Linking DNA methylation and histone modification: patterns and paradigms

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Abstract | Both DNA methylation and histone modification are involved in establishing patterns of gene repression during development. Certain forms of histone methylation cause local formation of heterochromatin, which is readily reversible, whereas DNA methylation leads to stable long-term repression. It has recently become apparent that DNA methylation and histone modification pathways can be dependent on one another, and that this crosstalk can be mediated by biochemical interactions between SET domain histone methyltransferases and DNA methyltransferases. Relationships between DNA methylation and histone modification have implications for understanding normal development as well as somatic cell reprogramming and tumorigenesis.

Histone
Protein component of chromatin that is involved in regulation of gene expression. Two of each of the core histones, H2A, H2B, H3 or H4, make up an octameric nucleosome, around which DNA winds. N-terminal tails of histones can be subject to covalent modification, including methylation and acetylation.

CpG island
A sequence of at least 200 bp with a greater number of CpG sites than expected given the average GC content of the genome. These regions are typically undermethylated and are found upstream of many mammalian genes.

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Although it is now accepted that chromatin structure has a large impact on the regulation of gene expression, little is known about how individual epigenetic marks are set up and then maintained through DNA replication and cell division. Chemical modification of DNA or of chromatin-associated proteins, particularly histones, has a major influence on chromatin structure and gene expression. In animal cells, DNA can be modified by methylation of cytosine residues in CpG dinucleotides, and the N-terminal tails of histone proteins are subject to a wide range of different modifications, including acetylation, methylation, phosphorylation and ubiquitylation. All of these chemical changes seem to have a substantial influence on chromatin structure and gene function, which differs depending on the type and location of the modification. In this Review we take advantage of evidence from recent genetic, biochemical and microarray studies to explore the relationship between DNA methylation and histone modification, particularly focusing on methylation of histone H3 at lysine 9 (H3K9) and 27 (H3K27), which are important modifications for gene repression.

Although DNA methylation and histone modification are carried out by different chemical reactions and require different sets of enzymes, there seems to be a biological relationship between the two systems that plays a part in modulating gene repression programming in the organism. We describe how DNA methylation and specific histone modifications influence each other during mammalian development. It seems that the relationship can work in both directions: histone methylation can help to direct DNA methylation patterns, and DNA methylation might serve as a template for some histone modifications after DNA replication. Recent evidence indicates that, at the molecular level, these connections might be accomplished through direct interactions between histone and DNA methyltransferases. We then discuss how histone modification and DNA methylation can have different roles in gene silencing, with histone modifications providing labile transcriptional repression and DNA methylation being a highly stable silencing mark that is not easily reversed. Finally, we address how understanding the relationship between these two types of modification can help us to decipher the epigenetic blocks that inhibit cellular reprogramming and to understand mechanisms of gene repression in cancer.

Generating modification patterns

Generation of the basal bimodal DNA methylation pattern. The basic methylation pattern of the animal genome is bimodal: almost all CpG dinucleotides are methylated, except those located in CpG islands, which are to a large extent constitutively unmodified. The DNA methylation pattern is erased in the early embryo and then re-established in each individual at approximately the time of implantation[1,2]. Differential methylation is established through two counteracting mechanisms: a wave of indiscriminate *de novo* methylation[3] and a mechanism for ensuring that CpG islands remain unmethylated. The precise details of how CpG islands are protected are not completely elucidated, but early studies using transgenic mice and transfection experiments in embryonic stem cells suggested that protection

Chromodomain
Initially identified in the *Drosophila melanogaster* heterochromatin protein 1 and Polycomb proteins, this is an ~50 amino acid, highly conserved domain that binds to histone tails that are methylated at certain lysine residues. Different classes of chromodomains have been implicated in binding histones, RNA and DNA.

Heterochromatin protein 1
(HP1). Conserved component of silent heterochromatic regions, which contains a chromodomain that binds nucleosomes containing histone H3 that is methylated on lysine 9.

might be directed by the recognition of common *cis*-acting sequences located in CpG islands[4–6] and mediated by active demethylation[7].

Recent studies strongly suggest that the establishment of the basic DNA methylation profile during early development might be mediated through histone modification[8] (FIG. 1). According to this model, the pattern of methylation of H3K4 (including mono, di and trimethylation, referred to here as H3K4me) across the genome might be formed in the embryo before *de novo* DNA methylation. H3K4 methylation might be directed by sequence-directed binding of RNA polymerase II, which recruits specific H3K4 methyltransferases[9]. As RNA polymerase II is bound mostly to CpG islands in the early embryo, only these regions are marked by H3K4me, whereas the rest of the genome is packaged with nucleosomes containing unmethylated H3K4. *De novo* DNA methylation is carried out by the DNA methyltransferase enzymes DNMT3A and DNMT3B complexed with DNMT3L[8,10], a closely related homologue that lacks methyltransferase activity[11]. DNMT3L recruits the methyltransferases to DNA by binding to histone H3 in the nucleosome, but contact between DNMT3L and the nucleosome is inhibited by all forms of methylation on H3K4 (REF. 8). As a result, *de novo* methylation in the embryo takes place at the majority of CpG sites in the genome, but may be prevented at CpG islands because of the presence of H3K4me. This model is consistent with the finding of a strong anti-correlation between DNA methylation and the presence of H3K4me in several cell types[12–15].

