THE DIVERSE FUNCTIONS OF HISTONE LYSINE METHYLATION

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Abstract | Covalent modifications of histone tails have fundamental roles in chromatin structure and function. One such modification, lysine methylation, has important functions in many biological processes that include heterochromatin formation, X-chromosome inactivation and transcriptional regulation. Here, we summarize recent advances in our understanding of how lysine methylation functions in these diverse biological processes, and raise questions that need to be addressed in the future.

SET DOMAIN
A sequence motif (named after Su(var)3–9, Enhancer of Zeste, Trithorax) that is found in several chromatin-associated proteins, including members of both the Trithorax group (trxG) and Polycomb group (PcG).

X INACTIVATION
A process of dosage compensation in mammals that is achieved by the transcriptional silencing of one of the X chromosomes in XX females.

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The DNA within our cells exists in the form of chromatin. The basic building block of chromatin is the nucleosome, a structure consisting of an octamer of four core histone proteins around which 147 bp of DNA is wrapped[1]. The histone proteins (H2A, H2B, H3 and H4) are each composed of a globular domain and an unstructured tail domain. A striking feature of core histones is that they are subject to a large number of covalent modifications including acetylation, methylation, phosphorylation and ubiquitylation[2]. Histone methylation occurs on arginine and lysine residues and is catalysed by enzymes belonging to three distinct families of proteins — the PRMT1 family, the SET-DOMAIN-containing protein family, and the non-SET-domain proteins DOT1/DOT1L[3–6]. Here, we focus on histone lysine methylation because of its prominence and its array of important functions (for more information on arginine methylation, see several recent reviews[6–8]).

Histone lysine methylation occurs on histones H3 and H4. So far, six lysine residues located on these two histones have been reported to be sites of methylation (FIG. 1a). Each of these lysine side chains can be mono-, di- or trimethylated. Unlike acetylation, which generally correlates with transcriptional activation, histone lysine methylation can signal either activation or repression, depending on the sites of methylation[6]. For certain processes, such as X INACTIVATION, methylation on the same site can lead to different outcomes depending on the number of methyl groups added. In the past few years, studies on different organisms have resulted in the identification of several enzymes that catalyse site-specific histone lysine methylation (FIG. 1a). The characterization of these enzymes has revealed important functions of histone methylation in many different biological processes that range from heterochromatin formation to transcription regulation. In this review, we summarize recent advances in this rapidly progressing field, including the discovery of histone demethylases, and outline questions to be addressed in the near future.

Histone methylation and the 'histone code'

In its extended form, chromatin appears as an array of nucleosomes, but in the nucleus, the chromatin fibres that form chromosomes undergo several levels of folding, resulting in increasing degrees of condensation[9]. It is known that the histone tails have an important role in this folding process[10]. Considering this and the fact that histone methylation primarily occurs in histone tails, it would be reasonable to propose that methylation might function to regulate chromatin structure directly by affecting the higher-order folding of the chromatin fibre. This could have important implications for chromatin-templated processes such as transcription and DNA repair, assuming that folding alters the accessibility of DNA to the proteins that mediate these processes. Although there is no evidence that lysine methylation directly affects chromatin dynamics, acetylation of lysine residues in histones

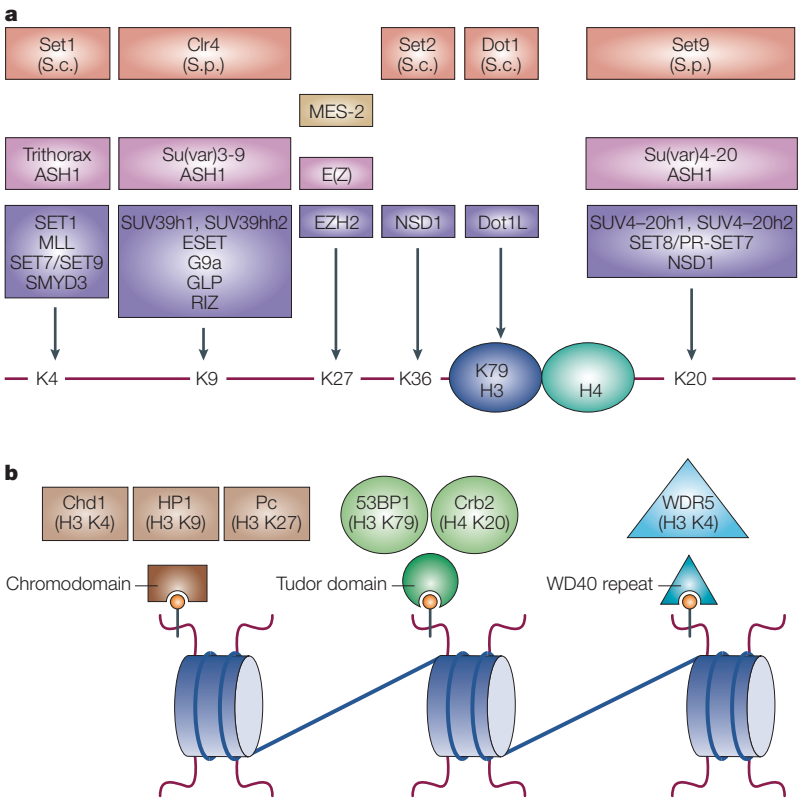


Figure 1 | **Histone lysine methyltransferases, their target sites and methyl-lysine binding domains. a** | The lysine methyltransferases are grouped according to the specific lysine residue targeted for modification and colour coded according to their origin (yeast, red; worm, yellow; fly, pink; mammalian, purple). Methyltransferases are indicated as S.c. and S.p. for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively. For the mammalian enzymes the name for only one species is given. The globular domains of the histones are shown as ovals and the tails are represented by straight lines. **b** | Methyl-lysine binding proteins contain one of three methyl-lysine binding domains: the chromodomain, the tudor domain or the WD40-repeat domain. These can not only interact with methyl-lysine, but also seem to discriminate between different methylated lysines. For example, the chromodomain of HP1 interacts specifically with methyl-H3-K9, whereas that of Polycomb (Pc) interacts specifically with methyl-H3-K27. This probably explains why different methylated lysines within histones H3 and H4 can have different biological outcomes.

BROMODOMAIN
A conserved acetyl-lysine binding domain found in several transcriptional regulatory proteins that are involved in gene activation.

CHROMODOMAIN
A conserved protein structure that is common to some chromosomal proteins. It interacts with chromatin by binding to methylated lysine residues in histone proteins.

TUDOR DOMAIN
A conserved protein domain that is found in several RNA-binding proteins and chromatin-associated proteins. Recent studies indicate this domain can bind to methyl-lysine or methyl-arginine.

is known to antagonize the folding of chromatin *in vitro*[9]. As acetylation neutralizes the positive charge of lysine, it has been suggested that this modification might operate through an electrostatic mechanism. Since methylation of lysine residues does not alter their charge, any direct effect of lysine methylation on chromatin folding would have to occur through a non-electrostatic mechanism (for example, through hydrophobic interactions).

