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Minireview

# Local DNA demethylation in vertebrates: how could it be performed and targeted?

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**Abstract In vertebrates, cytosine methylation is an epigenetic DNA modification that participates in genome stability and gene repression. Methylation patterns are either maintained throughout cell division, or modified by global or local de novo methylation and demethylation. Site-specific demethylation is a rather elusive process that occurs mainly in parallel to gene activation during development. In light of our studies of the glucocorticoid-dependent DNA demethylation of the tyrosine aminotransferase gene, we discuss the potential biochemical mechanisms allowing DNA demethylation and its targeting to specific sequences by transcription factors as well as possible links to DNA replication and chromatin remodelling.** 

*Key words:* DNA methylation; DNA demethylation; DNA replication; Chromatin; Transcription; Development

## 1. Introduction

In most vertebrates, 60–90% of the cytosines at CpG dinucleotides are methylated [1]. The presence of the methyl moiety on the base contributes generally to transcriptional repression by preventing regulators from binding to their target, or favouring the formation of inactive chromatin [2,3]. The methylation pattern is correlated with the activity of the gene and constitutes a stable epigenetic mark that is transmitted through DNA replication and cellular division. Methylation plays a functional role in genome organisation and stability [4,5], and in some aspects of gene expression, namely the silencing of mobile elements [6], genomic imprinting [7] and X chromosome inactivation [8]. Dysfunction of methylation control could be involved in cancer [9]. In mammals, modifications of methylation patterns occur during development but their importance for gene regulation is still discussed [10,11]. The methylation pattern of the mammalian embryo genome is established after fertilisation through several steps involving genome-wide demethylation and de novo methylation, followed by selective demethylation of regulatory elements occurring in parallel with their activation [12,13]. In other vertebrates, methylation could also participate in the control of gene expression during development even though it is not clear whether genome-wide modifications of methylation occur in all vertebrates [14,15]. Whereas the methylation process has become clearer since the identification of de novo and maintenance DNA methyltransferases (DNMT) [16], demethylation mechanisms have not yet been elucidated.

### *1.1. Global versus local demethylation*

Two kinds of demethylation events can be distinguished: global and site-specific demethylation. In mammals, a large portion of the genome undergoes demethylation during gametogenesis, early development of the embryo and in some differentiating cells [13,17]. In many vertebrates, site-specific demethylation affects tissue-specific genes in the tissue and developmental stage where they are expressed and may be involved in the absence of methylation of CpG islands [18]. Global and local demethylation are clearly distinct in their regulation and may be carried out by different mechanisms. Genome-wide demethylation could result from a global process acting on the cellular pool of enzymes involved in controlling DNA methylation, following either inactivation of DNMTs or activation of demethylating enzymes [19]. In contrast, local demethylation must be targeted (directly or not) to specific sequences by regulatory factors that could either recruit the activities involved in genome-wide demethylation, or use specific mechanisms better adapted to a precise action. Attempts to elucidate the demethylation event in vitro have generated diverging results, which may be due, in part, to different activities being analysed that are involved in different demethylation events. Clarification of the issue could be obtained through analysis of the biochemistry of the demethylation reaction actually taking place in vivo.

The elucidation of local demethylation mechanisms appears essential to establish whether DNA methylation plays a role during vertebrate development. We focus this review on the various mechanisms that could be involved in this elusive process and analyse two aspects: biochemical mechanism and targeting. We will discuss the various observations made throughout the last 10 years in light of the results we have obtained during the study of the regulation of a model tissue-specific gene.

### *1.2. Regulation of local DNA demethylation by the glucocorticoid receptor (GR)*

We are studying the mechanisms of transcriptional activation by nuclear receptors and we focus our studies on the glucocorticoid regulation of the model tyrosine aminotransfer-

