

Review

The cell biology of DNA methylation in mammals

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ABSTRACT

In this review, we will provide a brief reminder of epigenetic phenomena in general, and DNA methylation in particular. We will then underline the characteristics of the *in vivo* organization of the genome that limit the applicability of *in vitro* results. We will use several examples to point out the connections between DNA methylation and nuclear architecture. Finally, we will outline some of the hopes and challenges for future research in the field. The study of DNA methylation, its effectors, and its roles, illustrates the complementarity of *in vitro* approaches and cell biology.

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1. Introduction

1.1. DNA methylation is an essential epigenetic mark that controls gene expression

DNA methylation is an archetypal epigenetic mark [1,2]. It is borne by the genetic material but does not influence its sequence. It can regulate genomic activities, and can be maintained through mitosis and meiosis.

DNA methylation is essential in mammals: its loss leads to growth arrest or apoptosis in normal cells [3] as well as in cancer lines [4]. The presence of DNA methylation is absolutely required for embryonic development in mouse [5,6]. The key role of DNA methylation is to control gene expression, and methylated sequences undergo transcriptional repression [7].

Here we will review recent findings and concepts on the control of gene expression by DNA methylation, with an emphasis on cell biological aspects and a restriction to mammalian cells. We will first set the background with some reminders about the actors, the effectors, and the targets of DNA methylation.

2. The proteins that set up and interpret DNA methylation

The DNA of mammals can be methylated on cytosines within the CpG dinucleotides (Fig. 1). The added methyl groups protrude in the major groove of DNA. When the DNA is symmetrically methylated, both methyls face the same direction and are close to one another. The addition of methyl groups changes the biophysical characteristics of

the DNA and has two effects: it inhibits the recognition of DNA by some proteins and permits the binding of others [7].

The modification is brought about by enzymes called DNA methyltransferases (DNMTs). There are three such enzymes in mammals: DNMT1, DNMT3a, and DNMT3b (Table 1). DNMT3L is structurally related, but is catalytically inactive and serves as a cofactor for DNMT3a and DNMT3b [8]. The protein DNMT2 also has sequence similarity to these enzymes, but its function is quite different [9]; it will not be discussed further here. Extensive enzymology studies have yielded important insight into the function of these enzymes [10]. Notably, it was found that DNMT1 has preferential activity for hemi-methylated DNA over unmethylated DNA. It seems likely that, most of the time, DNMT3a and DNMT3b, aided by DNMT3L, set up the new imprints on previously naked DNA. For this they are called “*de novo*” methyltransferases. After DNA replication, methylated DNA becomes hemimethylated, and DNMT1 would be the main player in making it fully methylated again. It is thus called the “maintenance” enzyme (Table 1). This slightly oversimplified picture will suffice for our purpose here, but excellent detailed reviews are available to fill in the details [8,10].

The methyl mark is translated into transcriptional repression by the action of proteins that recognize methylated DNA and inhibit gene expression by creating a repressive chromatin structure [7]. Three families of proteins specifically recognize methylated DNA (Table 1) [11,12]. The first family contains MBD1, MBD2, MBD4, and MeCP2; these proteins share a related DNA binding domain called Methyl-binding Domain (MBD) [7]. The second family contains the Zinc-finger proteins Kaiso, ZBTB4, and ZBTB38 [13,14]. These proteins are bifunctional: they bind methylated DNA, but also some non-methylated consensus sequences. Finally, the third family comprises UHRF1 and UHRF2 (also known as ICBP90 and NIRF), which bind

