PROGRESS

Reversing histone methylation

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Histones package DNA, and post-translational modifications of histones can regulate access to DNA. Until recently, histone methylation—unlike all other histone modifications—was considered a permanent mark. The discovery of enzymes that reverse the methylation of lysines and arginines challenges our current thinking on the unique nature of histone methylation, and substantially increases the complexity of histone modification pathways.

In its 'naked' form, DNA is unwieldy and unmanageable for a cell to package. This problem is solved by histones, which compact and control DNA. The many different types of histone modifications (for example, acetylation, methylation, phosphorylation and ubiquitination; reviewed in refs 1–3) regulate DNA-based events in ways that were unimaginable a decade ago. Histone methylation, perhaps more than any other form of modification, has demonstrated the power of modifications over DNA-based functions, regulating fundamental processes such as gene transcription and DNA repair. Furthermore, since the discovery of the first histone methyltransferase[4], the potential for the methylation 'mark' to control epigenetic events has caught the imagination of workers in this field. However, the recent discovery that methylation can be reversed[5–7] has shaken the dogma that a 'permanent' methylation mark is necessary for epigenetic control.

Histones may be methylated on either lysine (K) or arginine (R) residues. It is possible that methylation induces alterations in chromatin architecture, either condensing or relaxing its structure. However, a methyl group is relatively small and its addition to lysine or arginine residues does not neutralize their charge, so it is unlikely that methylation alone will significantly affect chromatin structure. It is more likely that it creates binding sites for regulatory proteins that contain specialized binding domains.

Lysine side chains may be mono-, di- or tri-methylated, whereas the arginine side chain may be mono-methylated or (symmetrically or asymmetrically) di-methylated[3,8]. At present, there are 24 known sites of methylation on histones (17 are lysine residues and 7 are arginine residues). If we take into consideration all three possible methylation states of lysine and arginine, there are potentially 3×10^{11} distinct methylation states of histone proteins. Although all of this combinatorial specificity may not be used, this calculation highlights the vast potential for the regulation of function, and the enormity of the task of understanding how methylation works. Why are there such a huge number of possibilities? Are there specific functions that are controlled by a subset of modifications? Is this combinatorial specificity predictive, like a code? How do specific modifications give rise to appropriate biological outcomes? Here we review what is currently known about methylation and its control of chromatin function, and consider the implications of recent reports indicating that the methylation of histones is a dynamic process.

Methylation of lysines

The most-studied sites of lysine methylation lie in the amino termini of H3 and H4 histone proteins (Table 1). At a first level of characterization, these methylated sites are defined by their presence within a certain type of chromatin, either heterochromatin (a condensed and 'transcriptionally silent' chromatin) or euchromatin (a loosely packed and 'transcriptionally active' chromatin). In certain cases, the enzymes that mediate the methylation have been shown to direct the formation of specific chromatin states and to be responsible for transcriptional regulation (Table 1).

It is becoming clear that not all heterochromatin is the same with respect to the methylated histones that it contains. The methylated sites on the histones found within heterochromatin (H3K9, H3K27, H3K79 and H4K20) demarcate subdomains; tri-methylated H3K9 and tri-methylated H4K20 are enriched in pericentric heterochromatin, whereas tri-methylated H3K27 is enriched at the inactive X-chromosome[9–15]. This information could imply the existence of some sort of code, but whether this is predictive, with respect to the chromatin structure formed at these sites, remains to be established.

As with heterochromatin, not all euchromatin is the same. Genes within euchromatin have the potential to be active and are associated with methylated H3K4 and H3K36 histones. When a gene is expressed in yeast, further rounds of histone methylation appear in a localized fashion (enriched at the 5′ end of the gene) and in specific forms, primarily tri-methylation (reviewed in ref. 3). A large-scale analysis of human euchromatin indicates that a situation similar to the one in yeast may also occur in mammals[16].

The extent of our knowledge regarding the mechanistic and functional consequences of methylation is limited to the proteins and domains that recognize the modification. Repressive proteins, such as heterochromatin protein 1 (HP1) or the *Drosophila* Polycomb (PC) protein, contain a chromodomain that allows them to specifically recognize the appropriate repressive methylation mark (H3K9 and H3K27 respectively; reviewed in ref. 3), whereas the chromodomain helicase DNA-binding protein 1 (CHD1) activator protein from *Saccharomyces cerevisiae* uses its chromodomain to bind the activating methylated H3K4 (ref. 17). Therefore, the ultimate function of the methyl group is a reflection of the type of protein it has evolved to recruit—either an activator or a repressor of transcription (Fig. 1).

