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Genomic DNA methylation: the mark and its mediators

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Methylation of DNA at position five of the cytosine ring occurs at most CpG dinucleotides in the mammalian genome and is essential for embryonic viability. With several of the key proteins now known, it has become possible to approach the biological significance of this epigenetic system through both biochemistry and genetics. As a result, advances have been made in our understanding of the mechanisms by which DNA methylation is targeted to specific regions of the genome and interpreted by methyl-CpG-binding proteins. Recent studies have illuminated the role of DNA methylation in controlling gene expression and have strengthened its links with histone modification and chromatin remodelling.

Introduction

DNA methylation is found in the genomes of diverse organisms including both prokaryotes and eukaryotes. In prokaryotes, DNA methylation occurs on both cytosine and adenine bases and encompasses part of the host restriction system (reviewed in Ref. [1]). In multicellular eukaryotes, however, methylation seems to be confined to cytosine bases and is associated with a repressed chromatin state and inhibition of gene expression [2]. DNA methylation is essential for viability in mice, because targeted disruption of the DNA methyltransferase enzymes results in lethality [3,4].

There are two general mechanisms by which DNA methylation inhibits gene expression: first, modification of cytosine bases can inhibit the association of some DNA-binding factors with their cognate DNA recognition sequences [5]; and second, proteins that recognize methyl-CpG can elicit the repressive potential of methylated DNA [6,7]. Methyl-CpG-binding proteins (MBPs) use transcriptional co-repressor molecules to silence transcription and to modify surrounding chromatin, providing a link between DNA methylation and chromatin remodelling and modification [8–13].

In this review, we focus on recent advances in our understanding of the mechanisms by which DNA methylation is targeted for transcriptional repression and the role of MBPs in interpreting the methyl-CpG signal and silencing gene expression. We emphasize examples from mammalian systems, including studies on animal models, because several recent reviews have covered topics of DNA methylation and silencing in plants and fungi [14–18].

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Targeting DNA methylation *de novo*

DNA methyltransferases

Mammalian cytosine DNA methyltransferase enzymes fit into two general classes based on their preferred DNA substrate. The *de novo* methyltransferases DNMT3a and DNMT3b are mainly responsible for introducing cytosine methylation at previously unmethylated CpG sites, whereas the maintenance methyltransferase DNMT1 copies pre-existing methylation patterns onto the new DNA strand during DNA replication. A fourth DNA methyltransferase, DNMT2, shows weak DNA methyltransferase activity *in vitro* [19], but targeted deletion of the *DNMT2* gene in embryonic stem cells causes no detectable effect on global DNA methylation, suggesting that this enzyme has little involvement in setting DNA methylation patterns [20].

DNMT3L is a DNMT-related protein that does not contain intrinsic DNA methyltransferase activity, but physically associates with DNMT3a and DNMT3b and modulates their catalytic activity [21]. In combination, these *de novo* and maintenance methyltransferases seem to constitute the core enzymatic components of the DNA methylation system in mammals.

Mechanisms of targeting de novo *methylation*

Examples of global *de novo* methylation have been well documented during germ-cell development and early embryogenesis, when many DNA methylation marks are re-established after phases of genome demethylation [22]. It is difficult to study the mechanisms by which DNMT enzymes are recruited to the targeted DNA sequences at these times, owing to the small amounts of biological material available for molecular and biochemical study. Most current knowledge regarding targeting of DNA methylation *de novo* therefore comes from cell-culture model systems. These studies have suggested at least three possible means by which *de novo* methylation might be targeted: first, DNMT3 enzymes themselves might recognize DNA or chromatin via specific domains; second, DNMT3a and DNMT3b might be recruited through protein–protein interactions with transcriptional repressors or other factors; third, the RNA-mediated interference (RNAi) system might target *de novo* methylation to specific DNA sequences (Figure 1). Here, we consider evidence relating to these possibilities in turn.

In mouse cells, DNMT3 enzymes partially localize to regions of pericentromeric heterochromatin. Functional studies show that the conserved PWWP domain is