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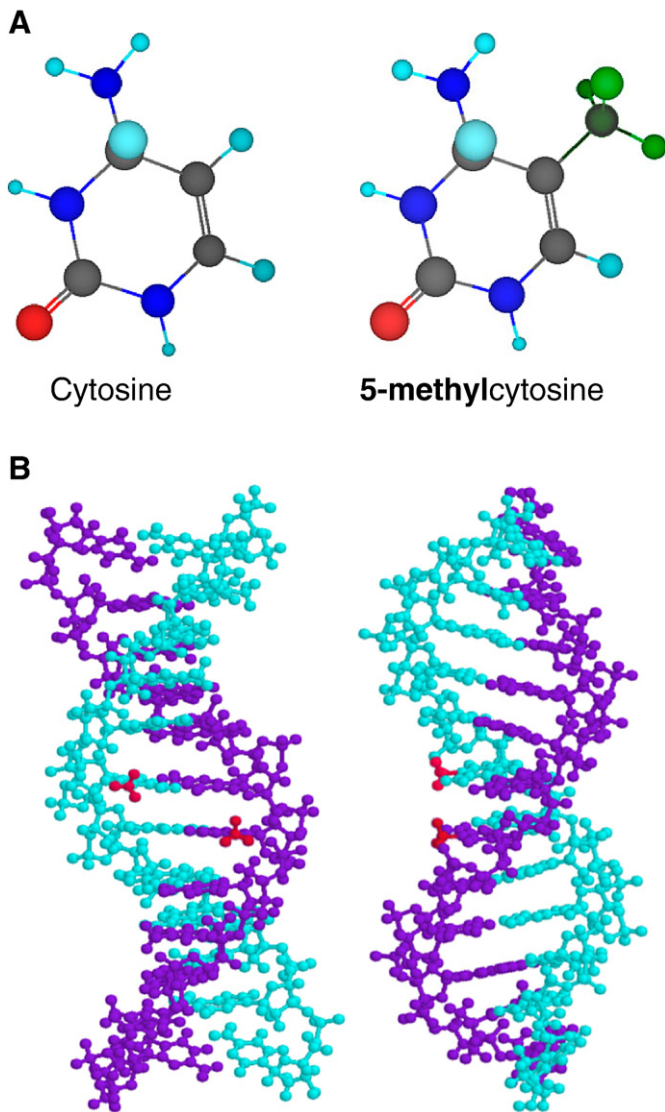


Fig. 1. The methylation of cytosines. (A) The chemical nature of DNA methylation. Sketch of a cytosine and its modified counterpart. (B) The methyl groups on modified cytosines are accessible in the major groove. The two strands of the DNA helix are colored blue and purple, and the methyl groups are highlighted in red. Two views of the same DNA are shown, they differ by a 90° rotation.

methylated DNA through their SET-and-RING-Finger-Associated (SRA) domain.

An important question, discussed at length in an excellent recent review [11] is that of the redundancy between methyl-binding proteins. Their degree of sequence specificity is poorly characterized, and it is unclear whether they can all bind the same target loci, or whether they have distinct targets. Even if the proteins do share some targets, they could be functionally different for other reasons. For instance, they could have different DNA-binding affinities. Also, the different proteins could be expressed at different times or places. Finally they could have different protein or nucleotide interactors that could possibly recruit them to different compartments of the nucleus.

3. The targets of DNA methylation differ in normal and cancer cells

In normal cells, three main types of targets are repressed by DNA methylation (Fig. 2). First: parentally imprinted genes, i.e. genes that are expressed differentially from the maternal and the paternal

chromosome. They are key regulators of embryonic development and adult life [15]. In most cases the inactive allele is marked by DNA methylation, and monoallelic expression is lost in the absence of methylation [16]. As an aside, recent data indicates that many genes may be expressed monoallelically in somatic cells, but it is yet unclear if this depends at all on DNA methylation [17]. Second: the transposons and other repeated sequences that constitute a large fraction of the mammalian genome [18]. Third: a number of genes are methylated in a tissue-specific manner [19]. An interesting subset of those are the Cancer/Testis (C/T) antigens, which are unmethylated and expressed in the testis, and methylated and repressed in all other tissues [20].

DNA methylation is deregulated in cancer (Fig. 2). Tumor cells often have an abnormal pattern of DNA methylation where some tumor suppressor genes are methylated and inactive [21]. Conversely, some normally methylated sequences, such as repeated DNA, imprinted genes, and C/T antigens, can become demethylated (Fig. 2). Abnormal DNA methylation is an early causal event during cellular transformation [22]. Demethylating agents can re-establish the expression of silenced tumor suppressor genes and have been approved for clinical use against some leukemias [23].

4. *In vitro* experiments do not reproduce some properties of the nucleus

In vitro work has been crucial in working out different elements of gene regulation by DNA methylation. It has yielded insight into the mechanisms of DNA methylation, the binding properties of methyl-binding proteins, and the way these factors regulate gene expression.

