Chapter 15
Regulation of Chromatin Structure and Transcription Via Histone Modifications

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15.1 Introduction

Eukaryotes package their genome into a highly organized structure, known as chromatin, composed of DNA and its intimately associated proteins. The nucleosome is the basic fundamental repeating unit of chromatin, a spherical-shaped macromolecule composed of approximately 146 base pairs of DNA wrapped twice around an octamer of four histone proteins – H2A, H2B, H3 and H4 (Fig. 15.1; Luger et al. 1997). Nucleosome assembly involves the association of an H3/H4 heterotetramer with DNA, and subsequent addition of two H2A/H2B heterodimers, facilitated by proteins known as histone chaperones (Park and Luger 2008). Chromatin is generally classified into either euchromatin or heterochromatin, depending on its level of compaction.

Euchromatin is 'open' and poised for gene expression, while heterochromatin is compact and refractory to transcription. Euchromatin is best described by the 'beads on a string' model, which is thought to represent the lowest level of chromatin compaction (10 nm fiber). Heterochromatin is formed by the addition of linker histone H1 and various non-histone proteins, which further compact nucleosomes into higher order structures (30 nm fiber and beyond). Finally, chromatin reaches its most condensed state during mitosis (Horn and Peterson 2002).

While chromatin plays a structural role, its regulation is highly dynamic. At least three critical factors contribute to chromatin dynamics – the post-translational modification of histones (see below), ATP-dependent chromatin remodeling, and the incorporation of specialized histone variants into chromatin. ATP-driven chromatin remodeling complexes rearrange or mobilize nucleosomes during cellular processes such as transcription, and their recruitment to chromatin can depend on the PTM-status of particular loci (Wu et al. 2009). Histone variants differ from canonical histones in amino acid sequence and therefore, are subject to distinct

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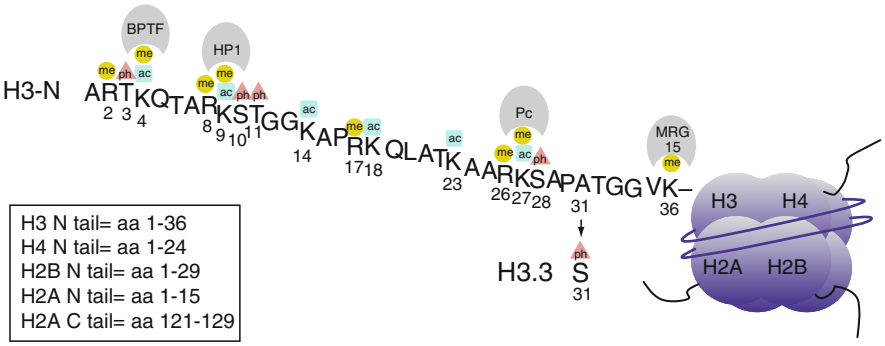

Fig. 15.1 The canonical nucleosome consists of 146bp of DNA wrapped around the histone octamer (two of each H2A, H2B, H3 and H4). The histone tails protrude beyond the nucleosomal core, are unstructured in nature, and highly post-translationally modified. See histone H3 tail as an example. Modified residues are numbered below the H3 tail sequence and PTMs are shown above; triangles represent phosphorylation (ph), squares represent acetylation (ac) and circles depict methylation (me). A single residue can be modified by different PTMs under different biological conditions (e.g. K9ac in transcription vs. K9me3 in transcriptional silencing). Representative readers of lysine methylation are shown above the methylation sites (see text for details). The H3.3 S31ph site is shown below the canonical H3 sequence (which contains an alanine at position 31). Lengths of histone tails are listed in the box below. Although not depicted here, PTMs are also found in the nucleosomal core, such as H3K79me

PTMs, resulting in unique biological outcomes (Bernstein and Hake 2006). Several key cellular pathways are regulated by histone variant PTMs, and these will be described where relevant.

PTMs are particularly abundant on the N-terminal histone tails, which protrude from the nucleosomal core. However, PTMs also exist within the core and on certain C-terminal tails, for example, H2A. Histone PTMs can result in 'on' or 'off' chromatin states regarding transcriptional status, including phosphorylation, acetylation, methylation and ubiquitination - the focus of this chapter. In order to simplify histone PTM terminology, we follow the nomenclature presented in Table 15.1 (Turner 2005).

The "histone code" hypothesis has been proposed to explain the complex and combinatorial pattern of PTMs and their biological consequences. This hypothesis states that PTMs can act through two mechanisms: (1) by structurally changing the chromatin fiber through internucleosomal contacts, thus regulating DNA accessibility, and (2) by serving as docking sites for effector molecules (generally referred to as 'readers') that initiate distinct biological processes (Strahl and Allis 2000; Turner 1993; 2000). As discussed throughout the chapter, histone PTMs are placed by enzymes referred to as 'writers' (e.g. methyltransferases), recognized and bound by 'readers' (e.g. HP1) and finally, removed by 'erasers' (e.g. demethylases), summarized in Table 15.2. Table 15.3 provides a list of the PTM-recognition domains of the readers discussed throughout the chapter. Finally, while the focus of the chapter is mammalian histone PTMs, groundbreaking studies in other organisms

Table 15.1 Histone posttranslational modification nomenclature

PTM	Abbreviation	Example
Phosphorylation	S/T/Y#ph	H3S10ph
Acetylation	K#ac	H4K16ac
Arginine methylation	R#me	
monomethyl-	me1	H4R3me1
dimethyl symmetric-	me2s	H4R3me2s
dimethyl asymmetric-	me2a	H4R3me2a
Lysine methylation	K#me	
monomethyl-	me1	H3K27me1
dimethyl-	me2	H3K27me2
trimethyl-	me3	H3K27me3
Ubiquitylation	K#ub	
monoubiquityl-	ub1	H2AK119ub1
polyubiquityl-	ubn	H2BK123ubn[a]

This nomenclature was put forth in 2005 (Turner 2005) and is used to describe PTMs throughout the chapter. The # represents the amino acid that is modified; examples are on right side of the table
[a]Polyubiquitylation of H2B was recently described in *S. cerevisae*; this K residue is conserved in mammals

have significantly contributed to our understanding of chromatin regulation and are mentioned where applicable.

