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DNA methylation dynamics in health and disease

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DNA methylation is an epigenetic mark that is erased in the early embryo and then reestablished in each individual, through a developmentally regulated program mediated by sequence information and *trans*-acting factors that direct the *de novo* DNA methylation and demethylation machinery to its targets in a dynamic manner. Once established, these patterns can be maintained in a stable manner over the lifetime of the organism. Additional changes in methylation that occur during aging and as part of disease processes may also be directed by similar molecular rules using the same complex machinery.

DNA methylation is a chemical marking system for annotating genetic information by causing gene repression through its ability to affect factor binding and chromatin structure. The genome-wide DNA methylation pattern in all cells of the body is basically bimodal, with the large majority of CpG sites modified at high levels (>85%) and CpG islands largely unmethylated (<10%). Unlike the DNA sequence itself, this overall profile is not inherited from the gametes. Rather, it appears that almost all methylation is erased in the very early embryo and a new bimodal pattern is then reestablished at the time of implantation. This 'clearing of the slate' is a key component of the entire epigenetic marking system, as it symbolizes erasure of germ-line programming as a prelude to resetting totipotency. Once set up, the basic bimodal methylation pattern is generally maintained through every cell division and serves as a global repression mechanism. Adjustments are made through targeted *de novo* methylation and demethylation events. Here we will review the dynamics of DNA methylation during normal development *in vivo*, starting from fertilization through embryogenesis and postnatal growth, as well as the abnormal methylation changes that occur in cancer.

Global demethylation before implantation

Early studies using methyl-sensitive restriction analysis already indicated that a large fraction of the genome is dramatically undermethylated in the preimplantation embryo[1], and analyses of individual genes confirmed this[2]. Results of more recent experiments in which a large number of different sequences were examined are consistent with this original observation[3]. The removal of methyl groups initially begins in the zygote, where specific sequences in the paternal nucleus are actively demethylated[4,5], and this is followed by more widespread demethylation, which may take place through a combination of active DNA-repair processes together with passive loss of methylation through replication[6].

Protection from demethylation before implanation. Although it has not been possible to obtain a complete genome-wide methylation map of blastocyst DNA at the nucleotide level, partial analysis indicates that a very large portion of the CpG residues are indeed unmethylated at this stage, but some tandem repeats as well as other DNA sequences retain some of their modification[3]. The mechanism for this protection is not known, but one possibility is that these loci have an inherent sequence-directed ability to recruit histone methyltransferases, which, in turn, can attract the *de novo* DNA methylase complex[7] and thus serve as a backup system that can compete with the general process of genome-wide demethylation. Satellite sequences that are known to recruit the histone methyltransferase Suv39h, for example, remain methylated in the preimplantation embryo[3,8].

Another type of DNA sequence that is known to remain methylated in the preimplantation embryo is that associated with imprinting centers[9]. These unique domains contain complex regulatory motifs that become methylated in one of the gametes but not in the other[10], thereby providing a mark to distinguish between the two alleles. This methylation ultimately serves to turn off the entire regional regulatory process on one chromosomal copy in somatic cells and in this way brings about the full imprinting phenotype at each locus. Clearly, this concept can only work if these special regulatory elements can survive the genome-wide demethylation that occurs during preimplantation development, and many studies have shown that this is indeed the case[3].

The detailed mechanism of this process is now beginning to be explored. One key factor appears to be ZFP57 (also known as KAP1), which is recruited exclusively to the methylated copy of imprinting centers, thereby preventing their demethylation in the early embryo[11,12]. A similar type of factor, PGC7, is necessary to protect maternal sequences from the demethylation that occurs in the zygote and thus could have a role in generating the paternal sequence–specific pattern of demethylation observed at this stage[13,14]. PGC7 apparently binds to its target sequences by recognizing the presence of H3K9me2 (ref. 15), suggesting that histone modification may cooperate with DNA methylation in marking sites that have to be protected from demethylation.

Function of demethylation before implantation. It is always assumed that demethylation before implantation may be important in resetting the genome after gametogenesis, but the details have not been worked out yet. One possibility is that this process is aimed at removing DNA

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methylation from CpG islands that specifically become modified during oogenesis or spermatogenesis[3]. If the methylation is not removed at this stage, these CpG islands would presumably still remain methylated at the time of implantation and into embryogenesis. In addition, the CpG island–like pluripotency genes such as *Pou5f1* (*Oct3/4*) and *Nanog* are known to be methylated in sperm DNA[16] and would thus be targets for demethylation before implanation, making this the key stage for reprogramming the genome to a pluripotent state.