However we should keep in mind that the inside of the nucleus differs from a test tube in several important ways [24]. We will mention three. First, the nuclear environment is densely packed with macromolecules. The protein concentration in some compartments is estimated at 500 mg/ml, and this macromolecular crowding has some kinetic and thermodynamic effects that are usually not taken into account *in vitro*. Crowding is a serious hindrance to diffusion-driven reactions, and kinetic constants measured *in vitro* almost always neglect this factor. This barrier to diffusion may be a reason why protein recruitment is such a frequently recurring theme. We illustrate this idea with the example of DNMT1 in section 7.

A second key issue is that nuclear proteins are not homogeneously distributed; many proteins are found in nuclear bodies as the result of homo- and heterotypic interactions [25]. This can have either positive or negative consequences on their activity. In some cases, such as transcription factories, the nuclear bodies are sites of concentration of active proteins. In other cases, the nuclear bodies are storage sites from which the proteins have to be released to reach their target. A significant consequence is that the activity of a protein can be regulated by directing it into or out of a given area of the nucleus. This is an interesting extension of the regulation of protein by nucleocytoplasmic shuttling.

Table 1

The proteins involved in setting up and interpreting the methylation mark

De novo	Maintenance	Cofactor
DNMT3a	DNMT1	DNMT3L
DNMT3b		
MBD	Zinc finger	SRA
MeCP2	Kaiso	UHRF1
MBD1	ZBTB4	UHRF2
MBD2	ZBTB38	
MBD4		

Top panel: the enzymes that methylate DNA in mammals.

Bottom panel: the three families of proteins that bind methylated DNA in mammals.

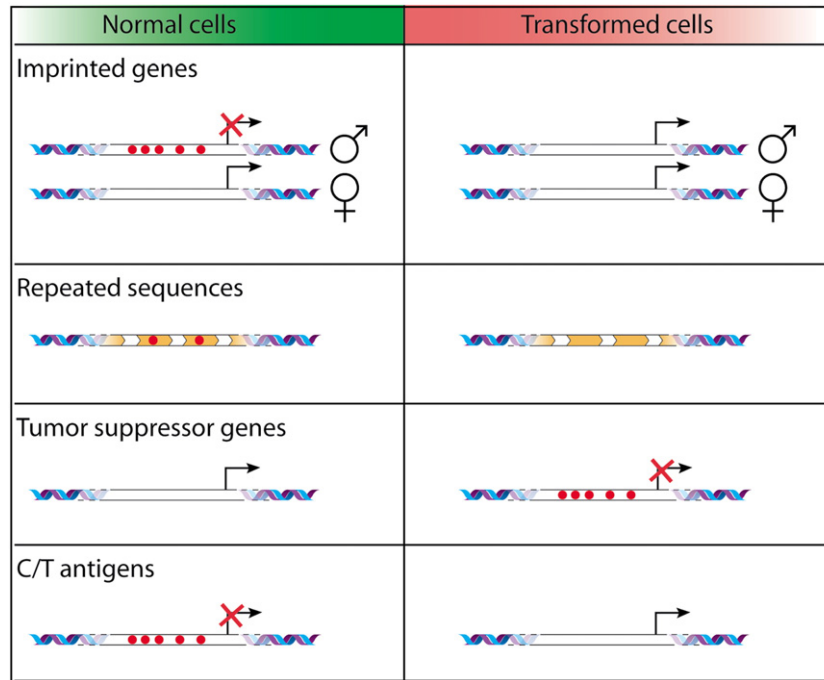


Fig. 2. The loci that undergo DNA methylation in mammals. At least three different types of loci can be methylated in normal cells (left half of the figure). Tumor suppressor genes, as well as the vast majority of genes with a CpG island promoter, are not methylated. In tumor cells (right half), the repeated sequences become demethylated, the imprinted genes become aberrantly demethylated (or methylated on both alleles, a situation not represented here). Some genes which were unmethylated can become methylated and transcriptionally silenced, and this is a frequent cause for the inactivation of tumor suppressor genes. Finally some genes that were methylated lose the mark, like the Cancer/Testis (C/T) antigens.

Thirdly, the genome is not arranged homogeneously and not all genes are equally accessible to protein regulators [25]. Chromosomes occupy given territories within the nuclear space. They have an interior and a surface, augmented by extruded loops and channel-like invaginations.