An alternative hypothesis proposes that specific histone modifications, including lysine methylation, are binding sites for different proteins that mediate downstream effects[11–13]. Consistent with this hypothesis, it has been shown that BROMODOMAINS can recognize acetylated lysines[14,15]. Recent studies on histone methylation identified at least three protein motifs — the CHROMODOMAIN[16–20], the TUDOR DOMAIN[21,22] and the WD40-REPEAT DOMAIN[23] — that are capable of specific interactions with methylated lysine residues (FIG. 1b). As described below, proteins that contain these motifs are recruited by specific methylated lysines, and this recruitment step seems to play an important part in the unique biological outcomes that are associated with different methylation events. However, additional levels of complexity exist: for example, as mentioned above, lysine residues can adopt one of three different methylation states. Also, the binding affinity of a protein for a particular modification might be affected by another adjacent modification. If true, these added complexities may support the 'histone code' hypothesis, which posits that different combinations of histone modifications mediate unique cellular responses[11–13].

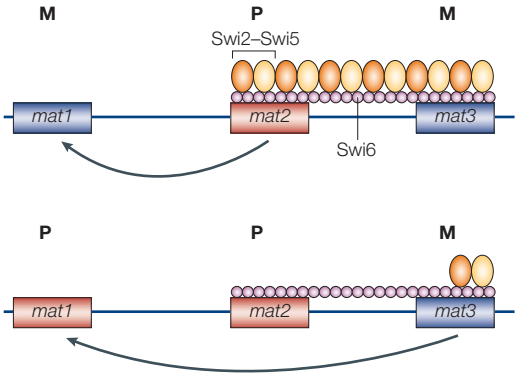
From the time that the hypothesis was first proposed, the existence of a histone code has been questioned[24]. Much of the debate seems to be rooted in semantics, as the existence of a code depends on the definition of the word 'code'. For example, Stephen Henikoff recently reviewed this topic and compared the histone code to a simple binary code, similar to a computer code consisting of permutations of 0 or 1 (REF. 25). On the basis of this strict definition, the three lysine residues (K4, K36 and K79) on each budding-yeast H3 molecule could generate eight possible methylation permutations (for simplicity, the methylation state is not considered). Hypothetically, each of these permutations might be recognized by a different effector protein, which would result in unique biological outcomes. Contrary to this expectation, recent data indicate that some histone modifications are closely correlated with each other[26,27]. This implies that the number of permutations that occur *in vivo* is limited, thereby diminishing the potential richness of a histone code. However, evidence for a certain level of 'cross-talk' between different covalent histone modifications does exist[28–30], although the generality of these observations are unclear.

In summary, on the basis of current evidence, it is difficult to reach a definitive conclusion concerning the existence of a strict histone code. Now that a more comprehensive list of histone modifications is available, correlative analyses of these modifications on a genome-wide scale will allow an estimate of the number of permutations that are used in nature. This, in turn, will allow conclusions to be drawn concerning the potential existence of a histone code. For the purpose of this review, however, it is sufficient to think of individual methylated lysines residues as simple docking sites that recruit different effector proteins.

Heterochromatin formation

Heterochromatin has been historically defined as chromosomal regions, such as CENTROMERES and TELOMERES, that remain condensed throughout the cell cycle[31]. The proper formation of heterochromatin is biologically important: for example, centromeric heterochromatin formation is required for the proper segregation of chromosomes during mitosis[32]. In addition, heterochromatin formation also plays a crucial role in recombination events that are associated with MATING-TYPE SWITCHING in fission yeast[33] (BOX 1).

Box 1 | **Function of H3-K9 methylation and Swi6 in mating-type switching**



Fission yeast can switch between two mating types, P and M, by replacing the genetic information that specifies one mating type with that from another. This occurs through a recombination mechanism in which one of two silent donor loci, containing genetic information that specifies the two different mating types, replaces the information at the mating-type locus. In fission yeast, the mating-type locus is called *mat1*, whereas the two silent donor loci are referred to as *mat2* (P) and *mat3* (M). Importantly, the choice of donor locus is non-random in that recombination almost always results in a switch in mating type. H3-K9 methylation and Swi6, the *S. pombe* homologue of mammalian HP1, seem to function in mating type-switching by facilitating the spreading of a complex composed of Swi2 and Swi5 over the *mat2* and *mat3* region. In cells of the P-mating type, the Swi2–Swi5 complex associates with the boundary of the *mat3* locus, but does not move inward towards *mat2* (see figure). By contrast, this complex spreads over the entire *mat2* and *mat3* region in cells of the M-mating type. In the absence of Swi6 function, the Swi2–Swi5 complex associates with the *mat3* boundary region, but does not move inward in M cells. In such cells, the *mat3* locus is incorrectly used to replace the genetic information at the mating-type locus, therefore preventing a switch in mating type. This indicates that cell-type-specific spreading of the Swi2–Swi5 complex is important for mating-type switching and that Swi6, and therefore heterochromatin, is required for spreading. The mechanism by which Swi6 facilitates spreading is unknown. One possibility is that spreading is the result of direct physical interactions between these proteins that occur in a mating-type-dependent fashion.

WD40-REPEAT DOMAIN
A protein motif that is composed of a 40-amino-acid repeat that forms a β-propeller sheet. Proteins that contain WD40 repeats function in a wide range of cellular functions, including G-protein-mediated signal transduction, transcriptional regulation, RNA processing, and regulation of vesicle formation and trafficking.

CENTROMERE
Region of a chromosome that is attached to the spindle during nuclear division.

TELOMERE
A segment at the end of each chromosome arm that consists of a series of repeated DNA sequences.

MATING-TYPE SWITCHING
A recombination event occurring in yeast that results in a 'switch' from one mating type, or sex, to another.

POSITION EFFECT VARIEGATION
A type of gene silencing that results from the translocation of normally active genes to locations that are proximal to heterochromatin. Silencing is initiated in a stochastic manner, but, once established, is stable and results in a variegated expression pattern.

SMALL INTERFERING RNA (siRNA). A non-coding RNA (~22 nucleotides long) that is derived from the processing of long double-stranded RNA during RNA interference. siRNAs direct the destruction or translation repression of mRNA targets that they hybridize with.

H3 lysine 9. In recent years, heterochromatin has been defined in molecular terms. This advance originated from the observation that the integration of transgenes into heterochromatic regions leads to silencing phenomena such as POSITION EFFECT VARIEGATION (PEV)[34]. Studies of PEV in *Drosophila melanogaster* have resulted in the identification of a number of PEV suppressors including Su(var)3–9 (REF. 35), and its human homologue SUV39H1 was later shown to be a histone lysine methyltransferase with a specificity for histone H3 lysine 9 (H3-K9)[36]. A role for SUV39H1 and its associated H3-K9 methyltransferase activity in heterochromatin function was indicated by the demonstration that it associates with the heterochromatin protein HP1 (REF. 37). Subsequent studies showed that methylation on H3-K9 provides a binding site for the chromodomain of the HP1 proteins[16,18].

