA regions can be mediated by other DNA binding proteins, such as transcription factors (6), or by binding to specific histone tail modifications (7). Site-specific methylation patterns can be generated by a CpG site-specific recruitment of Dnmts or CpG site-specific blocking, mediated by other proteins (8). The same principles could apply to demethylases. Abbreviations: Dnmt, DNA methyltransferase.

In summary, the stochastic nature of DNA methylation patterns, the lack of sufficient specificity of Dnmt1, specific DNA methylation changes observed in Dnmt knockout cells, the occurrence of non-CpG methylation, and the discovery of active DNA demethylation all call for an extension of the classical site-specific maintenance methylation model.

Targeting and regulation of Dnmts control DNA methylation

Research during the past several years has revealed the existence of sophisticated targeting and regulatory mechanisms of Dnmts (Figure 2). These mechanisms include the recruitment of the enzymes via specific interaction with histone tail modifications or through protein partners, as well as a role for chromatin remodeling, which controls the accessibility of the DNA for methylation and demethylation. Importantly, the targeting and regulation of Dnmts

can affect the activity of DNA methyltransferases at particular genomic regions, but not at individual CpG sites, indicating that the process of DNA methylation is more complex than assumed by the classical CpG site-specific maintenance model.

Dnmt3 enzymes have been shown to bind tightly to condensed and methylated heterochromatin regions. Such localization supports DNA methylation at these critical regions. At the same time, targeting to heterochromatin reduces the amount of freely available enzyme in the cell, preventing methylation at other sites [13,48–50]. This is partially mediated by the modification of histone tails. The ATRX-DNMT3A-DNMT3L (ADD) domains of Dnmt3a, Dnmt3b, and their regulator Dnmt3L prevent binding of the enzymes to H3 tails di- or trimethylated at K4 (H3K4me2/3), a common mark in active chromatin [51–53]. In addition, the PWWP domain of Dnmt3a directs the enzyme to H3K36me2/me3 [54], which is found in gene

bodies and heterochromatin. The Dnmt3L protein has been shown to stimulate Dnmt3a and Dnmt3b *in vitro* and *in vivo* [4]. In addition, it has been shown to alter the quaternary structure of Dnmt3a and mediate the release of Dnmt3a from heterochromatin [50]. Lacking a catalytic domain, Dnmt3L also has the ability to compete with Dnmt3 enzymes and reduce DNA methylation [55]. Furthermore, ubiquitin-like containing PHD and RING finger domain protein 1 (Uhrf1) interacts with Dnmt3 enzymes and connects DNA methylation with H3K9me3 [56,57]. Dnmt1 is recruited by proliferating cell nuclear antigen (PCNA) to the replication fork [58] and to hemimethylated DNA by the Uhrf1 protein [59,60]. These processes concentrate Dnmt1 at sites of replication, and thereby help to reduce its activity on other parts of the genome, where no hemimethylated sites are available. In addition to its targeting role, Uhrf1 stimulates the activity of Dnmt1 on hemimethylated DNA [61,62]. Recently, the regulation of Dnmt1 by long noncoding RNAs has been demonstrated as well [63].

Although chromatin modifications play a vital role for the targeting of DNA methylation, *in vitro* methylation studies have shown that DNA wrapped in nucleosomes is not a good substrate for Dnmts [64–66]. This enzymatic result is in agreement with the structures of Dnmt3a [12] and Dnmt1 [27], indicating that binding of DNA to nucleosomes would exclude binding to Dnmts. This apparent contradiction suggests that after recruiting of Dnmts, nucleosomes have to be removed or shifted by chromatin remodeling in order to allow the methylation of the DNA in the nucleosomal core region. Although it is conceivable that at least some of the maintenance methylation occurs rapidly after replication, when nucleosomes are not yet assembled, this is not possible in later phases and not the case for *de novo* methylation. Therefore, chromatin remodeling must play an important role in DNA methylation as well. Consequently, the lymphoid specific helicase (LSH) protein, which belongs to the family of switch sucrose non fermentable (SWI/SNF) chromatin remodelers has been found to play an essential role in *de novo* DNA methylation in mice [67,68], strengthening the connection between chromatin remodeling and DNA methylation.