Many of these features are dynamic. The proteinaceous nuclear bodies are stable entities but the proteins that constitute them are in flux. The bodies themselves move about in the nucleus, and this may be a mechanism for scanning the genome. The chromosomes

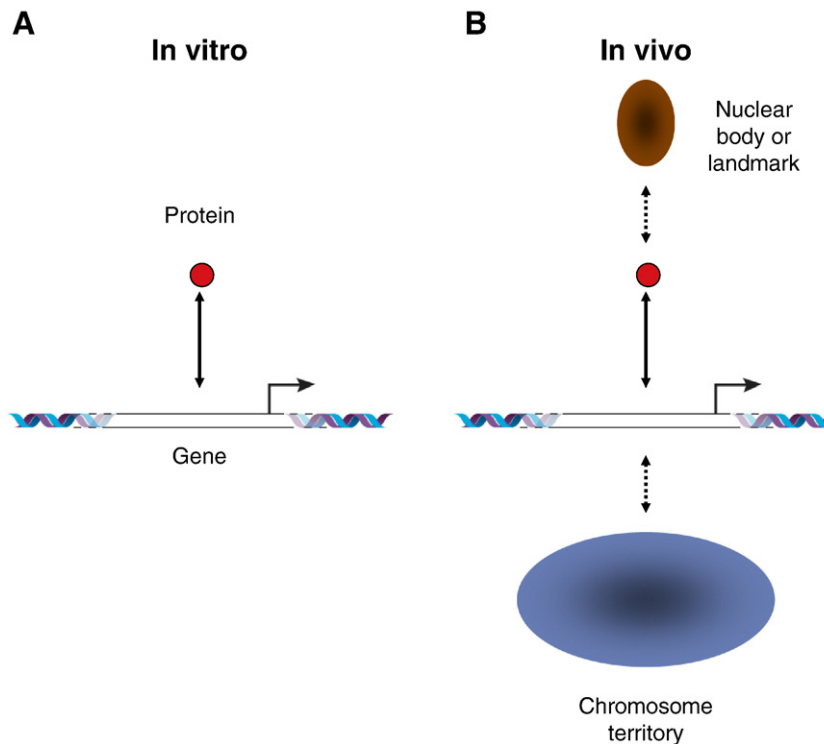


Fig. 3. Different parameters regulate DNA–protein interactions *in vitro* and *in vivo*. (A) *In vitro*, the interaction involves only two actors, and is determined only by their concentration and binding affinity. (B) In the nucleus of a cell, the situation is much more complex. The DNA-binding protein may interact with nuclear bodies or landmarks, such as the nuclear envelope or the nucleolus. The DNA locus is part of a chromosome and may be more or less accessible to the protein.

themselves are also mobile, yet their speed and range of diffusion are much inferior to that of nuclear bodies [25]. Smaller-scale movements such as the extrusion of a chromatin loop may have more functional significance.

The facts presented in this section bring us to an important conclusion (Fig. 3). Gene regulation depends on protein–DNA interactions. These can be easily modeled *in vitro*, where the only relevant parameters are the concentrations of the interactors and the binding constants. Yet the situation in living cells is considerably more complex: the organization of the nucleus is such that large scale interactions must also be taken into account. An added difficulty is their dynamic nature, the investigation of which demands appropriate technical approaches, as we will discuss later.

5. Kaiso: an example of regulation by nucleo-cytoplasmic shuttling?

The nucleo/cytoplasmic distribution of Kaiso is variable, and responds to intracellular signals. Indeed the activation of the Wnt pathway correlates with a movement of Kaiso out of the nucleus and into the cytoplasm [26]. The possibility that the Wnt pathway could, via its action on Kaiso, regulate the expression of methylated genes is tantalizing but has not yet been substantiated. For instance, it is not yet known whether the pool of Kaiso that shuttles in and out of the nucleus is the pool that is devoted to binding methylated DNA. It is possible that the methyl-binding population is immobile, whereas the fraction that binds unmethylated DNA is mobile. The generation of separation-of-function mutants of Kaiso, that could bind one type of target but not the other, would be useful to answer this question.

Kaiso knock-out mice display no obvious phenotype, nor reactivation of Kaiso target genes [27]. This suggests that other proteins have overlapping functions; ZBTB4 and/or ZBTB38 are the most likely candidates. This possible functional overlap suggests that these two proteins may be regulated in a similar fashion, but this has not yet been reported.