15.2 Histone Phosphorylation

Of all PTMs found on cellular proteins in general, phosphorylation is perhaps the most widely documented and well characterized. The ATP-dependent addition of a phosphate moiety to serine, threonine or tyrosine residues is mediated by kinases and removed by phosphatases. Historically, the interaction of phosphorylated proteins with downstream binding effectors has been recognized as an important step in controlling signal transduction cascades, for example, in the activation of receptor tyrosine kinases. Histone proteins are no exception to the action of kinases. Phosphorylation is found on all four core histones, the linker histone H1, as well as histone variants, and is carried out by a variety of kinases. Histone phosphorylation can broadly be divided into three classes – mitotic, gene transcription-induced, and apoptotic and DNA damage-induced phosphorylation. Interestingly, several individual phosphorylation sites play a role in more than one biological process.

15.2.1 Mitotic Phosphorylation

Histone phosphorylation occurs in a cell cycle-dependent manner and has long been recognized to play a role in mitotic chromosome condensation, however, the mechanisms remain unclear. Mitotic phosphorylation is well characterized on several

Table 15.2 Identified writers, eraser, and readers of the histone code and their effects on transcriptional readout

PTM	Writer(s)	Eraser(s)	Reader(s)	Transcriptional readout
Phosphorylation				
H3S10ph, H3S28ph	Aurora B (M); MSK1, 2, RSK2 (I)	[PP1] (M)	14-3-3 (I)	ON (I)
H3.3 S31ph	N/D	N/D	N/D	N/A
H2A.X S139ph	ATM/ATR, DNA PK	PP2C, PP4	MDC1	N/A
H2A.X Y142ph	WSTF	EYA	N/D	N/A
macroH2A S137ph	CDKs	N/D	N/D	[ON] (I)
Acetylation				
H3K9ac	CBP, p300	SIRT1, 2, 3	BAF180	ON
H3K14ac	GCN5, PCAF	HDACs	Brg1, BAF180, PCAF, TAFII250	ON
H4K16ac	GCN5, hMOF	SIRT1, 2, 3	GCN5	ON
Arginine methylation				
H3R2me1/2	CARM1	PAD4/JMJD6	N/D	ON/OFF
H4R3me1/2	PRMT1, PRMT5	PAD4/JMJD6	DNMT3A	OFF
Lysine methylation				
H3K4me1/2/3	MLL1-4 SET1A, B, SET7/9	LSD1/JARID1A, B, C, D	WDR5/CHD1, BPTF, ING2, JMJD2A	ON
H3K9me1/2/3	SUV39H1, 2, G9a, GLP, ESET, RIZ1	LSD1/JHDM2A, B, JMJD2A, B, C, D	HP1, CDY, CDYL2	OFF
H3K27me1/2/3	EZH1, EZH2	UTX, JMJD3	Pc	OFF
H3K36me1/2/3	SET2	JHDM1 A, B/JMJD2A, B, C	MRG15	ON
H3K79me1/2/3	DOT1L	N/D	53BP1	ON
H4K20me1/2/3	PR-SET7/SUV4-20H1, 2	N/D	L3MBTL1, L3MBT2, 53BP1/JMJD2A	OFF
Ubiquitylation				
H2AK119ub1	RING1A,B	2A-DUB, UBP-M, USP21	N/D	OFF
H2BK120ub1	RNF20	ATXN7L3, USP22, and ENY2	N/D	ON

This table categorizes many of the histone PTMs, readers, writers and erasers discussed in detail throughout the chapter. Proteins listed are all human homologs. Slashes (/) represent separation of different methylation states of the PTM and the readers, writers and erasers. Proteins or transcriptional readout in [] are likely candidates or functions, but not yet proven experimentally in humans. (M) represents mitosis and (I) represents interphase. N/D is not determined, N/A represents not applicable to transcriptional readout. While all possible histone regulatory molecules could not be discussed in the text, many other readers, writers, and erasers have been added to the table for completeness

Table 15.3 Domains responsible for histone code 'reading'

PTM	Reader domain(s)
Phosphorylation	14-3-3, Tandem BRCT domains
Acetylation	Bromodomain (BD)
Arginine methylation	
me0	WD40 repeats
me1	N/D
me2s	PHD finger
me2a	N/D
Lysine methylation	
me0	Plant homeodomain finger (PHD finger)
me1	Malignant brain tumor repeats (MBT)
me2	WD40 repeats, tandem tudor, MBT, PHD finger, chromo barrel
me3	Chromodomain (CD), double CD, chromo barrel, PHD finger, double tudor
Ubiquitylation	N/D

The readers of the histone code contain specific PTM-binding domains. These are summarized based on type of modification and methylation state. It is clear that many lysine methyl-binding domains have been discovered, while reading domains of other PTMs are less well characterized. While not discussed in the text, me0 is the unmodified state. Recently, PHD fingers have been shown to bind me0 histone tails (Taverna et al. 2007). N/D is not determined. See text for details

key residues of nucleosomal H3, including S10ph and S28ph (Cerutti and Casas-Mollano 2009). Linker H1 phosphorylation also peaks during mitosis, as does phosphorylation of S31 on the H3 variant H3.3 (van Holde 1988; Hake et al. 2005). Interestingly, S31 is one of five residues that differ between H3.3 and the canonical H3.1, suggesting unique histone variant PTM regulation (Fig. 15.1; Hake et al. 2005). Finally, other mitotic phosphorylation sites on the H3 tail include, T3ph and T11ph. Unlike S10ph and S28ph, T3ph and T11ph peak just prior to mitosis (Cerutti and Casas-Mollano 2009).

The enzymatic writers of H3S10ph and S28ph are the Aurora kinases (A and B), which are necessary for kinetochore assembly and microtubule attachment (Crosio et al. 2002). Aurora B is a component of the chromosome passenger complex (CPC), which includes the inner centromere protein, INCENP and survivin (Vader et al. 2006). This complex is required for proper mitotic execution. While the mammalian eraser of H3S10ph has yet to be reported, a study in the budding yeast, *S.cerevisiae*, identified a Protein Phosphatase 1 (PP1) homolog as the H3S10 eraser, implicating PP1 as the potential eraser in mammals (Hsu et al. 2000).