Recently, two domains that are distinct from the chromodomain were shown to bind methylated lysine residues. The Tudor domain within the DNA-repair checkpoint protein p53-binding protein 1 (p53BP1) recognizes methylated H3K79, a widely distributed histone modification in mammalian cells[18]. This finding fulfils the prediction for members of the larger Royal Family domain, which were thought to bind methylated lysine[19]. The WD40 repeats of the vertebrate transcriptional activator WDR5 also forms a binding site for a methylated lysine, in this case di- and tri-methylated H3K4 (ref. 20). The challenge in the future is to understand how the recruitment of specific proteins to methylated sites mediates the desired biological function.

Almost all methylation marks characterized to date have been shown to have a role in transcription. This monopoly of function is likely to be less a reflection of a unique role for methylation than of a bias in the current research. There is no reason to believe that other DNA functions, such as replication, recombination and repair, are

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not directly influenced by methylation. Recently, methylation of histones has been shown to be necessary for the establishment of a checkpoint control following DNA damage[18,21]. This checkpoint is necessary for the cell to arrest and repair its DNA. In mammals, p53BP1 is recruited to sites of DNA damage where it binds methylated H3K79 via its Tudor domian. The situation is similar in *Schizosaccharomyces pombe*, but here the Tudor domain of Cut5-repeat-binding protein 2 (CRB2; an orthologue of p53BP1 in *S. pombe*) mediates binding to methylated H4K20 (refs 18, 21). Without this methylation these proteins are mislocalized and the DNA checkpoint is then disrupted.

Methylation of chromatin at H3K9 is, in some instances, intimately linked to the methylation of DNA: H3K9 methylation is necessary for DNA methylation to take place (at least in *Neurospora crassa*), and a number of proteins involved in DNA methylation (DNA methyltransferases and methyl-binding proteins) directly interact with histone-methylating enzymes[22]. These data point to a convergence of the DNA and histone methylation pathways, which may cooperate to tightly switch off differentiation-specific or potentially oncogenic genes. Deregulation of DNA methylation is often seen in cancer cells. Recently, disruption of DNA methylation was linked to the loss of H4K20 methylation in cancer cells, and again highlights the tight connection between these two modifications[23].

Another process that is intimately linked with histone methylation is that of RNA interference (RNAi). Disrupting components of the RNAi machinery affects the formation of heterochromatin and the presence of methylated H3K9 and HP1 within heterochromatin[24–26]. The overlap between the two processes is revealed by the existence of a complex in *S. pombe* that contains components of the RNAi machinery as well as the chromodomain-containing protein, CHP1 (chromodomain protein in *Schizosaccharomyces pombe*; ref. 27). Targeting of this complex to chromatin may be through the recognition of specific methyl-lysines by the chromodomain of CHP1.

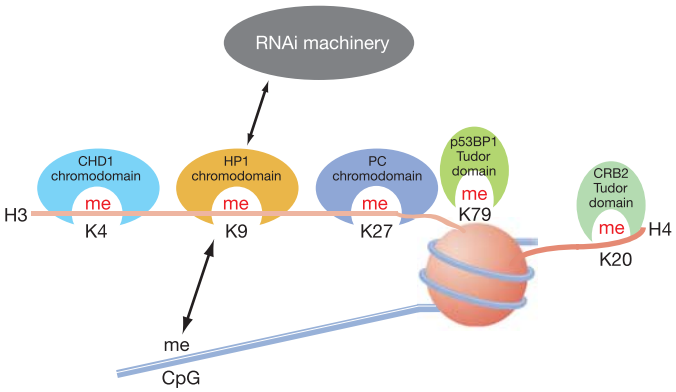

Figure 1 | Summary of the proteins that bind methylated histones. The protein domains that mediate binding to the respective histones are indicated. Histone proteins are shown in red, and DNA in blue. The lysine residues that are methylated (me) are also indicated. Arrows represent interactions between H3K9 methylation and the RNAi machinery and DNA methylation (meCpG). CHD1, chromodomain helicase DNA-binding protein 1; HP1, heterochromatin protein 1; PC, Polycomb protein; p53BP1, p53-binding protein 1; CRB2, Cut5-repeat-binding protein 2.

