

Chromatin Modifications and Their Function

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DOI 10.1016/j.cell.2007.02.005

The surface of nucleosomes is studded with a multiplicity of modifications. At least eight different classes have been characterized to date and many different sites have been identified for each class. Operationally, modifications function either by disrupting chromatin contacts or by affecting the recruitment of nonhistone proteins to chromatin. Their presence on histones can dictate the higher-order chromatin structure in which DNA is packaged and can orchestrate the ordered recruitment of enzyme complexes to manipulate DNA. In this way, histone modifications have the potential to influence many fundamental biological processes, some of which may be epigenetically inherited.

Chromatin is the state in which DNA is packaged within the cell. The nucleosome is the fundamental unit of chromatin and it is composed of an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped. The core histones are predominantly globular except for their N-terminal "tails," which are unstructured. A striking feature of histones, and particularly of their tails, is the large number and type of modified residues they possess. There are at least eight distinct types of modifications found on histones (Table 1). We have the most information regarding the small covalent modifications acetylation, methylation, and phosphorylation. However this Review tries to encompass as thoroughly as possible all modifications of the core histones, concentrating on recent literature. It covers the enzymes that mediate modifications, their mechanism of action, and their biological function. In the first few sections, some general issues regarding the analysis of modifications are discussed along with some general principles regarding their mechanism of action. Each class of modification is then reviewed more specifically under the heading of the function it regulates. The "Functions Regulated" part of Table 1 should act as a guide as to where a modification is mentioned in detail. At the end of this Review, the epigenetic nature of modifications is discussed.

Characterizing Histone Modification

Histones are modified at many sites. There are over 60 different residues on histones where modifications have been detected either by specific antibodies or by mass spectrometry. However, this represents a huge underestimate of the number of modifications that can take place on histones. Extra complexity comes partly from the fact that methylation at lysines or arginines may be one of three different forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines. This vast array of modifications gives enormous potential for functional responses, but it has to be remembered that not all these modifications will be on the same histone

at the same time. The timing of the appearance of a modification will depend on the signaling conditions within the cell.

The use of modification-specific antibodies in chromatin immunoprecipitations coupled to gene array technology (ChIP on CHIP) has revolutionized our ability to monitor the global incidence of histone modifications. Such global analysis has only been done on a subset of modifications (acetylation and lysine methylation), but the results clearly show that modifications are not uniformly distributed. Most of the information we have come from global analyses in budding yeast (Liu et al., 2005; Pokholok et al., 2005). Certain common features have come to light regarding the composition and enrichment of modifications on actively transcribed genes: acetylation is enriched at specific sites in the promoter and 5' end of the coding regions; within the promoter there are two nucleosomes flanking the initiation site that are hypoacetylated at certain lysines and are enriched in the H2A variant Hzt1 (Liu et al., 2005; Zhang et al., 2005; Raisner et al., 2005; Millar et al., 2006; Millar and Grunstein, 2006); the initiation site itself is devoid of nucleosomes; lysine trimethylation is enriched in the coding region; and each of the three known methylation sites in yeast (H3K4, H3K36, H3K79) has a specific distribution pattern. Thus there is a basic blueprint of modification patterning in yeast. Limited evidence from mouse and human tissues indicates that this is a conserved characteristic (Bernstein et al., 2005; see Review by B.E. Bernstein et al., page 669 of this issue).

However, the ChIP on CHIP approach does have a shortfall. It can detect the modification status over a range (2–3) of nucleosomes or even on a single nucleosome, but it cannot determine the modification status of different histones within the same nucleosome. So it is not possible to determine if both copies of a histone are identically modified within a single nucleosome or whether there is a distinct pattern on each. The only way to address this issue is to use mass spectrometry, but the fact that

Table 1. Different Classes of Modifications Identified on Histones

Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	K-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	K-me1 K-me2 K-me3	Transcription, Repair
Methylation (arginines)	R-me1 R-me2a R-me2s	Transcription
Phosphorylation	S-ph T-ph	Transcription, Repair, Condensation
Ubiquitylation	K-ub	Transcription, Repair
Sumoylation	K-su	Transcription
ADP ribosylation	E-ar	Transcription
Deimination	R > Cit	Transcription
Proline Isomerization	P-cis > P-trans	Transcription

Overview of different classes of modification identified on histones. The functions that have been associated with each modification are shown. Each modification is discussed in detail in the text under the heading of the function it regulates.

a protein has to be digested before such analysis can take place limits its potential. New methodology that uses a top-down proteomics approach (identify protein first and digest subsequently) gives promise that we may, in the future, look at the intact modification pattern of different histones in a given nucleosome (Macek et al., 2006).

Once global analysis of all histone modifications is done, a prediction would be that every single nucleosome would be found to be modified in some way. This picture is of course very static. The truth is that modifications on histones are dynamic and rapidly changing. Acetylation, methylation, phosphorylation, and deimination can appear and disappear on chromatin within minutes of stimulus arriving at the cell surface. Thus examining bulk histones under one specific set of conditions (with either antibodies or mass spectrometry) will identify only a proportion of the possible modifications.

There are also problems of detection that are specific for antibodies. Firstly, there are the obvious issues of specificity. These are difficult to avoid as there are no true controls for modifications in mammalian cells (unlike yeast) where it is impossible to mutate the residue to make sure reactivity is lost. In addition, an adjacent modification may disrupt the binding of the antibody or a protein may occlude its recognition, both of which may give a false reading. Similarly, there are problems of detection that are specific to mass spectrometry. Peptide coverage is not equivalent for all parts of the histone and this reduces the sensitivity of detection in these regions. These facts undoubtedly contribute to our underestimation of the extent of modifications present on histones.