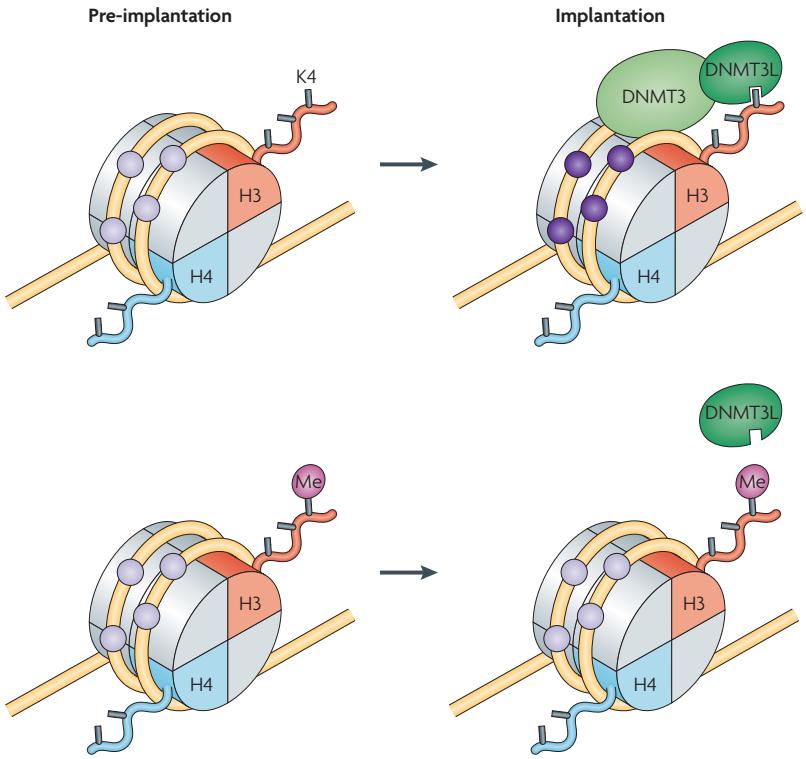

Figure 1 | **Establishment of bimodal methylation.** Before implantation, most CpGs in the embryonic genome are unmethylated (light purple circles), but some regions are packaged with nucleosomes containing methylated (Me) lysine 4 of histone H3 (H3K4), perhaps as a result of RNA polymerase binding. At the time of implantation, the methyltransferases DNMT3A and DNMT3B are expressed. DNA methylation (dark purple circles) is facilitated by the DNMT3 binding partner, DNMT3L, which binds to chromatin by recognizing the K4 residue on histone H3 (REFS. 8,10). If this histone moiety is methylated, however, the complex cannot bind and the underlying DNA region is thus protected from *de novo* methylation. This may be one of the mechanisms used to generate a bimodal methylation pattern characterized by methylation over most of the genome, but not at CpG islands.

Targeted* de novo *methylation in early development. Once the basal bimodal pattern of DNA methylation is established in the embryo at the time of implantation, this profile becomes subject to additional targeted alterations during development, including both *de novo* methylation and demethylation events[12,16]. A significant change that occurs in early development is the targeted repression and *de novo* methylation of genes that are necessary for preserving pluripotency, such as *Oct3/4* (also known as *Pou5f1*). This repression occurs at the time of gastrulation — when the embryo begins to separate into germ layers[17] and concomitantly loses the ability to maintain a pluripotent state.

Using embryonic stem cells as a model system, it has been shown that *Oct3/4* undergoes inactivation in a multistage process (FIG. 2). In the first stage, transcription seems to be turned off directly through the interaction of repressor molecules with the *Oct3/4* promoter[18–20]. This is followed by transcription factor-dependent recruitment of a complex that contains the histone methyltransferase G9a and enzymes with a histone deacetylase activity. This complex mediates local deacetylation of histones — a change that is associated with transcriptional repression. Deacetylation resets the lysine residues so that G9a can catalyse methylation of H3K9. This modification enables binding of the chromodomain protein heterochromatin protein 1 (HP1), which facilitates local formation of heterochromatin (heterochromatinization). In the final stage of silencing, the G9a-containing complex also recruits DNMT3A and DNMT3B, which catalyse *de novo* DNA methylation at the promoter[21]. This series of steps, mediated by the G9a-containing complex, seems to have a central role in post-implantation gene inactivation, with many other crucial genes (such as *Nanog* and *Dnmt3L*) also undergoing repression through this pathway[22].