The molecular events that are described above have been conserved during evolution. For example, in fission yeast, H3-K9 methylation is catalysed by the SUV39H1 homologue Clr4 (REF. 38). Clr4-mediated H3-K9 methylation serves to recruit Swi6, the fission yeast homologue of mammalian HP1 (REFS 16,18,38). Although both Clr4 and Swi6 are required for heterochromatin formation[39], the role of these two proteins in the functions associated with heterochromatin is not known. One possibility is that Swi6 mediates the recruitment of additional proteins that are required for the function of specific heterochromatic regions. A similar mechanism has recently been proposed for the fission yeast mating-type loci[33] (BOX 1).

Although the role of H3-K9 methylation in heterochromatin formation after Swi6 recruitment is uncertain, there has recently been much progress in understanding the mechanism by which Clr4 is targeted to heterochromatic regions. The first breakthrough in this area came with the discovery that the RNA interference (RNAi) pathway has a role in heterochromatin formation in fission yeast[40,41]. Central to the targeting mechanism are two protein complexes, known as RNA-induced transcriptional silencing (RITS) and RNA-directed RNA polymerase (RDRC)[42,43] (FIG. 2). RITS is composed of three protein subunits and is associated with SMALL INTERFERING RNA (siRNA) molecules that are homologous to repeat sequences that are found at sites of heterochromatin formation. According to the current models, the siRNA molecules target RITS to RNA transcripts that are associated with heterochromatic loci. This, in turn, leads to the recruitment of Clr4 and the methylation of H3-K9. With regard to the role of RDRC, evidence indicates that this complex is involved in the production of the siRNA molecules that associate with RITS. It seems that RDRC uses an intrinsic RNA polymerase activity to convert the heterochromatin-associated single-stranded transcripts to double-stranded RNA. These molecules then feed into the RNAi pathway to produce more siRNAs that, presumably, associate with RITS and reinforce the recruitment of this complex to centromeric repeats. Interestingly, RDRC targeting to sites of heterochromatin formation requires RITS, implying the existence of a self-reinforcing loop[44,45].

In general, this is an appealing model. However, similar to all tentative models, there are several aspects

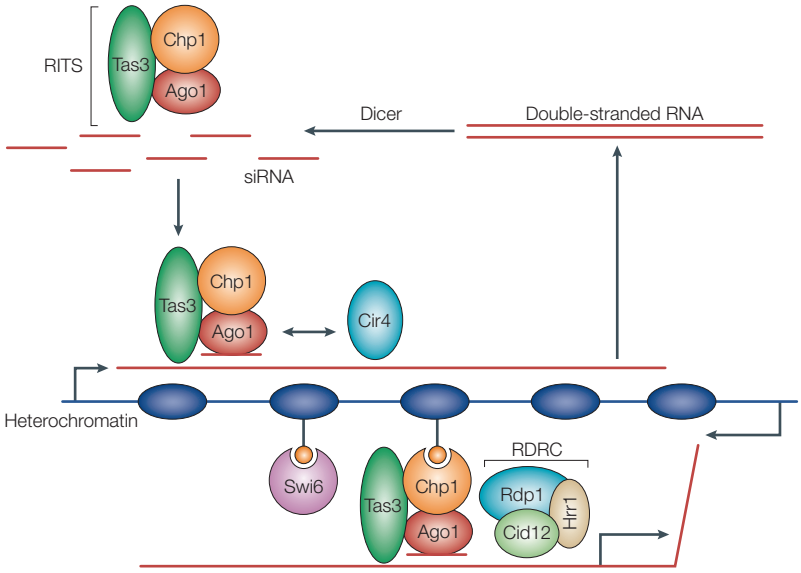


Figure 2 | **Heterochromatin formation and RNA interference.** The initiation of heterochromatin formation at specific chromosomal regions such as centromeres is thought to result from the bidirectional transcription of repeat sequences by RNA polymerase II[133]. This double-stranded RNA is converted into small interfering RNAs (siRNAs) by the enzyme Dicer and subsequently loaded onto the RITS complex, which is comprised of Chp1, Ago1 and Tas3. The siRNA most probably functions to target RITS to sites of heterochromatin, potentially as a result of base-pairing interactions with transcripts that are associated with repeat sequences. The targeting of RITS has at least two purposes. First, RITS is required for the recruitment of the histone methyltransferase Clr4 and subsequent H3-K9 methylation (orange circle on a stick), leading to the recruitment of Swi6, which is the fission yeast homologue of mammalian HP1. The chromodomain-containing protein Chp1, a component of RITS, also interacts with methyl-H3-K9 and this interaction might stabilize RITS binding. The second role of RITS is in the recruitment of another complex, RDRC, which is composed of Rdp1, Cid12 and Hrr1. This complex functions in the production of additional double-stranded RNA that feeds into the RNAi pathway. So, RITS reinforces its own recruitment to heterochromatic regions.

that require further clarification. For example, one of the components of RITS, Chp1, contains a chromodomain that interacts specifically with methyl-H3-K9 (REF. 46). In addition, Clr4, and presumably H3-K9 methylation, are required for the association of Chp1 and RITS with heterochromatin[47]. These observations seem to conflict with both the order of events laid out above and the concept that RITS functions to recruit Clr4. One possible explanation is that RITS is initially targeted to heterochromatin by a Clr4-independent mechanism, and that the subsequent Clr4 recruitment leads to the stabilization of RITS binding by virtue of the interaction between Chp1 and methyl-H3-K9 (REF. 44).

The pathway that leads to heterochromatin formation seems to be evolutionarily conserved. In mice, two Clr4 homologues, SUV39h1 and SUV39h2, are responsible for H3-K9 methylation at heterochromatic regions such as pericentric heterochromatin[18,48,49]. This modification recruits the Swi6 homologues HP1α and HP1β, in an analogous way to fission yeast[16,18]. However, the contribution of the RNAi pathway in mammalian heterochromatin formation has not yet been established.

EUCHROMATIN
A form of chromatin that is decondensed during interphase and that contains actively transcribing genes.

H4 lysine 20. In addition to H3-K9 methylation, H4-K20 methylation is also a marker of mammalian heterochromatin[50]. It has been shown that SUV4–20h1 and SUV4–20h2, two SET-domain-containing proteins that localize to pericentric regions, are responsible for this modification. Furthermore, mutations in the *D. melanogaster* homologue Su(var)4–20 impair heterochromatin formation as evidenced by the suppression of PEV. Interestingly, Suv4–20h1/Suv4–20h2 and specific HP1 isoforms are capable of directly interacting with one another, and H4-K20 methylation is abolished at pericentric regions in the absence of Suv3–9h1/Suv3–9h2 function[50]. These observations are important because they indicate that one function of H3-K9 methylation and HP1 binding might be to recruit Suv4–20h1/Suv4–20h2 to pericentric heterochromatin. However, it is important to note that this pathway is not conserved in fission yeast and might therefore be restricted to metazoans (BOX 2). In the future, it will be important to understand the mechanism by which H4-K20 exerts its effects. It is possible that this modification, similar to the methylation of H3-K9, is a binding site for an unknown protein involved in heterochromatin formation.