Demethylation of lysines

Until recently, the methylation of histones was thought to be an irreversible process. The dogma surrounding this irreversibility of

Table 1 | Characterized enzymes responsible for methylating and demethylating histones

Histone and residue	Methyltransferase	Demethylase/deiminase	Function
H3R2	CARM1 (Mm, Hs)	–	
	–	PADI4 (Hs)	
H3K4	ySET1 (Sc)	–	Activator/euchromatin
	SET7/Set9 (Hs)	–	Activator
	MLL (Hs)	–	Activator
	Ash1 (Dm)	–	Activator
	Smyd3 (Hs)	–	Activator
	–	LSD1 (Hs)	Repressor
H3R8	PRMT5	–	Repressor
	–	PADI4 (Hs)	
H3K9	SUV39h1/SUV39H1 (Mm, Hs)	–	DNA methylation/repressor/heterochromatin
	SUV39h2 (Hs)	–	DNA methylation/heterochromatin
	Clr4 (Sp)	–	Repressor/heterochromatin
	Dim5 (Nc)	–	DNA methylation
	Kryptonite (At)	–	DNA methylation
	G9a (Mm, Hs)	–	Imprinting/repressor
	Eu-HMTase1 (Hs)	–	Repressor
	ESET/SETDB1 (Mm, Hs)	–	Repressor/DNA methylation
	E(z)/EZH2 (Dm, Hs)	–	Repressor
	Ash1 (Dm)	–	Activator
	–	LSD1 (Hs)	Activator
H3R17	CARM1 (Mm, Hs)	–	Activator
	–	PADI4 (Hs)	
H3R26	CARM1 (Mm, Hs)	–	
	–	PADI4 (Hs)	
H3K27	E(z)/EZH2 (Dm, Hs)	–	Repressor
	Ezh2 (Mm)	–	X-chromosome inactivation/heterochromatin
H3K36	Set2 (Sc)	–	Activator
	NSD1 (Mm)	–	
H3K79	Dot1/DOT1L (Sc, Hs)	–	Repressor/DNA damage
H4R3	PRMT1	–	Activator
	–	PADI4 (Hs)	
H4K20	SET9 (Sp)	–	DNA damage
	Pr-SET7/Set8 (Hs, Dm)	–	Repressor
	SUV4-20 (Hs)	–	Heterochromatin
	Ash1 (Dm)	–	Activator
	NSD1 (Mm)	–	
H1K26	EZH2 (Hs)	–	

The enzymes (species indicated in parentheses) are listed according to the histone residue they methylate. Known functions of each modification are shown in the fourth column. Mm, *Mus musculus*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; Dm, *Drosophila melanogaster*; Sp, *Schizosaccharomyces pombe*; Nc, *Neurospora crassa*; At, *Arabidopsis thaliana*.

methylated lysines within histones came about from several lines of research. First, reports from over 30 years ago concluded that methylated lysines have the same half-life as histones. Second, the more recent discovery that methylation at H3K9 is responsible for forming and maintaining heterochromatin (a very stable and heritable chromatin state) bolstered the argument that methylation of histones is a permanent 'epigenetic' mark. Third, the mere fact that a demethylating enzyme had not been discovered, although many workers had searched for it, reinforced the view that methylation was a static process.

This view had a chink in its armour from the very beginning: an enzyme with demethylase activity had been reported by Paik and Kim in 1973 (ref. 28), although this activity was never attributed to a particular protein. Indeed, the reversibility of methylation became apparent a few years ago when antibodies against methylated arginine or methylated lysine residues were used in chromatin immunoprecipitations. These experiments revealed that the methylation of histone residues appeared to be reduced under certain conditions. This prompted the idea that demethylation was a likely possibility, and a proposal was put forward which suggested that such an enzymatic activity would function through an amine oxidase reaction (ref. 8 and Fig. 2).

Recently, the enzyme LSD1 (lysine-specific demethylase 1; also referred to as BHC110 or p110b) was identified, which is able to demethylate a specific lysine (K4) within histone H3 using an amine oxidase reaction[5]. This enzyme had previously been identified in a number of repressor complexes (refs 29, 30 and references therein), a fact that fits well with its ability to demethylate the activating methylation site at H3K4. However, demethylation by LSD1 is limited to mono- or di-methylated H3K4: it cannot demethylate tri-methylated H3K4. This is precisely as predicted for an amine oxidase reaction, yet it is the tri-methylated state that is most associated with active genes. Because the transcription of many genes is dynamic, enzymes capable of removing the tri-methylated state should exist. In addition, enzymes that mediate tri-methylation, such as enhancer of zeste homolog 2 (EZH2), are implicated in cancer, so it is probable that the cell has enzymes to reverse this methylation and counterbalance this potentially dangerous methylation state. Demethylation of tri-methylated lysine would require a distinct set of enzymes to the amine oxidases. Such enzymes will most probably function through a pathway involving a hydroxyradical attack[8]. As there are no apparent LSD1 homologues in *S. cerevisiae* (ref. 5), it is puzzling how this yeast deals with the high levels of H3K4 methylation it possesses. Is H3K4 methylation irreversible in *S. cerevisiae*? Is it reversed by distinct mechanisms? Or are there distantly related LSD1-like demethylases still to be discovered?