Interestingly, a link between H3S10ph and the mitotic loss of the heterochromatin protein HP1β, which binds the adjacent methylated K9 (Fig. 15.1), from chromatin has been demonstrated in mammals (Fischle et al. 2005). This is referred to as a 'methyl/phos switch', and suggests that an additional role of histone phosphorylation is to regulate the recruitment of effector proteins to chromatin. A recent study in the fission yeast, *S. pombe*, also reported this 'methyl/phos switch' and demonstrated that condensin, a factor required for chromosome condensation during mitosis, was present on chromosomes concomitant with H3S10ph (Chen et al. 2008). This implicates condensin, or factors required for its loading, as potential S10ph readers.

Finally, the cell cycle-dependent phosphorylation of the H2A variant, macroH2A was recently reported. MacroH2A is an unusually large histone variant, containing an H2A domain and an evolutionarily conserved macro domain. MacroH2A is phosphorylated at serine 137 (S137ph), and while this phosphorylation is detected throughout the cell cycle, it peaks during mitosis (Bernstein et al. 2008). In vitro kinase assays have implicated the cell cycle-regulated Cdk/cyclin complexes in writing this PTM (Bernstein et al. 2008). The reader(s) of this modification is currently unknown.

15.2.2 Phosphorylation in Gene Activation

Histone phosphorylation events exhibit complex relationships during the cell cycle. For example, while H3S10 and S28 are phosphorylated during mitosis, these residues are also phosphorylated during periods of immediate-early gene induction. Thus, one PTM can serve two opposing roles and suggests that PTMs should be studied in a context-dependent manner.

The phosphorylation of H3 was first noted to occur as a rapid 'nucleosome response' to external mitogenic stimulation by growth factors such as EGF or FGF. Immediate early genes, c-fos and c-jun, are transcribed following growth factor administration and their promoters acquire H3S10ph (Mahadevan et al. 1991). There is an extensive list of writers for H3S10ph during gene activation, and their activity largely depends upon the stimulus received by the cell. Among those characterized include MAP kinases MSK1 and MSK2, which respond to tumor promoting factor phorbol ester TPA, and Ribosomal S6 Kinase (RSK2), which responds to EGF (Bode and Dong 2005).

The readers of H3S10ph during gene activation are the phospho-serine binding 14-3-3 proteins (Winter et al. 2008; Taverna et al. 2007). 14-3-3's localize to H3 tails following phosphorylation of S10, but the biological significance of this interaction is unknown. The stability of 14-3-3's association with H3 tails bearing S10ph is enhanced when the H3 tail simultaneously bears acetylation on K9 or K14 (Winter et al. 2008). This phospho-acetyl motif serves to enhance the association of 14-3-3 and can overcome repressive chromatin marks that inhibit transcription. Thus, different histone PTMs can work together, and therefore, often need to be addressed in a combinatorial fashion.

15.2.3 Apoptotic and DNA Damage Induced-Histone Phosphorylation

The histone variant H2A.X is central to the cell's response to DNA double strand breaks (DSBs). In response to DSB-inducing ionizing radiation, H2A.X becomes rapidly phosphorylated at serine 139 (S139ph) in mammalian cells (Rogakou et al. 1998).

This modified form of H2A.X, referred to as γH2A.X, persists until DNA damage has been repaired and the cell is released from the damage-induced checkpoint. γH2A.X is a hallmark of DNA damage and can be used to assess DSB-induced genomic instability.

The writers of γH2A.X include two members of the phosphatidylinositol-3 kinase-like family (PI3KK), ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad3-related), as well as DNA PK (Motoyama and Naka 2004). These kinases recognize the highly conserved SQEX motif (X represents any amino acid) present on the C terminal tail of H2A.X. γH2A.X is highly conserved; in budding yeast, the predominant form of H2A is also phosphorylated in response to DNA damage.

Upon the induction of DNA damage, γH2A.X provides a template for factors involved in the DNA damage response, resulting in 'γH2A.X foci', which can extend megabases from the actual break sites. While numerous factors are found in γH2A.X foci, peptide association studies determined that ATM, the MRN complex (Mre11, Rad50, NBS1-responsible for repair via homologous recombination) and MDC1 (Mediator of DNA damage Checkpoint 1) directly bind to S139ph (Stucki et al. 2005). Among these potential readers, MDC1 binding was strongest and occurred via its tandem BRCT (BRCA1 C-Termini) domains. The factors present at γH2A.X foci, whether directly bound or recruited by readers, function to repair damaged DNA and activate the checkpoint in order to prevent cells from entering the next phase of the cell cycle prior to recovery from damage. Upon repair completion, dephosphorylation of H2A.X occurs. The erasers responsible for removing the phosphate moiety from S139 are the mammalian protein phosphatase 2A and 4 complexes (PP2A and PP4) (Bonner et al. 2008).

In addition to S139ph, phosphorylation of the very C-terminal residue of H2A.X, tyrosine 142 (Y142), was recently reported (Xiao et al. 2009). Interestingly, S139ph correlates with the loss of Y142ph and thus, appear to be mutually exclusive during the DNA damage response. The Y142ph kinase is WTSF (Williams-Beuren Syndrome Transcription Factor) and the eraser of this mark is the tyrosine phosphatase EYA (Eyes Absent) (Cook et al. 2009). An interesting interplay between these two modifications was suggested in the delineation between the DNA damage repair and apoptotic pathways. In the absence of Y142 desphosphorylation, the pro-apopotic stress response kinase, Jnk1, is recruited preferentially to break sites. Concomitantly, the pro-repair factor MDC1 is lost from break sites, suggesting that the chromatin signature at DSB sites can determine whether the cell responds to damage through repair mechanisms or by programmed cell death. Of interest, Y142ph is the first report of histone tyrosine phosphorylation (Xiao et al. 2009).