Erasure of methylation may also be involved in the activation of *Elf5*, a gene thought to be important for the development of the trophectoderm and extra-embryonic tissues. This gene becomes unmethylated in the blastocyst but then undergoes *de novo* methylation at the time of implantation. In keeping with its proposed function, it has been shown that embryonic stem (ES) cells cannot be induced to form trophoblast cells even though they are capable of differentiating into almost any embryonic lineage. Demethylating *Elf5* in these cells, however, permits the generation of trophoblast cells[17,18], suggesting that this gene has a major role in directing differentiation to an extra-embryonic phenotype. It thus may be that global demethylation in the early embryo not only serves to erase unwanted methylation from the gametes but also allows expression of *Elf5*, which in turn causes the differentiation of embryonic perimeter cells into trophectoderm. In this way, demethylation would be necessary for regenerating totipotency in the preimplantation embryo.

Methylation, a transgenerational marker? Although almost all methylation patterns in somatic and germ cells come about as a result of developmental genomic programming, it has been suggested that additional changes in methylation may occur as a result of biochemical 'environmental' influences or as a consequence of aging[19–21]. It has also been proposed that these changes may then be inherited in a transgenerational manner. In this way, events that impact the regulatory biology of an individual could be transferred, perhaps at the level of DNA methylation, to the offspring, and this may be viewed as a shortcut to evolution.

The basic foundations of methylation as we have summarized them here argue strongly against this modification serving as a transgenerational marker. First, it should be noted that epigenetic inheritance of this nature could only come from DNA of the germ line, and if these genes indeed contained environmentally induced alterations, there is a high probability that they would be erased during the global demethylation that takes place at the blastocyst stage[3]. It should also be noted that germ cells are the end product of gametogenesis, which, itself, is characterized by a global erasure of methylation that occurs early in development and, unlike the situation during preimplantation, even involves removal of imprinting methylation marks[2]. It thus appears that early embryonic development is actually designed to erase almost all of the epigenetic marks introduced specifically during gametogenesis, making it unlikely that DNA methylation can serve as a transgenerational signal if it is not specifically recognized as such. Nonetheless, it should be noted that there are indeed sequences that can retain their methylation despite this overall process of erasure[3].

Global *de novo* methylation in the implantation embryo

After erasure of DNA methylation in the early embryo, a new pattern is then established in each individual at about the stage of implantation. This is largely accomplished by the upregulation of the *de novo* methylases, Dnmt3a and Dnmt3b, together with Dnmt1, which bring about global methylation[22]. In coordination with this process, there is also a mechanism for protecting specific sequences, mostly CpG islands[23] (see below). Although this active process of *de novo* methylation appears to be restricted to a short window of time in early development, the resulting pattern of methylation is then maintained during all subsequent cell divisions[24,25]. Thus, the bimodal pattern of methylation seen in all somatic cells is a direct reflection of events that occurred at the time of implantation. It is very likely that the generation of this global profile has several different roles in development: (i) remethylation of sequences that underwent specific demethylation during gametogenesis promotes somatic cell survival[26], and (ii) setup of a general basal repression profile, which directs almost all areas of the genome to be silenced while allowing select windows, such as CpG islands, to remain in a relatively open structure for potential expression[27].

Protection of CpG islands. ES cells are an excellent system for studying the *de novo* methylation that occurs at the time of implantation. Non–CpG island sequences introduced into these cells appear to undergo *de novo* methylation, whereas CpG islands remain unmethylated in this system[23,28]. Early studies indicated that CpG islands are protected from methylation by virtue of nearby *cis*-acting sequences and not merely because they are rich in CpG dinucleotides[23]. Although these regulatory motifs have not been worked out completely, binding sites for transcription factor Sp1 appear to be involved[29]. Removal of Sp1 sites from a CpG island causes it to become methylated when such a template is transfected into ES cells, whereas the addition of these elements to a non–CpG island template results in protection from methylation. These concepts have also been confirmed *in vivo* using transgenic mice[30,31].