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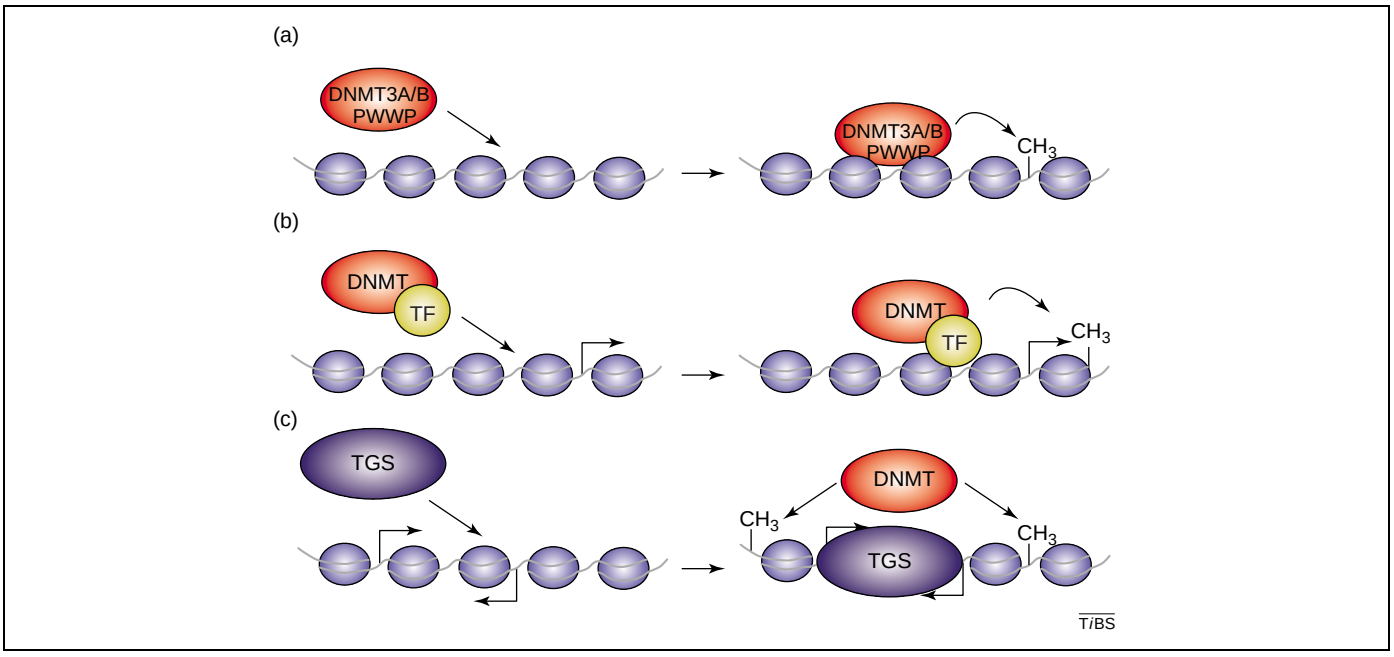

Figure 1. Targeting *de novo* DNA methylation. **(a)** The PWWP domain of the DNMT3a and DNMT3b enzymes is required for targeting DNA methyltransferase activity to regions of pericentromeric heterochromatin. The PWWP domain might function as a DNA- or chromatin-targeting motif. **(b)** Transcription factors (TF) have the capacity to interact with DNA methyltransferase enzymes (DNMT) and to recruit methyltransferase activity as a part of the molecular silencing repertoire used to shutdown gene expression. **(c)** *De novo* DNA methylation might be targeted by transcriptional gene silencing (TGS) pathways that respond to RNAi signals. The molecular mechanisms by which these silencing pathways are linked remain unknown.

required to target the catalytic activity to these regions of the genome (Figure 1a). The importance of this domain was highlighted by the discovery that a mutation in the PWWP domain of the human DNMT3b protein causes ICF syndrome, a severe autosomal recessive disease in humans [23]. The mutation abolishes normal chromatin binding by DNMT3b in tissue-culture cells [24] and causes a reduction in DNA methylation of classical satellite 2 DNA in affected individuals [23]. The crystal structure of the mouse Dnmt3b PWWP domain has been solved, and *in vitro* evidence indicates that this domain might interact with DNA in a sequence-independent fashion [25]. Notably, the PWWP domain shares structural similarities with the 'tudor' domain found in 53BP1, a protein that binds histone tails modified on lysine 79 of the H3 subunit [26,27]. It will be interesting to determine whether histone modifications are also involved in targeting DNMT3 proteins via their PWWP domains.

DNMTs can also be targeted to endogenous genes by interaction with site-specific transcriptional repressor proteins (Figure 1b). This idea was first suggested for the oncogenic fusion protein PML–RAR, which can recruit DNA methyltransferases and cause hypermethylation of target genes in cancer cells [28]. More recently, Brenner *et al.* [29] have shown that the Myc protein associates with DNA methyltransferase activity, and that a direct interaction between DNMT3a and Myc is required for efficient repression of the Myc target gene $p21^{cip1}$. Chromatin immunoprecipitation studies using tissue-culture cells have shown that Myc is required for recruitment of DNMT3a to the $p21^{cip1}$ promoter region, leading to *de novo* methylation of the $p21^{cip1}$ promoter. The DNA methyltransferase activity of DNMT3a is required for this gene-silencing event, because a point mutation in the catalytic domain alleviates silencing. The exact role of DNMT3a in regulating normal expression of $p21^{cip1}$ gene remains to be elucidated, but this model system has uncovered a potential mechanism by which *de novo* methylation is recruited by factors that repress transcription.

A second series of studies from Santoro *et al.* [30,31] has shown that DNA methylation has a role in repressing the expression of genes encoding ribosomal RNA (rRNA). This silencing event relies on *de novo* methylation of a single CpG dinucleotide in the promoter region of the rRNA gene. TIP5, a component of the NoRC repressor complex, associates with both DNMT3b and DNMT1, providing the link between NoRC silencing at the rRNA genes and the DNA methylation system. Chromatin immunoprecipitation analysis indicates that DNMT enzymes are actively recruited to the promoter region of the rRNA genes [31], and subsequent silencing is dependent on *de novo* methylation [30]. Together, these studies indicate that protein–protein interactions are important mediators of *de novo* DNA methylation, and that DNA methyltransferase enzymes can function as classical co-repressor molecules for some transcription factors.