A more accurate description of the histone modifications that are associated with mammalian heterochromatin would include information about the methylation status of H3-K9 and H4-K20 in these regions; that is, whether these residues are mono-, di- or trimethylated. In mice, for example, it has been shown that pericentric heterochromatin is specifically enriched in trimethyl-H3-K9 and H4-K20 (REFS 48–50). By contrast, mono- and dimethyl-H3-K9 and H4-K20 are found in non-heterochromatic regions, referred to as EUCHROMATIN (see below and BOX 2). The significance and generality of this observation is not certain. Arguably, the most intuitive answer is that the methylation status dictates the recruitment of specific methyl-binding proteins that are dedicated to unique functions. With regard to H3-K9 methylation, this is consistent with the different localization patterns that are exhibited by HP1 isoforms. So, whereas HP1α and HP1β are localized to pericentric heterochromatin, the HP1γ isoform is localized to euchromatin[51]. Whether methylation status or other factors dictates the localization of these proteins remains to be determined.

Transcriptional silencing

H3-K 9 methylation and euchromatic gene silencing. As noted above, H3-K9 mono- and dimethylation seems to be restricted to euchromatic regions in mammals[48,49]. Mice that are deficient in G9a or G9a-related protein (GLP), two highly related histone methyltransferases, exhibit severely reduced H3-K9 methylation in euchromatic regions, which indicates that G9a and GLP are the primary H3-K9 methyltransferases in these chromatin domains[52]. G9a/GLP-mediated H3-K9 methylation seems to function in the silencing of individual genes. In G9a-deficient cells, for example, several genes belonging to the MageA group are upregulated[53]. In addition to G9a and GLP, SUV39h1

Box 2 | **H4-K20 methylation: roles in cell cycle and DNA repair**

The histone methyltransferase SET8/PR-Set7 catalyses the monomethylation of H4-K20 (REFS 122–124). As part of the initial characterization of this enzyme, it was noted that both the levels of SET8/PR-Set7 and H4-K20 methylation fluctuate during the cell cycle. This observation indicates that SET8/PR-Set7 might be involved in some aspect of cell-cycle control. Indeed, loss of function of the *Drosophila melanogaster* homologue leads to cell-cycle arrest[125]. In addition, a recent study of the cell-cycle regulator HCF-1 (herpes simplex virus host-cell factor-1) showed that the C-terminal subunit of this protein represses SET8/PR-Set7 expression during mitosis[126]. Furthermore, small interfering RNA (siRNA)-mediated knockdown of the C-terminal subunit of HCF-1 leads to increased expression of SET8/PR-Set7 concomitant with chromosome segregation defects. Collectively, these findings indicate that progression through the cell cycle requires tight regulation of SET8/PR-Set7 and H4-K20 methylation.

In addition to the maintenance of heterochromatin and cell-cycle control, H4-K20 methylation has been linked to DNA repair. This finding is the result of studies in fission yeast aimed at understanding the function of the H4-K20 methylation. Surprisingly, deletion of SET9 — the enzyme responsible for H4-K20 methylation in this organism — does not result in defective heterochromatin formation as assayed by centromeric silencing[22]. Instead, a sensitivity to DNA double-strand breaks induced by ionizing radiation is observed. This phenotype seems to be caused by a failure to recruit Crb2, which is required for appropriate responses, to double-strand breaks. Interestingly, the mammalian homologue of Crb2, 53BP1, contains two tudor domains that mediate specific interactions with methyl-H3-K79 (REF. 21). This observation indicates that Crb2 might be recruited to methyl-H4-K20 through a similar interaction.

and SUV39h2 have also been linked to the silencing of specific genes located in euchromatin. For example, the retinoblastoma (Rb) protein has been shown to silence several S-phase genes by recruiting SUV39h1 and SUV39h2 to their promoters[54]. Importantly, these histone methyltransferases seem to repress expression only during terminal differentiation and do not have a role in the transient repression of these genes in cycling cells[55]. Consistent with the concept that SUV39h-mediated H3-K9 methylation serves as a binding site for HP1, HP1 is enriched at the promoter of the S-phase gene, *cyclin E*[54]. It is not clear how HP1 recruitment leads to gene silencing, but one possible mechanism could involve the targeting of DNA methyltransferases to these genes (see BOX 3 for a detailed discussion).

A recent study has challenged the generality of the association between H3-K9 methylation and gene silencing[56]. This provided evidence that, in several cell lines, the coding regions of a number of active genes are enriched in trimethyl-H3-K9 and the HP1γ isoform. These data are difficult to reconcile with the observations described above, but it might be possible to develop a model that accommodates all the data. For example, it has been proposed that H3-K9 methylation might have different functions depending on whether it occurs in coding regions or in promoters. So, K9 methylation targeted to the *cyclin E*- and *cyclin A2*-gene promoters might result in silencing, whereas K9 methylation targeted to the coding regions of certain genes may facilitate transcription. Another possibility is that the outcome of K9 methylation could be gene dependent. As mentioned above, the integration of transgenes into heterochromatic locations often results in silencing. However, certain genes that normally reside within heterochromatin can be active, despite presumably being enriched in H3-K9 methylation[57].

H3 lysine 27 methylation in Hox gene silencing. In addition to H3-K9 methylation, the methylation of histone H3-K27 has been linked to several silencing phenomena including homeotic-gene silencing, X inactivation and GENOMIC IMPRINTING[58]. At the core of this silencing system is the POLYCOMB GROUP (PcG) of proteins[59]. PcG-mediated gene silencing and histone methylation first converged when it was discovered that a complex of PcG proteins catalyses the methylation of H3 on lysine 27 (REFS 58,60–63). The reported composition of the complex varies, although the SET-domain-containing protein enhancer of zeste (E(Z); or its human homologue EZH2), extra sex combs (ESC; or its human homologue embryonic ectoderm development (EED)) and suppressor of zeste-12 (SUZ12) proteins are present in all complexes that have been isolated so far. Hereafter, the complex is referred to as the E(Z) or EZH2 complex.