Although the precise mechanism of CpG island protection is not understood yet, recent global analyses have helped shed some light on this process. In the human genome there are altogether about 13,000 CpG islands that are constitutively unmethylated[24] compared to the majority of sequences that are methylated in all tissues. Assuming that this pattern reflects decisions that were made at the time of implantation, one can use bioinformatics to detect sequence properties that distinguish between these two groups. A very high percentage of unmethylated islands contain known transcription start sites, and many specific motifs are also associated with these unmethylated regions, including transcription factor binding sites[24]. Transcription start sites are always packaged in nucleosomes containing H3K4me3 (refs. 32,33), and this mark may serve to inhibit the binding of *de novo* methylases[34,35]. Taken together, these analyses suggest a model whereby the binding of RNA polymerase or other proteins[36] in preimplantation cells may be important in preventing local *de novo* methylation during the transition to implantation (**Fig. 1**). This implies that the resulting basal methylation pattern simply reflects the potential transcription state of early embryos and, in this way, provides a mechanism to perpetuate this profile in a more stable manner.

Maintenance of the methylation pattern. Although implantation embryos have the capacity to set up the bimodal methylation pattern, the molecular machinery for carrying this out must be downregulated[22] very early in development, as somatic cells are no longer capable of global *de novo* methylation[37]. Nor do they seem to recognize CpG islands as sites to be protected[38]. Nonetheless, the overall initial pattern formed at the time of implantation is then maintained after each cell division[24]. This is accomplished through the action of Dnmt1 (ref. 39), which is constantly associated with the DNA replication machinery[40]. This enzyme is highly specific for hemimethylated CpG sites, like those generated during DNA synthesis, and it is this activity that perpetuates the methylation pattern present on the

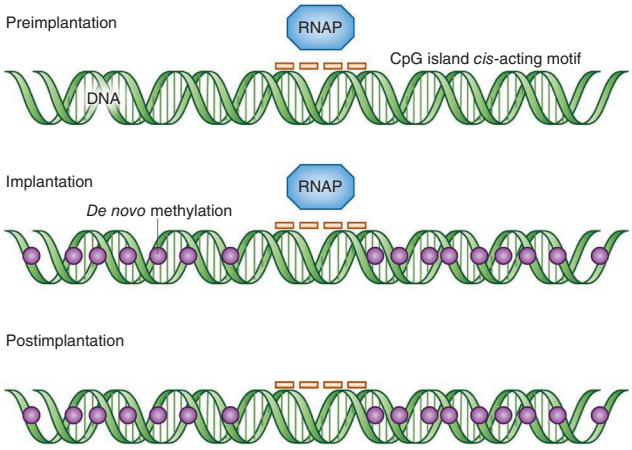

Figure 1 Protection of CpG islands from *de novo* methylation. In the preimplantation embryo, the RNA polymerase (RNAP) complex is stably bound to almost all CpG islands by virtue of *trans*-acting factors that recognize *cis*-acting motifs in these regions. This then serves as a template for protection against *de novo* methylation (purple circles) at the time of implantation. The methylation pattern is then maintained in subsequent divisions even in the absence of the original RNAP complex. Other proteins such as Cfp1 (not shown), which recruit the H3K4 methylase Set1, may also have a role in protecting CpG islands in ES cells[127].

original DNA strands[41]. At the same time, this enzyme has only very low *de novo* activity, so unmethylated sites remain in this same state during replication. Although this enzyme is assisted by additional factors present at the replication fork[42–45], Dnmt1 is the major force behind the ability of cells to maintain methylation patterns throughout cell division.

Functional role of global *de novo* methylation. In many simple organisms, the majority of genes are active in every cell[46] with only a few selective genes being repressed by the sequence-specific binding of transcriptional silencers. This situation is very different in animals, where more than 50% of all genes are repressed in every cell, and this clearly requires an alternate strategy to carry out the repression. The global methylation that occurs at the time of implantation provides an efficient way to silence almost the entire genome while leaving CpG islands relatively open. Note that this silencing takes place without the need to recognize specific sequence motifs.

The maintenance of DNA methylation patterns also serves an important function during development and aging. In general, gene expression patterns in any cell are determined by two key parameters: the availability of general and specific transcription factors as well as chromatin structure, which modulates local accessibility. Every time cells copy their genetic material as part of the cell division cycle, the replication machinery 'plows' through the DNA, thereby disrupting both chromatin structure and factor binding, and these must be rebuilt in every cell generation. In contrast, the underlying DNA methylation pattern is preserved throughout replication, and this serves as a template for guiding the repackaging of DNA without the need to completely rebuild these structures from scratch. In this way, DNA methylation serves as a mechanism for stabilizing gene expression patterns over the entire lifetime of the organism. It has been suggested that some histone methylases actually remain bound to DNA during replication and this may represent an additional element for facilitating chromatin restructuring[47].