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ase (*Tat*) gene. This gene is expressed specifically in liver cells, is turned on at birth and its transcription is activated by glucocorticoids in the adult. A key element in glucocorticoid response and developmental induction is an enhancer located at −2.5 kb consisting of multiple binding sites for the GR and accessory transcription factors [20]. Using genomic footprinting and chromatin immunoprecipitation, we determined the actual events occurring in cultured hepatoma cells and we could show that the GR uses a multistep mechanism to recruit successively the accessory DNA binding proteins that assist in the activation process [21]. Chromatin is first remodelled over a 350 bp region within 15 min of GR activation, allowing recruitment of transcription factor HNF-3 [22]. These events are reversible following hormone withdrawal. Prolonged glucocorticoid treatment elicits progressive demethylation of all four $^{Me}$CpGs located within the remodelled area; demethylation is complete within 3 days [23]. Nearby CpGs located outside the remodelled area remain methylated. Following demethylation, two additional transcription factors are recruited at sites overlapping the CpGs. In contrast to chromatin remodelling and transcription factor recruitment, demethylation is stable and persists for months following hormone withdrawal. As responses to subsequent glucocorticoid stimulation are stronger and faster, demethylation appears to provide memory of the first stimulation. During development, this demethylation occurs before birth, at a stage where the *Tat* gene is not yet inducible [23]. It might thus prepare the enhancer to allow the neonatal *Tat* gene induction that occurs in response to enhancer activation by hypoglycemia [20].

Glucocorticoid-dependent demethylation of the −2.5 *Tat* enhancer in cultured hepatoma cells is a well-defined system suitable for the study of a demethylation mechanism taking place in living cells on an endogenous gene in a natural chromatin context.

## 2. Biochemical mechanisms

### 2.1. Passive demethylation

$^{Me}$CpG dinucleotides display a symmetrical configuration that allows conservation of the methylation pattern following replication. The replication machinery incorporates unmethylated cytosine in the newly synthesised strand. The resulting hemimethylated sites are converted back to fully methylated sites by the maintenance methyltransferase DNMT1 that acts preferentially on hemimethylated CpGs (Fig. 1A). DNMT1 acts soon after replication because the enzyme is targeted to replication foci through its interaction with the DNA polymerase clamp PCNA [24]. Demethylation of $^{Me}$CpG could simply result from an absence of maintenance methylation after replication. This kind of mechanism would be a passive one since it does not involve any demethylating enzyme.

This passive process must take place during several DNA replication cycles to ensure that a significant part of the daughter molecules are effectively demethylated: three rounds of replication demethylate only 87.5% of the CpG. This figure is consistent with the kinetics of demethylation of the maternal genome that is observed during early mouse development [25], and that we observe for the local glucocorticoid-regulated demethylation of the −2.5 *Tat* enhancer [23]. Replication-dependent and therefore presumably passive demethylation of transfected DNA molecules has been observed in several instances [26,27].

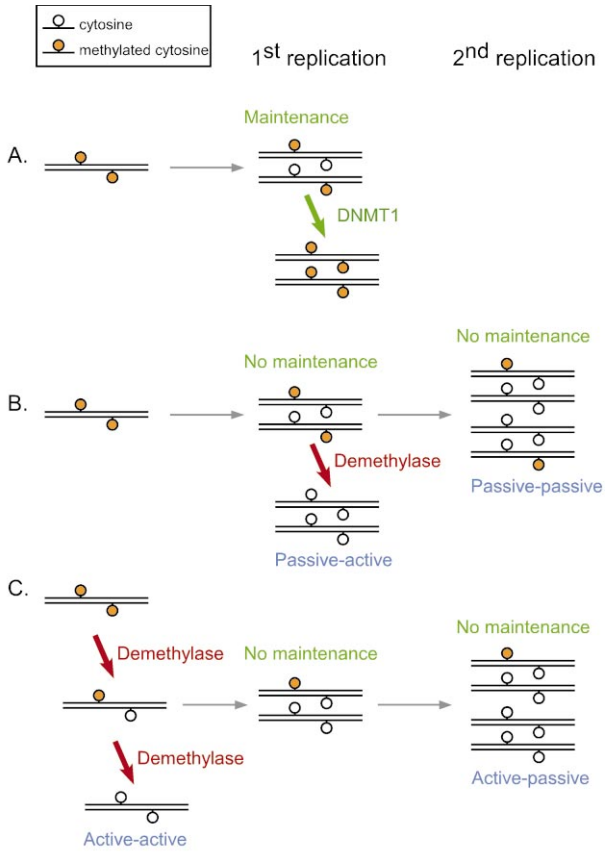


Fig. 1. Mechanisms of maintenance and modification of methylation patterns. (A) Maintenance of the methylation patterns by DNMT1 following DNA replication. (B) Possible demethylation events initiated by a passive step. (C) Possible demethylation events initiated by an active step.

### 2.2. Active demethylation

An active demethylation mechanism, i.e. demethylation in the absence of replication, has also been described. In mice, global demethylation of the zygotic paternal genome after fertilisation appears to occur by an active mechanism [28]. Local demethylation of transfected DNA molecules that do not replicate has also been observed [18,29]. Both active demethylation of fully methylated CpGs to produce hemimethylated molecules, and demethylation of hemimethylated sites have been observed with transfected DNA molecules [27,29].