In plants and some fungi, induction of the RNAi gene-silencing system results in both posttranscriptional and transcriptional silencing of gene expression. RNAi-mediated transcriptional silencing in plants often results in *de novo* methylation of the silenced gene [32]. Two independent studies have reported a similar mechanism of *de novo* methylation during RNAi silencing in mammalian cell-culture systems [33,34] (Figure 1c). When double-stranded RNA corresponding to the promoter sequence of a gene is introduced into mammalian tissue-culture cells, the target gene is efficiently silenced concomitant with

de novo DNA methylation of the corresponding promoter sequence. There is some controversy regarding the generality of this observation, because other reports detail RNAi-mediated silencing in the absence of DNA methylation [35–37].

This uncertainty is compounded by studies of mouse cells lacking the *Dicer* gene, an essential component of the RNAi system. In one study, deletion of *Dicer* resulted in transcription of normally silenced centromeric satellite repeats, which suggests that the RNAi system is required for the silenced state of these elements. In addition, in *Dicer*-null cells showing aberrant satellite transcription, there was a decrease in the amount of DNA methylation in the centromeric repeats [38], implicating the RNAi machinery in sustaining these methylation marks. By contrast, a separate study found no changes in satellite DNA methylation in a *Dicer*-null embryonic stem line [39]. Further work is evidently required to verify whether there is an RNAi-based transcriptional silencing pathway in mammals. Initial evidence supports its existence, but its effects seem to be much less obvious that those reported in plants.

Coupling epigenetic modification of DNA to gene expression silencing

DNA methylation is linked with transcriptional silencing of associated genes [6,40–42], and much effort has been invested in studying the mechanisms that underpin this relationship. Two basic models have evolved: in the first, DNA methylation can directly repress transcription by blocking transcriptional activators from binding to cognate DNA sequences [5]; in the second, MBPs recognize methylated DNA and recruit co-repressors to silence gene expression directly [9,43] (Figure 2a,b). For some promoters, repression mediated by DNA methylation is most efficient in a chromatin context, indicating that the 'active' component of this repression system might rely on chromatin modification [41,44]. In keeping with this observation, MBPs associate with chromatin remodelling co-repressor complexes [2,11,45,46].

Two unexpected facets of the DNA-methylation-mediated silencing system have recently become apparent: first, DNA methyltransferase enzymes themselves might be involved in setting up the silenced state in addition to their catalytic activities; and second, DNA methylation can affect transcriptional elongation in addition to its characterized role in inhibiting transcriptional activation.

DNA methyltransferases are transcriptional repressors associated with chromatin remodeling enzymes

As discussed, DNA methyltransferases can apparently be recruited by some transcriptional repressors during gene silencing, leading to methylation of DNA. In addition to this catalytic role, there is now evidence that DNMTs have a non-enzymatic role in transcriptional silencing [47–49]. DNMT-mediated silencing seems to rely on modification of chromatin, because each enzyme interacts biochemically with histone methyltransferases [50–52] and histone deacetylases [48–50,53–55]. One study also indicates that DNMT3b can interact with the ATP-dependent chromatin remodelling protein hSNF2H [50]. These findings raise the possibility that DNMTs are dual function proteins that silence gene expression both directly through transcriptional repression and indirectly through epigenetic modification of cytosine (Figure 2c).

DNA methylation and transcriptional elongation

Many studies have detailed the repressive effects that promoter-associated DNA methylation has on gene expression. Notably, a large fraction of normal genomic DNA methylation is found in genes, within both intronic and exonic regions. Experiments using plasmid-based reporter genes have indicated that methylation over the body of a gene can result in reduced gene expression [56].

Recent work has taken advantage of an integrated genomic reporter system to insert DNA methylation