We assume that each individual modification on histones leads to a biological consequence. However proof of a consequence is not always easy to provide and is often based on a correlation: a modification appears on a gene under certain conditions (e.g., when it is transcribed) and disappears when that state is reversed (e.g., when the gene is silent). Proving causality for a modification involves showing that the catalytic activity of the enzyme that mediates the modification is necessary

for the biological response. However we know that many of the histone-modifying enzymes have other nonhistone substrates. So the response may be going through another unidentified protein substrate. Furthermore, there may be signaling redundancy such that more than one enzyme may be capable of modifying a specific site. In this case, the effects of inactivating one enzyme may be masked by an upregulation in the activity of a second distinct but related enzyme. Showing that mutation of the modified residue gives the same output as mutating the enzyme is a second stringent test. However, this is not possible in humans due to many histone genes present in the genome, but it is possible in yeast.

So the truth is that we have “levels of confidence” regarding the causative nature of different modifications depending on how far the analysis has gone to prove the issue. We also have to be realistic and accept that, however far we go in proving that a histone modification is causative, we can never exclude the possibility that modification of other substrates by the same enzyme will play a parallel role in the biological response being monitored. The many other nonhistone substrates of chromatin-modifying enzymes are not covered in this Review.

Histone-Modifying Enzymes

The identification of the enzymes that direct modification has been the focus of intense activity over the last 10 years (Table 2). Enzymes have been identified for acetylation (Sterner and Berger, 2000), methylation (Zhang and Reinberg, 2006), phosphorylation (Nowak and Corces, 2004), ubiquitination (Shilatifard, 2006), sumoylation (Nathan et al., 2006), ADP-ribosylation (Hassa et al., 2006), deimination (Cuthbert et al., 2004; Wang et al., 2004b), and proline isomerization (Nelson et al., 2006).

Most modifications have been found to be dynamic, and enzymes that remove the modification have been identified. One major exception is methylation of arginines: although they are thought to be dynamic, a demethylating activity has not yet been found. Instead

Table 2. Histone-Modifying Enzymes

Enzymes that Modify Histones	Residues Modified
Acetyltransferase	
HAT1	H4 (K5, K12)
CBP/P300	H3 (K14, K18) H4 (K5, K8) H2A (K5) H2B (K12, K15)
PCAF/GCN5	H3 (K9, K14, K18)
TIP60	H4 (K5, K8, K12, K16) H3 K14
HB01 (ScESA1, SpMST1)	H4 (K5, K8, K12)
ScSAS3	H3 (K14, K23)
ScSAS2 (SpMST2)	H4 K16
ScRTT109	H3 K56
Deacetylases	
SirT2 (ScSir2)	H4 K16
Lysine Methyltransferase	
SUV39H1	H3K9
SUV39H2	H3K9
G9a	H3K9
ESET/SETDB1	H3K9
EuHMTase/GLP	H3K9
CLL8	H3K9
SpClr4	H3K9
MLL1	H3K4
MLL2	H3K4
MLL3	H3K4
MLL4	H3K4
MLL5	H3K4
SET1A	H3K4
SET1B	H3K4
ASH1	H3K4
Sc/Sp SET1	H3K4
SET2 (Sc/Sp SET2)	H3K36
NSD1	H3K36
SYMD2	H3K36
DOT1	H3K79
Sc/Sp DOT1	H3K79
Pr-SET 7/8	H4K20
SUV4 20H1	H4K20
SUV420H2	H4K20
SpSet 9	H4K20
EZH2	H3K27
RIZ1	H3K9

Table 2. Continued

Enzymes that Modify Histones	Residues Modified
Lysine Demethylases	
LSD1/BHC110	H3K4
JHDM1a	H3K36
JHDM1b	H3K36
JHDM2a	H3K9
JHDM2b	H3K9
JMJD2A/JHDM3A	H3K9, H3K36
JMJD2B	H3K9
JMJD2C/GASC1	H3K9, H3K36
JMJD2D	H3K9
Arginine Methylytransferases	
CARM1	H3 (R2, R17, R26)
PRMT4	H4R3
PRMT5	H3R8, H4R3
Serine/Thrionine Kinases	
Haspin	H3T3
MSK1	H3S28
MSK2	H3S28
CKII	H4S1
Mst1	H2BS14
Ubiquitilases	
Bmi/Ring1A	H2AK119
RNF20/RNF40	H2BK120
Proline Isomerases	
ScFPR4	H3P30, H3P38

Only enzymes with specificity for one or a few sites have been included, along with the sites they modify. Human and yeast enzymes are shown. The yeast enzymes are distinguished by a prefix: Sc (*Saccharomyces cerevisiae*) or Sp (*Saccharomyces pombe*). Enzymes that fall within the same family are grouped.

the process of deimination has been demonstrated to correlate with the disappearance of methyl-arginines, indicating that deimination has the potential to antagonize arginine methylation. There is no known enzyme that will convert peptidyl citrulline back to arginine, but evidence exists that this may be possible given the transient appearance of citrulline on promoters. Proline isomerization is by definition reversible as most isomerases have intrinsic ability to catalyze the formation of both *cis*- and *trans*-proline.

Of all the enzymes that modify histones, the methyltransferases and kinases are the most specific. This is perhaps the reason why methylation is the most characterized modification to date. Phosphorylation of histones is perhaps not as analyzed as methylation because distinct

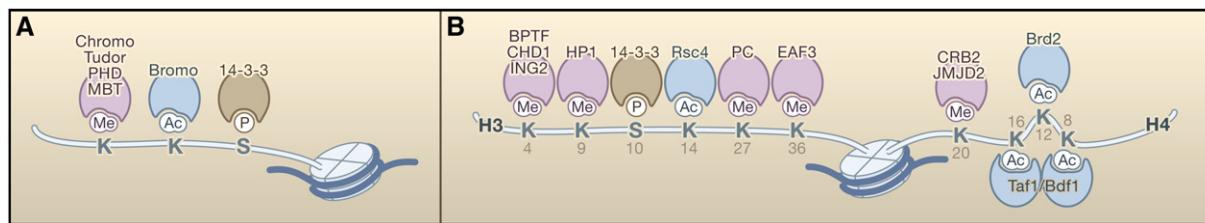


Figure 1. Recruitment of Proteins to Histones

(A) Domains used for the recognition of methylated lysines, acetylated lysines, or phosphorylated serines. (B) Proteins found that associate preferentially with modified versions of histone H3 and histone H4.

signaling pathways need to be activated to observe the modifications. In some cases, the specificity of enzymes that modify histones can be influenced by other factors: complexes in which enzymes are found may specify a preference for nucleosomal versus free histones (Lee et al., 2005a); proteins that associate with the enzyme may affect its selection of residue to modify (Metzger et al., 2005) or the degree of methylation (mono-, di-, or tri-) at a specific site (Steward et al., 2006).