In contrast to Kaiso, the MBD proteins seem to be constitutively nuclear [11]. Details regarding their localization at higher resolution are scarce, however. Sumoylation, which is known to regulate

subnuclear distribution in some proteins, occurs on MBD1 but has no effect on its localization [28]. In mouse cells, transfected MBD proteins are recruited to the chromocenters, i.e. the clusters of pericentric heterochromatin that are densely methylated. This is also the case for ZBTB4 (Fig. 4), but care should be taken when interpreting these experiments, as transfected proteins are usually overexpressed and do not necessarily reflect the behavior of the endogenous proteins. There are no chromocenters in human cells, in which the MBDs appear to be diffuse nuclear proteins [11]. Some questions are unresolved. Active genes tend to localize to “transcription factories”, whereas genes repressed by the Polycomb proteins are recruited to “Polycomb bodies” [29]. What about methylated genes? A mechanistic link between Polycomb repression and DNA methylation has been proposed [30], but methylated genes, and the MBD proteins, are not known to colocalize with Polycomb bodies. The presence of unbound proteins, which we will mention in the next section, may obscure the existence of subnuclear enrichments of the different methyl-binding proteins.

More generally, it is unclear if methylation results in gene repression because it drags to the locus to a repressive environment, or because it removes it from a permissive environment, or both, or neither. Having a better idea of where the methyl-binding proteins localize in relation to nuclear structures would help answer this question.

6. Much remains to be learnt about the dynamics of the methyl-binding proteins

The *in vivo* dynamics of some heterochromatin proteins has been well studied. Work on HP1 yielded the surprising result that, even though heterochromatin is functionally stable, the heterochromatin proteins themselves are in rapid flux: they exchange in and out of the chromatin domains quickly. For HP1, 50% recovery after photobleaching of heterochromatic regions occurred in about 2.5 s [31]. The knowledge of these kinetic parameters gives insight into the mechanism by which these proteins act, and the possible means by which they may be regulated.

In contrast, relatively little is known about the dynamics of methyl-binding proteins.

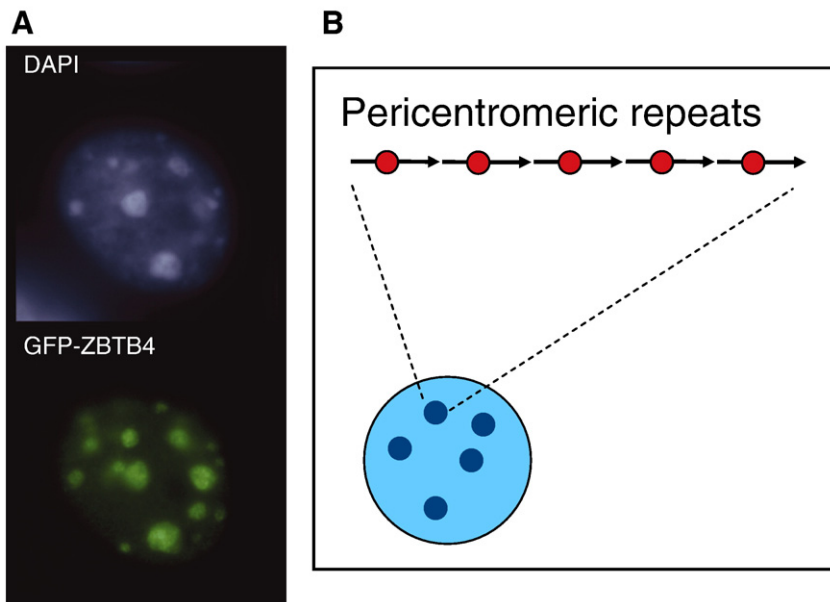


Fig. 4. Localization of the methyl-binding protein ZBTB4 in mouse cells. (A) Mouse 3T3 fibroblasts were transiently transfected with an expression vector for ZBTB4 fused to the green fluorescent protein GFP. Top panel: the nucleus, stained with DAPI. Bottom panel: the green fluorescence signal, which colocalizes with the DAPI-dense foci. (B) Interpretative scheme. The DAPI-dense foci, known as chromocenters, correspond to the clustering of peri-centromeric repeats (arrows) from several chromosomes. These repeats are heavily methylated (red circles).