Even though it is clear that active demethylation occurs in some instances, the nature of the enzyme(s) responsible for this modification has not been unequivocally established to date. Three main biochemical mechanisms have been proposed but none has yet either been proven to operate under relevant physiological conditions or gained wide acceptance. They differ in the first step performed by the putative enzyme named demethylase: direct replacement of the methyl moiety by a hydrogen atom, excision of the methylated base, or excision of the methylated nucleotide, possibly with a few surrounding nucleotides (Fig. 2). The second and third mechanisms imply further steps to repair the DNA lesion initiated by the demethylases, either base or nucleotide excision repair [30].

An enzymatic activity that is believed to demethylate without altering the phosphodiester backbone was isolated from human cells [31,32]. The demethylase was suggested to be

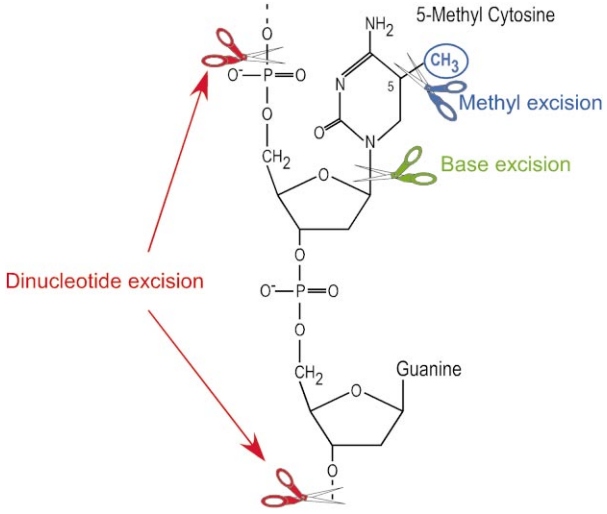


Fig. 2. Possible levels of action of the demethylases.

MBD2b, which appeared to demethylate DNA in vitro, but this claim is contested and could not be independently reproduced [33,34]. In addition, the amazing biochemistry of the reaction and the purification steps have raised widespread concerns [5,35]. Indeed, the biochemical properties of the putative demethylase activity varied widely during purification through successive ion exchange columns and the enrichment factors were surprisingly high even when the activity was eluted in the non-resolved fractions (see discussions in [5,35]). Furthermore, the proposed demethylation mechanism is in conflict with generally admitted thermodynamic and chemical reaction theories as previously discussed [5]. The inactivation of the MBD2 gene should allow to establish whether this activity is involved in DNA demethylation in vivo.

The mechanism involving base excision is more documented. Using chicken embryo nuclear extracts that can promote active demethylation, a demethylase has been purified by Jost and colleagues [36,37]. The enzyme, a $^{Me}$C-DNA-glycosylase, acts preferentially on hemimethylated CpGs and initiates demethylation by breaking the glycosidic bond of $^{Me}$C, thus leaving an abasic site that should be further processed by an AP-endonuclease and other DNA repair enzymes [30]. Throughout purification, the enzyme copurified with the DNA repair enzyme G/T mismatch DNA-glycosylase (TDG) and it turned out in the end that TDG was able to carry out the $^{Me}$C-DNA-glycosylase reaction [36,37]. It is not fully clear whether TDG is the initially described activity. Furthermore, several aspects of the enzymatic assays used are worrying. Neither recombinant TDG, nor the purified chicken demethylation complex showed efficient $^{Me}$C-DNA-glycosylase activity as they converted only a minor proportion of the substrate in 1 h reactions that contained a 10-fold molar excess of enzyme over substrate [36,37]. In addition, RNA was found to be essential for $^{Me}$C-DNA-glycosylase activity of the purified chick embryo enzyme, whereas recombinant TDG was active in the absence of RNA [37–39]. The putative RNA requirement has been inferred following RNase treatment of the purified chicken enzyme. Reactions were carried out using similar amounts of glycosylase and RNase, both enzymes being in 100-fold excess over substrate DNA. At these excesses, RNase has been shown to coat the substrate DNA protecting it unspecifically from the demethylase [40]. Addition of RNA at a 1000-fold molar excess over substrate relieved the inhibitory effect of RNase, and some RNA sequence specificity of this effect was observed [37–39]. Another G/T mismatch glycosylase, MBD4, which preferentially acts on G/T mismatch resulting from $^{Me}$C deamination within the $^{Me}$CpG dinucleotide [41], has also been shown by Jost and colleagues to possess a weak $^{Me}$C-glycosylase activity on hemimethylated substrates [42]. In contrast, Bird, Jiricny and colleagues found that MBD4 had no $^{Me}$C-DNA-glycosylase activity [41]. It is not clear whether these discrepancies are due to differences in the excess of enzymes to substrate used, to deamination of some of the $^{Me}$C within the substrate creating unappreciated G/T mismatches, or to other variations in the experimental conditions. It would be important to show, in particular through genetic experiments, whether these glycosylases operate in vivo to demethylate $^{Me}$CpG.