Targeted *de novo* methylation after implantation

After implantation, there are no additional global changes in DNA methylation, and all alterations, whether they involve *de novo* methylation or demethylation, appear to occur through sequence-specific targeting. One of the first developmental events of this nature is the methylation and silencing of genes responsible for pluripotency, such as *Oct3/4* or *Nanog*[48,49]. Studies in ES cells and in mice have demonstrated that these genes become inactivated in a three-step manner. First, transcription is turned off by direct interactions with repression factors. In the second stage, the histone methylase G9a is recruited to these gene loci; this complex, in coordination with histone deacetylases and H3K4 demethylases, systematically removes all histone-activating modifications from local nucleosomes and then brings about methylation of histone H3K9, which in turn binds HP1, leading to formation of heterochromatin. Finally, G9a itself can recruit Dnmt3 molecules and cause *de novo* DNA methylation, an event that occurs with slower kinetics, even *in vivo*[50,51] (**Fig. 2a**).

It is clear from this example that DNA methylation itself does not initiate the silencing of pluripotent genes but is rather a secondary or even tertiary effector. This raises the question of what might be the function of *de novo* methylation in this case. Experiments in ES cells have shown that after differentiation *in vitro*, cells lose their ability to revert back to a pluripotent state even if placed in selective medium. In *G9a*$^{-/-}$ (*Ehmt2*$^{-/-}$) cells, differentiation still takes place normally, but the resulting derivatives can be easily induced to return back to a state of pluripotency. Additional genetic manipulations on the key factors involved in this process ultimately showed that the lack of plasticity is actually caused by DNA methylation, with heterochromatin itself playing only a minor role[51]. These results suggest that although DNA methylation is not required for initiating gene silencing, it may be important in maintaining the repressed state over many cell generations, even covering the entire lifespan of the organism.

Another example of *de novo* methylation after implantation is that which accompanies X-chromosome inactivation in female embryos. Random X-chromosome inactivation occurs in each cell concomitantly with differentiation. This process, which is directed by *Xist* expression on the chosen allele, involves chromosome-wide changes, including a shift to late replication, deacetylation of histones[52], methylation of H3K27 by the Polycomb complex[53,54] and inactivation of

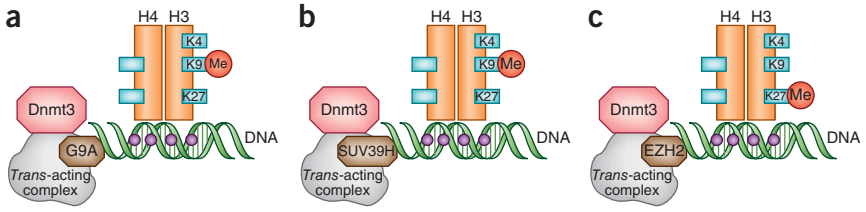

Figure 2 Targeted *de novo* methylation. (**a**–**c**) Site-specific *de novo* methylation (purple circles) often involves the initial binding of *trans*-acting complexes (gray) capable of recruiting histone methylases (brown) that not only modify specific lysine residues on histone H3 but also recruit Dnmt3, which then methylates the underlying DNA. For example, G9A directs *de novo* methylation of pluripotency genes such as *Oct-3/4* and *Nanog* after implantation (**a**); SUV39H, a H3K9 methylase is responsible for *de novo* methylation of satellite sequences (**b**); and the Polycomb complex containing the H3K27 methylase EZH2 is responsible for *de novo* methylation of CpG islands in specific tissues in cancer and perhaps after X-chromosome inactivation (**c**).

many genes. Once again, *de novo* methylation of promoters located in CpG islands appears to be a very late event as measured both in differentiating ES cells as well as *in vivo*[55,56]. There is no question that the inactivation of X chromosome–linked genes can be accomplished in the absence of DNA methylation as actually occurs in extra-embryonic tissues and in marsupials. The added layer of methylation in animal cells apparently provides long-term stability, making it almost impossible to reactivate genes on the inactive X chromosome in somatic tissues[57]. In contrast, X chromosome–linked genes in marsupials readily undergo derepression over the lifetime of these animals[58].