15.3 Histone Acetylation

While most histone PTMs result in single-site specific effects, acetylation is a more global affair with multiple lysine residues becoming acetylated at once, mainly on H3 and H4 (Fig. 15.1). The first indication that histone acetylation positively regulated

transcription came from incubation studies of chromatin with acetic anhydride, which provides an acetyl moiety. This resulted in increased global histone acetylation and transcriptional activity (Csordas et al. 1984). Enzymes of the histone acetyltransferase (HAT) family add an acetyl group from acetyl-CoA to the ε-amino group of lysines, which in turn, are removed by an enzyme family known as histone deacetylases (HDACs) (Roth et al. 2001). This section focuses on histone acetylation, HAT and HDAC activity, and their functional readout on histones.

Histone acetylation is broadly classified as a charge-altering modification. The structure of the nucleosome is largely based upon the physical attraction between the negatively charged DNA phosphate backbone and the positively charged histones. Acetylation adds a negative charge to positively charged lysines, reducing the strong interaction between DNA and the histone octamer, thus facilitating the unwinding of DNA from the octamer. The protein machinery that mediates fundamental cellular processes such as transcription, DNA replication and repair, require access to naked DNA, and acetylation plays a critical role during these events. On a global scale, histone acetylation and deacetylation are involved in the dynamic structural alterations of the chromatin template. Acetylation is found mainly in euchromatin and deacetylation predominates in condensed chromatin, contributing to open and closed chromatin states, respectively (Roth et al. 2001).

15.3.1 Histone Acetyltransferases

HATs can be divided into two main classes – Class A HATs acetylate lysine residues on nucleosomal histones and generally function during transcriptional activation, whereas Class B HATs acetylate free histones, which are thought to become incorporated into the nucleosome during DNA replication (Sterner and Berger 2000; Roth et al. 2001). Due to the focus of this chapter on transcriptional control, we discuss Class A HATs below.

While much of the histone acetylation work has been done in *S.cerevisiae*, the first report of HAT activity came from studies in the ciliated protozoan *Tetrahymena thermophila* in 1995 (Brownell and Allis 1995; Shahbazian and Grunstein 2007). This activity was subsequently attributed to Gcn5, a factor originally identified in *S.cerevisiae* to positively regulate transcription (Georgakopoulos and Thireos 1992; Brownell et al. 1996). Gcn5 has specificity for histones H3 and H4 (H3K14, H4K8 and H4K16) and optimal activity requires additional subunits, which are components of the yeast SAGA and ADA HAT complexes (Roth et al. 2001).

Class A HATs consists of the GNAT (*G*cn5-related *N a*cetyl*t*ransferase) and MYST (named after *M*OZ, *Y*bf2/Sas3, *S*as2 and *T*ip60) families (Sterner and Berger 2000). Both contain a HAT domain, but have variable domains, contributing to their unique function. For example, GNAT members contain a lysine acetyl-binding motif at their C-termini, known as a bromodomain (BD) (Table 15.3). This domain allows GNAT members to read the modifications they write, suggesting a mechanism by which HATs remain at sites of their activity for prolonged function.

MYST family members, on the other hand, often contain a zinc finger motif (ZF) and/or methyl-lysine binding chromodomains (CD) (Roth et al. 2001).

The well-characterized mammalian Class A HATs include p300, CBP (*C*REB-*B*inding *P*rotein), PCAF (*p*300/*C*BP *A*ssociated *F*actor) and GCN5. Interestingly, p300 and CBP contain both a BD and a ZF motif, which are peculiar to both the GNAT and MYST families (Roth et al. 2001). Both p300 and CBP can acetylate all core histones in the context of the nucleosome, and have overlapping substrate specificities with equal activity on H3K9, H3K14 and H4K5. However, p300 acetylates H4K8 to a higher degree than CBP, while CBP has greater activity on H4K12 (McManus and Hendzel 2003). PCAF is a GCN5 ortholog and like GCN5, it physically interacts with p300 and CBP. Both PCAF and GCN5 have been shown to acetylate both free and nucleosome-bound H3K14 (Table 15.2; Yang et al. 1996).

Mouse knockout experiments have demonstrated that the loss of p300, CBP or GCN5 results in embryonic lethality, indicating that histone acetylation plays a crucial role during mammalian development (Roth et al. 2001). Moreover, it suggests that despite the similarity between HATs and their targets, they are not functionally redundant. Mutations in CBP or p300 have been implicated in Rubinstein-Taybi Syndrome, a developmental disorder characterized by mental retardation (Roth et al. 2001). Genetic loss-of-function mutations and chromosomal translocations of CBP and p300 have also been detected in various tumors (Wang et al. 2008).

In the context of transcription, HAT complexes are recruited to gene promoters by transcriptional activators. For example, GCN5 interacts with the transcriptional activator hADA2 to regulate the transcription factor Pax5, which determines B cell lymphopoiesis and midbrain patterning (Barlev et al. 2003). AML1/RUNX1, a transcription factor required for hematopoietic gene expression, physically interacts with p300 and CBP to stimulate transcription (Aikawa et al. 2006). It is thought that these interactions allow the specific acetylation of target gene promoters.

15.3.2 Histone Deacetylases

HDACs are crucial to the formation and maintenance of heterochromatin. In mammals there are five classes of HDACs (Class I, IIa, IIb, III and Class IV), and while all classes contain a conserved HDAC domain, they differ in structure, function and tissue specific expression patterns. Mammals contain a class of NAD-dependent HDACs, known as the Sirtuins or Class III HDACs, which have been implicated in aging (Haberland et al. 2009).

Class I HDACs (HDAC1, 2, 3 and 8) are homologs of the yeast HDAC, Rpd3. They are relatively simple in structure; all have a conserved deacetylase domain, and with the exception of HDAC8, are subunits of repressor complexes. For example, HDAC1 and 2 are components of the Sin3 complex (Sin3A/Sin3B), which has various roles, including gene repression during development (Cunliffe 2008). Class IIa HDACs (HDAC4, 5, 7 and 9) have an extended conserved N-terminal region involved

in protein-protein interactions. They have minimal catalytic activity and the mechanism by which they function is relatively unknown (Haberland et al. 2009). Class IIb and Class IV HDACs make up much smaller classes, with only two members in Class IIb (HDAC6 and 10) and one known member in Class IV (HDAC11). The precise roles of these members are still unclear; however, HDAC6 functions mainly in the cytoplasm and has two deacetylase domains, distinguishing it from other HDACs.