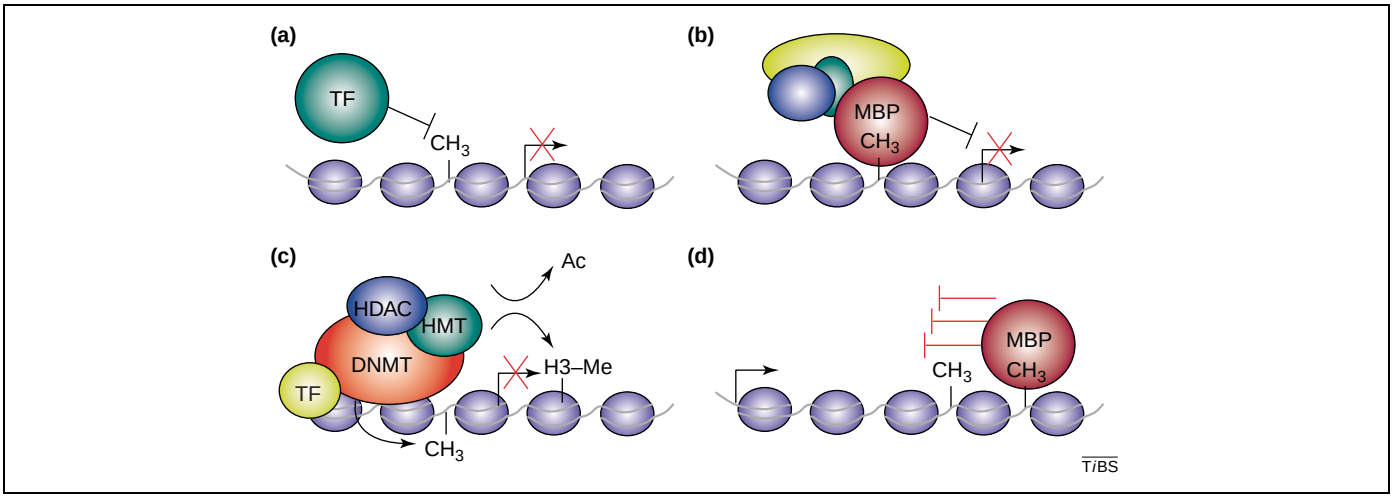

Figure 2. Mechanisms of DNA-methylation-mediated repression. **(a)** DNA methylation in the cognate DNA-binding sequences of some transcription factors (TF) can result in inhibition of DNA binding. By blocking activators from binding targets sites, DNA methylation directly inhibits transcriptional activation. **(b)** Methyl-CpG-binding proteins (MBPs) directly recognize methylated DNA and recruit co-repressor molecules to silence transcription and to modify surrounding chromatin. **(c)** In addition to their DNA methyltransferase activities, DNMT enzymes are also physically linked to histone deacetylase (HDAC) and histone methyltransferase (HMT) activities. In this case, the addition of methyl groups to DNA is coupled to transcriptional repression and chromatin modification. **(d)** DNA methylation within the body of genes can also have a dampening effect on transcriptional elongation. MBPs might be involved in inhibiting elongation, either directly or by their effects on the surrounding chromatin structure.

specifically distal to the start site of transcription [57] (Figure 2d). This study has found that reduced expression of the reporter is not caused by the effects of DNA methylation on initiation of transcription or promoter clearance. Instead, there is a reduction in RNA polymerase II (Pol II) occupancy and reduced chromatin accessibility over the body of the methylated gene in comparison to identical unmethylated control plasmid integrants. Precisely how DNA methylation in the body of a gene modulates expression remains poorly understood, but its presence can clearly dampen the capacity of Poll II to transcribe through methylated regions.

Proteins that recognize methyl-CpG and repress transcription

A family of methyl-CpG-binding proteins

Biochemical activities that specifically recognize methyl-CpG were identified more than a decade ago [58,59]. Characterization of the methyl-CpG-binding domain (MBD) – the protein motif responsible for binding to methylated CpG dinucleotides – facilitated bioinformatic identification of a family of proteins that share this domain [7] (Figure 3). With the exception of MBD3, which contains amino acid substitutions that prevent binding to methyl-CpG, the mammalian MBD proteins (named MBD1–MBD4) and the founding member, MeCP2, all specifically recognize methyl-CpG. A novel MBP named Kaiso lacks the MBD, but recognizes methylated DNA through zinc-finger domains [60].

All MBPs can mediate silencing of gene expression. They achieve this by targeting chromatin remodelling co-repressor complexes to regions containing DNA methylation [8–13,45,61,62]. In contrast to other MBPs, MBD4 is best characterized for its role in DNA repair [63–65], but a recent report suggests that it might also function as a transcriptional repressor [66]. Because the basic properties of MBPs have been reviewed elsewhere [2,18,46], in the following sections we highlight some of the more recent findings regarding the functions of these proteins in gene silencing.

Kaiso: the odd one out

Kaiso is a bifunctional DNA-binding protein that can recognize DNA sequences containing two methylated CpG dinucleotides or a cognate sequence that does not contain methyl-CpG DNA [60,67]. Kaiso recognizes methyl-CpG using zinc-finger domains present in the C terminus and mediates repression of methylated genes by associating with the histone-deacetylase-containing N-CoR co-repressor complex [45].

In *Xenopus* embryos, depletion of Kaiso results in upregulation of methylated genes before the mid blastula transition, confirming the role of this MBP as a methylation-dependent transcriptional repressor [68]. *Xenopus* Kaiso also represses expression of factors involved in the Wnt signalling pathway via its non-methyl-CpG-binding activities [69]. Mice lacking the *Kaiso* gene show no overt phenotype, however, they show resistance to intestinal tumorigenesis [70]. Further analysis of this atypical MBP is needed to determine its relative contribution to methylation-dependent repression in mammalian systems.

MBD1 can set up repression during DNA replication

Sarraf and Stancheva [11] have uncovered a novel silencing role of MBD1 during S phase of the cell cycle. In HeLa cells, MBD1 can interact with the histone H3 lysine 9 (H3K9) methyltransferase enzyme SETDB1 [71], coupling recognition of DNA methylation to modification of the surrounding chromatin by histone methylation (Figure 4). During S phase of the cell cycle when DNA replication occurs, MBD1–SETDB1 forms a transient complex with the chromatin assembly factor CAFp150, which in turn associates with the DNA replication machinery. This suggests a scheme in which MBD1–SETDB1 is targeted to methylated CpG sites during DNA replication.