In summary, DNA methylation is controlled by regulation and targeting of Dnmts, which includes interaction with chromatin marks. Furthermore, the accessibility of the DNA influenced by chromatin remodeling regulates DNA methylation.

A unified stochastic model of DNA methylation

All the experimental findings discussed so far illustrate that the classical maintenance methylation model needs an overhaul. Therefore, we developed a revised model that incorporates the cooperation of Dnmt3 and Dnmt1 in *de novo* methylation and the role of Dnmt3a and Dnmt3b in the maintenance of DNA methylation. Although Dnmt1 certainly plays a main role in the maintenance process, because of its high activity and its preference for hemimethylated target sites, the Dnmt3 enzymes are nevertheless needed, in particular at CpG rich repeat sequences. In our new model, DNA methylation is guided by an epigenetic network, in which DNA modifications, histone tail modifica-

tions, and other epigenetic marks influence each other and function in a synergistic fashion (Figure 2). These marks recruit Dnmts and DNA demethylases to DNA regions, which are targets of methylation and demethylation, simultaneously reducing their binding to other parts, and regulate the activity of the enzymes. Importantly, the coupling of DNA methylation with histone tail modifications cannot define the methylation states of individual CpG sites, because one nucleosome contains about 200 base pairs of DNA (including the linker region), which statistically contain about 3–10 CpG sites depending on the level of CpG depletion. Therefore, the average DNA methylation level of DNA regions is inherited rather than the methylation state of individual CpG sites. The preference of Dnmt1 for hemimethylated CpG sites in the revised model is just one of the mechanisms to increase and stabilize the methylation of certain DNA regions. Notably, in this model, both *de novo* and maintenance methylation basically use the same mechanisms, except that the preference of Dnmt1 for hemimethylated CpG sites supports the maintenance process. Furthermore, this model can also account for stable non-CpG methylation in other species and human cells, achieved by the combined effects of ongoing *de novo* methylation and demethylation.

Riggs and colleagues provided an important conceptual advancement and introduced a dynamic model of DNA methylation that is the basis of our model [7]. It assumes that the average methylation of each CpG site is determined by a stochastic process that requires ongoing *de novo* methylation in addition to the maintenance activity. We developed their model into a general stochastic model of DNA methylation in the biological context, which in addition to maintenance and *de novo* DNA methylation incorporates the rate of cell division as well as passive and active demethylation, which is applicable also for non-CpG methylation. In this dynamic model of DNA methylation, the change in the fraction of methylation (θ_m^i) at any site (i) is given by the difference between the rate of DNA methylation and rates of loss of methylation by replication and active demethylation at this site (Box 2). Note that that the corresponding rates are different at different target sites:

$$\frac{d\theta_m^i}{dt} = r_{met}^i \times (1 - \theta_m^i) - \left(\frac{1}{2} D(1 - f_{main}^i) + r_{demet}^i \right) \times \theta_m^i \quad [1]$$

With: r_{met}^i , rate of *de novo* DNA methylation at site i (dimension: 1/time); D, rate of cell division (dimension: 1/time), f_{main}^i , efficiency of maintenance methylation at site i (as a fraction) and r_{demet}^i , rate of DNA demethylation at site i (dimension: 1/time). In the original maintenance methylation model f_{main} would be 1.0 and r_{met} and r_{demet} zero at all sites. The non-CpG methylation is integrated in our model by considering that f_{main} is zero in this case, because maintenance methylation based on the second strand methylation does not occur.

The rates of methylation (r_{met}^i) or demethylation (r_{demet}^i) are dependent on the local concentration of the corresponding enzymes, their catalytic activity, as well as properties of the target sequence (Figure 2). The local enzyme concentration depends on the overall expression of the enzyme

Box 2. A unified stochastic DNA methylation model

The *de novo* methylation at any CpG (or non-CpG) site *i* is determined by the corresponding rate r_{met}^i multiplied by the fraction of unmethylated sites $(1 - \theta_m^i)$, which represents the concentration of the available substrate. The active demethylation at site *i* is given by the corresponding product of the demethylation rate at that site r_{demet}^i multiplied by the fraction of methylated sites θ_m^i . The passive demethylation at site *i* is determined by the rate of cell division D multiplied by the fraction of failure of maintenance methylation at site *i* $(1 - f_{main}^i)$, multiplied by the fraction of methylated sites θ_m^i and multiplied by 0.5, because DNA replication leads to the loss of the methylation in only one DNA strand.