The role of HDACs in the formation of heterochromatin is well characterized at the telomeres of *S.cerevisiae*. Telomeres are heterochromatic and their formation begins in late S phase following replication of the genome. The key regulators of this process are the Silent information regulator (Sir) proteins including the HDAC, Sir2. Sir2 is an NAD-dependent enzyme that is recruited to telomeres by telomeric DNA-binding proteins, and subsequently deacetylates K16 on H4 (Shahbazian and Grunstein 2008). This deacetylation event serves to allow other Sir proteins to bind to chromatin, increasing the levels of chromatin compaction.

The recruitment of HDACs occurs in a similar fashion to that of HATs. HDACs are recruited to gene promoters by transcriptional repressors where they deacetylate histones and allow chromatin compaction. For example, the tumor suppressor Rb interacts with HDAC1 targeting it to promoters of genes required for cell cycle progression (Luo et al. 1998). Additionally, the yeast histone deacetylase 1 (Hda1) is directed to chromatin by the transcriptional repressor Tup1, while Ume6, a sequence specific repressor, recruits Rpd3. Deletion of either Hda1 or Rpd3 results in the hyperacetylation of chromatin and aberrant gene expression (Shahbazian and Grunstein 2007). Interestingly, it has also been observed that transcriptional activators can recruit HDACs to chromatin. In the case of growth factor stimulation in mammalian cells, which leads to the expression of immediate early genes, Sin3A gets recruited to these gene promoters by the transcriptional activator Elk1 (Yang et al. 2001). This mediates the rapid repression of genes following activation and is crucial to the temporal regulation of gene expression.

15.4 Histone Methylation

Histones are modified via the enzymatic addition of methyl groups through the donor, S-adenosylmethionine (SAM). This reaction occurs on the ε-nitrogen of lysines and arginines, and is mainly found on histones H3 and H4 (Shilatifard 2006; Ruthenburg et al. 2007). Mass spectrometry and biochemical studies have confirmed that lysines can be mono-, di-, or trimethylated in vivo (Ruthenburg et al. 2007; Taverna et al. 2007). Similarly, methyl groups can be added to arginine residues in order to generate monomethyl, symmetrical dimethyl or asymmetric dimethyl states (Wysocka et al. 2006). Symmetrical and asymmetrical dimethylation refers to the addition of either one methyl group to each nitrogen or two methyl groups to one nitrogen of the guanidinium group, respectively.

Importantly, in contrast to acetylation, methylation does not influence the net charge of the affected residues. This suggests that methylation primarily serves as

an 'information-storage' mark. In support of this hypothesis, studies indicate that both the degree of methylation and lysine residue in question, are differentially "read" by specific binding proteins (Fig. 15.1). This in turn leads to particular downstream effects, including gene activation and repression (Taverna et al. 2007). In recent years, considerable progress has been made in identifying and characterizing the enzymatic machinery involved in adding and removing histone methylation (Shilatifard 2006; Ruthenburg et al. 2007; Shi and Whetstine 2007). The distinct proteins responsible for 'writing', 'reading' and 'erasing' histone methylation will be discussed (see Table 15.2).

15.5 Reading, Writing and Erasing Histone Arginine Methylation

Histone methyltransferases (HMTs) are grouped into three classes: PRMTs (Protein Arginine Methyltransferases), SET domain-containing lysine HMTs (KMTs) and non-SET KMTs. PRMTs catalyze arginine methylation and to date, at least four mammalian PRMTs are known to catalyze histone methylation: PRMT1, PRMT5, PRMT6 and CARM1. Type I PRMTs include PRMT1, PRMT6, and CARM1 (cofactor associated arginine methyltransferase), which generate Rme1 and Rme2a marks. PRMT1 methylates H4R3 (me2a), PRMT6 methylates H3R2, while CARM1 methylates H3R2, H3R17 and H3R26 (Wysocka et al. 2006; Litt et al. 2009). Type II PRMTs include PRMT5, which generate Rme1 and Rme2s; specifically PRMT5 methylates H3R8 and H4R3 (me2s) (Wysocka et al. 2006).

While histone arginine methylation is involved in multiple chromatin events, its role as a transcriptional coactivator through nuclear hormone receptors (NR) has been particularly explored (Xu et al. 2003). For example, CARM1 cooperates with PRMT1 and p300/CBP in NR-mediated transcriptional activation (Koh et al. 2001). PRMT5 and 6, on the other hand, have been shown to be associated with transcriptional repression. PRMT5-mediated H4R3me2s is required for subsequent DNA methylation in mammals. In fact, DNMT3A, a *de novo* DNA methyltransferase, reads H4R3me2s via its PHD (Plant Homeodomain) finger domain, providing a mechanism by which this repressive PTM and DNA methylation are coordinated (Zhao et al. 2009). PRMT6-catalyzed H3R2me prevents transcriptional activation by abrogating H3K4me3 readers from binding (see below; Litt et al. 2009). A reader for PRMT6-mediated H3R2 methylation has yet to be identified.

Removal of arginine methylation is performed by at least two possible mechanisms. In the first, peptidylarginine deiminases (PADs) convert methyl-arginine to citrulline while releasing methylamine, known as demethylimination (Wysocka et al. 2006). Mammals encode four PADs, of which the nuclear PAD4, was shown to carry out demethylimination with broad specificity (H3R2, R8, R17 and R26 and H4R3) (Wysocka et al. 2006). Like PRMT1 and CARM1, PAD4 is recruited to hormone-induced gene promoters where its presence correlates with loss of arginine methylation and disengagement of RNA polymerase II (Wysocka et al. 2006).