The exception is MeCP2, which has been studied by FRAP (Fluorescence Recovery After Photobleaching) in living mouse cells [32]. Two populations of MeCP2 can be identified. One, localized in the nucleoplasm, is highly dynamic with a $t_{1/2}$ of 0.1 s, suggesting that there is a pool of unbound protein. The second population is bound to the chromocenters. There, its residence time is higher ($t_{1/2}=25$ ss, consistent with earlier reports [33]). This is much longer than the residence time of HP1, and it may reflect additional interactions that retain the protein to chromatin. The other MBDs have not been similarly studied and, to the best of our knowledge, no information is yet published regarding UHRF1, UHRF2, or the Zinc finger proteins Kaiso, ZBTB4, and ZBTB38.

The results of immunofluorescence experiments performed on fixed cells are available for almost all of these proteins, but they only provide a snapshot of the protein distribution at a given time. They do not inform us about the dynamics: the same immunofluorescence picture could actually reflect two very different situations. A very dynamic situation, with rapid association and dissociation, would give the same appearance as a very static situation in which movement is rare. These two extreme situations impose very different characteristics on the way the proteins can act and can be regulated.

7. The recruitment of DNMT1 for DNA replication and transcriptional repression

In order to maintain the epigenetic information, the DNA methylation marks have to be reproduced after each round of DNA replication. A link between replication and the DNA methylation machinery was found early on when it became apparent that DNMT1 interacts directly with the sliding clamp PCNA [34]. It was also found

that DNMT1 colocalizes with the replication machinery in S-phase, and this gave rise to a model in which the DNA was remethylated by DNMT1 concurrently to its synthesis (Fig. 5, left panel). However, kinetic studies later made it clear that the speed of DNA methylation is much smaller than the speed of DNA replication, which questioned the validity of this simple model [10]. This yielded a refined picture, in which DNMT1 is assumed to be loaded onto DNA by the moving replication fork, and then to re-methylate DNA at its own speed (Fig. 5, right panel).

This model is probably true but incomplete. Indeed, DNMT1 can be loaded onto chromatin outside of S-phase [35]. Moreover, the PCNA-interacting region of DNMT1 can be removed without major consequences for DNA remethylation [36,37]. The missing link for DNMT1 targeting was very recently identified in two independent papers [38,39]. The methyl-binding protein UHRF1 was shown to i) bind hemi-methylated DNA ii) recruit DNMT1 and iii) be necessary for the maintenance of methyl marks. It is likely that most DNMT1 molecules are brought to hemimethylated sites via UHRF1, as depicted in Fig. 5.

It is worth stressing that, even though DNMT1 has clear affinity for and activity on hemimethylated sites *in vitro*, this is not sufficient to target it to these sites *in vivo*. The affinity of the protein for its sites may be too low in relation to their relative concentrations, or the obstacles to diffusion may too great, or both. In any event, this is an interesting illustration of the principle outlined in Fig. 4.

While the reproduction of marks by DNMT1 is relatively well understood, little is known about the mechanisms by which DNMT3a and DNMT3b establish their imprints at given loci. The enzymes have some degree of sequence specificity towards specific arrangements of CpGs [40], and a preference for regions lacking euchromatin marks