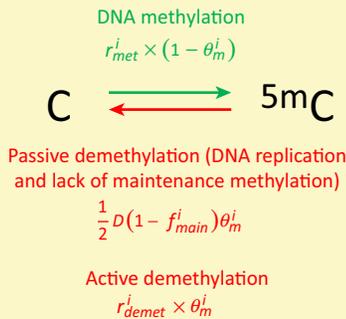
θ_m^i fraction of methylation at site *i*

r_{met}^i rate of *de novo* DNA methylation at site *i*

r_{demet}^i rate of DNA demethylation at site *i*

D rate of cell division

f_{main}^i efficiency of maintenance methylation at site *i* (as a fraction)



and its targeting to the region. The activity of the enzyme can be regulated by other bound proteins or RNAs, or by post-translational modifications, for example, phosphorylation. Finally, the sequence environment of the target CpG site, its methylation state and its accessibility can influence the local enzyme activity.

Another conceptual problem of the original CpG site-specific maintenance methylation model is that it does not offer any possibility for proofreading or repair, because any failure in maintenance methylation or aberrant *de novo* methylation would be propagated in all following cellular generations. Given that no biological system is fully accurate and perfectly efficient, the information stored in the methylation pattern would be unavoidably lost over time in a gradual manner [69]. By contrast, the stochastic model represented by equation 1 is stabilized by an inherent negative feedback, leading to a stable steady-state methylation level of each site. The feedback regulation is achieved, because any transient increase in methylation will cause a decline in the first term and an increase in the second term of equation 1, such that the methylation rate will drop and demethylation rate increases. As a consequence, the methylation level will decrease until the steady-state level is reached again. Similarly, a transient loss of methylation will cause a counteracting response of the system. Based on this the steady-state methylation level at site *i* ($\theta_m^{i,ss}$) is given by:

$$\theta_m^{i,ss} = \frac{r_{met}^i}{\frac{1}{2}D(1 - f_{main}^i) + r_{demet}^i + r_{met}^i} \quad [2]$$

The crosstalk between different chromatin modifications, including DNA methylation, provides additional

reinforcement loops of epigenetic states, because repressive histone modifications generally recruit DNA methylation, whereas activating modifications reduce DNA methylation. Therefore, large domains of repressed chromatin alternate with domains of active chromatin (Figure 3).

Applications and implications of the stochastic model of DNA methylation

The stochastic methylation model can recapitulate one of the key DNA methylation changes observed in cancer cells. In most tumors, a global reduction of DNA methylation has been observed that is accompanied by local increases in methylation at particular gene promoters [70,71]. Although local hypermethylation can be explained by adaptive advantages of the cancer cells due to the silencing of tumor suppressor genes, the origin of global hypomethylation in cancer cells is less clear. However, this process can be easily understood in the context of equation 2, because the steady-state methylation level depends on the activities of Dnmts (implicit in r_{met} and f_{main}) and demethylases (implicit in r_{demet}), as well as on the rate of cell division (D). If the cell division rate increases without adjustments of the methylation machinery (such that r_{met} , f_{main} and r_{demet} are not changed), which is a reasonable assumption for an early-stage cancer cell, the global DNA methylation level will drop automatically. Furthermore, the increased significance of the Dnmt3 enzymes in the maintenance of DNA methylation can explain the important contribution of Dnmt3a mutations to cancer, which is indicated by the strong enrichment of somatic Dnmt3a mutations in cancer tissues [72,73]. Finally, it has been recently reported that changes in the S-adenosyl-L-methionine level (the coenzyme that functions as methyl group donor in Dnmts) regulate DNA methylation in Schwann cells, which is in line with our stochastic model of DNA methylation [74].