Mechanisms of Histone Modification Function

There are two characterized mechanisms for the function of modifications. The first is the disruption of contacts between nucleosomes in order to “unravel” chromatin and the second is the recruitment of nonhistone proteins. The second function is the most characterized to date. Thus, depending on the composition of modifications on a given histone, a set of proteins are encouraged to bind or are occluded from chromatin. These proteins carry with them enzymatic activities (e.g., remodeling ATPases) that further modify chromatin. The need to recruit an ordered series of enzymatic activities comes from the fact that the processes regulated by modifications (transcription, replication, repair) have several steps. Each one of these steps may require a distinct type of chromatin-remodeling activity and a different set of modifications to recruit them. Below is a more detailed description of the different mechanisms by which modifications work.

Modifications may affect higher-order chromatin structure by affecting the contact between different histones in adjacent nucleosomes or the interaction of histones with DNA. Of all the known modifications, acetylation has the most potential to unfold chromatin since it neutralizes the basic charge of the lysine. This function is not easy to observe *in vivo*, but biophysical analysis indicates that intern-nucleosomal contacts are important for stabilization of higher-order chromatin structure. Thus, any alteration in histone charge will undoubtedly have structural consequences for the chromatin architecture. Furthermore, the recent development of strategies to make recombinant nucleosomes modified at specific sites has allowed this question to be addressed *in vitro*. By chemically ligating modified tail peptides onto recombinant histone core preparations, it has been possible to show

that acetylation of H4K16 has a negative effect on the formation of a 30-nanometer fiber and the generation of higher-order structures (Shogren-Knaak et al., 2006; also see Minireview by D. Tremethick, page 651 of this issue). Phosphorylation is another modification that may well have important consequences for chromatin compaction via charge changes. The role of this modification has not been demonstrated rigorously *in vitro* but demonstrations of its role in mitosis, apoptosis, and gametogenesis are suggestive of such a role (Ahn et al., 2005; Fischle et al., 2005; Krishnamoorthy et al., 2006).

Proteins are recruited to modifications and bind via specific domains (Figure 1A). Methylation is recognized by chromo-like domains of the Royal family (chromo, tudor, MBT) and nonrelated PHD domains, acetylation is recognized by bromodomains, and phosphorylation is recognized by a domain within 14-3-3 proteins.

A number of proteins have been identified that are recruited to specific modifications (Figure 1B). The recent isolation of several proteins that recognize H3K4me has highlighted the fact that their purpose is to tether enzymatic activities onto chromatin. BPTF, a component of the NURF chromatin-remodeling complex, recognizes H3K4me3 via a PHD domain. This recruitment tethers the SNF2L ATPase to activate HOXC8 gene expression (Wysocka et al., 2006; Figure 3A). The PHD-finger protein ING2 tethers the repressive mSin3a-HDAC1 histone deacetylases complex to highly active, proliferation-specific genes after the exposure of cells to DNA-damaging agents (Pena et al., 2006; Shi et al., 2006). This finding represents a new mechanism of active shut-off of highly transcribed, H3K4-methylated genes. Two other H3K4me-binding proteins JMJD2A and CHD1 also tether enzymatic activities to chromatin, but in these instances the enzymatic activity resides within the methyl-binding protein: JMJD2A is a histone lysine demethylase that binds via a tudor domain and CHD1 is an ATPase that binds via a chromodomain (Huang et al., 2006; Pray-Grant et al., 2005; Sims et al., 2005). One other protein, WDR5, has been demonstrated to bind H3K4me1 and H3K4me2 (Wysocka et al., 2005). However, structural analysis of this interaction does not support a purely methyl-recognition-based interaction but suggests that this protein binds most avidly to the residues preceding H3K4 and in particular to H3R2 (Couture et al., 2006). Perhaps this protein

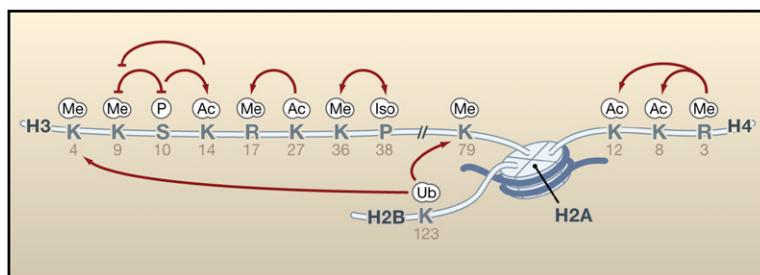


Figure 2. Crosstalk between Histone Modifications

The positive influence of one modification over another is shown by an arrow and the negative effect by a dash-line.

provides an adaptor function, augmenting the recognition of H3K4me (Ruthenburg et al., 2006).

Proteins that bind other modified residues also deliver enzymes: H3K27me recruits the chromodomain containing polycomb protein PC2, which is associated with ubiquitin ligase activity specific for H2A; the chromo-containing HP1 protein binds H3K9me and is associated with deacetylase activity and methyltransferase activity.

Equally important may be the effectiveness of histone modifications in preventing the docking of nonhistone proteins onto chromatin. The study of such pathways is less detailed, but examples include H3K4me disrupting the binding of the NuRD complex and H3T3ph preventing the binding of the INHAT complex. Both complexes have a repressive capability for transcription, so their occlusion by positively acting modifications makes sense (Margueron et al., 2005).