A further example of how *de novo* DNA methylation might be linked to histone modification in early development is the heterochromatinization of pericentromeric satellite repeats. At these satellite sequences, it is the SET domain-containing histone methyltransferase enzymes SUV39H1 and SUV39H2 that are responsible for trimethylating H3K9 and heterochromatinization. These proteins are also required to recruit DNMT3A and DNMT3B in order to methylate CpG sites in the satellite sequences[23,24]. Interestingly, this heterochromatinization process seems to be initiated by a Dicer-mediated mechanism that recognizes RNA duplexes that are naturally formed at satellite sequences. The resulting RNA-induced silencing complex (RISC) is then specifically targeted back to pericentromeric regions where it probably recruits SUV39H1 and SUV39H2,

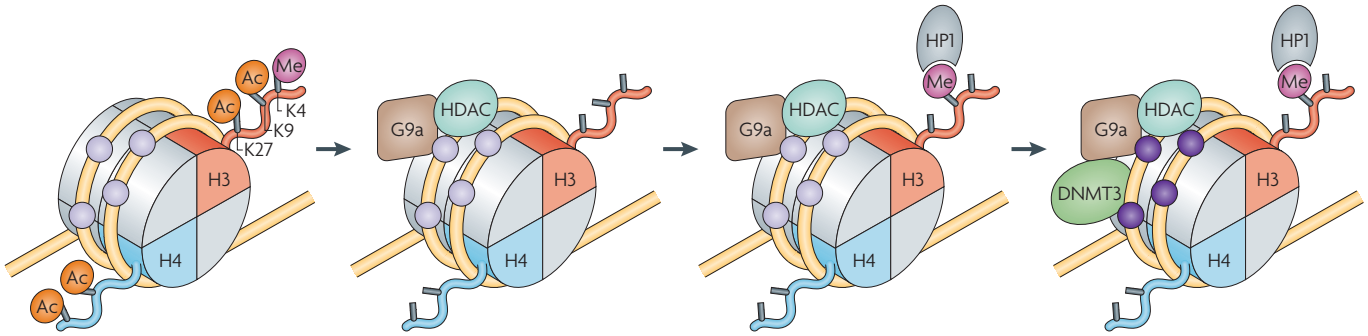

Figure 2 | **Turning off pluripotency genes.** In embryonic stem cells, pluripotency genes such as *Oct3/4* and *Nanog* have unmethylated CpG islands (light purple circles) and are packaged with acetylated (Ac) histone H3 and H4 and methylated (Me) lysine 4 of histone H3 (H3K4). With the onset of differentiation the SET domain-containing histone methyltransferase G9a is recruited, together with a histone deacetylase (HDAC), and this causes deacetylation of local histones. In addition, H3K4 is demethylated, but the enzymatic machinery responsible for this has not yet been identified. In the next step, G9a catalyses the methylation of H3K9, and this modification serves as a binding site for the chromodomain protein heterochromatin protein 1 (HP1), thus generating a form of local heterochromatin. Finally, G9a recruits the methylases DNMT3A and DNMT3B, which mediate *de novo* methylation (dark purple circles) of the underlying DNA[21,22].

Heterochromatin
Highly compacted regions of chromatin, in which transcription is repressed. Constitutive heterochromatin is a common feature of highly repetitive DNA sequences.

Satellite repeat
DNA that contains many tandem repeats of a short basic repeating unit. Both the major and minor satellite repeats are located at pericentromeric heterochromatin.

SET domain
An evolutionarily conserved sequence motif that was initially identified in the *Drosophila melanogaster* position effect variegation suppressor Su(var)3–9, the Polycomb-group protein Enhancer of zeste, and Trithorax (a Trithorax group protein). It is present in many histone methyltransferases and is required for enzyme activity.

Dicer
An RNA endonuclease that cleaves double-stranded RNA into small interfering RNAs of approximately 21 bp.

RNA-induced silencing complex
(RISC). A complex made up of an Argonaute protein and small RNA, which inhibits translation of target RNAs through degradative or non-degradative mechanisms.

Imprinted locus
A locus at which the expression of an allele is different depending on whether it is inherited from the mother or the father.

which are the most important components in this heterochromatin pathway[25–28]. Indeed, non-coding RNA may also play a part in recruiting histone methylases in other cases of gene inactivation, such as at imprinted loci and during X chromosome inactivation[29–31].

These two examples of pluripotency-associated gene silencing and satellite sequence repression illustrate how histone modification and DNA methylation can have a cooperative relationship in the early embryo. These studies, in animal cells, indicate that there is an intimate relationship between DNA and histone methylation, and this is strongly supported by genetic manipulation experiments. Indeed, studies in *Neurospora crassa*[32], *Arabidopsis thaliana*[33] and animal cells[21–23] show that knockdown of certain SET domain histone methyltransferases causes a decrease in DNA methylation in specific genomic regions. Conversely, the tethering of the histone methyltransferase G9a to a random region of the DNA in animal cells seems to cause histone methylation and DNA methylation at nearby sequences[34].