The role of the E(Z) complex and H3-K27 methylation in PcG-mediated gene silencing was first studied in the context of homeotic-gene regulation, although it is now clear that PcG proteins also regulate many other genes[59]. The homeotic genes encode transcription factors that specify the identity of embryonic tissues[59]. Silencing of these genes by PcG proteins restricts the expression of HOX GENES to specific cell types in the developing embryo. Studies that have examined the recruitment of PcG proteins to the *Ubx* gene, a *D. melanogaster* homeotic gene, lend support to a stepwise silencing model[64] (FIG. 3a). In the first step, the DNA-binding proteins Pleiohomeotic (Pho) and Pleiohomeotic-like (PhoL) are recruited to a specific DNA sequence, which is known as a Polycomb response element (PRE), and is located upstream of the *Ubx* gene. Pho and PhoL, in turn, recruit the E(Z) complex and its associated methyltransferase activity through protein–protein interactions, which leads to the methylation of H3-K27. Analogous to the proposed function of methyl-H3-K9, methyl-H3-K27 seems to be a binding site for a chromodomain-containing protein, Polycomb (Pc), which is a component of the Polycomb repressive complex-1 (PRC1)[17,19,65]. The significance of this interaction was unknown until recently, when it was discovered that a PRC1-like complex possesses a UBIQUITIN E3 LIGASE activity that is specific for histone H2A[66]. These observations indicate that H3-K27 methylation and Pc binding might function to recruit a protein with E3 ligase activity to Hox genes, which would result in the ubiquitylation of H2A. This hypothesis is supported by studies showing that Pc binding and H2A ubiquitylation at PRE elements is abolished in the absence of E(Z) function[66]. The main question that these observations do not answer is how H2A ubiquitylation is related to silencing. One possibility is that this modification, like other histone modifications, allows the recruitment of an effector protein that mediates silencing. Alternatively, H2A ubiquitylation might directly interfere with a specific

GENOMIC IMPRINTING
A genetic mechanism by which genes are selectively expressed from the maternal or paternal chromosomes.

POLYCOMB GROUP
(PcG). A class of proteins — originally described in *Drosophila melanogaster* — that maintain stable and heritable repression of a number of genes, including the homeotic genes with which they are associated.

HOX GENES
(Homeobox genes). A group of linked regulatory genes that are involved in patterning the animal body axis during development.

UBIQUITIN E3 LIGASE
An enzyme that catalyses the covalent attachment of ubiquitin to lysine residues in proteins.

Box 3 | **H3-K9 methylation and DNA methylation**

In addition to the link between H3-K9 methylation and heterochromatin formation, H3-K9 methylation has been connected to DNA methylation, another type of epigenetic modification that is involved in gene silencing. The methylation of cytosine bases in DNA occurs to varying degrees in a wide range of organisms, ranging from plants to mammals[127]. Gene silencing that is mediated by DNA methylation is known to have important roles in many biological processes, including genomic imprinting and X inactivation. Interestingly, genetic studies have shown in a number of organisms that H3-K9 methylation is a prerequisite for DNA methylation[128–130]. The obvious question that these experiments raise concerns the nature of the link between the two modifications. Consistent with the possibility that HP1 might recruit DNA methyltransferases through protein–protein interactions[130], HP1 is essential for DNA methylation in *Neurospora crassa*[131]. Alternatively, in some systems the DNA methyltransferases themselves seem to be directly recruited to loci of H3-K9 methylation and its associated modifications[28].

Although these results indicate that histone methylation controls DNA methylation, this relationship seems to be reversed in some instances. For example, it was observed that H3-K9 methylation is drastically reduced at a tumour suppressor gene in cells that were deficient in DNA methyltransferases[132]. So, in this case, H3-K9 methylation seems to require DNA methylation. The connection between histone and DNA methylation gained further support by research on the function of MBD1, which is a protein that specifically binds to methylated DNA sequences[133]. MBD1 was found to be associated with the H3-K9 methyltransferase SETDB1, which is the human homologue of mouse ESET (FIG. 1). In addition to SETDB1, MDB1 interacts with CAF1, which is responsible for depositing histones onto newly replicated DNA. On the basis of several experiments, it was proposed that methylated DNA is a signal that directs the methylation of H3-K9 during DNA replication, resulting in the silencing of gene expression.

step of transcription, such as the transition from initiation to elongation.

X inactivation and genomic imprinting. In addition to homeotic-gene silencing, PcG proteins have been implicated in another form of silencing — female X chromosome inactivation[67]. Central to the mechanism of X inactivation is *Xist*, a non-coding RNA that is expressed from the X chromosome that is destined to become silenced (FIG. 3b). This RNA transcript coats the entire inactive X chromosome (Xi) and leads to chromosome-wide gene silencing. The process of X inactivation can be divided into two separate classes, imprinted X inactivation and random X inactivation.

The term 'imprinted' relates to silencing phenomena in which one allele — or chromosome in the case of X inactivation — from either parent is preferentially silenced. Early in mouse development, imprinted X inactivation, in which the paternal chromosome is inactivated, occurs in all cells of the embryo[68,69]. The paternal X chromosome remains silenced in those cells that contribute to extra-embryonic tissues, whereas X inactivation is reversed in the cells that form the embryo proper. Subsequently, X inactivation is re-established in these cells, but in this case inactivation is randomly initiated on either the maternal or paternal X chromosome.

With regard to the role of PcG proteins in this process, it has been observed that components of the mouse EZH2 and the PRC1 complexes are transiently recruited to the inactive X chromosome during the initiation stage of both imprinted and random X inactivation[70–73]. Furthermore, recruitment of these proteins coincides with the appearance of methyl-H3-K27 and ubiquitylated H2A on Xi. These findings, together with the fact that X inactivation is impaired in the absence of EED[74] (a component of the mouse EZH2 complex), indicate that similar mechanisms might mediate both homeotic-gene silencing and X inactivation. In addition to these forms of silencing, the EED protein is also required for silencing of a subset of imprinted genes[75]. Interestingly, the *Kcnq1ot1* non-coding transcript has been proposed to function in the recruitment of the EZH2 complex and H3-K27 methylation to imprinted genes[76,77]. These observations, in combination with the recent finding that transcription through PREs is required for reversal of PcG-mediated Hox gene silencing[78], raise the possibility that non-coding RNA could have a general role in PcG-mediated gene silencing.

Regulation of H3 lysine 27 methylation. With the development of tools that allow the methylation state of different lysines to be reliably determined, there has been an effort to analyse the distribution of H3-K27 mono-, di- and trimethylated residues both at large chromosomal domains and at individual genes. Based on the analyses of entire chromosomes as well as individual genes, it seems that trimethyl-H3-K27 is enriched at silenced homeotic genes, the inactive X-chromosome and imprinted genes[64,72,73,76,77]. By contrast, it has been observed that monomethyl-H3-K27 is localized to pericentric heterochromatin in mammalian cell lines[48,49]. Furthermore, whereas all forms of H3-K27 methylation require EED[79], SUZ12 seems to be dispensable for the monomethylation of H3-K27 *in vivo*, although it is indispensable for the enzymatic activity *in vitro*[80,81]. This observation indicates the existence of an alternative SUZ12-independent EZH2 complex that might mediate H3-K27 monomethylation and pericentric heterochromatin formation. To complicate matters further, it was also reported that specific isoforms of EED have the capability of switching the substrate specificity of the human EZH2 complex such that the enzyme methylates lysine 26 within the linker histone H1 (REF. 82). However, the potential function of this modification is presently unclear. Taken together, these data indicate that different EZH2 complexes with distinct functions might exist *in vivo*.