***De novo* methylation: a backup maintenance mechanism?** It should be noted that *de novo* methylation can also serve as a backup maintenance mechanism, as may be the case for the *Oct3/4* gene in somatic cells. Although G9a originally binds to this gene locus during early post-implantation development, this factor appears to remain bound to the gene even in somatic cells and presumably continues to recruit Dnmt3 (ref. 50). Using a *p53* (*Trp53*) knockout line, fibroblasts with low levels of Dnmt1 were shown to lose over 95% of all their DNA methylation, probably because there is not enough maintenance activity to fill in all the complementary methylations required during replication[59]. Notably, however, the *Oct3/4* gene promoter still remains methylated in these cells, presumably because it can use its affinity for *de novo* methylases to back up for the loss of methylation that would normally occur during replication and cell proliferation[50]. Indeed, it has been demonstrated, in general, that *de novo* methylases can cooperate with Dnmt1 in the maintenance of DNA methylation[60,61].

One of the most interesting concepts to have emerged from these studies is that targeted *de novo* methylation is almost always mediated by histone methylases[7] that are recruited by local regulatory factors. As noted above, this is true for *Oct3/4* methylation, which is directed by the H3K9 methylase G9a, and is also the case for methylation of satellite DNA, which is mediated by a different H3K9 methylase, Suv39h (**Fig. 2b**). In addition to these events, it has been shown that different CpG islands undergo tissue-specific *de novo* methylation during development, with each individual cell type having its own set of targeted genes[62]. Chromain immunoprecipitation–high-throughput sequencing (ChIP-seq) analysis has shown that a very high percentage of these CpG islands are actually binding sites for the Polycomb complex[24], which includes Ezh2, a histone methylase specific for H3K27 that can recruit Dnmt3 (refs. 63,64; **Fig. 2c**). It appears that these histone-modifying proteins have evolved as a self-contained 'machine' programmed to bring about the epigenetic closure of local gene sequences, and this is accomplished at two different levels: first, by bringing about heterochromatin formation, and then by covalently attaching methyl groups to the DNA, allowing this mark to be stably maintained over many cell generations[7]. It should be noted that other regulatory factors may also be used to recruit methylases to specific sites in the genome[65].

Targeted demethylation

Many tissue-specific genes are methylated in most cell types but are unmethylated in the tissue where each of these genes is expressed[66]. As these gene sequences are initially methylated in the implantation embryo, it is clear that this difference is generated through a process of demethylation. Using DNA-mediated gene transfer in cultured cells, it has been shown that many specific cell types retain their ability to recognize and demethylate gene sequences that are normally expressed in the same cell, and reverse-genetics studies have made it possible to actually identify the *cis*-acting sequences that enable these genes to be recognized for demethylation. This has been shown to be the case, for example, for the α-actin gene in myoblasts[38,67] as well as the *Igk* locus in B lymphocytes[68], and has also been confirmed for spermatogenesis-specific demethylation using transgenes *in vivo*[69].

These observations suggest that there must be tissue-specific *trans*-acting factors that recognize these motifs and can recruit the demethylation machinery. One clear-cut example is NF-κB, which binds the *Igk* enhancer and has actually been proven necessary to demethylate this region in B cells before rearrangement of the locus[70,71], but the involvement of transcription factors in the demethylation process appears to be of a general nature[72]. In keeping with this, data from the Encyclopedia of DNA elements (ENCODE) project have revealed a straightforward correlation between tissue-specific demethylation and the binding of transcription factors at these same sites[73].

Mechanism of gene-specific demethylation. Early studies demonstrated that demethylation is an active event and does not occur passively as a result of DNA replication in the absence of methyl maintenance. This was originally demonstrated for the δ crystalline gene that undergoes demethylation in the lens even though these cells are not replicating[74], and other tissue-specific genes behave in the same manner. In addition, transient transfection of methylated substrates into muscle cells definitively showed that the original exogenous DNA strands become demethylated[67], ruling out the possibility that demethylation takes place through a passive process.

For many decades, the biochemical mechanism of demethylation was unknown. One attractive proposal was that this reaction involves direct removal of the methyl group from cytosine and its conversion to methanol[75], but this mechanism has never been validated, and several studies did not reproduce these results[76]. Using another approach, it was shown that demethylation could take place through a repair-like process involving glycosylation to remove the base followed by excision repair (**Fig. 3**). Early experiments *in vitro* indicated that this reaction could occur[77,78], and labeling studies *in vivo* also supported the concept that demethylation involves base removal[79].