Effect of DNA methylation on histone modification. The examples discussed above illustrate how histone modifications might play a role in establishing the patterns of DNA methylation, but there is also evidence that DNA methylation is important for maintaining patterns of histone modification through cell division. After the bimodal methylation profile is established in the pluripotent embryo, the enzymatic machinery needed for this process is then downregulated[35] and, following differentiation, cells generally lose both their *de novo* methylation activity and their ability to recognize and protect CpG islands. Nonetheless, the basic DNA methylation pattern that is generated at the time of implantation is maintained throughout development through the action of DNMT1, which is associated with the replication complex[36]. Recent studies indicate that DNMT1, together with the E3 ubiquitin-protein ligase UHRF1 (also known as Np95 or ICBP90), specifically recognizes the methylated CpG residues of the hemimethylated DNA that is generated during DNA replication and methylates the opposite strand[37–39], thus reproducing a faithful copy of the methylation profile that is present in the parent cell.

Despite the importance of chromatin conformation in moulding transcription patterns, it is likely that chromatin structures are disrupted as the replication fork progresses along the DNA, so mechanisms are needed to reproduce chromatin conformation after replication has occurred. The DNA methylation pattern might be one of the main markers that are used for reconstructing the epigenetic state of the genome following cell division. Regions that have a methylated profile are reassembled in a closed conformation, whereas unmethylated DNA tends to get repackaged in a more open configuration[40,41]. Using chromatin immunoprecipitation (ChIP), it has been shown that unmethylated DNA is largely assembled in nucleosomes that contain acetylated histones, which are associated with open chromatin, whereas the presence of methyl groups on identical DNA sequences correlates with assembly of nucleosomes containing non-acetylated histone H3 and H4, leading to more compact chromatin[42,43].

This relationship between DNA methylation and histone modification might be partially mediated through methylcytosine-binding proteins, such as MECP2 or MBD2, that are capable of recruiting histone deacetylases to the methylated region[44,45]. It is probable that the presence of DNA methylation also directs H3K9 dimethylation, which is a mark of repressive chromatin[43], perhaps through the interaction of G9a and DNMT1 with the replication complex[46]. There is also evidence that DNA methylation inhibits H3K4 methylation[43,47] and, in plants, excludes the histone variant H2AZ from nucleosomes[48] — both of these marks are associated with active transcription. However, the mechanisms underlying these processes are not known. Thus, it seems that the DNA methylation profile that is established during development might act as a template to maintain transcriptional repression patterns at many genomic

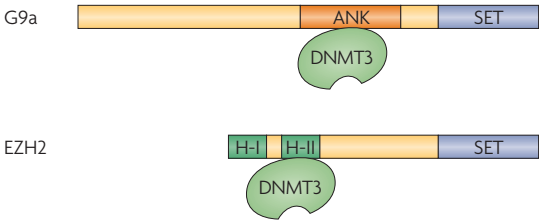

Figure 3 | **SET domain-containing histone methyltransferases interact with DNMT3A and DNMT3B.** Two examples of SET domain histone methyltransferases that are involved in heterochromatinization and in targeted *de novo* DNA methylation are shown. G9a recruits DNMT3A and DNMT3B through its ankyrin (ANK) domain. EZH2 has been shown to interact with DNMT3A, DNMT3B and DNMT1 *in vitro* through a homology domain[51] (H-II).

sequences throughout cell division, without the need to recognize specific sequences or genes after each round of DNA replication.

Interrelationships through enzyme interactions. Using a combination of biochemical and genetic approaches, it has now been shown that the connection between histone and DNA methylation is generated at the level of enzyme interactions. In the case of G9a, for example, histone methyltransferase activity and the link with DNA methyltransferase activity seem to be carried out by different protein domains (FIG. 3). As a result, point mutations in the SET domain can eliminate H3K9 methylation without affecting DNA methylation[22,49,50]. This suggests that DNA modification is not dependent on histone modification *per se*; instead it seems that the G9a enzyme is responsible for recruiting DNMT3A and DNMT3B. Biochemical studies indicate that this physical interaction is carried out by the G9a ankyrin domain[22]. Similarly, biochemical analysis has shown that DNA methyltransferases bind to the histone methyltransferase EZH2 through a domain that is independent of the SET domain responsible for H3K27 methylation[51]. The mediation of DNA methylation by SUV39H1 (REF. 24) at pericentric heterochromatin[23], or by SETDB1 (also known as ESET)[52], also seem to involve direct interactions between these histone methyltransferases and DNMT3A and DNMT3B. There is also evidence of a similar interaction for the histone methyltransferase SUVH4 (also known as KRYPTONITE) in *A. thaliana*[33]. In addition to these SET domain proteins, it is possible that HP1 has the ability to recruit DNMT proteins[53], and this may serve as an auxiliary mechanism leading to DNA methylation of heterochromatic regions. Indeed, HP1 seems to be an essential component for DNA methylation in *N. crassa*[54].

The examples discussed in this section summarize the current body of evidence supporting the notion of bidirectional crosstalk between histone modifications and DNA methylation. In many of these cases it seems that these relationships operate at the level of protein effectors, rather than through the modifications themselves. In the following section we discuss how the relationship between DNA methylation and histone modifications influences gene silencing in a number of biological situations.