The abundance of modifications on the histone tail makes "crosstalk" between modifications very likely (Figure 2). Mechanistically such communication between modifications may occur at several different levels. Firstly, many different types of modification occur on lysine residues (Table 1). This will undoubtedly result in some form of antagonism since distinct types of modifications on lysines are mutually exclusive. Secondly, the binding of a protein could be disrupted by an adjacent modification. The best example of this being that of phosphorylation of H3S10 affecting the binding of HP1 to methylated H3K9 (Fischle et al., 2005). Thirdly, the catalytic activity of an enzyme could be compromised by modification of its substrate recognition site; for example, isomerization of H3P38 affects methylation of H3K36 by Set2 (Nelson et al., 2006). Fourthly, an enzyme could recognize its substrate more effectively in the context of a second modification; the example here is the GCN5 acetyltransferase, which may recognize H3 more effectively when it is phosphorylated at H3S10 (Clements et al., 2003). Communication between modifications can also occur when the modifications are on different histone tails. The best studied example is the case of ubiquitination of H2B being required for methylation of H3K4me3.

Functional Consequences of Histone Modifications

Simplistically, the function of histone modifications can be divided into two categories: the establishment of global chromatin environments and the orchestration of DNA-based biological tasks. To establish a global chromatin

environment, modifications help partition the genome into distinct domains such as euchromatin, where DNA is kept "accessible" for transcription, and heterochromatin, where chromatin is "inaccessible" for transcription. To facilitate DNA-based functions, modifications orchestrate the unravelling of chromatin to help the execution of a given function. This may be a very local function, such as transcription of a gene or the repair of DNA or it may be a more genome wide function, such as DNA replication or chromosome condensation. All these biological tasks require the ordered recruitment of the machinery to unravel DNA, manipulate it and then put it back to the correct chromatin state. The term "histone code" has been loosely used to describe the role of modifications to enable DNA functions. This term, although useful in defining the need for a specific set of modifications for a given task, is unlikely to truly reflect the presence of a predictable "code" in the strictest sense of the word (Liu et al., 2005).

Below is a brief description of the two categories of functions associated with histone modifications, starting with the establishment of genomic chromatin environments followed by the orchestration of processes such as transcription, repair, replication, and chromosome condensation. (For a detailed discussion of chromatin function during transcription, DNA replication, and repair, see Reviews by B. Li et al. and A. Groth et al., pages 707 and 721 of this issue, respectively).

Establishing Global Chromatin Environments

Grossly speaking, there are two different types of chromatin environments in the genome, silent heterochromatin and active euchromatin. Each of these is associated with a distinct set of modifications. In mammals, demarcation between the different environments is set up by boundary elements, which recruit enzymes to modify the chromatin. The CTCF transcription factor is an example of a boundary element binding protein that delivers the modifying enzymes. Experiments in fission yeast have shown that heterochromatin boundaries are maintained by the presence of methylation at H3K4 and H3K9 in adjacent euchromatic regions. Thus one critical function of chromatin modifications is that they dictate the different chromatin environments and preserve these two types of domains.

Heterochromatin is an important structure, which can determine the protection of chromosome ends and the separation of chromosomes in mitosis. In mammals the

silent heterochromatic state is associated with low levels of acetylation and high levels of certain methylated sites (H3K9, H3K27, and H4K20). The recruitment of PC2 to H3K27me is thought to be involved in the maintenance of the inactive X chromosome, whereas the recruitment of HP1 to H3K9me is thought to play an important role in the maintenance of pericentric heterochromatin.

Methylation at H3K27 seems to be missing in both budding and fission yeast. However H3K9 is present in fission yeast where heterochromatin is more similar to higher organisms. In fission yeast there is evidence that the nucleation of heterochromatin (rather than its spreading) involves the production of small interfering RNAs (siRNAs) from transcripts emanating from centromeric repeats. The dicer-mediated siRNAs are packaged into the RITS complex, which then delivers H3K9 methylation to the sites of heterochromatin formation. Recruitment of HP1 (*Swi6* in *pombe*) then allows spreading and maintenance of the heterochromatic state (Zhang and Reinberg, 2006).

Euchromatin represents a large proportion of the genome. In this environment DNA has flexibility in biological output. Genes can be turned on or kept off, DNA can be “unravelled” for repair or replication. Thus the modification pattern in euchromatin has to reflect this “open choice” scenario. In the transcriptionally inactive state, low levels of acetylation, methylation, and phosphorylation can be detected on genes, but these are insufficient to elicit transcription. Further enzymatic activities are necessary for transcription to take place and typically, actively transcribed euchromatin has high levels of acetylation and is trimethylated at H3K4, H3K36, and H3K79.

Recently bivalent domains have been found that possess both activating and repressive modifications, which somewhat shatters our simplistic view that activating versus silencing modifications dictate distinct types of chromatin environments (Bernstein et al., 2005). Bivalent domains were discovered during the analysis of numerous highly conserved noncoding elements in mouse embryonic stem cells. The use of ChIP on CHIP technology revealed that two methylation sites with conflicting output (H3K27me and H3K4me) coexist in these bivalent domains (Azuara et al., 2006; Bernstein et al., 2005). Classically H3K27 methylation is implicated in silent chromatin and H3K4 methylation is involved in active chromatin. The enrichment of these opposing modifications within bivalent domains correlated with low-level expression of developmental transcription factors. However, when ES cells were made to differentiate, the bivalent domains tended to preserve either the repressive H3K27me or the activating H3K4me modification, but not both. The interpretation of these results is that transcription factors that control certain differentiation processes are kept in a poised, low-level expression within ES cells by having a bivalent cluster of modifications. This finding has important implications for the preservation of pluripotency in ES cells. The hope would be that the differentiation of stem cells can be manipulated by the selective regulation of modification pathways.

Orchestration of DNA-Based Processes

Transcription

The regulation of gene expression within euchromatin requires the delivery of chromatin-modifying enzymes by DNA-bound transcription factors. Following the appearance of a stimulus, transcription factors bind to the promoter of specific genes and initiate a cascade of modification events, which result in the expression or silencing of the gene.