By isolating MBD1-binding sites *in vivo*, Sarraf and Stancheva [11] were able to monitor the effect of MBD1 depletion on gene silencing and chromatin modification after cell division. As expected, the MBD1-bound regions of chromatin were hypomethylated on histone H3K9 when MBD1 was deficient. In one instance where the MBD1-

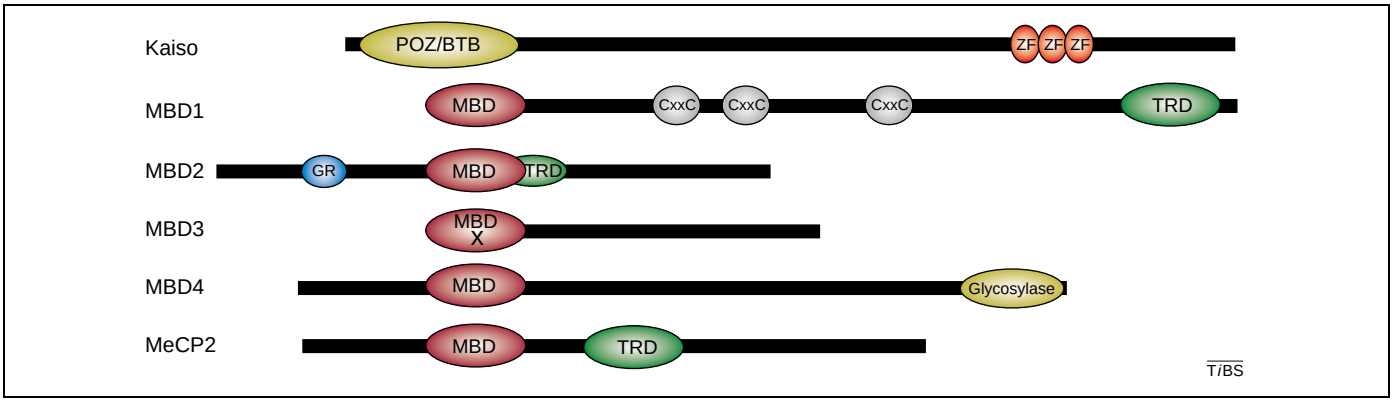

Figure 3. A family of methyl-CpG-binding proteins (MBPs). Six mammalian MBPs have been characterized so far. Kaiso is an atypical MBP, because it depends on a zinc-finger domain (ZF) to recognize methylated DNA and a POZ/BTB domain to repress transcription. MBD1 uses its methyl-binding domain (MBD) to bind methylated DNA sequences. In addition, MBD1 contains three zinc-binding domains (CxxC), one of which binds specifically to non-methylated CpG dinucleotides, and a C-terminal transcriptional repression domain (TRD). MBD2 possesses an MBD that overlaps with its TRD domain, and a GR repeat at its N terminus. MBD3 contains a well-conserved MBD domain that does not recognize methylated DNA owing to crucial amino acid changes. MBD4 binds methylated DNA through an MBD domain and has a C-terminal glycosylase domain that is important for is function in DNA repair. MeCP2 is the founding member of the MBD protein family and contains a conserved MBD domain and an adjacent TRD domain.

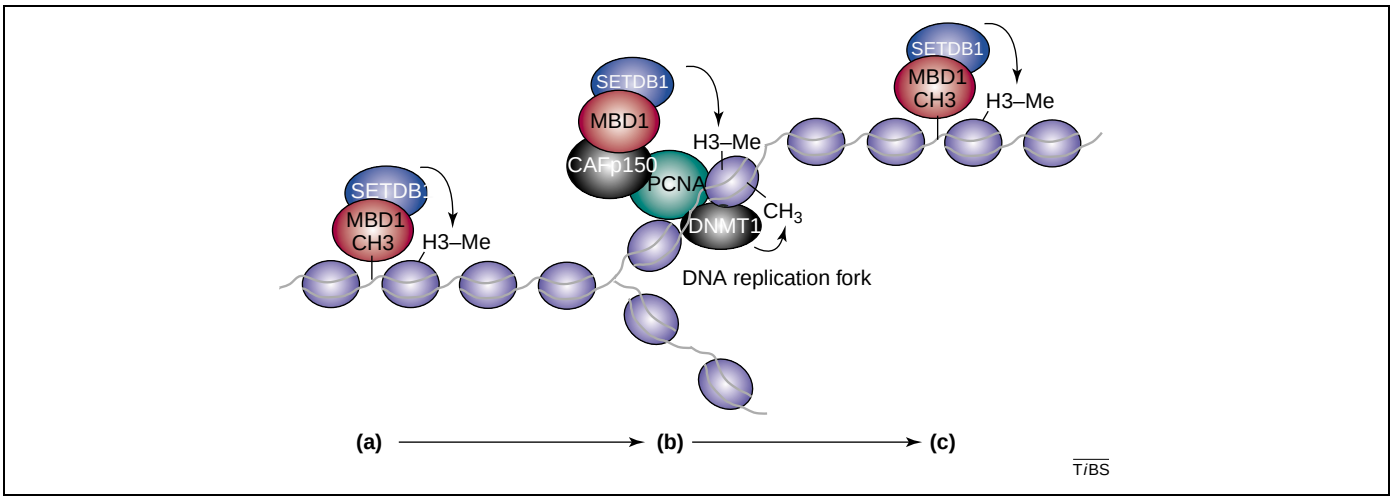

Figure 4. MBD1 directs histone methylation to methylated DNA during the cell cycle. **(a)** MBD1 associates with the histone methyltransferase SetDB1, thereby coupling DNA methylation to histone methylation. **(b)** During S phase of the cell cycle, DNMT1 associates with proliferating-cell nuclear antigen (PCNA) and tracks with the DNA polymerase complex. There is evidence that MBD1 associates with the replication-fork-associated factor CAFp150, facilitating the application of histone methylation marks (H3-Me) to newly replicated regions of methylated DNA. **(c)** According to this scheme, MBD1 is involved in bridging cell-cycle events to re-establish histone methylation marks at loci containing DNA methylation.

binding site was associated with the promoter region of a gene, depletion of MBD1 resulted in reactivation of this normally silenced gene. These data indicate that MBD1 might have a special role during the cell cycle in linking epigenetic marks on DNA to histone modification and establishment of gene silencing.