Transcriptional activation

In addition to transcriptional repression, histone lysine methylation has been shown to function in transcriptional activation. The main sites of lysine methylation that have been associated with gene activity include K4, K36 and K79 of histone H3. Interestingly, the methylation of all three sites seems to be directly coupled to the transcription process. In the case of H3-K4 and H3-K36 methylation, the enzymes responsible for both modifications have been shown to physically associate with RNA polymerase II (RNAPII) during elongation (see below), resulting in histone methylation in the coding regions[83–86] (FIG. 4). In addition

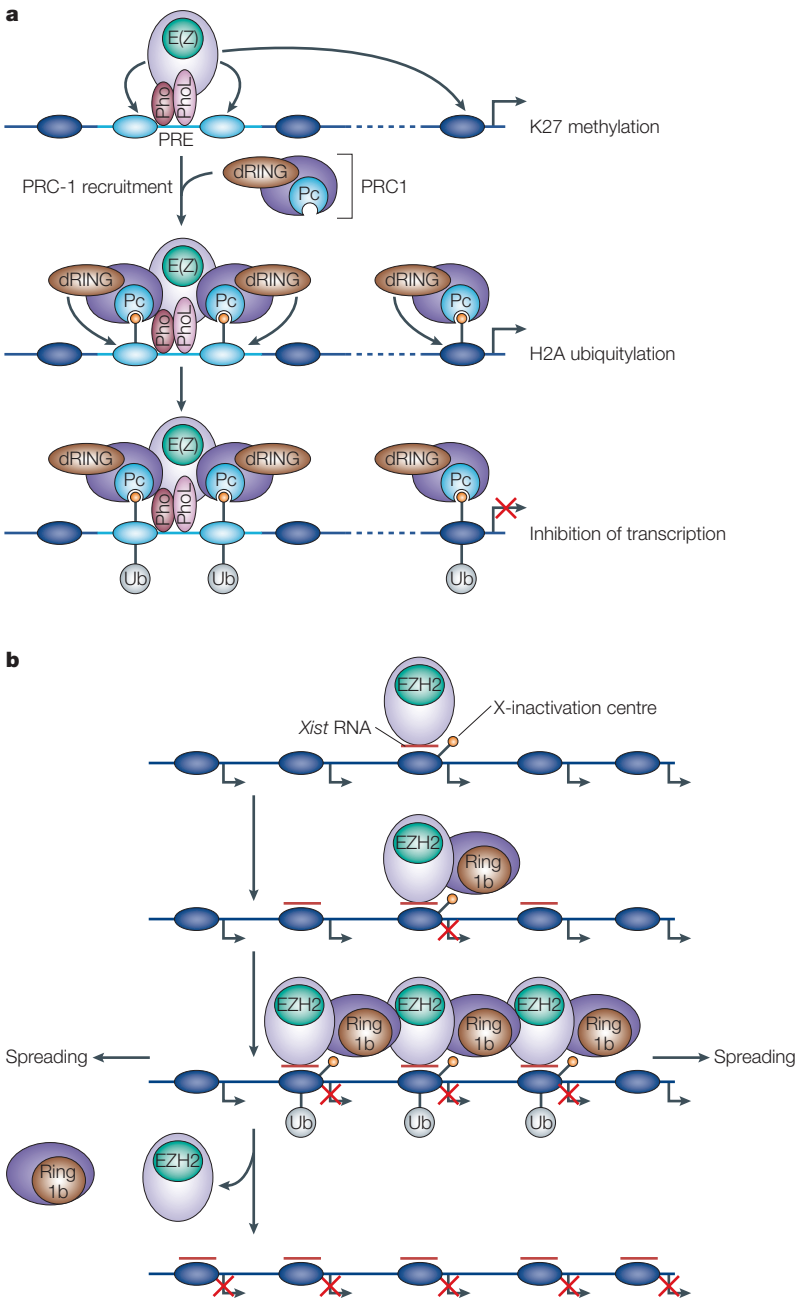


Figure 3 | **H3-K27 methylation in Polycomb silencing and X inactivation. a** | Two enzyme complexes with different histone-modifying activities are recruited to homeotic genes in a stepwise fashion. DNA-binding proteins, such as Pho and PhoL, interact with the Polycomb response element (PRE). These proteins recruit the E(Z) complex, which methylates H3-K27 (orange circle on a stick), forming a binding site for the Polycomb (Pc) protein. Pc exists as part of a complex known as PRC1 and recruits PRC1 to the PRE through interactions with methylated K27. The E3 ligase activity contributed by dRING, another component of PRC1, leads to H2A ubiquitylation. Through unknown mechanisms, PRC1, possibly as a result of H2A ubiquitylation, causes the silencing of homeotic genes. **b** | The events surrounding the inactivation of the X chromosome are shown. *Xist* RNA (red) is transcribed at the X inactivation centre and coats the X chromosome, starting at the X-inactivation centre and spreading outward. *Xist* RNA, possibly through direct interactions, recruits the EZH2 complex and its associated methyltransferase activity. Coincident with the appearance of methyl-K27 is the recruitment of Ring1a/b (Ring1b is shown) and subsequent H2A ubiquitylation. Both types of histone modifications are transient — they are present only during the initiation phase. Although all these events correlate with X inactivation, EED is the only PcG protein shown so far to be required for this process[74]. How H3-K27 methylation and H2A ubiquitylation contribute to the silencing process is unknown.

to lysine methyltransferases, enzyme complexes that mediate the ubiquitylation of H2B are also tethered to RNAPII[87]. H2B ubiquitylation is required for the methylation of H3-K4 and H3-K79, implying that the latter is also coupled to gene transcription[30,88–90].

Studies in budding yeast indicate that gene-specific activators recruit a ubiquitylation complex that is composed of Rad6 and Bre1 to gene promoters[91–93]. This complex is then loaded onto RNAPII and subsequently carried into the coding region of transcribed genes. It is unknown how H2B ubiquitylation facilitates histone methylation. However, 'wedge' models have been proposed in which the relatively large ubiquitin molecule, which is ~50% of the molecular weight of the average histone, acts to open the nucleosome structure and make it accessible to methyltransferases[94]. Alternatively, it is possible that the ubiquitin modification serves to recruit other protein(s) that are required for methylation. Candidates for this role include Rpt4 and Rpt6, two proteosomal ATPases that are required for H3-K4 and H3-K79 methylation[95]. It has been proposed that these proteins might function as chaperones by utilizing energy derived from ATP hydrolysis to unfold histone H3, thereby increasing its accessibility.