For the purposes of transcription, modifications can be divided into those that correlate with activation and those that correlate with repression. Acetylation, methylation, phosphorylation, and ubiquitination have been implicated in activation whereas methylation, ubiquitination, sumoylation, deimination, and proline isomerization have been implicated in repression. However the truth is likely to be that any given modification has the potential to activate or repress under different conditions. For example, methylation at H3K36 has a positive effect when it is found on the coding region and a negative effect when in the promoter. Methylation at H3K9 may be the same: negative in the promoter and positive in the coding region (Vakoc et al., 2005). The more we look in to modifications, the more it will become clear that context is everything.

In the following few sections, each type of modification is considered separately regarding its role in transcription, with emphasis on recently defined functions.

Acetylation. This modification is almost invariably associated with activation of transcription. Acetyltransferases are divided into three main families, GNAT, MYST, and CBP/p300 (Sterner and Berger, 2000). In general these enzymes modify more than one lysine but some limited specificity can be detected for some enzymes (Table 2). Most of the acetylation sites characterized to date fall within the N-terminal tail of the histones, which are more accessible for modification. However, a lysine within the core domain of H3 (K56) has recently been found to be acetylated. A yeast protein SPT10 may be mediating acetylation of H3K56 at the promoters of histone genes to regulate gene expression (Xu et al., 2005), whereas the Rtt109 acetyltransferase mediates this modification more globally (Han et al., 2007; Driscoll et al., 2007; Schneider et al., 2006). The K56 residue is facing toward the major groove of the DNA within the nucleosome, so it is in a particularly good position to affect histone/DNA interactions when acetylated.

Deacetylation. The reversal of acetylation correlates with transcriptional repression. There are three distinct families of histone deacetylases: the class I and class II histone deacetylases and the class III NAD-dependant enzymes of the Sir family. They are involved in multiple signaling pathways and they are present in numerous repressive chromatin complexes. In general these enzymes do not appear to show much specificity for a particular acetyl group although some of the yeast enzymes have specificity for a particular histone: Hda1 for H3 and H2B; Hos2 for H3 and H4. The fission yeast class III deacetylase Sir2 has some selectivity for H4K16ac, and

recently the human Sir family member SirT2 has been demonstrated to have a similar preference (Vaquero et al., 2006).

Phosphorylation. Little is known about histone phosphorylation and gene expression. MSK1/2 and RSK2 in mammals, and SNF1 in budding yeast, have been shown to target H3S10. A role for H3S10 phosphorylation has been demonstrated for the activation of NFkB-regulated genes and also “immediate early” genes such as *c-fos* and *c-jun*. Concomitant with this phosphorylation is the appearance on chromatin of a phosphor-binding protein 14-3-3 (Macdonald et al., 2005). Recently, a global ChIP on CHIP analysis of many kinases in budding yeast has shown that they are present on the chromatin of specific genes (Pokholok et al., 2006). This has important implications regarding signal transduction. It suggests that the mainly cytoplasmic protein phosphorylation cascades that have dominated signal transduction processes for many years may have a more direct effect on gene expression through the phosphorylation of chromatin.

Lysine Methylation. Lysine methyltransferases have enormous specificity compared to acetyltransferases (Table 2). They usually modify one single lysine on a single histone and their output can be either activation or repression of transcription (Bannister and Kouzarides, 2005).

Three methylation sites on histones are implicated in activation of transcription: H3K4, H3K36, and H3K79. Two of these, H3K4me and H3K36me, have been implicated in transcriptional elongation. In budding yeast H3K4me3 localizes to the 5' end of active genes and is found associated with the initiated form of RNA Pol II (phosphorylated at serine 5 of its C-terminal domain). H3K36me3 is found to accumulate at the 3' end of active genes and is found associated with the serine 2 phosphorylated elongating form of RNA pol II. One role for H3K36me is the suppression of inappropriate initiation from cryptic start sites within the coding region (Carrozza et al., 2005; Cuthbert et al., 2004; Joshi and Struhl, 2005; Keogh et al., 2005). To achieve this, methylation at H3K36 recruits the EAF3 protein, which in turn brings the Rpd35 deacetylase complex to the coding region. Deacetylation then removes any acetylation that was placed in the coding region during the process of transcription, thus resetting chromatin into its stable state. This “closing up” of chromatin, following the passage of RNA pol II, prevents access of internal initiation sites that may be inappropriately used. Very little is known about the function of methylation at H3K79. We do know that it is involved in the activation of HOXA9 and it has a role in maintaining heterochromatin, probably indirectly, by limiting the spreading of the Sir2 and Sir3 proteins into euchromatin.

Three lysine methylation sites are connected to transcriptional repression: H3K9, H3K27, and H4K20. Methylation at H3K9 is implicated in the silencing of enhromatic genes as well as forming silent heterochromatin mentioned above. Repression involves the recruitment of methylating enzymes and HP1 to the promoter of repressed genes. Delivery of these components of meth-

ylation-based silencing is mediated by corepressors such as RB and KAP1. The dogma, that H3K9 methylation and HP1 recruitment are always repressive, has recently been challenged by the finding that H3K9me3 and the γ isoform of HP1 are enriched in the coding region of active genes (Vakoc et al., 2005). The explanation for this difference is not clear. One possibility is that H3K9me within the coding regions is activatory whereas H3K9me in the promoters is repressive.

H3K27 methylation has been implicated in the silencing of HOX gene expression. A similar mechanism is likely to be operational for the involvement of H3K27me in silencing of the inactive X chromosome and during genomic imprinting. Very little is known regarding the repression functions of H4K20 methylation. It has a role in the formation of heterochromatin and has a role in DNA repair. Recently a protein has been identified that may mediate its functions. The JMJD2A lysine demethylase has been demonstrated to bind H3K20me (Huang et al., 2006; Kim et al., 2006) via a tudor domain. The implications of this interaction are not clear especially given that JMJD2A can also bind the positively acting methylation site at H3K4.