Mbd1-null mice are relatively healthy, but show some mild defects in neurogenesis, spatial learning and long-term potentiation in the dentate gyrus of the hippocampus [72]. It remains to be seen whether the phenotype of *Mbd1*-null mice reflects the role of MBD1 in chromatin modification that has been established in tissue-culture cells.

MBD2 in intestinal cancer and lineage commitment in T cells

Mbd2-null mice are healthy and fertile, but for unknown reasons they fail to nurture their offspring [73]. At the molecular level, fibroblast cell lines derived from *Mbd2*-null mice have reduced capacity to silence methylated reporter genes, confirming that MBD2 has a role in DNA-methylation-mediated silencing [73]. Genetic studies in which *Mbd2*-null mice have been crossed onto a mouse model for intestinal cancer indicate that MBD2 is required for tumour formation, because in its absence tumorigenesis is markedly reduced [74]. These findings raise the possibility that pharmaceutical targeting of MBD2 could antagonize colorectal cancer.

Although *Mbd2*-null mice show a mild phenotype under laboratory conditions, deficiencies in methylation-mediated silencing and the essential role of MBD2 in progression intestinal cancer suggest that other defects, perhaps not apparent at the gross morphological level, exist in *Mbd2*-null mice. In keeping with this hypothesis, a thorough analysis of the differentiation of T-helper cells into Th1 and Th2 lineages in the *Mbd2*-null mice has revealed a striking defect in cytokine production [75]. Normally, naïve T-helper cells are induced to differentiate into Th1 or Th2 effector cells when stimulated by antigen-presenting cells. Th1 cells express interferon-γ (*Ifnγ*) and Th2 cells express interleukin-4 (*Il4*) but not visa versa. T-helper cells from *Mbd2*-null mice show a substantial disorganization of *Il4* and *Ifnγ* gene expression during polarized differentiation of T-helper cells. A similar defect in cytokine gene expression has been observed in naïve T cells when DNMT1 is conditionally deleted [76], suggesting that MBD2 interprets the DNA methylation signals that are required for normal cytokine expression.

Molecular studies aimed at delineating the role of MBD2 in maintaining *Il4* gene silencing have detected MBD2 bound to hypo-acetylated chromatin surrounding its promoter region [75]. Ectopic expression of *Il4* occurs in

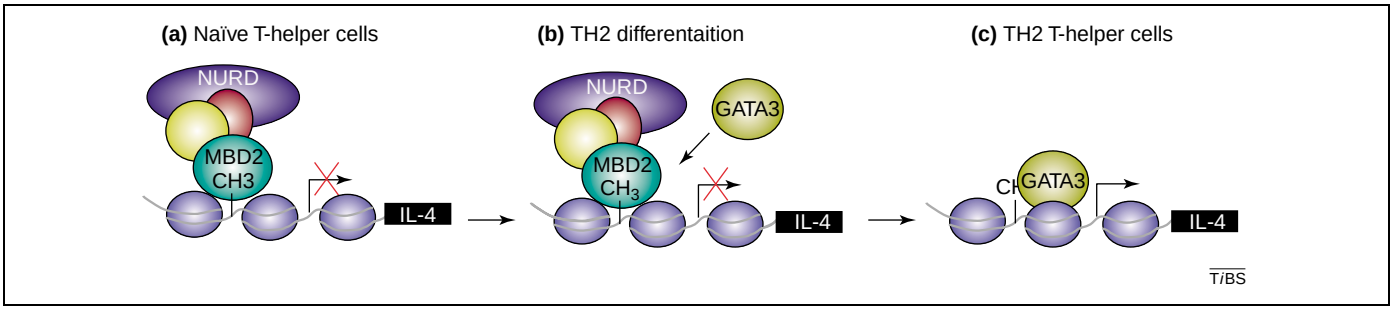

Figure 5. MBD2 acts as a repressor of gene expression at the *Il4* gene. **(a)** MBD2 binds the methylated promoter region of the *Il4* gene in naïve T-helper cells, maintaining the silenced state of gene. **(b)** On differentiation into Th2 T-helper cells, the transcriptional activator GATA3 is expressed. **(c)** GATA3 competes with MBD2 for binding to the *Il4* promoter, leading to loss of MBD2 and efficient transcription of *Il4*.