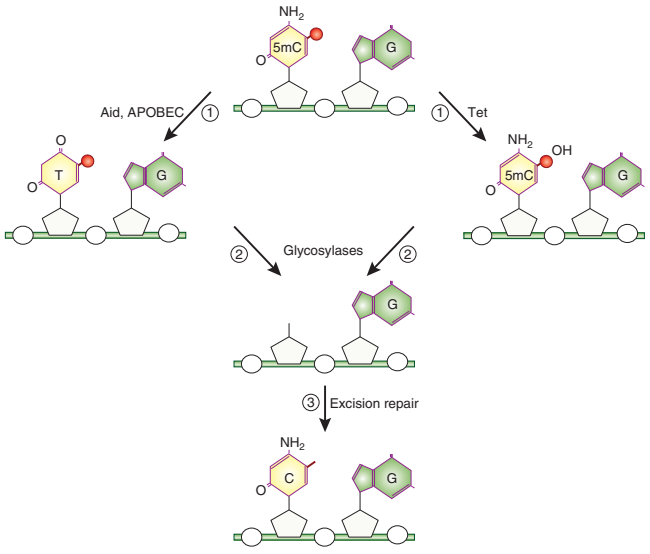

Figure 3 Pathways of demethylation. It is likely that step 1 of the demethylation pathway involves modification of 5mC through deamination (Aid, APOBEC) or hydroxymethylation (Tet). These modified bases are removed by glycosylases, which generate apyrimidinic acid (step 2) that is subsequently removed by excision repair and replaced with cytosine (step 3). It should be noted that 5hmC can be further oxidized to 5fmC or 5caC, which can also be removed by glycosylation (not shown).

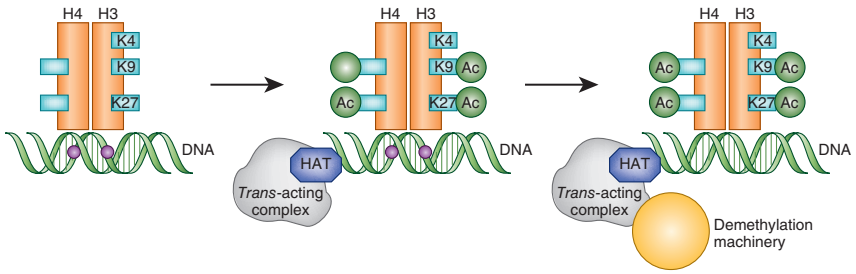

Figure 4 Targeted demethylation. The actual pathway for targeted demethylation has not been characterized, but it is possible that it is directed by histone acetylases (HATs). In this scheme, sites in the genome that are methylated (purple circles) and packaged with unacetylated histones are recognized by specific *trans*-acting complexes, which recruit HATs and other histone activation enzymes that can further recruit the demethylation machinery.

Over the past few years, much of the enzymology of demethylation has become clarified. A major advance in our understanding of this process came with the discovery of the Tet family enzymes that can convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC)[13,80,81], and this appears to represent a major intermediate in the demethylation pathway (**Fig. 3**). A good example emphasizing the importance of this enzyme is the active demethylation of sequences in the paternal genome that occurs in the zygote immediately after fertilization. Not only does this process involve the conversion of 5mC to 5hmC, but knockdown of Tet3 actually prevents demethylation[13,82]. Although this reaction may account for the first step, the subsequent biochemical events have not been fully clarified yet. One possibility is that 5hmC is further oxidized to 5-formylcytosine (5fC) or 5-carboxylcytosine (5caC), which can be removed by known glycosylation enzymes[83,84]. Alternatively, 5hmC, which is not recognized by the Dnmt1 maintenance activity[85,86], could become diluted out during replication.

The main principle underlying this pathway appears to be that 5mC must be modified before it can be removed by glycosylation and excision repair. It has been suggested, for example, that deamination may be involved in this process, and this is supported by evidence showing that Aid or APOBEC may be necessary for demethylation in the brain and during spermatogenesis[87–89]. Another possibility is that 5mC is converted to 5hmC, which is then deaminated to 5hmU, a good substrate for several known glycosylases[90], but it is still not clear whether 5hmC can actually serve as a substrate for deamination *in vitro* or *in vivo*[91] (**Fig. 3**).