The association of SET1 and SET2 with RNAPII indicates that H3-K4 and H3-K36 methylation might be the result of gene activity, at least when a gene initially becomes active. These observations and the fact that methylated lysines within histones are relatively stable indicate that K4, K36 and K79 methylation might mark genes that are active. This could have several important implications for gene regulation. For example, it is possible that these modifications might function to maintain transcription in situations where the factors that initiated transcription are down-regulated or no longer function. In this scenario, the different methylated lysines could be thought of as a kind of molecular 'door stop' to keep genes turned on. Investigations into the functions of H3-K4 methylation in particular indicate that this could occur through the recruitment of factors that are involved in the regulation of gene transcription[20,23].

H3 lysine 4. Analyses of the different H3-K4 methylation states and their distribution in various organisms indicate that both the dimethyl- and trimethyl-H3-K4 modifications are enriched at actively transcribed genes[27,83,96,97]. These different methylation states, however, do not overlap completely. While the dimethyl modification seems to be generally distributed across the body of active genes, the trimethyl modification is localized specifically to the 5′ end of these genes. This general methylation pattern was confirmed by a high-resolution genome-wide study[98].

In *S. cerevisiae*, a lysine methyltransferase named Set1 is responsible for all three H3-K4 methylation states, implying that these states and their distinct spatial patterns must result from the regulation of the enzymatic activity and/or targeting of Set1 (REFS 99,100). Indeed, the pattern of H3-K4 trimethylation seems to be linked to the association of Set1 with a specific

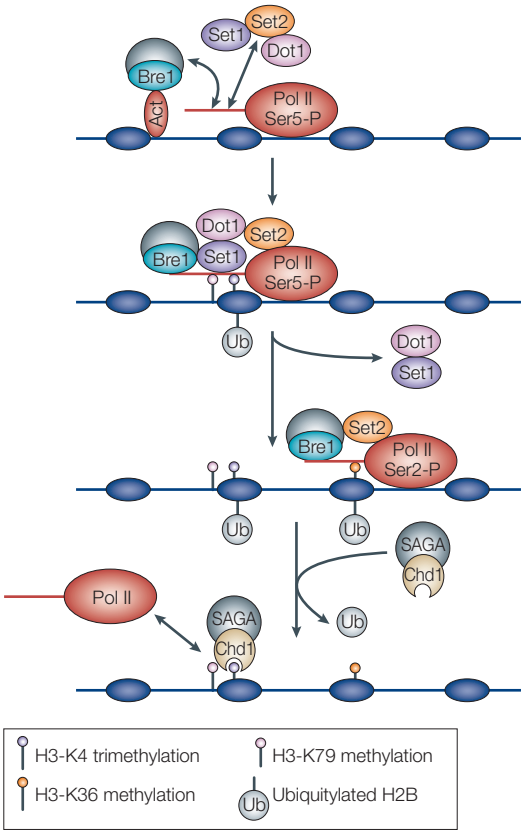


Figure 4 | **Histone lysine methylation and transcription.** Gene-specific activators bind to promoters and activate transcription by recruitment of the basal transcriptional machinery or co-activators required for transcription. In the model, one function of the activator (Act) is to recruit the Rad6–Bre1 complex, which is loaded onto RNA polymerase II (Pol II) at the promoter along with the H3-K4 and H3-K36 methyltransferases Set1, Set2 and, possibly, Dot1. Rad6–Bre1 then catalyses the ubiquitylation of H2B, an event that is required for the methylation of K4 and K79 on H3. At some point during elongation, the C-terminal domain of RNAPII becomes phosphorylated on Ser2. Concomitant with this event, Set1 dissociates from RNAPII. After the first round of transcription, the gene is marked by K4, K36 and K79 methylation. H3-K4 methylation serves as a binding site for the recruitment of the SAGA complex through a specific interaction between methyl-K4 and the chromodomain of Chd1. Histone acetylation by the SAGA complex leads to transcription activation.

phosphorylated form of elongating RNAPII (FIG. 4) that is confined to the 5′ regions of genes[83]. One possibility is that interactions between SET1 and its associated proteins cause a conformational change in the catalytic site of SET1, converting the enzyme into a trimethylase. A precedent of such a protein is mouse ATFa-associated modulator (mAM), which, when associated with ESET, converts ESET from a H3-K9 dimethylase into a trimethylase[101]. A potential region within Set1 that might be involved in such hypothetical interactions is a recently identified auto-inhibitory domain that seems to specifically inhibit H3-K4 trimethylation[102]. Another possibility is that Set1 is subject to a post-translational modification that converts the enzyme into a trimethylase when associated with RNAPII. As it has been demonstrated that the Bur1 kinase complex is required for H3-K4 trimethylation in *S. cerevisiae*[103], it is tempting to take this hypothesis one step further and speculate that a phosphorylation event might be involved.

The realization that methyl-H3-K9 and methyl-H3-K27 serve as binding sites for different proteins that mediate the biological effects of these modifications led to speculation that this model would also hold true for methyl-H3-K4. Supporting this model, Isw1, a subunit of the Isw1 ATP-dependent chromatin remodelling complex, is recruited to a specific set of genes in a manner that is dependent on H3-K4 methylation[104]. However, Isw1 does not possess a methyl-binding domain, which indicates that the interaction with methylated H3-K4 is probably indirect.

More recently, it has been shown that two different proteins, Chd1 and WDR5, interact specifically and directly with methylated H3-K4 (REFS 20,23). Chd1 is a component of SAGA (Spt–Ada–Gcn5–acetyltransferase), a histone acetyltransferase (HAT) complex that is involved in transcription of a large group of genes in *S. cerevisiae*[105]. Only one of the two chromodomains in Chd1 mediates interactions with methylated K4, and this interaction seems to have a role in the recruitment of SAGA and its associated histone acetyltransferase activity to gene promoters. It is worth noting that SAGA possesses an H2B deubiquitylation activity that is required for the activation of SAGA-regulated genes[91]. Although there is no evidence to support a role for this activity in SAGA recruitment, it is possible that the ubiquitin moiety must be removed subsequent to H3-K4 methylation in order for SAGA to bind methylated H3-K4.

The second identified methylated-K4-binding protein, WDR5, is a component of a mixed-lineage leukemia (MLL) complex that possesses H3-K4 methyltranferase activity[23,106]. Unlike Chd1, WDR5 interacts with methyl-K4 through a novel methyl-binding motif, known as a WD40-repeat domain. Interestingly, WDR5 binds preferentially to dimethyl-K4 and is required for maintaining global trimethyl-H3-K4 levels. Although the exact underlying mechanism is unclear, it has been proposed that the recruitment of the MLL methyltransferase complex to dimethyl-H3-K4 as a result of WDR5 binding might convert dimethyl-H3-K4 to the trimethyl state[23].