Paradigms of repression

Long-term repression plays an important part in the programming of gene expression profiles in the developing organism. By mapping DNA methylation and histone modification across the genome, it seems that there are a number of different molecular strategies involved in long-term repression. Furthermore, different types of epigenetic marks might have specific biological roles *in vivo*. Many regions of the genome adopt a closed chromatin structure owing to *de novo* methylation that occurs very early in development, and they are kept in this state through the maintenance of DNA methylation and chromatin structure following every cell division. This is a global process that encompasses a large portion of the genome, including many repeated sequences and transposons, and seems to be unique to higher organisms. Many of these regions contain genes that can then become activated in specific cell types in a process that involves targeted gene recognition followed by alterations in chromatin structure and removal of DNA methylation[55].

Another strategy for silencing involves large protein complexes that bind near target genes and cause repression through a combination of enzymatic and structural activities that lead to the closure of local chromatin, mainly by affecting histone modifications. Examples include the complex that contains NRSF (also known as REST), which recognizes specific DNA sequences near genes that are destined to be expressed in neuronal cells[56], and the Polycomb repressive complex 2 (PRC2) (discussed in more detail below), which represses a wide variety of genes that have key roles during development[57–59]. In these cases, repression can be maintained over multiple cell divisions because the complexes are present constitutively and can readily rebind their target sequences following DNA replication[60]. Although not truly global in its scope, each of these complexes recognizes multiple gene regions and therefore represents a general mechanism for repression of specific sets of genes. This form of repression might be particularly important at stages in which DNA methylation is erased, such as in primordial germ cells or pre-implantation embryos[61].

Polycomb targets and DNA methylation. The Polycomb target genes provide our first example of how histone modification and DNA methylation cooperate to achieve silencing. In this case the mechanism of repression involves the generation of local heterochromatin: the SET domain histone methyltransferase EZH2, as part of the PRC2 complex, catalyses trimethylation of H3K27 (H3K27me3) on surrounding nucleosomes; these methyl moieties then serve as 'landing sites' for the heterochromatin-like chromodomain protein PC (also known as HPC), which is associated with additional chromatin structure-modifying activities as part of the

X chromosome inactivation
The process that occurs in female mammals by which gene expression from one of the two X chromosomes is downregulated to match the levels of gene expression from the single X chromosome that is present in males. Inactivation involves changes in DNA methylation and histone modifications.

Chromatin immunoprecipitation
(ChIP). A technique that is used to analyse the genomic location of DNA-associated proteins that involves crosslinking DNA–protein complexes then immunoprecipitation using an antibody against a protein of interest. This is followed by analysis of the recovered DNA sequences.

Polycomb repressive complex
(PRC). A group of repressive chromatin proteins that maintain states of gene expression throughout development. Originally identified in *Drosophila melanogaster* as genes in which mutations caused homeotic transformations.

PRC1 complex[62,63]. One of the main characteristics of Polycomb-induced repression is that it is easily reversible. Almost all Polycomb target genes are marked by both the repressive H3K27me3 modification and the activating modification H3K4me3 in embryonic stem cells[64–68]. This so-called bivalent modification pattern is predicted to confer the potential for a gene to be driven either to its active or inactive state. Thus, genes that are silenced by this mechanism maintain the possibility of being readily activated during differentiation, whereas genes in their active conformation might easily revert to the repressed state.

Genes targeted by Polycomb complexes are generally associated with CpG island promoters and, as such, are protected from *de novo* methylation at the time of implantation[4]. Thus, most EZH2 target genes actually remain constitutively unmethylated throughout development[13]. Nonetheless, a number of these genes might become targets for *de novo* DNA methylation under certain circumstances. It was recently shown, for example, that during differentiation of embryonic stem cells to neural precursors, many gene sequences undergo *de novo* methylation, and a large portion of these are initially marked by the Polycomb complex[13,14]. In addition, several other genes become methylated during later developmental stages, and these sites have also been identified as targets of Polycomb proteins[69]. Although the significance of adding DNA methylation as an additional layer of repression is not clear, it is likely that the Polycomb complex plays a part in mediating the DNA methylation reaction. This might be mediated by EZH2, which interacts with DNMT3A and DNMT3B *in vitro*[51]. However, it is clear that other factors must also be involved in triggering this cell-type specific *de novo* methylation.

X inactivation. A good example for understanding the role of DNA methylation in long-term repression is the X chromosome in female mammalian cells. Following random selection, one X chromosome in each cell undergoes region-wide inactivation at an early stage of development. Initially this involves changes in chromatin structure that restrict accessibility of DNA to protein factors, and this seems to be sufficient to silence all of the target genes on the chromosome[70]. Many of these sequences then undergo *de novo* methylation at a later post-implantation stage[71], but it is clear that this takes place after the X chromosomal genes are already silenced.

Despite the fact that the DNA methylation event is secondary, it probably contributes an additional level of repression by providing long-term stability. Indeed, when X inactivation takes place without DNA methylation, such as in marsupials or in extra-embryonic tissues of mammals, genes on the inactive X chromosome slowly become reactivated as a function of age[72,73]. This is in contrast to X inactivation in somatic cells of the mammal, in which reactivation is extremely rare. The addition of DNA methylation has also been shown to cause irreversible repression of viral sequences in embryonic cells[74].