Functional role of targeted demethylation. Demethylation of tissue-specific genes is always associated with activation of these genes during development, but it is not always clear whether demethylation itself is actually required for activation. As noted above, the specificity of this process must be directed by factors that recognize nearby *cis*-acting elements, implying that demethylation itself is not the primary event. In some cases, demethylation occurs before transcription and may even be necessary for the gene to become activated. One example of this is in the immune system, where demethylation appears to be a necessary step in the process of rearrangement[92,93]. In other instances, however, RNA synthesis already occurs before demethylation, as has been seen for several different liver-specific genes[94–97]. These results suggest that both demethylation and transcription are driven by the same or similar set of *trans*-acting factors and take place in a coordinated manner.

These studies clearly indicate that demethylation does not represent the primary event controlling gene activation during development. Rather, it appears to provide a secondary mechanism for making sure the target gene stably remains in an open conformation. Indeed, when tissue-specific genes are inserted into a non-expressing cell type by DNA-mediated gene transfer, unmethylated copies are transcribed at a basal level, whereas methylated templates are further inhibited and are expressed at very low levels, similar to that of the parallel endogenous gene in these cells[38,98]. The same is true for tissue-specific genes that have been programmed to be constitutively unmethylated in transgenic mice[31,99]. These experiments clearly show that even after bypassing the activation step, one can still observe the effect of undermethylation on long-term expression patterns.

Taken together, these studies show that both specific *de novo* methylation, as well as specific demethylation, operate through similar overall strategies, with targeting being accomplished by interactions between *cis*-acting sequences and *trans*-acting recognition factors. In the case of *de novo* modification, it was demonstrated that methylation enzymes are almost always recruited by local histone methylases[7]. In a similar manner, it is possible that site-specific demethylation of DNA is associated with the presence of histone acetylases or demethylases[100] (**Fig. 4**). Although there is no direct evidence that these enzymes themselves actually recruit the DNA demethylation machinery, it is clear that gene activation may require both the action of histone demethylases to decondense local chromatin structure as well as the removal of methyl groups from the DNA itself. If correct, this would suggest that the manipulation of these two epigenetic marks is actually coordinated *in vivo* at the level of genome programming.

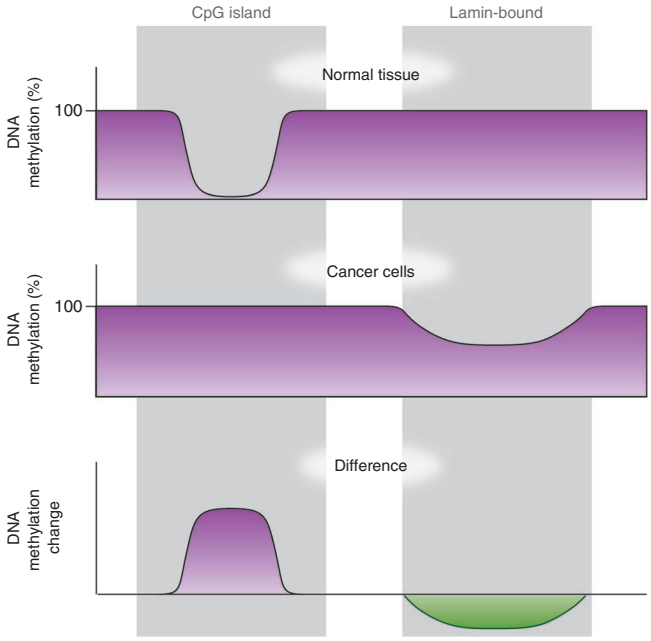

Figure 5 DNA methylation pattern in cancer. In normal tissue, almost the entire genome is highly methylated with only CpG islands left unmethylated. In cancer cells, some CpG islands undergo *de novo* methylation, whereas regions associated with the nuclear lamin become demethylated, as illustrated in the difference graph.