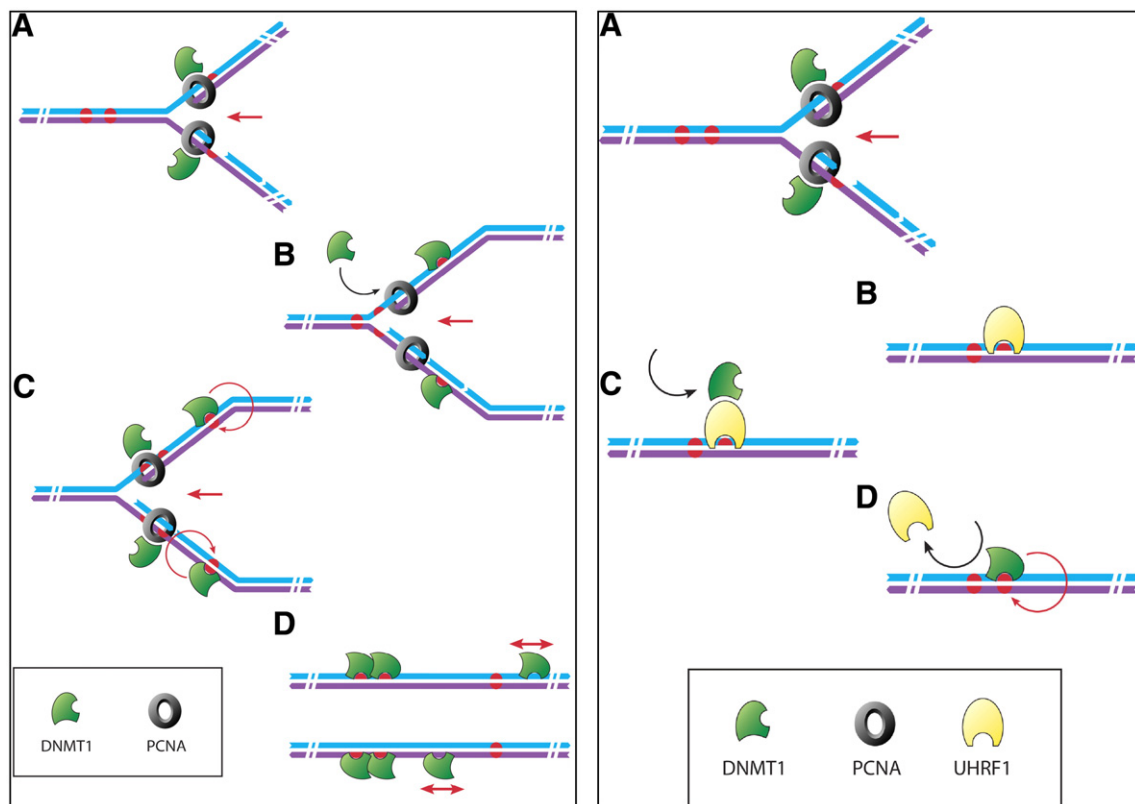


Fig. 5. Two recruitment events insure faithful DNA remethylation after replication. Left panel. A. As DNA is replicated, the sites that were previously symmetrically methylated (full red circle) become hemimethylated (red semi-circle). The DNA methyltransferase DNMT1 interacts with the replication protein PCNA, which slides along the DNA. B. As the replication fork progresses, DNMT1 is transferred to the hemimethylated sites, and new protein is loaded onto PCNA. C. DNMT1 remethylates the hemimethylated site it is bound to. D. Finally, the loaded DNMT1 can track along the genome to remethylate sites that have been ignored. Right panel. A second mechanism for targeting DNMT1 to hemimethylated sites does not depend on DNA replication (A). Instead, it involves the protein UHRF1, which has specific affinity for hemimethylated sites (B), and which can directly recruit DNMT1 (C). UHRF1 no longer interacts with the fully methylated sites re-created by DNMT1 (D).

[41], but additional targeting mechanisms are likely to exist. One aspect of the regulation may be that some regions are made accessible to, or are protected from the enzymes by their respective localization in the nuclear space, but this has not yet been shown.

The recruitment of DNA methyltransferases also occurs in the context of transcriptional regulation. DNMT1 serves as a corepressor for certain transcription factors, such as p53 [42] and Daxx [43]. The heterochromatin protein HP1, which is a global repressor, also recruits DNMT1 [44]. C-myc represses some of its target genes by recruiting DNMT3a [45]. Transcriptional inhibition in all these cases results in DNA methylation and may cause a repression that is more stable than what is obtained by histone modifications only. Again, it is unclear if methylation systematically causes the target locus to join a heterochromatic area of the nucleus.

8. Is MeCP2 a structural component of the nucleus in differentiated cells?

The amount of MeCP2 in cells increase as they differentiate, and it reaches surprisingly high levels. It has been estimated that there are on average 6×10^6 molecules of MeCP2 per brain cell [46]. This is close to the estimated number of nucleosomes in the cells, which is 3×10^7 [47]. If MeCP2 binds only nucleosomes in heterochromatin, and if heterochromatin is estimated to constitute one third of the genome, then there is almost one molecule of MeCP2 for every heterochromatic nucleosome. This raises the possibility that MeCP2, in addition to, or maybe instead of, being a gene-specific transcriptional repressor, might function as a structural component of the nucleus in neurons. In this respect it might play a role similar to that of SATB1. This protein is a structural component of nuclei in the lymphocytic lineages, where it regulates the expression of many genes [48].