Another glycosylase acting on $^{Me}$C within fully methylated CpGs has been observed in vitro [43], but the validity of this result has been questioned [44]. The original authors have later reiterated their claim using partially purified enzyme preparation [45]. More extensive characterisation is necessary to ascertain the existence of this activity.

Finally, demethylation through excision of the dinucleotide $^{Me}$CpG appears to occur in vitro [46]. This activity was observed upon analysis of the conversion from fully methylated to hemimethylated substrate in extracts of cells able to promote such conversion of transfected DNA. It was first thought that RNA has an active role in the demethylation reaction as an acceptor of the dinucleotide but this was later reevaluated following demonstration of the aforementioned protective coating of the substrate by RNases [40]. Despite this reevaluation, the demethylase activity per se has been observed independently in this critical study. This demethylase has not yet definitively been characterised.

In conclusion, the presently available data are far from clear, the biochemical approaches have been rather frustrating and the demethylation mechanism remains to be firmly established. In favour of an active demethylation mechanism causing DNA strand breaks during the process, we have observed such breaks during the glucocorticoid-induced demethylation of the *Tat* enhancer (unpublished results).

### 2.3. Respective advantages of the different mechanisms

Two steps are necessary to demethylate a $^{Me}$CpG on both strands, and any combination of active or passive mechanisms could theoretically occur (Fig. 1B,C). However, the relative efficiencies of these combinations differ. An exclusively passive mechanism is inefficient, as it requires several rounds of DNA replication to achieve significant demodification (Fig. 1B). Such a mechanism appears poorly adapted if DNA methylation participates effectively in the control of gene expression during development, as it would not allow rapid reprogramming of gene expression following a single DNA replication event. In contrast, an entirely active mechanism could permit a rapid demethylation of both strands (Fig. 1C). However, as the best established active demethylation mechanisms available to date provoke nicks or gaps in DNA, double-stranded breaks could occur if one $^{Me}$CpG, or two or more nearby $^{Me}$CpGs are simultaneously processed on the two DNA strands. To avoid such deleterious events, demethylation should only occur on one strand at a time. With an active–

active process, this could be ensured in either of two ways: (a) the demethylase machinery occupies the site as long as it is not fully repaired and prevents cleavages at nearby $^{Me}$CpGs; (b) the demethylase machineries are not abundant and are not recruited efficiently, thus lowering to a negligible level the probability of simultaneous processing of nearby sites. In addition, if different demethylases process the fully methylated and the hemimethylated sites, they could act asynchronously. As discussed previously, such demethylases have been observed in vitro.

A passive–active mechanism would efficiently demethylate both strands of $^{Me}$CpGs in a single replication event while preventing double-stranded break formation (Fig. 1B). Indeed, all $^{Me}$CpGs that would be actively demethylated after replication are on the same DNA strand. The existence of such a mechanism is supported by the identification of a $^{Me}$C-glycosylase which catalyses demethylation preferentially on hemimethylated molecules [17,36], and by a two-step demethylation event involving a single replication event [27].

Finally, an active–passive mechanism could also occur because, as previously described, there appears to be a demethylase converting fully methylated CpGs to a hemimethylated site [29,46]. However, such a mechanism is not much more efficient than a fully passive one, as only one of the two daughter molecules is demethylated on both strands after a single replication event (Fig. 1C).

## 3. Targeting

DNA demethylation of regulatory elements of housekeeping and tissue-specific genes is often restricted to regions where transcription factors bind and where chromatin is hypersensitive to DNase I cleavage. It is likely that these events target the demethylation process. Methylation can sometimes spread over several kilobases beyond the regulatory regions as observed for the immunoglobulin genes. In this case, the enhancer is sufficient for enhancer-proximal demodification, but particular regulatory sequences, with 'matrix attachment' properties, are required to allow demethylation of enhancer-distal sites [47,48]. In the absence of precise knowledge of the demethylation machinery, the mechanisms allowing targeting of demethylation to specific sites are far from clear, but we will attempt to review the various possibilities.