Methylation in cancer

It is well documented that tumor cells are characterized by an abnormal pattern of DNA methylation, with many non–CpG island regions becoming demethylated and some CpG islands undergoing *de novo* methylation[101,102]. Much emphasis has been placed on the methylation of CpG islands. Originally it was thought that DNA methylation in cancer may be 'leaky' and therefore lead to random methylation at all unmethylated sites in the genome, with growth selection determining the final pattern, as is the case with mutations[103]. By using genome-wide approaches to map DNA methylation in tumors, however, it soon became apparent that many CpG islands consistently undergo *de novo* methylation and that most of these events occur at genes that are not involved in growth selection[104–106] (**Fig. 5**). Many studies have now confirmed that this methylation is indeed targeted and that these *de novo* events are predominantly specific for CpG islands marked with the Polycomb repressor[107–109]. As one of the main components of this complex is Ezh2, it is likely that in this case as well a histone methylase may be responsible for recruiting *de novo* methylases[63,64] (**Fig. 2c**).

The timeline of DNA methylation in cancer is not fully understood. In colon cancer, high levels of modification are already present in polyps[105], suggesting that this modification may be an early event in the tumor transformation process. Furthermore, many of the sites that become methylated in cancer have been found to be partially methylated in normal tissues via a process that appears to increase with age[19,110,111], and this is consistent with observations that normal tissue surrounding tumors also appears to have a strong *de novo* modification pattern[112,113]. It thus seems likely that much of the age-related and tumor-related methylation comes about through a common mechanism involving site-specific Dnmt3 targeting. It should be noted, however, that many mutations in cancer take place in genes involved in chromatin structure and modification[114,115], and this may also contribute to the abnormal epigenetic landscape.

Demethylation in cancer. In addition to this *de novo* methylation, tumor cells are also characterized by a high degree of demethylation. Initial observations gave the impression that this undermethylation is global and therefore may include most non–CpG island regions of the genome that are normally methylated in every cell type[116]. Recent bisulfite sequencing studies, however, have better defined the topography of this demethylation, and it now appears that this occurs mainly in regions that are associated with the nuclear envelope lamina[117,118] (**Fig. 5**). These areas of the genome are characterized by late replication and relative gene repression. Although the mechanism for this demethylation is not known, it has been suggested that this may occur as a passive process with maintenance methylation unable to keep up with rapid replication. As all of the free Dnmt proteins in the cell appear to be capable of forming a single enzymatic complex, it is thought that in tumors much of the Dnmt1 may get abnormally recruited to *de novo*–targeted CpG islands, and this could, in turn, reduce the amount of Dnmt1 maintenance activity at the replication fork, thereby bringing about passive demethylation[64]. This effect would be most influential at the lamina-associated regions because they alone undergo replication late in S phase. If correct, this mechanism would provide an integrated explanation for why tumor cells show both targeted *de novo* modification as well as general demethylation.

Pathophysiological role of DNA methylation. It is not yet clear, however, how DNA methylation changes actually affect tumor cell biology. By using cancer cell lines in culture, it has been demonstrated that *de novo* methylation may operate by repressing tumor-suppressor genes[119–122]. Considering the large number of sites that are targeted in tumors, however, it is likely that this abnormal modification may have multiple additional effects, including the inhibition of differentiation, maintenance of proliferation and repression of genes needed for repair or apoptosis[105].

There is no question that abnormal methylation is important in tumorigenesis. By inhibiting DNA methylation in a mouse model for intestinal tumors, for example, it is possible to dramatically decrease the occurrence of adenomas[119], and the same is true for other cancer types as well[123,124]. In contrast, certain forms of leukemia in mice are actually promoted by inhibiting DNA methylation[125], suggesting that for some tumors, it is demethylation[126] that has a dominant influence.

Outlook

DNA methylation is a unique form of gene regulation because, unlike other gene-control mechanisms based on protein-DNA interactions, it involves covalent changes to the genome that provide long-term stability. In this Review we outlined how these methylation patterns are initially erased and then set up anew during a multistep process that takes place during development. The instructions for where to place methyl groups in time and place are all programmed into the genome, and although some of these DNA signals have been deciphered, there is still a great deal of work that must be done to understand the full extent of this programming language. Proving the function of this language will involve manipulating the control elements *in vivo* by reverse-epigenetics approaches. In addition to the programmed planning of modification that occurs normally during development, it is commonly thought that DNA methylation changes may occur during the lifetime of the organism as a result of internal or external environmental stimuli. Many studies are now under way to detect methylation changes of this nature. In light of our understanding of how methylation is programmed *in vivo*, it is reasonable to assume that additional changes come about as a result of specific protein-DNA interactions, and this could serve as a general outline for the discovery of nonscheduled epigenetic modifications during disease.

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