Lysine Demethylation. For a number of years following the discovery of histone methyltransferases, the existence of demethylases was contentious. The discovery of the first histone demethylase LSD1 (Shi et al., 2004) has opened the way for the discovery of many other such enzymes (Table 2). So far there are two types of demethylase domain, with distinct catalytic reactions: the LSD1 domain and the JmjC domain. LSD1 acts to demethylate H3K4 and repress transcription (Shi et al., 2004). However when LSD1 is present in a complex with the androgen receptor, it demethylates H3K9 and activates transcription (Metzger et al., 2005). H3K9 can also be demethylated by JHDM2A (Yamane et al., 2006), JMJD2A/JHDM3A (Tsukada et al., 2006; Whetstone et al., 2006), JMJD2B (Fodor et al., 2006), JMJD2C/GASC1 (Cloos et al., 2006), and JMJD2D (Shin and Janknecht, 2006). Methylation at H3K36 can be reversed by JHDM1 (Tsukada et al., 2006; Whetstone et al., 2006), JMJD2A/JHDM3A (Klose et al., 2006), and JMJD2C/GASC1 (Cloos et al., 2006). Structural analysis of JMJD2A has shown that three distinct domains, in addition to the JmjC domain, are necessary for catalytic activity (Chen et al., 2006).

It is too early to know the precise function of all these new demethylases. What is clear is that they will antagonize methylation by being delivered to the right place at the right time (Yamane et al., 2006). Also, the activity of the enzymes are under the influence of the proteins they bind, as in the case of LSD1/BHC110, which acts on nucleosomal substrates in the presence of CoREST (Lee et al. 2005a). A very important part of the specificity of these new demethylases also comes down to the state of methylation they act on. Their selectivity for mono-, di-, or trimethylated lysines allows for a larger functional control of lysine methylation (Shi and Whetstone, 2007).

Arginine Methylation. Like lysine methylation, arginine methylation can be either activatory or repressive for

transcription, and the enzymes (protein arginine methyltransferases, PRMT's) are recruited to promoters by transcription factors (Lee et al., 2005b). The most studied promoter regarding arginine methylation is the estrogen-regulated pS2 promoter. A very interesting observation regarding this promoter is that modifications are cycling (appear and disappear) during the activation process (Metivier et al., 2003). The reason for this is not known, and certainly this is not a behavior represented at most other genes. The reason may be that estrogen-regulated genes have to respond to outside stimuli very rapidly, so their chromatin has to be in "a state of alert" for impending shutdown of transcription. There are no proteins yet identified that can bind specifically to arginine-methylated histones and no enzymes that can reverse arginine methylation.

Deimination. This involves the conversion of an arginine to a citrulline. Arginines in H3 and H4 can be converted to citrullines by the PADI4 enzyme. Deimination has the potential to antagonize the activatory effect of arginine methylation since citrulline prevents arginines from being methylated (Cuthbert et al., 2004; Wang et al., 2004a). In addition, in vivo data demonstrate that mono- (but not di-) methylated arginines can be deiminated (Wang et al., 2004a). In vitro analysis of the PADI4 enzyme suggests that the reversal of monomethyl arginine to citrulline is not carried out by the recombinant enzyme when methylated peptides are used as substrates, suggesting that a cofactor may be necessary in vivo (Hidaka et al., 2005). Converting citrulline to arginine has not been described, although citrulline is cyclic on the pS2 promoter, so reversal may be possible (Bannister and Kouzarides, 2005).

Ubiquitylation. This very large modification has been found on H2A (K119) and H2B (K20 in human and K123 in yeast). Ubiquitylation of H2AK119 is mediated by the Bmi/Ring1A protein found in the human polycomb complex and is associated with transcriptional repression (Wang et al., 2006). This modification is not conserved in yeast. In contrast, H2BK120 ubiquitylation is mediated by human RNF20/RNF40 and UbcH6 and in budding yeast by Rad6/Bre1 and is activatory for transcription (Zhu et al., 2005). A role for this modification has been demonstrated in transcriptional elongation by the histone chaperone FACT (Pavri et al., 2006). How ubiquitylation functions is unclear; it is likely to recruit additional factors to chromatin but may also function to physically keep chromatin open by a "wedging" process, given its large size.

Deubiquitylation. In budding yeast, two enzymes (Ubp8 and Ubp10) have been identified that antagonize ubiquitylation of H2BK123. The Ubp8 enzyme (subunit of the SAGA acetyltransferase complex) is required for activation of transcription, indicating that both the addition and removal of ubiquitin is necessary for stimulation of transcription. The Ubp10 deubiquitylase functions in transcriptional silencing at heterochromatic sites in budding yeast (Emre et al., 2005; Gardner et al., 2005).

Sumoylation. Like ubiquitylation, sumoylation is a very large modification and shows some low similarity to ubiquitylation. This modification has been shown to take place on all four core histones, and specific sites have been identified on H4, H2A, and H2B (Nathan et al., 2006, #2). Sumoylation antagonizes both acetylation and ubiquitylation, which occur on the same lysine residue, and consequently this modification is a repressive one for transcription in yeast.

ADP Ribosylation. This histone modification is ill defined with respect to function. ADP ribosylation can be mono- or poly-, and the enzymes that mediate it are MARTs (Mono-ADP-ribosyltransferases) or PARPs (poly-ADP-ribose polymerases), respectively (Hassa et al., 2006). In addition the Sir family of NAD-dependent histone deacetylases have been shown to have low levels of this activity, so they may represent another class of this family. There are many reports of ADP ribosylation of histones, but only one site, H2BE2ar1, has been definitively mapped. Although the function of the enzymes has often been linked to transcription, evidence that the catalytic activity is involved has been lacking. Recently a role for PARP-1 activity in transcription has been demonstrated but only under conditions where DNA repair is induced. Double-strand breaks mediated by Topoisomerase II β activate the PARP-1 enzyme, which then directs chromatin changes to the estrogen-regulated PS2 gene (Ju et al., 2006).

Proline Isomerization. Prolines exist in either a *cis* or *trans* conformation. These conformational changes can severely distort the polypeptide backbone. Recently an enzyme, FPR4, has been identified in budding yeast that can isomerize prolines in the tail of H3 (Nelson et al., 2006). FPR4 isomerizes H3P38 and thereby regulates the levels of methylation at H3K36. The appropriate proline isomer is likely to be necessary for the recognition and methylation of H3K36 by the Set2 methyltransferase. In addition, it is possible that demethylation of H3K36 is also affected by isomerization at H3P38 (Chen et al., 2006). The catalytic cleft of the JMJD2 demethylase is very deep and may necessitate a bend in the polypeptide (mediated by proline isomerization) to accommodate the methyl group at H3K36.