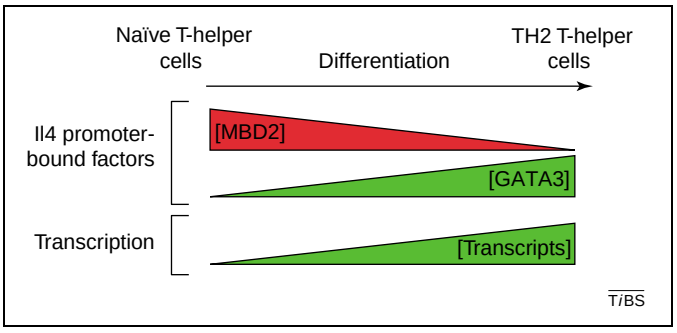

Figure 6. GATA3 acts in a dose-dependent manner to overcome MBD2-mediated repression. Model experiments in which the concentrations of MBD2 and GATA3 are manipulated artificially [75] indicate the level of *Il4* transcription is proportional to occupancy of the promoter by GATA3, but is inversely proportional to occupancy by MBD2.

naïve T cells that lack the transcriptional activator protein GATA-3, which is normally required for *Il4* expression. Thus, MBD2 seems to be a key silencer of *Il4* expression in the uninduced state (i.e. in the absence of appropriate transcriptional activators) through recognition of methylated DNA in the promoter region of the gene (Figure 5). If so, then GATA-3 might be instrumental in overcoming the silencing effect of MBD2 during activation of the gene in the Th2 lineage. In agreement with this hypothesis, ectopic expression of GATA-3 in wild-type Th1 cells causes displacement of MBD2 from the *Il4* gene and dose-dependent activation of the normally silenced gene. This observation supports the view that activators must overcome a threshold of epigenetically imposed silencing to transcribe their target gene efficiently (Figures 5 and 6). To test the generality of this model, other cell lineages will need to be examined in similar detail.

Targeted binding of MeCP2 in regulation of neuronal genes

Mutations in the human *MECP2* gene cause Rett syndrome, a debilitating neurological disease that affects ~1 in 10 000 female live births (reviewed in Ref. [77]). A mouse model with many features that are reminiscent of Rett syndrome has been created by deletion of the *Mecp2* gene in mice [78,79]. Microarray-based gene expression analysis in *Mecp2*-null mouse brain tissue, where the Rett phenotype is manifested, shows subtle changes in gene expression [80,81].

Within the group of upregulated genes are two glucocorticoid-inducible genes (*Sgk* and *Fkbp5*) and a gene that is involved in the glucocorticoid signalling pathway (*Pomc*) [80]. Importantly, the defects in *Sgk* and *Fkbp5* gene expression are observed before presentation of the Rett-like phenotype in *Mecp2*-null mice, indicating that the changes are not a secondary consequence of symptoms. It is thus conceivable that MeCP2 is involved in regulating expression of genes involved in the glucocorticoid signalling pathway in the brain. The finding that MeCP2 is directly associated with the methylated promoter regions of both genes in wild-type mice (Figure 7) is compatible with this view. Further work is needed to assess the extent of MeCP2 involvement in regulating glucocorticoid target genes and to establish the relevance of this altered gene expression to the human disorder.

Because DNA methylation is important in the regulation of imprinted gene expression, it has been proposed that MBP-deficient mice might show misregulation of imprinted gene expression. Initial studies failed to identify such defects [73,79], but recently several imprinted genes, including *Dlx5*, *Dlx6* and *Ube3A*, have been reported to show loss of normal expression in brain tissue of *Mecp2*-null mice [82–84]. MeCP2 binds to a region in the body of the *Dlx6* gene and recruits histone deacetylase 1 to create a region of silenced chromatin [82]. Moreover, binding of MeCP2 to the *Dlx6* locus correlates with the formation of looped chromatin structures that might be required for silencing the flanking genes.

Using a candidate gene approach, two groups [85,86] have identified the Ca^{2+}-inducible neuronal gene for brain-derived neurotrophic factor (*Bdnf*) as a direct target for repression by MeCP2 in cultured neurons. MeCP2 binds near an inducible promoter of *Bdnf*, where it associates with the co-repressor molecule Sin3a, presumably to maintain the repressed state of the *Bdnf* gene. On Ca^{2+} signalling, MeCP2 becomes phosphorylated and is liberated from the promoter as the *Bdnf* gene is activated (Figure 7). In cultured neurons from *Mecp2*-null mice, basal (uninduced) expression of *Bdnf* is upregulated