Pluripotency genes. The role of histone methylation, as opposed to DNA methylation, in repression stability is well illustrated by the pluripotency genes, which undergo repression through a series of three steps (see also the section on targeted *de novo* methylation in early development). In the first step, repressor molecules induced by differentiation cues bind to the gene promoter region and turn off transcription. This form of repression seems to be completely reversible once the initial inducer is removed.

In the second step, a G9a-associated complex coordinates histone deacetylation followed by local methylation of H3K9, thereby generating local heterochromatin. This change in chromatin structure provides a new layer of repression that is much more stable than repressor binding alone, as shown by its ability to prevent gene reactivation even after removal of the original differentiation factors. Heterochromatinization by itself, however, does not seem to be a sufficient barrier against reprogramming: differentiated cells in which pluripotency genes are silenced by histone modification alone can still be converted back to an embryonic phenotype by exposing them to appropriate growth conditions[21,22].

In the final step of the inactivation process, the promoters of these key genes undergo DNA methylation, mediated through the G9a-containing complex. Once this occurs, reprogramming becomes almost impossible without artificially altering key factors in the cell. This example clearly puts into perspective the differences in the developmental potential of different forms of gene silencing — from a labile and flexible repressor-based mechanism to a highly stable inactivation that is maintained by DNA methylation.

Somatic cell reprogramming

The relationship between DNA methylation and histone modification, discussed above for a number of physiological situations, is also relevant to understanding how somatic cells can be reprogrammed to a pluripotent state — the formation of induced pluripotent stem (iPS) cells, for example. As turning off the genes that maintain pluripotency involves both histone and DNA methylation in a programmed, coordinated manner (see above), it is expected that reprogramming of somatic cells to pluripotency takes place by reversing this process, combining the removal of repressive histone marks with DNA demethylation.

One method for reprogramming somatic cells is through induction, which uses a combination of exogenously introduced key pluripotency transcription factors[75–78]. When these transcription factors are introduced, the somatic cells undergo a step-wise process in which the endogenous pluripotency genes slowly convert from their repressed state to an active conformation (FIG. 4). When examined in this system, reprogramming seems to occur by reversal of the initial inactivation, with changes in histone modifications taking place in the early stages and demethylation occurring late in the reprogramming pathway[79].

Although formation of iPS cells is initiated by the external addition of protein factors that are known to

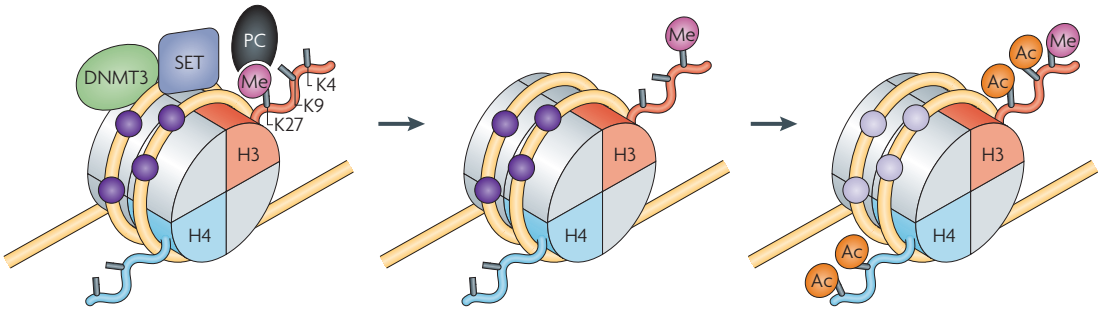

Figure 4 | **A model of somatic cell reprogramming.** Pluripotency genes in somatic cells have methylated CpG islands (dark purple circles) and are packaged with nucleosomes containing non-acetylated histones and methylated (Me) lysines (histone H3 trimethylated at lysine 27, for example), which bind chromodomain proteins such as Polycomb proteins (PC). These marks seem to be maintained by the presence of both SET domain-containing proteins (SET) and DNA methyltransferases, such as DNMT3A and DNMT3B. Reprogramming through the generation of induced pluripotent stem cells takes place in two steps. In the first step, the repressive histone methylation marks are removed, and this is then followed at a much later stage by removal of DNA methylation (light purple circles) and activation of the gene and its overlying chromatin structure[79]. Ac, acetylation.

be involved in pluripotency, these exogenous components are only required transiently to trigger an intrinsic programme for resetting the key genes[75–78]. It has been shown that inhibition of G9a[80,81], or the inclusion of DNA or histone demethylating agents[79,82], stimulates reprogramming and can even reduce the need for some of the initial factors. This presumably works because G9a plays a part in maintaining both histone and DNA methylation. Knockdown of G9a has also been shown to stimulate the reprogramming that can be induced by the fusion of somatic cells into an embryonic stem cell environment[82]. It should be noted that normal reprogramming that takes place *in vivo* during the formation of primordial germ cells or in the early post-fertilization embryo also involves a combination of heterochromatin removal and demethylation[1,61].