DNA Repair

Phosphorylation. Chromatin generates a barrier for the repair of DNA damage. Modifications on histones assist in the recognition and accessibility of sites where DNA repair needs to take place. One of the earliest recognized responses to DNA damage is the phosphorylation of the histone variant γ -H2AX in mammalian cells (Fillingham et al., 2006). This phosphorylation extends over many kilobases around the site of the damage. In budding yeast phosphorylation of H2AX has been shown to recruit the INO80 complex, which possesses ATP-dependant remodeling activity (Van Attikum et al., 2004). Two phosphorylation sites on this histone have a role in double-strand break repair via nonhomologous end joining: H2AS129 mediated by Mec1 (Downs et al., 2000) and H4S1 mediated by Caesin kinase II (Cheung et al., 2005).

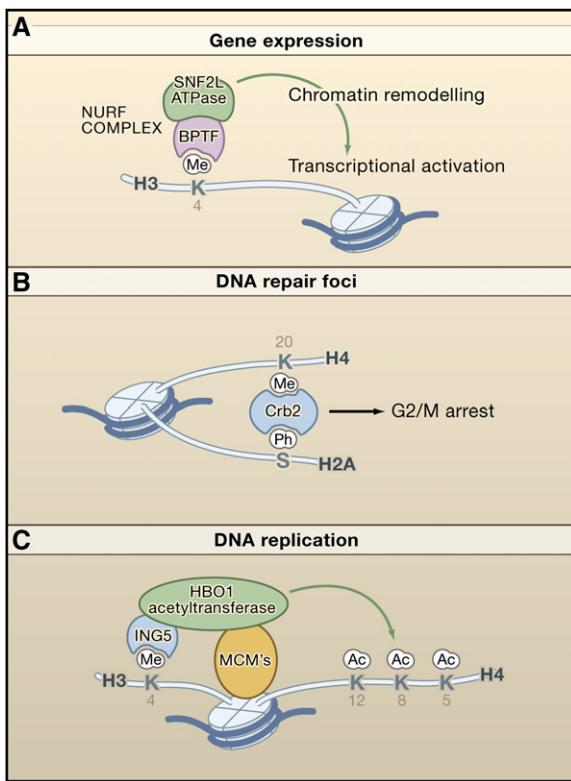


Figure 3. Functional Consequences of Histone Modifications

(A) Gene-expression changes are brought about by the recruitment of the NURF complex, which contains a component BPTF recognizing H3K4me and a component-remodeling chromatin.

(B) The Crb2 protein of fission yeast is recruited to DNA-repair foci during a DNA-repair response. Crb2 is partly tethered there by association with methylated H4 and phosphorylated H2A.

(C) The HBO1 acetyltransferase is an ING5-associated factor and is therefore tethered to sites of replication via methylated H3K4. HBO1 also binds to the MCM proteins found at replication sites. Evidence exists that HBO1 augments the formation of the preinitiation complex and is required for DNA replication.

Methylation. In fission yeast, ionizing radiation-induced DNA damage generates nuclear foci at sites of DNA repair, which contain methylated H4K20 and the cell-cycle checkpoint protein Crb2 (Sanders et al., 2004). This protein signals a G2/M arrest in order for the DNA to be repaired (Figure 3B). Crb2 recruitment to DNA repair foci is dependant on the recognition of methylated H4K20 via the double tudor domains of Crb2 (Botuyan et al., 2006). Methylation at H4K20 is present throughout the genome. During DNA damage it becomes “apparent” at the sites of DNA repair but appears absent elsewhere. So how does Crb2 recruitment take place so specifically at these sites? The answer may lie in a second modification, a phosphorylation of H2AX that Crb2 recognizes at these sites via its BRCT domain. This phosphor-binding domain may recognize the DNA-damage-induced phosphorylation site and then stabilize itself on chromatin via the recognition of H4K20me (Du et al., 2006). In human cells,

p53BP1, the homolog of Crb2, may operate in a very similar way. Although this protein may have some affinity for H3K79 methylation (Huyen et al., 2004), recent structural and functional studies suggest that this protein recognizes H4K20 methylation very avidly and is recruited to sites of DNA via H4K20 methylation (Botuyan et al., 2006). Interestingly, Crb2 and p53BP1 only recognize the mono- and dimethyl forms of H4K20, which opens the possibility that the trimethyl form may function to regulate a different step in DNA repair, or it may be involved in a completely different function in the absence of DNA-damage signaling.

Acetylation. In budding yeast acetylation of H3K56 is deposited on newly synthesized histones during S phase. In the absence of damage, H3K56 acetylation disappears in G2. However, in the presence of DNA damage the deacetylases for H3K56, Hst3, and Hst4 (two paralogs of Sir2) are downregulated and the modification persists (Celic et al., 2006; Maas et al., 2006). The Rtt109 enzyme, which acetylates H3K56, has recently been implicated in genome stability and DNA replication (Driscoll et al., 2007; Han et al., 2007; Schneider et al., 2006). The yeast acetyltransferase Hat1 is another enzyme that is implicated in DNA repair. This enzyme is recruited to sites of DNA repair and acetylates H4K12 (Qin and Parthun, 2006).

Ubiquitination. This is the most recent modification to be linked to DNA repair. UV-induced DNA repair signals ubiquitination of H3 and H4 by the CUL4-DDB-Roc1 complex (Wang et al., 2006). Misregulation of this ubiquitin ligase complex by downregulation of CUL4A prevents the recruitment of the XPC repair protein to DNA-damage foci. Monoubiquitylation of H2A is also implicated in UV-induced repair (Bergink et al., 2006). In this case, the Ring2 ubiquitin ligase mediates the modification. The monoubiquitylation of H2A is coincident with H2AX phosphorylation but is independent of it. Instead, a DNA-damage-specific kinase, ATM, seems to be necessary for this modification to take place.