The local activities and efficiencies of DNA methylation and demethylation depend largely on the target sequence, its chromatin environment, as well as targeting and regulation of Dnmts and demethylases, therefore, this model implies that different genomic loci in the same cell have different steady-state methylation levels. This concept explains the locus-specific response of genomic methylation levels observed in several studies with Dnmt inhibitors, such as 5-azacytidine [24,75,76]. After application of such inhibitors, the global activity of Dnmts will drop, but the response of particular genomic loci will depend on the local targeting of the methyltransferases. Regions with strong targeting of Dnmts will experience smaller changes in methylation rates and lower demethylation than regions with an inefficient targeting mechanism. In addition, local reinforcement mechanisms will help to keep higher Dnmt activity in some regions, again leading to a locus-specific response to global Dnmt inhibition. Local activities of methylation and demethylation also explain how the targeting or regulation of Dnmts can directly lead to changes in the methylation level.

In the light of our revised model, the site-specific methylation of a few CpG sites cannot be considered a model for epigenetic inheritance. This point needs to be strongly considered, particularly in epigenetic studies investigating

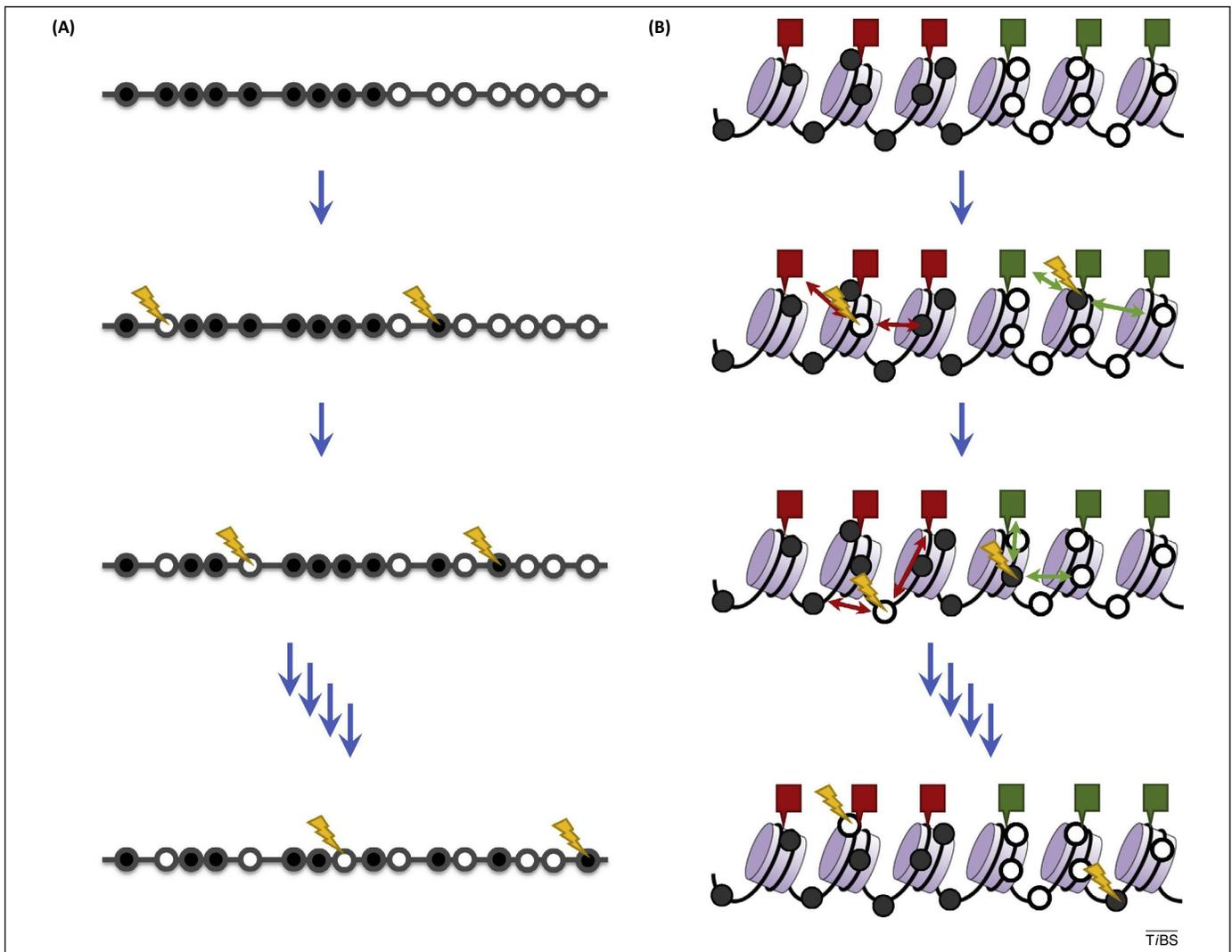


Figure 3. Schematic drawing of the reinforcement of chromatin states by crosstalk of DNA methylation and histone modifications during the maintenance of DNA methylation. **(A)** In the classical model of CpG site-specific maintenance methylation there are no mechanisms of error correction. Hence, methylation mistakes (indicated by yellow flashes) that arise either from aberrant *de novo* methylation or failure of maintenance will be propagated and the methylation pattern finally lost. **(B)** In the stochastic model, mistakes of maintenance methylation will occur as well. However, the crosstalk between different chromatin marks will lead to the correction of methylation mistakes. Repressive chromatin marks (red flags) and existing DNA methylation will recruit Dnmts and stimulate their activity (red arrows). Conversely, activating chromatin marks (green flags) will reduce the methylation of corresponding regions and keep CpG sites in an unmethylated state (green arrows). These processes allow the formation of domains of repressed and active chromatin as well as their stable propagation. After replication histone modifications are also diluted and need to be re-established, based on the existing chromatin marks, including DNA methylation. Blue arrows indicate one cell generation.