DNA methylation in cancer

Understanding the relationship between DNA methylation and certain histone modifications is also providing insights into the aberrant gene expression patterns observed in cancer. Many studies have shown that cancer cells are subject to abnormal *de novo* methylation compared with their normal counterparts, and new evidence suggests that this process may be linked to histone modification. Early experiments that concentrated on individual gene promoters indicated that cancer-associated DNA methylation is restricted to tumour suppressor genes, and these findings gave rise to the theory that these methylation patterns must be generated through a process of 'selection'[83]. Preliminary evidence suggested that some cancer cells express an abnormally high concentration of methyltransferases[84,85], and this could cause a low level of stochastic *de novo* methylation over all CpG islands in the genome. One model based on this evidence argues that *de novo* methylation of tumour suppressor genes would inhibit their function and thus promote increased cell proliferation, thereby providing a strong selective advantage for cells with methylated tumour suppressor promoters. This model thus predicts that growth selection would result in a specific pattern of *de novo* methylation.

With the advent of microarray methodologies for assessing DNA methylation on a genome-wide scale, it has become possible to examine global patterns of *de novo* methylation in cancer without sampling biases. These studies indicate that a large number of CpG islands can become *de novo* methylated at an early stage of tumorigenesis[16,86]. Many of these methylation events occur at the promoters of genes that are not tumour suppressors, and the large majority of these genes (>90%) are actually already repressed in the normal tissue, before transformation[86]. This clearly indicates that the *de novo* methylation profile in tumours is not formed as a result of selection. Rather, it seems that the precise locations of *de novo* methylation may be determined by a pre-programmed targeting mechanism. Indeed, several studies now show that a significant proportion of *de novo* methylated CpG islands are target sites for Polycomb protein binding[87–89]. Thus, in normal cells these loci are probably bound by PRC2 through the SET domain protein EZH2.

Although these CpG islands remain largely unmethylated during normal development[13,14], there seems to be some trigger that causes them to undergo *de novo* methylation in cancer. This might be mediated by the interaction of EZH2 with DNA methyltransferases[51] (FIG. 5). This model suggests that, in a manner similar to that occurring during normal development, histone methyltransferases are involved in enabling *de novo* methylation in cancer. One possibility is that changes in the overall levels of EZH2 (REF. 90), DNMT3A or DNMT3B lead to an altered equilibrium at the sites of Polycomb target genes, and this might be mediated through microRNAs[91–93].

Interestingly, it has recently been shown that many of the genes that become methylated *de novo* in cancer actually undergo a decrease in Polycomb marking in the same tumour cells[94]; it seems that the DNA methylation partially replaces the previous heterochromatinization that was mediated by histone methylation (FIG. 5). The DNA methylation might then be maintained by DNMT1, even though the original factors that triggered

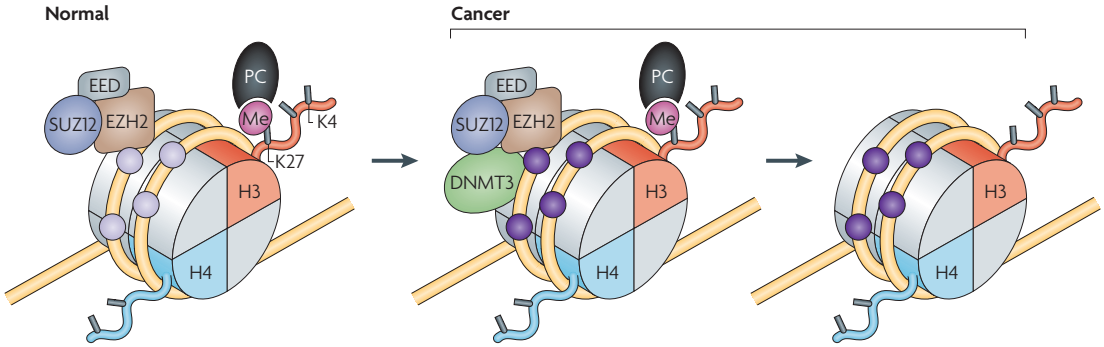

Figure 5 | **A model of *de novo* methylation in cancer.** In normal cells, Polycomb protein target genes are repressed by the presence of Polycomb repressive complex 2 (PRC2), which contains the histone methyltransferase EZH2 and other proteins. PRC2 maintains histone H3 lysine 27 trimethylation (Me) and leads to heterochromatinization through the binding of PRC1, which contains the chromodomain protein PC. Although the genes are repressed, most of them have unmethylated CpG islands (light purple circles). In cancer, some of these genes are targets of *de novo* methylation (dark purple circles)[87–89], possibly by interaction between EZH2 and the methyltransferases DNMT3A and DNMT3B[51]. After the DNA is methylated, some of these genes then lose their Polycomb repressive proteins[94], but they remain inactive because of DNA methylation, which is maintained by DNMT1 (not shown).

de novo methylation have been removed[95]. As almost all of these genes are constitutively repressed both in the normal tissue and in the cancer cell line, it is not completely clear how DNA methylation affects these genes in cancer. It is possible that this change prevents differentiation-associated gene activation or brings about long-lasting stability, thus causing a decrease in gene programming flexibility. Although this phenomenon was detected in tumour cells, a similar type of epigenetic switching has also been observed at imprinted loci in normal cell types[96].