The LSD1 demethylase is not part of a big family and does not have many obvious homologues. This is rather surprising as there are many methylated lysines in histones and LSD1 seems to be very specific for H3K4. The answer to this problem may be found in proteins that associate with LSD1. The androgen receptor appears to alter the specificity of LSD1 from H3K4 to H3K9, and thereby converts the demethylase from a repressor to an activator of transcription[31]. Thus, at least for androgen receptor target genes, an H3K9 demethylase has been identified. But what about at other sites of lysine methylation? Are there other LSD1 binding factors that alter the specificity of this demethylase? And what about the demethylation of trimethylated H3K9 in heterochromatin, which is apparently stable? One way to resolve this issue of stability is to evoke a dynamic demethylation of mono- and di-methylated H3K9 (by LSD1-like enzymes) but a relatively 'stable' tri-methylated state. In other words, the enzymes that demethylate tri-methylated H3K9 might be tightly controlled or allosterically inhibited so the modification appears stable.

A final point to make concerning LSD1 is that it has the potential to reverse the methylation of H3K4 performed by the oncogenic mixed-lineage leukaemia 1 (MLL1) methyltransferase[3], whose gene is found to be rearranged in leukaemia. Thus, it may be that LSD1 is itself involved in cancer. The logic behind this statement comes from the analogy with the histone acetylation pathway. Acetylases (like p300/CBP) are found to be rearranged in cancer cells, and the enzymes that reverse acetylation (deacetylases) are found to be overexpressed in cancer cells.

Arginine methylation

This modification has been relatively difficult to detect *in vivo*, although the existence of a number of protein arginine methyltransferases (PRMTs) suggest that this is a relatively prevalent modification[32,33]. Mass spectrometry has shown that arginine methylation is present on purified histones in the mono-methylated state[34]. *In vitro*, however, enzymes such as coactivator-associated arginine methyltransferase 1 (CARM1; also known as PRMT4) can further catalyse the reaction to a di-methylated form. Whether this di-methylated state is deposited on histones *in vivo* is still unclear. Antibodies have been raised that can recognize di-methylated arginine by chromatin immunoprecipitation but it is now clear that many, if not all, commercially available antibodies cross-react with mono-methylated arginine (our unpublished observations). So it is still unclear whether di-methylation takes place *in vivo*.