However, PAD4 only converts the mono-methyl state into citrulline in vitro. This, combined with its broad specificity, leaves several questions unanswered, including how the removal of the dimethyl state is carried out. Recently, JMJD6 was identified to have *bone fide* demethylation activity (the second mechanism) with specificity for H3R2me2 and H4R3me2, and additional arginine demethylases will likely continue to be identified (Litt et al. 2009).

15.5.1 Reading, Writing and Erasing Histone Lysine Methylation

Like arginine methylation, lysine methylation contributes to both active and repressive chromatin functions and varies considerably depending on the modified lysine residue (Shilatifard 2006; Taverna et al. 2007). Lysine methylation is likely the most complex modification of histones. In most cases, the biochemical machinery devoted to each individual methylation site is unique and methylation of lysines only a few residues apart, can have distinct biological readouts. The methylation of H3K4, 9, 27, 36 and H4K20 catalyzed by SET-domain containing KMTs, and H3K79me written by a non-SET domain containing KMT, will be discussed.

15.5.1.1 Transcriptional Activation by Lysine Methylation

Methylation of residues H3K4, H3K36 and H3K79 are associated with transcriptionally active chromatin. In the cases of H3K4 and H3K36 methylation, the responsible KMTs are associated with RNA polymerase II during transcriptional initiation and elongation, resulting in methylation of promoter and coding regions, respectively (Shilatifard 2006; Ruthenburg et al. 2007). Importantly, the methylation state (me1, me2, me3) also plays a key role in transcriptional readout. Analyses of the distribution of different H3K4 methylation states using high-resolution genome-wide studies indicate that H3K4me2 and H3K4me3 are found predominantly at active loci, however H3K4me2 can also be present on poised inactive genes (Ruthenburg et al. 2007). In *S. cerevisiae*, a single KMT, Set1, is responsible for all three states of H3K4 methylation, while humans encode multiple H3K4 KMTs including MLL (mixed lineage leukemia) 1-4, hSET1A/B, SET 7/9, among others (Table 15.2; Shilatifard 2006; Ruthenburg et al. 2007). These KMTs exist in multi-subunit complexes that often contain H3K4me binding proteins (Ruthenburg et al. 2007).

Considerable attention has been given to proteins that read H3K4me2/3 (Ruthenburg et al. 2007; Taverna et al. 2007). At least four factors associated with transcriptionally active genes have been reported to interact with H3K4me2/3. This includes the CD-containing CHD1 (Chromo-helicase/ATPase-DNA binding protein 1), a core component of ATP-dependent chromatin remodeling complexes, and WDR5, a component of MLL complexes. WDR5 interacts with H3K4me2 through its WD40 repeats to promote H3K4me3, and has therefore been referred to as a

'presenter' rather than a reader (Table 15.3; Ruthenburg et al. 2007; Taverna et al. 2007). Another well-characterized chromatin remodeling complex, NURF, associates with H3K4me3 through its subunit BPTF (Bromodomain PHD finger Transcription Factor; Fig. 15.1). BPTF recognizes H3K4me3 via its PHD finger to assist NURF-mediated chromatin remodeling for transcriptional activation (Ruthenburg et al. 2007; Taverna et al. 2007). The ING (Inhibitor of Growth) family of tumor suppressors has also been identified to bind H3K4me3 through its PHD fingers (Ruthenburg et al. 2007; Taverna et al. 2007). ING2 binds H3K4me3 upon DNA damage, and stabilizes the Sin3A-HDAC complex at the promoters of proliferation genes (Ruthenburg et al. 2007).

Other methylation sites associated with gene activation include H3K36me and H3K79me. Set2 is responsible for K36 methylation in *S. cerevisiae* and is associated with elongating RNAPII (Shilatifard 2006). Set2 differs from Set1 as it remains associated with RNAPII throughout the body of the transcribed gene. Specifically, the presence of H3K36me2 within ORFs correlates with the 'on' state of transcription. A subunit of the yeast Rpd3 HDAC complex, Eaf3, reads H3K36me2/3 in yeast. Its human homolog MRG15, which associates with Sin3A-HDAC complexes, also reads H3K36me2/3 through its chromo barrel domain, which has similar structure to CDs (Sun et al. 2008).

DOT1 proteins are responsible for H3K79me, and are the only KMTs that lack an identifiable SET domain (Shilatifard 2006). In contrast to other methylated lysines, no specific function has been attributed to the different methylation states of H3K79 in mammals, which all seem to localize along the length of active genes, suggesting overlapping functions (Frederiks et al. 2008). Mice deficient for DOT1L display a global loss of H3K79me. Interestingly, reduced levels of heterochromatin marks at centromeres and telomeres were observed, accompanied by aneuploidy, telomere elongation and proliferation defects (Jones et al. 2008). Thus, the role of H3K79me is thought to prevent binding of heterochromatic proteins in euchromatin. When H3K79me is lost, heterochromatic proteins can bind euchromatin, resulting in a reduction and dilution of these factors in heterochromatin and a concomitant loss of silencing (Shilatifard 2006; Jones et al. 2008).

15.5.1.2 Transcriptional Silencing and Heterochromatin Formation by Lysine Methylation

Of the histone methylation sites found primarily in transcriptionally silent chromatin**,** H3K9, H3K27 and H4K20 are best characterized. The first SET-domain containing KMT was identified in *Drosophila*, Su(var)3-9; its human homolog, SUV39H1, was later shown to have specificity for H3K9 (Tschiersch et al. 1994; Rea et al. 2000). The role of SUV39H1 and H3K9me in heterochromatin formation was indicated through its association with Heterochromatin Protein, HP1. Subsequent studies demonstrated that H3K9me3 provides a binding site for the CD of HP1 (Fig. 15.1; Maison and Almouzni 2004). H3K9me3 is specifically enriched in pericentric heterochromatin, while H3K9me1 and H3K9me2 are often found in

euchromatic regions (Maison and Almouzni 2004; Rice et al. 2003). While SUV39H1 is responsible for heterochromatic methylation, mouse knock out studies suggest that G9a and G9a related protein (GLP) are the primary H3K9 KMTs in euchromatin (Tachibana et al. 2005).