DNA Replication

Acetylation. A role for acetylation in DNA replication was suspected some time ago when an acetyltransferase, HB01, was isolated as a binding partner for an origin recognition complex protein. More recently a very central role for HB01 in DNA replication has emerged. In the process of analyzing the stoichiometric partners of the ING family of proteins, HB01 was found in a complex with ING4 (a tumor suppressor) and ING5 (Doyon et al., 2006). Depletion of ING5 and depletion of HB01, although less severe, causes a reduction of DNA synthesis and affects progression into S phase. In a separate study HB01 is shown to augment the assembly of the pre-replicative complex and the recruitment of MCMs to chromatin (Iizuka et al., 2006). In *Drosophila*, the HB01 homolog, Chameau, is found to increase the firing of replication origins (Aggarwal and Calvi, 2004). Together, these findings suggest that HBO1, via its ability to acetylate H4, is required for S phase initiation and fixing of replication origins (Figure 3C).

Chromosome Condensation

Phosphorylation. Condensation and decondensation of chromatin are important processes during the replicative cell cycle. Two phosphorylation events in mammalian cells may play an important role in these processes during mitosis. The first is phosphorylation of H3S10 during mitosis by the Aurora B kinase. Recent data suggest that one of the mechanisms by which H3S10 phosphorylation may function is via the displacement of HP1 from H3K9me, which normally compacts chromatin (Fischle et al., 2005). The second phosphorylation event is at H3T3 (Dai et al., 2005). This modification is mediated by the Haspin kinase and is required for normal metaphase chromosome alignment. A number of other phosphorylation sites have been implicated in this process in budding yeast. Phosphorylation of H4S1 regulates sporulation (Krishnamoorthy et al., 2006), and phosphorylation of H2BS10 regulates peroxide-induced apoptosis (Ahn et al., 2005). The latter modification is on a residue that is not conserved in mammals. However, phosphorylation of mammalian H2BS14 by Mst1 is thought to play an analogous function.

Acetylation. In vitro experiments provide a role for H4K16Ac in chromatin decondensation (Shogren-Knaak et al., 2006). A class III deacetylase SirT2, which has specificity for H4K16Ac, may have the ability to induce the condensation of chromatin in vivo (Vaquero et al., 2006). Consistent with this idea is the finding that SirT2 localizes to chromatin during G2/M transition when chromatin has to be recondensed.

Are Histone Modifications Truly Epigenetic?

Histone modifications have been implicated in a number of epigenetic phenomena. The classic definition of epigenetics is the study of heritable phenotype changes that do not involve alterations in DNA sequence. The use of the term "heritable" has been dropped in recent usage, allowing the term epigenetic to mean the information carried by the genome (e.g., on chromatin) that is not coded by DNA. However the classic term, that includes heritability, is important to maintain as it defines a nongenetic memory of function that is transmitted from generation to generation. A number of cellular phenotypes are transmitted in this way, including imprinting, X chromosome inactivation, aging, heterochromatin formation, reprogramming, and gene silencing. In addition there are environmentally induced changes, which are passed on from generation to generation, without the need for the original stimulus (most studied in plants). There is no disputing that histone modifications are involved in epigenetic processes. The question is, do modifications pass on the memory of a given chromatin state or do they merely implement the memory, once the memory is passed on via a distinct process?

If epigenetic memory is mediated by one or more of the histone modifications, then there should be a mechanism for the transmission of such modifications onto the chromatin of the replicating DNA. Such a mechanism has been proposed for H3K9 methylation in the transmission

of the heterochromatin: recruitment of HP1 brings in further H3K9-methylating activity that modifies nucleosomes on the daughter strand, thus ensuring the transmission of the H3K9me mark. This mechanism of transmission, along with the observation that H3K4me3 patterns persist, have given lysine methylation an epigenetic status. The issue that remains, however, is whether the modification pattern inherited by the daughter chromatin is sufficient to impose the correct chromatin structure originating from the mother cell. Is methylation of lysines dictating the memory of chromatin structure?

The argument that histone methylation is a permanent mark is now on shaky ground, given the discovery of demethylases. Are other types of histone modifications epigenetic? Do we expect the complicated chromatin structure of the entire genome to be perpetuated by a few inherited histone modifications? Are there other determinants likely to transmit information for the assembly of a correct local chromatin structure?

One such determinant is RNA. Work in fission yeast has shown that small RNAs are associated with chromatin-modifying complexes and can deliver histone-modifying enzymes to chromatin (Verdel et al., 2004). Deletion of the enzyme Dicer that processes small RNAs can also affect heterochromatin formation, methylation of H3K9, and recruitment of HP1 (Fukagawa, 2004; Kanelloupolou et al., 2005; also see Review by M. Zaratiegui et al., page 763 of this issue).

The case for RNA as a determinant is certainly appealing, and some evidence exists that it acts in such a way. Recent work in mice has shown that small RNAs present in sperm can be transmitted to offspring where they mediate an epigenetic phenotype called paramutation, a process first identified in plants (Rassoulzadegan et al., 2006). Perhaps this mechanism is more widespread than we think. Small RNAs may emanate from many loci in the genome and once transmitted to the next generation, these RNAs may deliver chromatin-modifying complexes to specific genes or to specific locations, thus generating the pattern of chromatin that we observe (Verdel et al., 2004; Buhler et al., 2006). One appealing aspect of this model is that small RNAs are likely to be highly precise in their delivery since their guiding system is nucleic acid.

Only time will tell whether such speculative mechanisms exist for the widespread transfer of chromatin information. The model proposed implies that RNA may be perfect as a molecule to transmit the memory of a specific chromatin state. However, such an RNA-mediated mechanism does not imply that histone modifications are unnecessary for epigenetic events. It merely points out that histone modifications may be the executors of the epigenetic phenomenon rather than the carriers of the memory.

ACKNOWLEDGMENTS

I thank Andy Bannister for helpful discussions. T.K. is a director of Abcam plc.

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