the role of DNA methylation with respect to its somatic and transgenerational inheritance. Instead, the average DNA methylation levels of particular DNA regions are stably inherited through the combined action of the chromatin modification network. Indeed, epigenetic inheritance in plants was shown to depend of the methylation of larger regions of DNA [77]. Specific methylation states of certain loci can be created by the targeting of Dnmts or DNA demethylases to these sites and by increasing the accessibility of the chromatin for DNA methylation or demethylation through corresponding chromatin modifications or remodeling activity (Figure 2 ①–⑦). Specific methylation states of individual CpG sites can be generated as well by targeting Dnmts or demethylases to specific CpG sites or by DNA-binding proteins protecting individual CpG sites from methylation or demethylation (Figure 2 ⑧). In the stochastic methylation model, such a situation would be described by large differences in the specific rates of methylation or demethylation between neighboring CpG sites.

However, it is not plausible to assume that Dnmt1 alone might propagate CpG site-specific methylation patterns with perfect efficiency, such that the targeting mechanism must be permanently active to perpetuate site-specific methylation. This view is in agreement with recent discoveries at imprinted loci, indicating that specific enhancement processes for DNA methylation are acting at these sites to ensure the inheritance of the methylation mark [78].

Concluding remarks

The classical CpG site-specific DNA methylation maintenance model does not explain many experimental results. A stochastic model of DNA methylation is presented here, in which DNA methylation at each site is determined by the local rates of methylation and demethylation. The stochastic model has inbuilt feedback regulation, which stabilizes a steady-state methylation state. These local rates depend on the targeting and regulation of Dnmts

and demethylases, such that DNA methylation is controlled by the network of epigenetic modifications. Interaction with other chromatin marks provides a second layer of feedback, which leads to the stable establishment of larger repressed and activated chromatin domains. The stochastic DNA methylation model explains *de novo* and maintenance methylation in a unified approach, as well as non-CpG methylation in human cells and other organisms. It has important implications, as single methylation events are not stably inherited, but only the combined methylation state of larger DNA regions. The new view on DNA methylation can recapitulate the global hypomethylation of cancer cells and the prominent role of Dnmt3a somatic cancer mutations. Finally, it can explain the locus specific response of genomic methylation levels to the application of DNA methyltransferase inhibitors that has been observed in many studies.

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