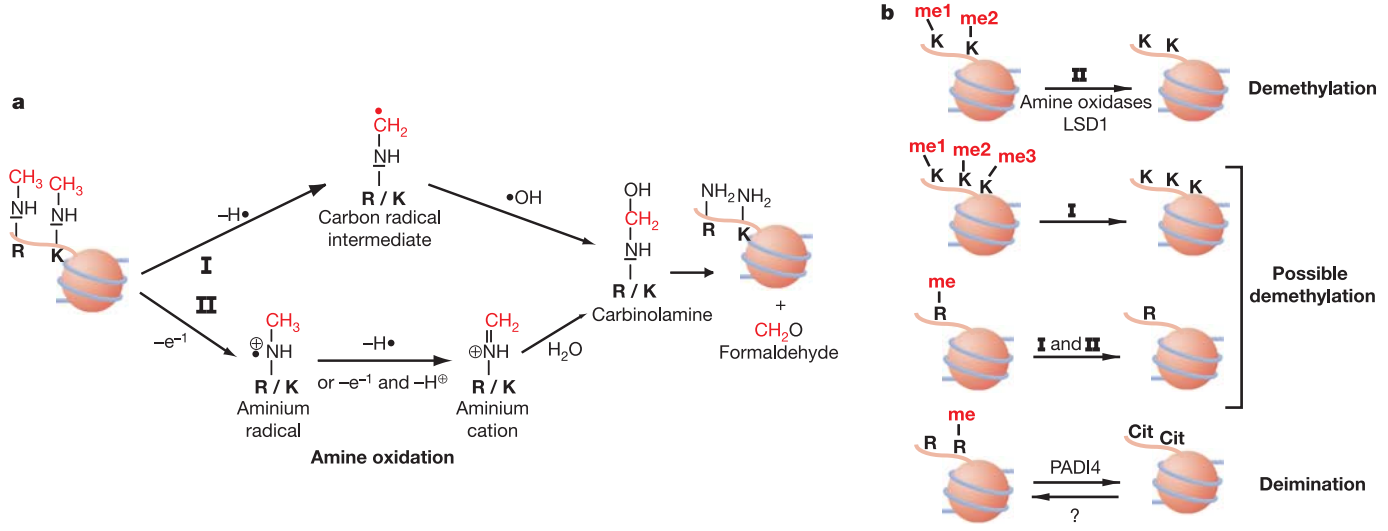

Figure 2 | Reaction mechanisms for methyl group removal. **a**, Two potential chemical reactions (I and II) for the removal of methyl groups (shown in red) from lysine (K) and/or arginine (R) side-chains (adapted from ref. 8). A methylated amine group from the side-chain of each amino acid is shown. **b**, Representation of different mechanisms (possible and actual) for removing methyl groups through specific demethylation and deimination processes. I and II relate to the reactions outlined in **a**. me1, me2 and me3 represent mono-, di- and tri-methylated states, respectively; Cit, citrulline.

The methylation of arginine residues has only been linked to active transcription because this modification is only found on chromatin when genes are actively transcribed[33]. This modification has been best studied as part of the oestrogen signalling pathway. During transcriptional activation by the oestrogen receptor, arginine methylation of H3 appears transiently and in a cyclical manner[35]. It is unclear why these cycles of modification take place, but they suggest the existence of enzymes that reverse arginine methylation. It is also unclear how arginine methylation is involved in the activation signal. A methyl-arginine binding protein has yet to be discovered.

Removal of arginine methylation

The search for arginine demethylases over the last few years has been fruitless. However, the fact that lysine demethylases such as LSD1 exist, makes it much more likely that there is an arginine demethylase. The amino oxidase reaction, through which LSD1 works, is predicted to be compatible with the demethylation of methyl-arginines as well as methyl-lysines (Fig. 2). However, an alternative pathway for the reversal of arginine methylation has been proposed[8] and recently shown to be operational on histones in mammalian cells[6,7]. This pathway involves the removal of a methyl group from an arginine by the conversion of the methyl-arginine residue into citrulline. This process is termed deimination, since the methyl group is removed along with the imine group of arginine. The enzyme that mediates this reaction, peptidyl arginine deiminase 4 (PADI4), converts unmodified arginine and mono-methylated (but not di-methylated) arginine to citrulline at specific sites on the tail of H3 and H4. This activity of PADI4 is linked to the repression of an oestrogen-controlled gene, *pS2*.

The regulated deposition of citrulline in histones raises a number of issues. First, what is the functional consequence of conversion to citrulline? Is it just a way of removing a methyl group, or does the citrulline itself have a positive role to play in transcription repression? One can imagine proteins being recruited that recognize the citrullinated histones specifically, or perhaps the conformation of the histone being altered. Second, citrulline deposition appears to be transient during gene expression[6,7], so how does citrulline get converted back to arginine or methyl-arginine? Is there a rapid replacement of histones by unmodified variants, or are there specific enzymes that mediate the reverse reaction? Enzymes that convert non-peptidyl citrulline to arginine are known to exist, so peptidyl amino transferases may function on citrullinated histones.

Discussion

Deimination and demethylation are both processes that reverse methylation but they are unlikely to be redundant. Even though methylation of arginines and lysines is evolutionarily conserved from mammals to yeast, deiminating enzymes appear to be restricted to higher organisms. The tissue-specific expression pattern of deiminases, and the connection between citrulline and human disease[36], points to a specialized role for deimination in controlling developmental processes. Perhaps the post-translational deposition of a non-coded residue (citrulline) in place of a modified residue (methyl-arginine) may be a process that has evolved to provide an additional level of control to a complex organism. If this is so, then post-translational conversion of amino acids other than arginine may well take place. In contrast to deimination, demethylation is likely to be an activity that is needed for all organisms in which chromatin modifications are found. The identification of many new demethylases is on the horizon, given the plethora of different methylation sites. Indeed, since arginine methylation occurs in lower organisms, but deimination does not, the chances are also high that arginine demethylases will also be discovered. One thing is for certain—if there is a barrel of enzymes that modify histones, we have not yet reached the bottom.

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