Methylation of H3K27 is linked to the silencing of Hox genes, X-chromosome inactivation (Xi) in female mammals and genomic imprinting (Cao and Zhang 2004; Whitcomb et al. 2007; Reik 2007). Originally discovered in *Drosophila* as being essential for proper body patterning, the Polycomb Group (PcG) family of proteins lies at the heart of this silencing system (Ringrose and Paro 2004). Biochemical and genetic evidence has identified at least two major complexes of PcG proteins that are important for H3K27me-dependent silencing (Ringrose and Paro 2004; Whitcomb et al. 2007). These are referred to as PRC1 and PRC2 (Polycomb Repressive Complex). *Drosophila* PRC2 contains Enhancer of Zeste (E(Z); human homologs EZH1 and EZH2), which catalyzes H3K27me3, Extra Sex Combs (ESC; human homolog EED) and Suppressor of Zeste-12 (SUZ12) (Whitcomb et al. 2007). Both EED and SUZ12 are essential for efficient H3K27 methylation and the integrity/stability of the complex (Whitcomb et al. 2007). Analogous to HP1 binding of H3K9me3, H3K27 is bound by the CD-containing protein Polycomb (Pc) which itself is a component of PRC1 (Fig. 15.1; Cao and Zhang 2004; Whitcomb et al. 2007). PRC1 also contains Ring1B, which possesses ubiquitin E3 ligase activity specific for H2A (discussed below), linking H3K27me and H2A ubiquitylation (Wang et al. 2004).

Methylation of H4K20 also marks mammalian heterochromatin. H4K20 methylation states also demarcate different genomic regions, with H4K20me3 found in constitutive heterochromatin and H4K20me2 or H4K20me1 occurring in non-overlapping fashion throughout the euchromatic regions (Yang and Mizzen 2008). SUV4-20H1 and SUV4-20H2, two SET-domain containing proteins are responsible for di- and trimethylation, while PR-Set7 is mainly responsible for H4K20me1 (Yang and Mizzen 2008). H4K20me1 has also been linked to transcriptional repression and X inactivation (Kohlmaier et al. 2004; Karachentsev et al. 2005). Intriguingly, H3K9me3 is required for the induction of H4K20me3 at constitutive heterochromatin and like SUV39H1, the SUV4-20H KMTs also interact with HP1 isoforms (Schotta et al. 2004).

Recent studies have uncovered a link between H4K20me and the DNA damage checkpoint - H4K20me is required for localization of the repair protein, 53BP1, to DNA DSBs. The tandem tudor domains of 53BP1 read H4K20me2, but not H4K20me3 (Taverna et al. 2007). Readers for HK20me1 include transcriptional repressors L3MBTL1 and L3MBTL2, which contain three tandem MBT (Table 15.3; Malignant Brain Tumor) repeats (Yang and Mizzen 2009; Guo et al. 2009).

15.5.1.3 Histone Lysine Demethylation

The identification of enzymes responsible for removing lysine methylation has lagged behind the discovery of histone methyltransferases until recently (Klose and

Zhang 2007; Shi and Whetstine 2007). For many years, it was believed that histone methylation was static and could only be removed by histone exchange or by cleavage of the methylated histone tail, which, in fact, has recently been shown for mammalian H3 (Duncan et al. 2008). The tremendous amount of effort put into identifying lysine demethylases (KDMs) has led to discovery of novel enzymes including families of amine oxidases and hydroxylases (Klose and Zhang 2007; Shi and Whetstine 2007).

The first KDM identified was LSD1 (lysine-specific demethylase 1), which acts on H3K4 methylation. LSD1 is a FAD-dependent amine oxidase that produces hydrogen peroxide and formaldehyde in its reaction (Shi and Whetstine 2007). Since the reaction mechanism requires a protonated nitrogen to initiate demethylation, LSD1's activity is limited to me1 and me2. Interestingly, LSD1 can function both as an activator or repressor, depending on the complex it associates with. When LSD1 associates with the transcriptional repressor complex Co-REST, it acts as an H3K4me1/me2 demethylase contributing to transcriptional repression. However, LSD1 association with the androgen receptor converts it into an H3K9me1/2 demethylase, allowing it to function as a transcription activator (Shi and Whetstine 2007).

The Jumonji family of proteins containing the JmjC domain has also been shown to function as KDMs. JmjC is similar to the bacterial AlkB catalytic domain and is capable of carrying out hydroxylation on methylated lysines (Shi and Whetstine 2007). There are 27 JmjC domain-containing genes within the human genome, 15 of which whose protein products demethylate specific lysines or arginines in the H3 tail (Klose et al. 2006). The first JmjC domain protein identified as a demethylase, JHDM1, targets H3K36me1/2 (Shi and Whetstine 2007). The apparent inability to demethylate H3K36me3, despite using a different reaction mechanism than LSD1, was surprising. JHMD2, like JHMD1, was also specifically shown to demethylate me1 and me2 states of H3K9, but not H3K9me3. The JHDM3 family was subsequently shown to demethylate both H3K9me3 and H3K36me3, demonstrating the reversibility of the trimethyl mark (Klose and Zhang 2007)**.** These enzymes require a JmjN domain in addition to JmjC domains to efficiently catalyze trimethyl demethylation. Their C-termini often contain lysine methyl-binding PHD and Tudor domains, which likely contribute to their activity and specificity in chromatin (Shi and Whetstine 2007). Finally, the JARID1 subfamily of JmjC proteins is capable of demethylating H3K4me2 and me3, but fails to initiate demethylation of H3K4me1 (Secombe and Eisenman 2007). Thus, the counterpart of JARID1 in full K4 demethylation is LSDI.

KDM activity for H3K27 methylation has been attributed to UTX and JMJD3. Their activity is specific for H3K27me2/3 and interestingly, UTX is found in MLL complexes allowing for concomitant H3K4 methylation and H3K27 demethylation (Lee et al. 2007). JMJD3 plays a role in neural lineage commitment by regulating the expression of neurogenesis genes (Burgold et al. 2008). The demethylases for H3K79me and H4K20me have yet to be identified and it remains plausible that these marks are relatively static or alternative mechanisms are utilized to erase them (Klose and Zhang 2007).