### *3.1. Targeting of the passive mechanism (Fig. 3A)*

If demethylation results from the inhibition of maintenance methylation, how could such a transient inhibition be exerted locally? One model, based on the observation that virus-derived episomal vectors are demethylated at sites where transcription factors bind with high affinity, relies on steric hindrance: transcription factors protect their cognate site from DNMT1 action (Fig. 3A) [27,49]. The model requires the protein to reoccupy its site quickly after being displaced by the replication fork as DNMT1 follows it through its interaction with PCNA. This presumably necessitates a relatively high concentration of high affinity transcription factors. Such a model does not account for the demethylation that occurs at sites that are distinct, and eventually remote, from the sites triggering the demethylation [29,47,48]. In addition, it could not account for the glucocorticoid-regulated demethylation of the *Tat* enhancer, because we have observed, using genomic footprinting, that occupancy of the CpG-containing transcription factor binding sites rather follows than precedes their demethylation [23]. The recruitment of an active demethylation mechanism recognising hemimethylated sites could also be responsible for the exclusion of DNMT1, as suggested previously [17]. However, in such a case, the half-life of the hemimethylated intermediates should be very short, in contrast to what has been observed with replicating episomal vectors [27].

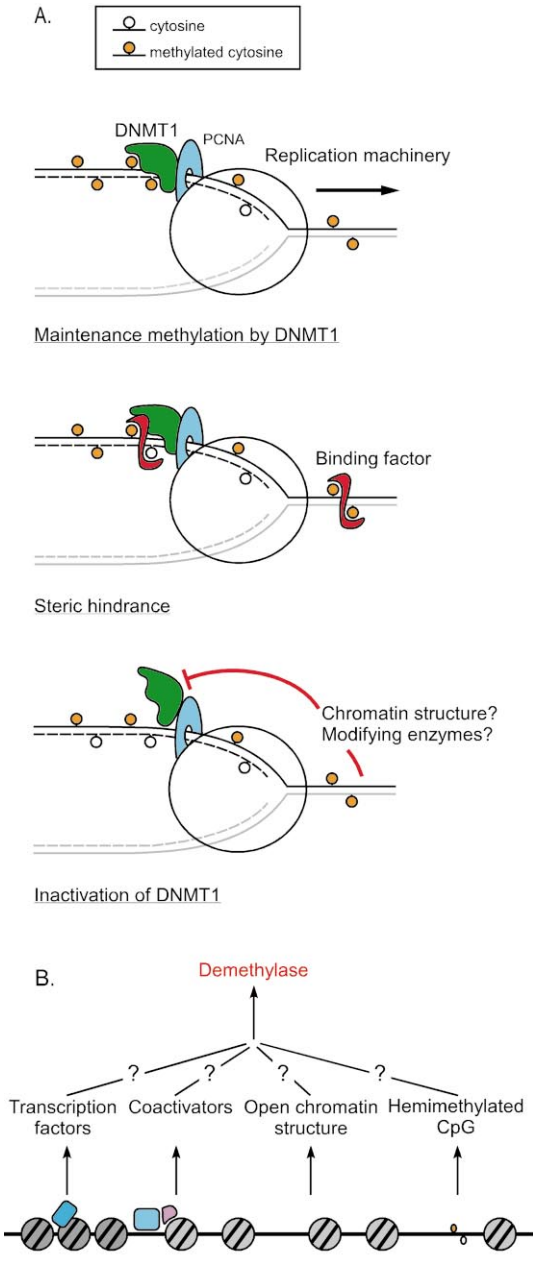


Fig. 3. Possible levels of targeting of demethylation. (A) Targeting of passive demethylation. (B) Targeting of active demethylation.