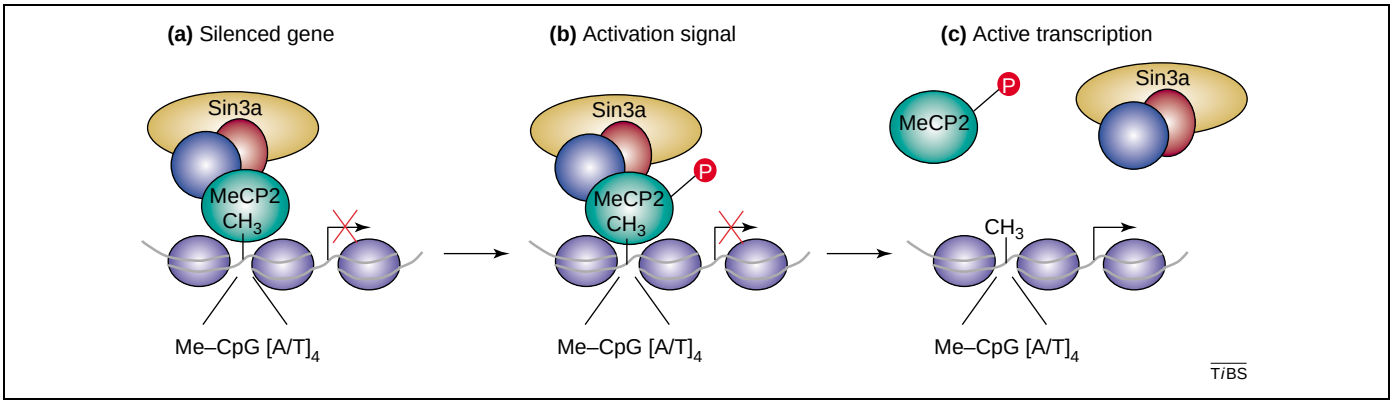

Figure 7. MeCP2 is a dynamic repressor of neuronal genes. **(a)** MeCP2 binds methylated DNA near promoter regions of several neuronal genes and some imprinted genes, maintaining silencing through recruitment of the Sin3a–HDAC co-repressor complex. Binding of MeCP2 to specific methyl-CpG dinucleotides in target genes relies on a run of four or more A/T bases, $(A/T)_4$, adjacent to methyl-CpG. **(b)** In the case of *Bdnf* gene activation in stimulated neuronal cells, signalling cascades result in phosphorylation (P) of MeCP2. **(c)** This phosphorylation causes the MeCP2–co-repessor complex to dissociate from the promoter, which is followed by gene activation.

twofold, indicating that MeCP2 makes a direct contribution to silencing of this promoter. The level of *Bdnf* expression in calcium-induced cells seems to be unaffected by the absence of MeCP2. A study of several other genes during neuronal differentiation in cell culture suggests that MeCP2 has a widespread role in modulating neuronal gene expression [87].

Analysis of gene expression by microarray [81] indicates that MeCP2 is not a global regulator of gene expression, because few genes are deregulated in its absence. Instead, MeCP2 seems to modulate a distinct subset of methylated genes. A recent study aimed at understanding MBD protein occupancy determined that MeCP2 binds to distinct loci not occupied by other MBPs *in vivo* [88]. Thus, MeCP2 does not seem to compete with other MBPs for access to methyl-CpG nucleotides, but is confined to a few genomic binding sites.

Analysis of MeCP2-binding properties using an *in vitro* DNA-binding site selection assay [88] has established that MeCP2 requires methyl-CpG sequences flanked by a run of four or more A/T base pairs, $(A/T)_{\geq 4}$, for efficient DNA binding (Figure 7). DNA fragments associated with MeCP2 *in vivo* also contain the additional $(A/T)_{\geq 4}$ motif, indicating that this recognition sequence is used in cells. Furthermore, MeCP2-binding sites in two of its known target genes, *Bdnf* and *Dlx6*, contain methyl-CpG flanked by $(A/T)_{\geq 4}$ motifs. Therefore, at least part of the target gene specificity of MeCP2 seems to stem from this additional sequence specificity. A requirement for DNA sequence in addition to methyl-CpG might apply to other MBPs, because a major form of MBD1 contains a second DNA-binding domain [89]. In addition, Kaiso requires not one but two consecutive methyl-CpG dinucleotides for efficient methylation-specific DNA binding [60]. So far, only MBD2 seems to be specific for methyl-CpG sites alone [88].

Ongoing efforts to understand the role of MeCP2 both in the defects seen in *Mecp2*-null mice and in the aetiology of Rett syndrome in humans are complemented by studies in amphibians. *Xenopus laevis* embryos depleted of MeCP2 do not survive past the neurulation stage and show defined defects in neuronal gene expression, suggesting that MeCP2 has a role more central to developmental in amphibians than in mammals [90]. MeCP2 binds to the promoter region of the *Xenopus Hairy2a* gene and participates in its repression. When MeCP2 is deficient, expression of *Hairy2a* is increased, leading to excessive neurogenesis in the embryo. Whether misregulation of the mammalian *Hairy2a* orthologue is also a consequence of MeCP2 deficiency is not yet known. In general terms, the phenotypes of *Mecp2*-null mice could be due to the aberrant function of a single molecular pathway or an aggregate of minor defects in several cellular processes.

The possibility that MeCP2 has novel functions that are unrelated to transcriptional repression must also be kept in mind. Indeed, a recent report implicates MeCP2 as a modulator of alternative splicing via an interaction with the RNA-binding protein YB1 [91]. It seems likely that a rational approach to future medical intervention in Rett syndrome will depend on continuing efforts to understand the defects in *Mecp2*-mutant mice.

Concluding remarks

The absolute requirement for an intact system for DNA methylation in mice suggests that this epigenetic modification has a vital role in development; however, studies aimed at understanding the scope of the impact that DNA methylation has on development have barely scratched the surface. The molecular characterization of enzymes and proteins involved in placing and interpreting the DNA methylation signals and, more recently, the availability of mouse knockout lines for these components have provided the tools with which to manipulate and to analyse the normal function of the DNA methylation systems *in vivo*. The information that is rapidly unfolding from using these reagents is likely to be of great importance to our understanding of basic epigenetic silencing mechanisms and in the future could have broad implications for the treatment of inherited diseases and cancer.

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