15.6 Histone Ubiquitylation

Ubiquitin (ub) is a 76 amino acid protein and since its discovery 34 years ago, a variety of ubiquitin-dependent cellular processes have been identified (Hochstrasser 1996). These include protein degradation, cell-cycle regulation, protein trafficking and transcriptional regulation. Depending on the nature of ubiquitylation, it can either be a signal for specific downstream effects (mono-ubiquitylation) or result in protein degradation (poly-ubiquitylation). Several years after the discovery of ubiquitin, it became clear that a single ubiquitin moiety is conjugated to both H2A and H2B in mammals (Osley 2006). However, it took many years to decipher the role and regulators of these PTMs. Consistent with other PTMs, monoubiquitylation of histones is reversible and can be removed from target histones by a class of thiol proteases known as ubiquitin specific proteases (called UBPs in yeast and plants; USPs in mammals) (Weake and Workman 2008).

15.6.1 H2B Ubiquitylation

H2B is mono-ubiquitylated at K120 in mammals (H2BK120ub1), which corresponds to K123 in *S. cerevisiae*, K119 in *S. pombe* and K143 in *Arabidopsis* (Osley 2006). H2Bub is a mark of transcriptional activation and accumulating evidence has demonstrated a crosstalk between H2B ubiquitylation and H3 methylation, including H3K4me3 and K79me3 (Shilatifard 2006). For example, in the absence of H2Bub, H3K4 KMT complexes are only able to monomethylate H3K4.

Rad6 is the E2 ubiquitin conjugase that together with the E3 ubiquitin ligase Bre1, is required for H2B monoubiquitylation in yeast (Weake and Workman 2008). Rad6/Bre1 mediated H2B ubiquitylation is a prerequisite for H3K4 and H3K79 methylation (Shilatifard 2006). Humans have two sequence homologs of Rad6: HR6A and HR6B that might act redundantly, and the Bre1 homolog RNF20 regulates H2Bub in vivo (Weake and Workman 2008).

Deubiquitylation of H2Bub is carried out by two UBPs in *S. cerevisiae*: UBP8 and UBP10. UBP8 is a component of the SAGA complex and deubiquitylates H2B in vivo. UBP10, functions independently of SAGA and appears to affect distinct pools of H2Bub in vivo (Weake and Workman 2008). Recently, the *Drosophila* homolog of UBP10, *scrawny*, was shown to be required in germline, epithelium, and intestinal stem cells to repress the premature expression of key differentiation genes (Buszczak et al. 2009). Loss of the *Arabidopsis* homolog, UBP26, results in reactivation of transgenes and transposons by altering DNA methylation and H3K4 and H3K9 levels at these loci (Sridhar et al. 2007).

15.6.2 H2A Ubiquitylation

H2A ubiquitylation is catalyzed by the E3 ubiquitin ligase, Ring1B, a component of PRC1 (Table 15.2, Wang et al. 2004). PRC1 contains three RING domain-containing

subunits Ring1A, Ring1B and Bmi-1, with only Ring1B possessing in vitro E3 ligase activity towards H2A (Wang et al. 2004). RNAi-mediated knockdown of Ring1B and its homolog, Ring1A, depletes H2Aub from the Xi in mice, consistent with its role in silencing (Whitcomb et al. 2007). It has been suggested that H2Aub might directly contribute to transcriptional repression by regulating higher order chromatin structure (Weake and Workman 2008). Of note, the corresponding lysine residues in the H2A variants H2A.Z and macroH2A1 are also subjected to mono-ubiquitylation (Weake and Workman 2008).

Specific deubiquitylases for H2Aub have been identified including Ubp-M, USP21 and 2A-DUB (Weake and Workman 2008). H2A deubiquitylation by Ubp-M is required for Hox gene expression and cell-cycle progression, while USP21 relieves the repression of transcription initiation caused by H2Aub-mediated inhibition of H3K4me. Finally, 2A-DUB interacts with the HDAC, PCAF, and preferentially deubiquitylates hyperacetylated nucleosomes in vitro (Zhu et al. 2007).

While histone monoubiquitylation was discovered decades ago, only two sites on H2A and H2B have been well characterized – H2Aub1 in transcriptional repression and H2Bub1 in transcriptional activation. Both are involved in crosstalk with other modifications including methylation and acetylation on the H3 tail. Of note, recent evidence suggests that H2B in yeast can be polyubiquitylated; however, its function is currently unknown (Geng and Tansey 2008). Future work focused on the discovery and characterization of novel sites of histone ubiquitylation, as well as the role of histone polyubiquitylation, will be of interest.

15.7 Concluding Remarks

While the sole purpose of the nucleosome was once thought to be the packaging of DNA, it is now clear that the nucleosome and its associated histone PTM profile play an active role in numerous DNA-templated cellular processes. Chromatin is an extremely dynamic template and we are just at the brink of understanding the complexity of histone PTMs. PTMs are numerous and diverse, they crosstalk with one another, and interact with various histone modifying complexes to bring about additional PTMs, remove PTMs and remodel chromatin. However, this complexity must be tightly regulated. It is becoming increasingly apparent that chromatin-modifying factors are involved in disease states, which has led to great interest in targeting these molecules for therapeutic purposes. In fact, the inhibition of HDACs (HDACi) is a current focus of cancer therapy (Haberland et al. 2008).

While beyond the scope of this chapter, it should be noted that histone PTMs are only one means of altering the chromatin template. Other factors include DNA methylation, histone variants, and non-coding RNAs. This chapter has focused on well-characterized PTMs, however, other regulatory PTMs exist, including sumoylation, ADP-ribosylation, proline isomerization, formylation, glycosylation and biotinylation (van Holde 1988; Kouzarides 2007). There is no doubt that this list will continue to grow and that new mechanisms for regulating histone PTMs, their

contribution to the histone code, and downstream biological effects, will come into light – just like discovery of the histone demethylases, only a few years ago. It will be exciting to watch the world of chromatin 'unfold'.

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