The identification and initial characterization of Chd1 and WDR5 supports the concept that methyl-K4 serves as a binding site for proteins involved in gene transcription, but leaves many questions unanswered. For example, how is the dimethyl state converted to the trimethyl state? Do dimethyl-and trimethyl-K4 dictate the recruitment of distinct proteins, which, in turn, mediate different consequences? An alternative possibility is that the significance of different methylation states lies in their inherent stability. It is possible that trimethyl-H3-K4 is turned over at a slower rate than dimethyl-H3-K4. In this case, the trimethyl modification that was established by initial rounds of transcription might linger during a period of silence in which no new H3-K4 methylation

occurs. This is significant because it would mean that trimethylation could serve as a 'memory' of previous transcriptional activity[83]. In other words, histone methylation could be the basis for a system in which previous gene activity modulates future activity. In one possible scenario, transcriptional memory could manifest itself through the increased recruitment of co-activators such as SAGA to genes that have been transcribed in the past. This might then increase the probability that such genes will be transcribed by lowering the threshold required for activation.

H3 lysine 36 and lysine 79. Compared with H3-K4 methylation, little is known about the functions of K36 and K79 methylation. In *S. cerevisiae*, Set2 is responsible for K36 methylation and, similar to Set1, this enzyme is physically associated with elongating forms of RNAPII[84–86]. Set2, however, differs from Set1 in that it remains associated with RNAPII throughout the body of transcribed genes. The significance of this is unknown, but indicates that Set2 and K36 methylation might be dedicated to the regulation of different steps of gene transcription than Set1.

With regard to H3-K79 methylation, the responsible enzymes are Dot1 or its homologues, which are the only lysine methyltransferases that have been identified so far that lack an identifiable SET domain[107–110]. Dot1 is required to prevent the spread of HISTONE DEACETYLASES, which are enzymes that are involved in gene silencing, into active chromosomal regions[108,111]. Some insight into the possible function of H3-K79 function was realized recently when it was shown that this modification serves as a binding site for the mammalian protein 53BP1 (REF. 21). This protein interacts with methyl-H3-K79 through a tudor domain at sites of DNA damage. While this could indicate a role for H3-K79 methylation in DNA repair, currently there is no evidence that 53BP1 is involved in gene transcription, which implies the existence of additional methyl-K79 binding proteins. The identification of such proteins will be crucial to understanding the role of Dot1 and H3-K79 methylation in transcription.

In addition to the regulation of gene expression under normal conditions, H3-K79 methylation in certain genes has been linked to cancer. It was shown recently that the mammalian homologue of yeast Dot1 plays a role in leukemogenesis mediated by the MLL–AF10 fusion protein[112]. It was found that hDOT1L is recruited to MLL–AF10 target genes, such as *HoxA9*, through an interaction between hDOT1L and AF10. Methylation of H3-K79 by hDOT1L leads to upregulation of HOXA9 expression, resulting in leukaemic transformation. The fact that the enzymatic activity of hDOT1L is required for leukaemic transformation raises the possibility of a therapeutic intervention that targets hDOT1L methyltransferase activity.

HISTONE DEACETYLASES
Enzymes that modify histones by removing acetyl groups, a chemical modification that is implicated in the regulation of gene expression and chromatin structure.

Histone demethylation

The identification of enzymes that are responsible for demethylating lysine within histones has lagged behind the identification of the histone methyltransferases. In fact, the very existence of such enzymes was questioned[113]. The first breakthrough in this area came with the identification of an enzyme that can convert methyl-arginine to citrulline[114,115]. Shortly after this observation was made, it was shown that LSD1 (also known as BHC110), a protein that was previously identified as a component of several histone deacetylase complexes, can demethylate mono- and dimethylated H3-K4 in an amine oxidase reaction[116]. Similar to many histone methyltransferases, the enzymatic activity of LSD1 can be regulated by its associated proteins, such as CoREST[117]. Furthermore, it was observed that downregulation of LSD1 correlates with increased H3-K4 methylation and upregulation of LSD1 target genes. While these experiments indicate that LSD1 functions in transcriptional repression, a recent study of hormone-regulated gene expression has shown that LSD1 also functions in gene activation[118]. These conflicting observations seem to be explained by the existence of interacting proteins that can modulate the substrate specificity of LSD1. Association with the androgen receptor was shown to be capable of conferring H3-K9 demethylase activity to LSD1 (REF. 118). The mechanism underlying the switching of substrate specificity remains to be determined.

Because of inherent limitations in the amine oxidase reaction, it is not possible for LSD1 to demethylate trimethylated lysines. This might indicate that the trimethyl mark, such as the H3-K4 trimethyl modification that is associated with transcriptional activation, is stable. Alternatively, a different class of enzymes might be dedicated to the reversal of trimethylation. Potential candidates include proteins that contain JmjC domains[119], a motif that is predicated to have the capacity of reversing lysine trimethylation through a hydroxylation reaction[120]. It is interesting to note that in the studies mentioned above, androgen stimulation resulted in a loss of trimethyl-H3-K9 that was insensitive to an LSD1 inhibitor, supporting the existence of an alternative enzyme specific for the trimethyl state.

Concluding remarks

Five years ago, it was known that histones were methylated, but the significance of this post-translational modification was unclear. Since then, the field of histone methylation, and particularly histone lysine methylation, has flourished with the identification of numerous methyltransferases that participate in diverse biological processes. The pace of discovery in this field can partly be attributed to previous genetic studies, which firmly established the genes that encode many of these enzymes in pathways that affect chromatin function. Although the progress in the field has been impressive, many questions remain to be addressed.

One challenge is to define the molecular events that link histone methylation with specific biological outcomes, such as transcriptional activation or repression. This can be achieved by understanding the upstream regulatory pathways of individual enzymes and the downstream events that occur after methylation. The current paradigm is that histone methylation serves as

a molecular mark for the subsequent recruitment of effector proteins. We anticipate that future studies will uncover new protein domains that recognize individual or combinational modifications. Subsequently, it will be necessary to delve into the mechanistic details of how these methyl-binding proteins might functionally interact with downstream effectors such as the basal transcription machinery.

In addition to a mechanistic understanding, we anticipate that great progress will be made in linking the various histone modification patterns to different biological processes such as cell-lineage determination and maintenance. In this regard, it will be important to define cell-lineage-specific histone modification patterns of key regulators that are involved in lineage specification. We anticipate that chromatin immunoprecipitation (ChIP)-coupled microarrays will have an important role in this area of study.

The third area of interest is the potential role of histone methylation in various diseases, particularly cancer. While many histone lysine methyltransferases are linked to cancer, EZH2 and hDOT1L are probably the best examples[112,121]. If the involvement of these histone methyltransferases in cancer is verified in animal models or cancer patients, they will be excellent targets for therapeutic treatment.

Finally, a question that will surely attract great attention is the extent and biological significance of histone demethylation. To answer this question, it will be necessary to identify and characterize more potential demethylases. We predict that the use of novel unbiased activity-based assays coupled with biochemical approaches will lead to the discovery of novel demethylases. We have every reason to believe that the years ahead will be even more exciting in the field of histone methylation.

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Competing interests statement
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