Future directions

In this Review we have summarized what is known about the relationship between histone methylation and DNA methylation. Although each of these modifications seems to have its own role in the regulation of gene expression, there is clearly a built-in connection between them. The presence of DNA methyl groups, for example, can affect histone modification in overlying nucleosomes in a process that might be mediated by methyl binding proteins. Conversely, the presence of certain types of histone methylation marks can be associated with underlying DNA methylation. This connection is probably mediated by interaction between SET domain histone methyltransferases and DNA methyltransferases; although recent results suggest that histone arginine methylation may actually be able to recruit DMNT3A directly[97]. Crosstalk between the different types of modification serves to coordinate these prominent epigenetic effectors that are involved in many aspects of gene expression regulation during development.

There are still many mechanistic details of these schemes that need to be clarified. For example, although it is known that the presence of methyl groups in DNA affects chromatin packaging, it is still not known how the DNA methylation pattern is actually translated to produce the correct histone modification profile. Although methyl binding proteins might well play a part in modulating histone acetylation, the proteins involved in the DNA methyl-mediated control of histone methylation at H3K4 or H3K9 have not yet been identified. These are important links that are required to understand how DNA methylation affects chromatin structure. In addition, it should be noted that histone and DNA methylation may also be connected by indirect interactions[98].

There are also many mysteries about how the formation of histone methylation patterns may affect *de novo* DNA methylation. Although we have cited a number of examples of SET domain histone methyltransferases that are capable interacting with DNA methyltransferases, it must be noted that the presence of histone methylation at H3K9 or H3K27 does not always lead to *de novo* methylation. This suggests that there are additional factors required for triggering the recruitment of DNMT3 molecules specifically at sites that ultimately undergo DNA methylation. This is particularly obvious in cancer, in which hundreds of Polycomb target genes become methylated *de novo*, even though these same sites are completely unmethylated in normal cell types.

In this Review, we have emphasized how histone methylation and DNA methylation combine to induce and then maintain gene repression during development, and we have discussed the general molecular pathways that have been identified. It is still not clear, however, how these modifications are removed as part of the gene activation process. Both in the case of tissue-specific activation and reprogramming to pluripotency, in which these events have been followed sequentially, it seems that histone demethylation and accompanying histone acetylation take place before demethylation of the underlying DNA[79,99]. The initial changes probably occur through the targeting of histone demethylases[100] by factors that recognize specific gene sequences, and this must then be followed by local demethylation. It would be interesting to understand how these two demethylation events — histone and DNA demethylation — are coordinated at the molecular level.

Although the biochemical mechanism for histone demethylation has been deciphered[101,102] it is still not clear how methyl groups are removed from DNA, even though it is known that this can occur in an active manner[103]. Several studies now suggest that active DNA demethylation might be accomplished through a process of DNA repair[104,105] that involves nucleotide exchange[106,107], replacing 5-methylcytosine with unmodified cytosine, and it is possible that this is the physiological mechanism that operates during normal development *in vivo*[108].

One important aspect of long-term repression is the ability to maintain localized silencing through many cell divisions. Maintenance of silencing is particularly crucial in light of the fact that many properties of chromatin structure are disrupted by the process of DNA synthesis, and must be reconstructed following each round of replication. DNA methylation, which has an autonomous mechanism for being maintained, clearly plays a part in this process. The repression states in unmethylated regions of the genome, such as those targeted by the Polycomb complex, might maintain their unique heterochromatin structure through DNA sequences that recruit the machinery necessary for methylating H3K27, a mark that then serves as the landing site for heterochromatinization proteins. A key question in this field is whether histone modifications can serve as templates for autonomously reproducing these same structures on newly incorporated nucleosomes following replication. Although one recent study suggests that this may be the case[60], much additional work is necessary to clarify this fundamental question of epigenetic inheritance.

Studies on the interrelationships between DNA and histone modification help put into focus the general question of how epigenetic regulation is coordinated and what its role is during normal development. Early embryogenesis and gametogenesis are characterized by global alterations in the epigenetic structure of the genome. During gametogenesis, for example, overall demethylation of the DNA is initiated before the formation of new chromatin structure[61], and the wave of *de novo* methylation in the implantation embryo also seems to occur as an independent event. In contrast to these global effects, DNA regions that are targeted for epigenetic change during later stages of development seem to occur in the opposite order, with changes in histone modification preceding alterations in DNA methylation, regardless of whether this involves gene repression[21] or gene activation[99]. We argue that, unlike global changes that occur in early development, these events must be initially directed by factors that recognize specific sequences, with DNA methylation having a secondary role that, nonetheless, ultimately contributes to long-term stability.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Oct3/4
UniProtKB: http://www.uniprot.org
EZH2 | SETDB1 | SUV39H1 | SUV39H2 | UHRF1

FURTHER INFORMATION

The Cedar laboratory: http://www.md.huji.ac.il/depts/humangenetics/cedar/cedar.html

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