The lack of MeCP2 in neurons causes Rett syndrome [49]. Intriguingly, the symptoms can be reversed in mice by reintroduction of an active copy of MeCP2 [50]. The hypothesis most frequently used to explain the syndrome is that lack of MeCP2 leads to the inappropriate re-expression of some methylated genes, which then impair neuronal function. Curiously, however, only a handful of genes have been shown to be repressed by MeCP2 in neurons in a methylation-dependent manner, and they probably do not account for the severe phenotype observed. As an alternative explanation, MeCP2 has been reported to influence splicing [51]. However MeCP2 has not been observed to be a stoichiometric component of spliceosomes, at least in cultured cells [52]. A third possibility would be that MeCP2 indeed plays the role of a scaffold. The lack of MeCP2 might result in a structural weakening of the nuclear architecture, and/or a global loosening of the transcriptional program of neurons. Neither alteration could be clearly diagnosed using the methods currently employed (microarray and differential expression). Because mRNA splicing is intimately linked to nuclear export, structural defects in the absence of MeCP2 could also account for the splicing abnormalities observed. If this situation were true, it would present an interesting parallel to the human diseases named “laminopathies”, in which mutations of the lamins affect the structure of the nucleus and result in severe phenotypes [53].

The possibility that MeCP2 could be a scaffold that links nucleosomes is supported by some biochemical experiments [54]. It is also consistent with the fact that MeCP2 overexpression causes chromocenters to cluster [55]. However, it seems to conflict with two other pieces of data. First, neurons lacking MeCP2 have a morphologically normal nucleus. This contrasts with nuclei from laminopathy patients, which are misshapen. Of course, morphology is a coarse readout which may not reflect subtle defects. A second observation is the above mentioned fact that MeCP2 is fairly mobile in the cell [32]. MeCP2 behaving dynamically seems hard to reconcile with the idea that it may be a structural component. One caveat of the *in vivo* studies, however, is that they were done using fibroblasts, not neurons.

9. Technical challenges and advances

As for many other fields, our understanding of DNA methylation in the context of the nucleus is critically dependent upon the tools we have.

Live-cell imaging has yielded data which was unobtainable by the study of fixed cells. The DNMTs and MeCP2 have been well studied in live cells, and hopefully the other methyl-binding proteins will also be investigated by these methods. To be maximally significant, a study should use cells in which the fluorescent proteins are expressed at levels lower than the endogenous counterpart, so as not to interfere with its distribution. Also, this should be done in a physiologically relevant cell type. This can be done by stable transfection and selection of clones, or, ideally, by fusing a fluorescent tag to the endogenous protein by homologous recombination (knock-in). These requirements may be difficult to fulfill, but the rewards can be great.

Microscopy is not the only way to examine the architecture of the nucleus. Molecular biology approaches have been very fruitful as well. The identification of methylated sequences has been greatly facilitated by a variant of chromatin-immunoprecipitation called MeDIP [56]. Other techniques give insight into the possible 3D arrangement of these loci. The DamID technique is an elegant approach to map the organization of loci relative to nuclear structures [57]. The Chromosome Conformation Capture (3C) assay is also powerful, especially in combination with the use of microarrays [58]. We can be confident that the wide-spread use of these techniques in well characterized cellular systems will greatly enhance our comprehension of the architecture of methylated loci.

10. Questions for the future

The architecture of the nucleus is a key parameter for the function of the genome. One of the elements that regulates gene expression is DNA methylation. How it ties in with the three dimensional organization of the nucleus is partly understood at best, and many questions remain. Are methylated loci recruited to a specific repressive compartment, or are they pulled away from permissive regions? Do genes become methylated just because their location, or their chromatin organization, makes them accessible to methyltransferases? Conversely, are genes protected against methylation because they are “in the right place at the right time”? Insulators delimit euchromatic and heterochromatic regions, and have strong links to DNA methylation [59]. How do they work in space and time?

The correct pattern of DNA methylation on genes, including those that are parentally imprinted, is critical for the embryonic development of mammals. This pattern undergoes dramatic remodelling steps in the fertilized egg and in germ cells [60]. How is this connected to the architecture of chromatin, which is in flux at the same time too [61]? The expression of MBD proteins, and the distribution of the methylated chromocenters, are dynamic during development, but do they only respond to differentiation, or do they actually drive change by acting on gene expression? The different cells in our bodies have different epigenomes [62]. Are those dictated by their different nuclear organizations? A similar question applies to cancer cells, as opposed to normal ones.

Finally, and most intriguingly, what about DNA demethylation? This has been shown to occur, at least in cell lines, and the reaction could be reconstituted *in vitro* [63]. When and where does this take place in the nucleus? Fortunately, there is no lack of interesting questions for the future.

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