Replication-dependent demethylation of specific sites in *Xenopus* embryo is strongly stimulated by the *trans*-activation domain of the triggering transcription factor [26]. Activation domains could simply facilitate transcription factor interaction with DNA or act directly, for example through modifications of some components of the replication machinery to prevent methylation. The most direct way could be through

altered recruitment of DNMT1 to the replication fork. For example, it has been shown that $p21^{WAF1}$ can compete with DNMT1 for interaction with PCNA [50]. Such a mechanism appears better adapted for a large-scale than for a local demethylation, the latter requiring very dynamic interactions between PCNA, DNMT1 and inhibitors. Foci of 'replication factories' are rather static, but it is still possible that the interactions of proteins with PCNA within the factories are dynamic enough to allow for such a local demethylation [24]. Alternatively, DNMT1 associated to the replication machinery could be transiently inactivated through either covalent modifications or recruitment of an inhibitor. A number of the coactivators recruited by transcription factors that are able to trigger DNA demethylation, like GR, can phosphorylate, acetylate, or methylate other proteins [51]. Yet, it has not been shown whether such modifications of DNMT1 could inactivate it. Small molecular weight inhibitors of DNMT have been found in tissues [52], but it is not known if such compounds could be transiently targeted by regulatory factors.

An alternative possibility is that the replication machinery could be able to monitor chromatin structure and to demethylate in response to the detection of an active chromatin state. Indeed, evidences are accumulating suggesting that the replication machinery contributes to epigenetic inheritance and to the restoration of the chromatin state as it existed prior to replication [53]. This implies that the replication machinery has a chromatin-monitoring ability. Demethylated CpGs are most often found at sites where chromatin remodelling creates DNase I hypersensitivity. As mentioned previously, GR triggers chromatin remodelling of the *Tat* enhancer followed by demethylation of all CpGs within the 350 bp long remodelled area [21,23]. Histone acetylation is unlikely to be responsible for this demethylation for two reasons. First, treatment of cells with trichostatin A, a drug inducing histone hyperacetylation, does not trigger demethylation [23]. Second, chromatin immunoprecipitation analysis reveals that GR-induced histone acetylation spreads beyond the area where demethylation occurs ([21]; M. Flavin and T.G., unpublished results). However, there is a perfect overlap between the areas where CpGs are demethylated and where chromatin is remodelled by activities presumed to be members of the Swi/Snf family of ATPases.

Another type of reprogramming of the replication machinery has been proposed to allow DNA demethylation [54]. This model, based on the observation that CpG islands are often associated with replication origins, suggests that there is a particular replication machinery initiating replication that differs from the elongating machinery by its inability to maintain methylation. Transcription factors would trigger demethylation by inducing a DNA replication initiation event in the vicinity of their binding sites. This is an attractive model but it necessitates the existence of different classes of replication origins with different abilities to methylate DNA. Indeed, whereas some replication origins are located within methylation-free CpG islands, others are enriched in $^{Me}$CpGs and even appear to require CpG methylation to function [55,56].

Finally, instead of locally and transiently modifying the replication machinery, the locus to be demethylated could also be relocated in a nuclear area devoid of maintenance activity upon a specific signal. This is not an attractive possibility for most local demethylation events, as it implies very rapid and transient relocation. However, such a mechanism could operate when local demethylation spreads over several kilobases [47,48].

### *3.2. Targeting of the active mechanism (Fig. 3B)*

The targeting of active demethylation amounts to locally recruiting the demethylase. Such recruitment could be direct, some transcription factors interacting with DNA demethylase complexes, or indirect, through chromatin remodelling that could allow demethylase interaction the way it allows transcription factor recruitment (e.g. [22]). A passive–active mechanism would not necessarily need the targeting of a demethylase. Indeed, the demethylation of hemimethylated sites created by a passive step could be completed by a demethylase that would demethylate any hemimethylated substrate met in the genome. However, if the demethylase follows the replication machinery, as does the maintenance methyltransferase, transcription factors or chromatin remodelling could instruct the replication machinery to either remethylate or fully demethylate CpGs. Such a replication machinery with multiple DNA modification potentials would ensure integration of rapid and precise demethylation in a single replication event.

## 4. Conclusions

Numerous DNA demethylation mechanisms have been observed in vitro and in transfection assays but only a subset of the responsible enzymes have been identified and it is not clear whether they are involved in demethylation in vivo. The properties of the demethylation processes observed differ widely and it is likely that there is more than one mechanism. This diversity may allow fine-tuning of demethylation by different classes of regulatory factors. The mechanisms allowing targeting of the demethylation to specific sequences are far from clear. It is urgent to define which mechanisms are really at work in living cells and to identify the responsible enzymes, as this should allow to definitively establish whether the control of DNA methylation patterns is important for vertebrate development. In addition, as deregulation of the control of methylation patterns appears to participate to tumourigenesis, this could lead to the identification of novel tumour-promoting or -suppressing genes.

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